

**MASARYKOVA UNIVERZITA
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ÚSTAV PATOLOGICKÉ FYZIOLOGIE**

**MIKROPROSTŘEDÍ NÁDORŮ HLAVY A KRKU A JEHO
DIAGNOSTICKÝ POTENCIÁL**

Habilitační práce

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Abstrakt

Úvod. Nádory v oblasti hlavy a krku jsou jedny z nejzávažnějších onkologických onemocnění v ČR a ve světě. Hlavním prognostickým indikátorem pacientů s těmito nádory a současně nástrojem volby léčebné strategie je zařazení do klinického stádia; prognostické biomarkery pro nádory hlavy a krku neexistují a současně odpovědávost na léčbu i v rámci jednoho stádia je velmi heterogenní. Cílem této práce je tuto heterogenitu uchopit pomocí studia nádorového mikroprostředí a na základě získaných dat navrhnout klinicky aplikovatelné závěry.

Metodika. Mikroprostředí bylo studováno víceúrovňově – na úrovni séra a tkáňových vzorků pomocí analýzy přežití, ale také na úrovni jednotlivých subpopulací buněk získaných z nádorových vzorků pacientů. Bylo využito metod analýzy genové exprese, metod studia buněčné migrace, invazivity, buněčného růstu a byla analyzována míra reparace dvouřetězcových zlomů DNA.

Výsledky. Bylo zjištěno, že transkriptom tkáně přiléhající k tumoru byl v mnoha ohledech „podobnější“ nádorové tkáni a bylo možné jej využít k predikci přítomnosti nádoru. Byl také prokázán prognostický význam BAX, BCL2, TP53 a EGF. V heterogenním prostředí nádorů hlavy a krku byly studovány populace s povrchovými antigeny CD44 a CD90. Populace CD44 je významně zapojena do patogeneze prostřednictvím své účasti v molekulárně-biologických procesech de-diferenciace, proliferace a apoptózy. Populace CD90+ „s nádorem asociovaných fibroblastů“ zásadně podporuje růst vlastní nádorové populace a propůjčuje rezistenci k radioterapii jiným subpopulacím a je tak významným podpůrným článkem nádorového mikroprostředí.

Závěr. Přístup k vývoji nových diagnostických a (zejména pak) léčebných strategií musí heterogenitu mikroprostředí reflektovat, protože právě toto je jedna z cest – možná cesta zásadní – pro personalizovanou medicínu potenciálně schopnou radikálního zlepšení prognózy pacientů s pokročilými nádory.

Klíčová slova

nádory hlavy a krku; prognóza; biomarker; nádorové mikroprostředí; CD90; CD44; migrace; radioterapie; dvouřetězcové zlomy DNA; miRNA

Abstract

Introduction. Head and neck cancers are one of the most serious oncological diagnoses in the Czech Republic and globally. The main prognostic indicator and a therapy-planning tool is based on staging of tumours. Prognostic biomarkers for head and neck tumours do not exist, and concurrently therapy response is very heterogeneous even within one stage. The aim of the thesis is to propose clinically applicable conclusions by studying the tumour microenvironment

Methods. The microenvironment was studied at the level of serum, tissue samples using survival analysis, but also at the level of individual subpopulations of cells obtained from tumour samples of patients. Methods of gene expression analysis, analysis of cell migration, invasiveness, cell growth were used and the analysis of repair of two-strand DNA breaks were used.

Results. It has been found that tumour-adjacent tissue transcripts have been in many respects "more similar" to tumour tissues, and this could be used to predict the presence of the tumour. Also the prognostic importance of BAX, BCL2, TP53 and EGF was revealed. In the heterogeneous environment of head and neck tumours, cells with surface antigens CD44 and CD90 were studied. The CD44 population is significantly involved in pathogenesis through its participation in molecular-biological processes of de-differentiation, proliferation and apoptosis. "Tumour-associated fibroblasts" – the CD90+ cells strongly support the growth of tumour population and provides resistance to radiotherapy to other subpopulations and are thus significant supportive element of the tumour microenvironment.

Conclusions. Development of new diagnostic and (especially) treatment strategies must reflect the heterogeneity of the microenvironment, as this is one of the paths – perhaps the most important – for personalized medicine potentially able to radically improve the prognosis of patients with advanced tumours.

Keywords

head and neck tumours; prognosis; biomarker; tumour microenvironment; CD90; CD44; migration; radiotherapy; double strand DNA breaks; miRNA

Děkuji za podporu kolegům tvořícím podnětné zázemí pro práci.
Díky patří také rodině.

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1 Úvod

Nádory v oblasti hlavy a krku jsou jedny z nejzávažnějších onkologických onemocnění v rámci České republiky a také ve světě. Označením „nádory hlavy a krku“ se rozumí nádory dutiny ústní (rty, první dvě třetiny jazyka, sliznice dutiny ústní), slinných žláz, faryngu (nazofarynx, orofarynx, hypofarynx) a laryngu. V naprosté většině se jedná o nádory spinocelulární, „head and neck squamous cell carcinoma“, HNSCC (výjimku tvoří právě např. nádory slinných žláz)^{1,2}.

Vzhledem ke svému umístění jsou nádory hlavy a krku, resp. jejich léčba, zejména v pokročilých stádiích, významně mutilující diagnózou. Podobně jako u jiných onkologických malignit je časný záchyt klíčovým předpokladem příznivé prognózy^{2,3}. Ukazatelem pokročilosti je klasifikace TNM a zařazení do klinického stádia I-IV. I přes specifika jednotlivých lokalit platí, že zatímco lokalizovaná stádia I-II vykazují dobrou prognózu, lokálně pokročilý nádor je terapeuticky zvládnutelný obtížně s pětiletým přežitím 25% a 4% pro stádia IVa a IVb⁴; přibližně u poloviny diagnostikovaných s pokročilým tumorem dochází během prvních let k recidivám^{5,6}. Prognosticky nepříznivý je také fakt, že v 60 % dochází k primozáchtům právě v těchto pozdních stádiích⁷.

1.1 Prognostické biomarkery

Hlavním prognostickým indikátorem pacientů s nádory hlavy a krku a současně nástrojem volby léčebné strategie je zařazení do klinického stádia⁸. Nicméně odpovědnost na léčbu a prognóza pacientů i v rámci jednoho stádia jsou velmi heterogenní. Klinicky využitelné prognostické biomarkery podobné PSA pro nádor prostaty, či estrogenovému receptoru či HER2 pro nádor prsu^{9,10} pro nádory hlavy a krku neexistují.

V prvních částech práce bylo cílem ověřit prediktivní, resp. prognostickou roli molekulárních markerů v rozvoji HNSCC (kapitoly 4.1, 4.2, 4.3 a 4.5). Tyto markery byly z literatury vytypovány na základě jejich významné funkce v jednom z následujících zásadních kroků kancerogeneze: (1) autonomní signalizace, zvýšená míra proliferace, (2) buněčná smrt, regulace buněčného cyklu, (3) odpověď na zvýšený (oxidační) stres v nádorovém mikroprostředí, (4) angiogeneze, (5) schopnost zakládat metastázy a (6) odpověď na radioterapii.

1.1.1 Markery klíčových procesů kancerogeneze

V současnosti byly popsány desítky molekul s prediktivním/prognostickým potenciálem pro HNSCC. Ty jsou systematicky shrnuty a rozčleněny v kapitole Raudenská et al. „HNSCC Biomarkers Derived from Key Processes of Cancerogenesis“ v knize „Targeting Oral Cancer“ (dostupná na str. 25), resp. v přehledovém článku Polanska et al¹¹ dostupném na str. str. 14. V textu níže jsou zmíněny ty „kandidátní biomarkery“, které byly předmětem našich experimentálních prací.

Nezbytným krokem růstu buněk je proliferační signalizace. Toho je u nádorových buněk docíleno aktivací proto-onkogenů¹², díky čemuž se tyto buňky stávají nezávislé na exogenních růstových faktorech. Pro nádory hlavy a krku je klíčovým proto-onkogenem receptor pro epidermální růstový faktor (EGFR). Jedná se o transmembránovou tyrozin kinázu patřící do rodiny ErB/HER¹³⁻¹⁵. K jeho fyziologické aktivaci dochází při navázání ligandu EGF, TGF- α , či amphiregulinu, čímž dojde k dimerizaci EGFR a autofosforylaci tyrozin-kinázové domény EGFR. Takto aktivovaný receptor aktivuje zejména signální dráhy RAS a PI3K a tím ovlivňuje proliferaci¹⁶, viz obrázek na str. 28.

Na základě studií je popsáno, že zvýšená míra exprese EGFR se vyskytuje až u 90 % všech spinocelulárních nádorů hlavy a krku a bývá popsána již u premaligních lézí¹⁷⁻¹⁹. Studie popisují spojitost s nepříznivou prognózou^{11,20,21}, s vysokou mírou proliferace, s angiogenezí a s vysokou mírou invazivity nádorových buněk. Ve spojitosti s EGFR, resp. s rezistencí oproti inhibitorům novými terapeutiky, je také studován tyrozinkinázový receptor pro hepatocytární růstový faktor, c-Met. Jeho fyziologická úloha je spojena s klíčovými procesy během embryogeneze a hojení ran, jmenovitě např. schopnost migrujících buněk odloučit se od extracelulární matrix, uniknout anoikis a zůstat v novotvořené tkáni²² (viz kapitola 4.2).

Další klíčovou vlastností nádorových buněk je únik buněčné smrti¹². Mezi nejzásadnější mechanismy ovlivnění buněčné smrti patří mutace supresoru *TP53*, snížení exprese apoptózu vyvolávajících faktorů (BAX, BIM aj.)²³, zvýšení exprese anti-apoptotických proteinů (např. BCL-xL, BCL2)²⁴, či narušení regulace buněčného cyklu²⁵. U HNSCC bylo popsáno, že zvýšená exprese BCL-xL a BCL2 se významně podílí na rozvoji tohoto nádoru²⁶.

Jakmile populace nádorové masy přesáhne velikost řádově jednoho mm³, difuze kyslíku a nutrientů přestává buňkám stačit a v případě jejich dlouhodobého nedostatku tento proces vyústí v nekrózu buněk. Nádorové buňky, podobně jako tomu dochází při hojení ran, jsou schopny indukovat novotvorbu cév. Jedním ze zásadních působků je vaskulární endoteliální růstový faktor A (*VEGFA*). Ten je indukován zejména transkripčním faktorem indukovaným hypoxií (*HIF1A*) či prostřednictvím EGFR^{27,28}. Studie zabývající se HNSCC potvrzují, že míra exprese

VEGFA koreluje s rezistencí vůči chemoterapii a tím s horší prognózou pacientů a také s pokročilostí nádoru a s horším celkovým přežitím²⁹⁻³⁴.

Počáteční fáze kancerogeneze jsou doprovázeny procesy, které se podobají hojení ran. Doprovodným jevem bývá chronický zánět a zvýšená míra oxidativního stresu³⁵. Existuje celá řada metod studia redoxního stavu buněk – mikroskopicky na základě fluorescenčních barviv, na základě antioxidační kapacity, na základě míry exprese genů zodpovědných za syntézu antioxidantů (glutathion reduktáza, peroxidáza, aj.) či na základě exprese antioxidačně působících proteinů, např. metalothioneinu (MT)³⁶⁻³⁸. Tento protein byl ve vztahu ke spinocelulárním nádorům hlavy a krku studován v řadě studií³⁹⁻⁴¹ a na základě námi provedené meta-analýzy Gumulec *et al.* byla prokázána vyšší exprese v nádorových tkáních (odds ratio, OR 9,95; 95% CI 5,82-17,03)⁴².

Vztah tkáňové exprese genů *EGF*, *EGFR*, *MKI67*, *BCL2*, *BAX*, *FOS*, *JUN*, *TP53*, *VEGF*, *FLTI*, *MMP2*, *MMP9*, *MT1A* a *MT2A* a spinocelulárních karcinomů je popsán ve studii Raudenska *et al.* na str. 103. Jejich vztah s HPV statusem pacientů byl zkoumán v práci Polanska *et al.* na str. 147. Vztah sérových hladin EGFR byl asociován s prognózou, viz studie Polanska *et al.* na str. 140, kapitola 4.5.

1.1.2 microRNA jako marker metastáz

Extenzivně studovanými HNSCC biomarkery jsou také microRNA (miRNA). MiRNA jsou skupinou malých nekódujících RNA molekul s významnou funkcí v regulaci genové exprese na post-transkripční úrovni. Jejich velikost se pohybuje okolo 20 nukleotidů. MiRNA se nepřekládají do proteinové struktury a svým regulačním působením ovlivňují buněčné procesy jako je proliferace, diferenciace, apoptóza, metastázování, angiogeneze a imunitní odpověď. Mnohé studie potvrdily, že miRNA je dobrým biomarkerem, protože je rezistentní ke štěpení ribonukleázami, vydrží bez trvalého poškození extrémní pH i teplotu a jejich distribuce v tkáních a tělních tekutinách se mění v závislosti na druhu, stupni a stádiu nemoci⁴³.

V této práci byla studována exprese miR-200b-5p, miR-29c-3p, miR-34a-5p a miR-375, viz práce Hudcova *et al.* na str. 123 a metodická studie Hudcova *et al.* na str. 131. Signální dráha TGF- β /ZEB/miR-200 je zapojena do regulace epiteliálně-mezenchymální tranzice, miR-29 funguje v roli nádorového supresoru, protože potlačuje migraci a invazivitu nádorových buněk prostřednictvím inhibice signální dráhy laminin-integrin, mezi další potenciální nádorově-supresorové faktory patří též miR-34a-5p⁴⁴ a miR-375⁴⁵.

1.1.3 Markery odpovědi na (radio)terapii

Nádory hlavy a krku jsou typicky agresivními neopláziemi s vysokou mírou rekurence a nepříznivou prognózou. Kvůli jejich fyzikální blízkosti k vitálním strukturám je užití radikálního chirurgického přístupu často nemožné, neboť může způsobit výrazné snížení kvality života pacientů. V těchto případech jsou preferovány nechirurgické postupy (chemo/radioterapie). Častou limitací těchto přístupů je obtížně predikovatelná radiorezistence, vyskytující se přibližně u 50 % spinocelulárních karcinomů hlavy a krku⁴⁶. Selhání radioterapie dramaticky zhoršuje výsledky sekundárně volené „salvage“ chirurgie v porovnání s primárně provedenou operací⁴⁷.

Správné rozhodnutí první terapeutické modality je proto klíčovým krokem dramaticky ovlivňujícím pacientovo přežití. V současnosti neexistují biomarkery umožňující radiosenzitivitu predikovat dříve, než započne léčba. Je tomu zejména z důvodu výrazné genetické a fenotypové heterogenity nádorové populace, charakteristické zejména pro HNSCC; nádory hlavy a krku se často pohybují ve spektru obou extrémů radiorezistence-radiosenzitivita⁴⁸. Ačkoli v minulosti proběhly studie poukazující na geny s potenciálem predikovat rezistentní nádory, „founder“ mutace těchto genů nejsou známy, a tak je výpovědní hodnota takovýchto markerů oslabena⁴⁹. Radioterapie je tak často aplikována na základě odezvy na neoadjuvantní chemoterapii s očekáváním obdobné reakce.

Míru citlivosti k radioterapii je nicméně možné nepřímou odhadovat na základě komplexních odpovědí buněk na poškození DNA, zejména pak na základě míry aktivace opravných mechanismů dvouřetězcových zlomů, typicky vyvolaných právě radioterapií či některými typy chemoterapie. Mimo tento mechanismus jsou studovány další mechanismy – rezistence k apoptóze⁵⁰, poškození regulace buněčného cyklu⁵¹, schopnost dělit se s poškozením genomu⁵², či schopnost navrátit se do buněčného cyklu i přes aktivaci senescence⁵³. Situaci komplikuje fakt, že mechanismus radiorezistence je rozdílný nejen u různých nádorů, ale dokonce u různých subpopulací v rámci jednoho nádoru (a vzájemnými interakcemi mezi těmito subpopulacemi)⁵⁴ V další části této práce (viz studie Falk *et al.* na str. 175) byla zaměřena pozornost právě na objasnění asociace mezi mírou reparace dvouřetězcových zlomů s přežíváním buněk *in vitro* a s prognostickými ukazateli pacientů.

1.2 Etiologické faktory nádorů hlavy a krku

Nejzásadnější rizikové faktory spojené s rozvojem HNSCC jsou konzumace tabáku, nadužívání alkoholu, rizikové sexuální chování a v neposlední řadě také genetická predispozice. Některé

dědičné choroby, jako například Fanconiho anemie, vysoce predisponují svého nositele k brzkému rozvoji HNSCC⁵⁵. V 80. letech dvacátého století byl odhalen vliv lidského papillomaviru (HPV) v incidenci HNSCC⁵⁶. Výskyt HPV indukovaného HNSCC vykazuje významné geografické vlivy; ve Švédsku jde o 90 % nádorů, v zemích s vysokou konzumací tabáku je podíl indukovaného HNSCC výrazně menší, přibližně 20%⁵⁷. Existuje více než sto typů HPV⁵⁸, ve vztahu ke spinocelulárním nádorům jsou nejrizikovější subtypy HPV16 a 18^{59,60}. Viry přednostně napadají epitel tonzilárních krypt a jsou zdrojem proteinů E6 a E7. Ty inaktivují klíčové tumor supresory – TP53, RB1, P21 aj. a tím umožní průchod buněčným cyklem¹¹ (bližší obrázek na str. 15), ovlivňují buněčnou diferenciaci⁶¹, genovou nestabilitu⁶² a antivirovou obranu⁶³. Biologicky jsou proto HPV⁺ a HPV⁻ nádory hlavy a krku velmi odlišná onemocnění, byť se jedná o stejný histologický typ tkáně^{11,64-66}. HPV⁺ HNSCC pacienti jsou většinou mladší a bez předchozí zkušenosti se zneužíváním alkoholu. K dispozici je mnoho prospektivních i retrospektivních důkazů podporujících hypotézu, že podskupina HPV⁺ pacientů s karcinomem orofaryngu má příznivější prognózu ve srovnání s pacienty s HPV⁻ formou tohoto onemocnění⁶⁷⁻⁷⁰. Důvodem může být větší radiosenzitivita HPV⁺ tumorů. HPV⁺ tumory mají sice snížené hladiny nádorového supresoru TP53 v důsledku degradace zprostředkované virovým proteinem E6, ale zbylý TP53 bývá funkční a nemutovaný a pokud radiační poškození buňky zvýší expresi TP53, může dojít k zástavě buněčného cyklu a spuštění buněčné smrti. Naopak u HPV⁻ tumorů bývá TP53 často mutovaný a nefunkční za jakýchkoli podmínek⁷¹. Další odlišnosti HPV⁺ a HPV⁻ tumorů viz přehledový článek Polanska *et al* na str. 14.

Druhá část této práce je dedikována biologické a klinické charakterizaci HPV⁺ a HPV⁻ HNSCC tumorů (viz práce Polanska *et al.* na str. 147).

1.3 Mikroprostředí nádoru

V mnoha studiích byla exprese vybraných markerů v nádorové tkáni porovnávána s jeho expresí v okolní histologicky normální tkáni. Vzhledem k tomu, že nádorové buňky a histologicky normální tkáň v okolí nádoru sdílejí stejné mikroprostředí, mohou být tyto „zdravé“ tkáňe ovlivněny působky produkovanými nádorem⁷², a tak mohou být jen stěží považovány za ideální „kontrolní“ tkáň. Některé cytokiny a růstové faktory podílející se na procesu neoplastické transformace jsou produkovány nenádorovými buňkami vyskytujícími se v okolí nádoru⁷³. Naopak parakrinní účinky faktorů produkovaných nádorem mohou narušit homeostázu okolní tkáňe doprovázenou zánětlivou reakcí a zvýšenou angiogenezi⁷⁴. Z těchto důvodů je velmi důležité charakterizovat vztah a komunikaci nádorových buněk s jejich okolím. Takto nabyté poznatky

mohou zásadně ovlivnit management léčby HNSCC pacientů. Dále předpokládáme, že expresní vzorce histologicky normálních buněk sousedících s nádorem mohou mít prediktivní význam.

1.3.1 Heterogenita mikroprostředí

Jednou ze zásadních příčin selhání nechirurgických léčebných modalit je skutečnost, že solidní nádory nevznikají klonálně, ale jsou heterogenní populací různých typů buněk s různými vlastnostmi⁷⁵. Ty se liší rychlostí proliferace, odpovědí na léčbu, schopností indukovat zánět, či schopností založit sekundární ložiska⁷⁶. Charakterizace buněčných populací mikroprostředí je tak možnou cestou personalizované terapie. Široce studovanými buněčnými populacemi nádorů hlavy a krku jsou ty s povrchovými antigeny THY1 (CD90) a CD44⁷⁷⁻⁷⁹. Na základě studií je patrné, že buňky CD44⁺ jsou schopny iniciace kancerogeneze, protein se účastní buněčné migrace a prostřednictvím napojení na signální dráhu EGFR zasahuje do regulace proliferace⁸⁰⁻⁸⁷. Buňky exprimující CD44 bývají proto označeny jako „cancer stem cells“^{83,88-90}. THY molekula se vyjma své role v diferenciaci lymfocytů vyskytuje na povrchu mezenchymálních buněk, tedy zejm. fibroblastů^{77,91,92}. Její výskyt na povrchu epiteliálních buněk může proto indikovat proces epiteliálně-mezenchymální tranzice⁹³.

Ve třetí části této práce (viz práce Svobodova *et al.* na str. 157) bylo cílem vytvořit primární nádorové linie odvozené z karcinomu hlavy a krku a charakterizovat jednotlivé subpopulace na základě různé exprese povrchových CD-znaků (CD44⁺/CD90⁻, CD44⁻/CD90⁻, CD44⁺/CD90⁺, CD44⁻/CD90⁺). Cílem bylo porovnat růstové charakteristiky, tumorigenicitu, invazivitu a schopnost migrace u těchto buněčných subpopulací. Dále bylo cílem studovat genové expresní profily u těchto subpopulací. Cílem bylo porovnat agresivitu jednotlivých buněčných subtypů identifikovaných uvnitř nádoru, ale též určit přínos, který by konkrétní subpopulace mohla mít v rámci rozvoje tumoru. Stimulace růstu benigních, či méně kooperativních subpopulací by mohla být totiž zajímavou strategií pro léčbu nádorových onemocnění.

1.4 Přehledové články

V této sekci jsou uvedeny přehledové články dále rozšiřující tematiku stručně nastíněnou v úvodu.

1.4.1 Klinická významnost biomarkerů HNSCC – přehledový článek

V předchozích kapitolách byly zmíněny základní molekulární mechanismy rozvoje spinocelulárních karcinomů hlavy a krku aplikovatelné jako prognostické či prediktivní biomarkery. Tato problematika byla podrobněji rozpracována v následujícím¹¹ přehledovém článku na str. 14. „Klinická signifikance“ je dána faktem, že v práci jsou zmíněny pouze studie provedené na pacientech se spinocelulárními nádory hlavy a krku, experimentální práce zmiňující molekulární mechanismy ověřené na buněčných či zvířecích modelech, byť s potenciálem stát se biomarkery, jsou zmíněny až v následující práci⁹⁴ na str. 25.

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Review

Clinical significance of head and neck squamous cell cancer biomarkers



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SUMMARY

Head and neck tumors belong among the six leading causes of cancer death worldwide. The predominant type of head and neck tumors consists of squamous cell carcinomas (HNSCC). Early detection of primary tumor and relapse is a key factor for enhancing the survival rate of HNSCC patients, because high rates of cases are recognized at advanced stages. Accordingly, biomarkers suitable for the early detection of HNSCC are sorely needed to improve patient outcomes. HNSCC evolve through a multistep process by the accumulation of genetic and phenotypic changes. Searching for specific biomarkers capable of characterizing each degree is therefore really essential.

In this review, genomic and gene expression alterations of HNSCC are summarized and associated with HPV status, clinicopathological conditions, and patient history from the perspective of potential biomarker utilization. The emphasis is placed on non-invasive markers detectable from saliva and blood and clinically relevant studies are mentioned in particular. These include analyses of tumorous tissues, saliva, and blood from patients with histologically defined tumors; cell culture- and other in vitro-based studies with no clinical correlations are rather excluded.

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Introduction

Head and neck cancers include several types of cancer originating from the head or neck region, not including thyroid or skin cancers. The predominant (95%) type consists of squamous cell carcinomas whilst 4–5% are salivary gland (adeno) or other carcinomas [1]. Head and neck squamous cell carcinomas (HNSCC) belong among the six most common cancers worldwide [2]. HNSCC develop from the mucosal linings of the upper respiratory tract. Major risk factors associated with the development of HNSCC are smoking or tobacco chewing, alcohol consumption, use of smokeless tobacco products, and genetic predisposition. Tobacco smoking and alcohol consumption have a synergistic effect [3].

Furthermore, human papillomavirus (HPV) infection was identified as one of the primary causes of HNSCC. About 40–80% of oropharyngeal tumors are inflicted by HPV infection in the USA, whereas HPV cancer incidence in Europe changes from 90% in Sweden to approximately 20% in countries with the highest tobacco

consumption [4]. Epstein-Barr virus (EBV) can also be a causative agent of nasopharyngeal carcinoma [5]. Eventually, some inherited disorders, such as Fanconi anemia, predispose to HNSCC [6].

Together with progress in treatment, early detection of primary tumor and relapse is a key factor for improving the survival of patients with HNSCC, because high rates of cases are recognized at advanced stages. Deeper understanding of the molecular biology of HNSCC can provide new insights into its development and progression; it also provides various biomarkers with a potential application for cancer screening and monitoring of the response to therapy. Although there is a number of reviews regarding HNSCC biomarkers [7–11], none of them currently provides a general overview of the topic. There are rather exhaustive reviews dedicated to specific issues, which include e.g. HNSCC and miRNAs, HPV status, molecular characteristics, and others. In this review, genomic and gene expression alterations of HNSCC are summarized and associated with HPV status, clinicopathological conditions, and patient history from the perspective of potential biomarker utilization. The emphasis is placed on non-invasive markers detectable from saliva and blood and clinically relevant studies are mentioned in particular. These include analyses of tumorous tissues, saliva, and blood from patients with histologically defined tumors; cell culture- and other in vitro-based studies with no clinical correlations are rather excluded.

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HPV in head and neck carcinogenesis

HNSCC is a heterogeneous disease containing leastways two divergent groups: (a) tumors caused by HPV infection, and (b) tumors caused by other mechanisms. Approximately 20–25% of HNSCC are HPV-positive, generally arising in the oropharynx [11,12]. A majority of HPV-induced HNSCCs are caused by HPV-16 [13]. HPV-positive HNSCC patients tend to be younger, with no former experience of tobacco and heavy alcohol consumption. Moreover, HPV-positive HNSCC can also be sexually transmitted; a significant association was revealed between HPV-16 positive HNSCC and oral sex [14].

HPV-associated HNSCCs mostly emerge in the lingual and palatine tonsils, because HPV targets preferably the extremely specialized reticulated epithelium of tonsillar crypts [15]. Active HPV infection results in several alterations in key cell signaling pathways that promote tumorigenesis. In particular, the expression of E6 and E7 viral proteins leads to the inactivation of two key tumor suppressors, p53 and Rb (retinoblastoma protein). The E6 protein is a small polypeptide that contains two zinc-binding domains [16] and stimulates p53 degradation [12]; a significant decrease in the expression of p53 and p21 was observed in the HPV 16/18 positive sinonasal-inverted papilloma compared with the HPV 16/18 negative sinonasal-inverted papilloma [17]. Besides, the HPV-16 E6 protein can also activate telomerase [18]. Similarly as the E6 protein, the E7 protein is also a small, nuclear polypeptide. The carboxyl-terminus of E7 contains a zinc-binding domain. By contrast to E6, E7 binds to Rb. The underphosphorylated Rb binds E2F and thus prevents the E2F-mediated S-phase induction (Fig. 1) [4,19]. Under physiological conditions, the intracellular accumulation of p16 protein inhibits the progression of cell cycle through cyclin D1 and CDK4/CDK6-mediated events. By contrast, HPV E7 overrides this important cell cycle control, pushing the cells from G1 into S phase [20], because the disrupted binding of E2F to Rb allows E2F to bind DNA and induce cell growth and proliferation [21]. In sum, both E6 and E7 promote cell cycle progression through its activity at different points of cell cycle regulation. In addition, the E7 protein induces abnormal centrosome duplication, resulting in multipolar, abnormal mitoses, aneuploidy and genomic instability [22].

In the context of HPV positivity or negativity, other molecular changes should be assessed. In Fischer et al. study, p21WAF1/Cip1 was highly expressed in HNSCC samples from larynx and

pharynx. Its higher expression was correlated with lymph node metastases, decreased survival rate, and locoregional relapse [23]. HPV status was not given in this study. In a more accurate study, high p21WAF1/Cip1 expression was associated with better outcome in HPV-positive HNSCC [24]. Furthermore, in HPV-negative HNSCC, p53 is often mutated, Rb levels are normal, and p16 protein is decreased. Other known differences include the frequent hypermethylation of 14-3-3 σ and RASSF1A promoters and the cyclin D gene amplification in HPV-negative HNSCC [25–27]. The result of the hypermethylation of these genes is similar: it abolishes the cell cycle arrest. RASSF1A is a tumor suppressor, which binds to microtubule-binding proteins and regulates the cell cycle and apoptosis in response to mitogenic or apoptotic impulses. The repression of cyclins A and D1 by RASSF1A results in the cell cycle arrest [28]. Protein 14-3-3 σ negatively regulates the cell cycle progression by inhibiting activities of cyclin-dependent kinases and Akt oncogenic signaling [29,30]. Furthermore, it was demonstrated, that inactivation of 14-3-3 σ by promoter methylation correlates with metastases in nasopharyngeal carcinoma [31].

In addition, the selective upregulation of TCAM-1 (testicular cell adhesion molecule 1) in HPV-positive HNSCC tissue samples was observed by multiple researchers [32,33].

Major differences between head and neck squamous cell carcinomas (HNSCCs) according to the human papillomavirus (HPV) status are listed in Table 1.

Precursor lesions and genetic progression of HNSCC

Malignant transformation of the mucosal lining is a complex genetic mechanism ensuing from the accumulation of multiple genetic alterations, which influence the probability and rate of progression to invasive carcinoma, see Fig. 2 [7]. Cancerogenesis is a multistep process; numerous studies pointed out the fact that individual steps could be characterized by specific genetic or molecular alterations. Therefore, potential biomarkers with regard to tumor progression are mentioned in this chapter.

Precursor lesions

Genetic analysis of surgical margins indicated that HNSCC frequently develops in the field of genetically altered epithelial

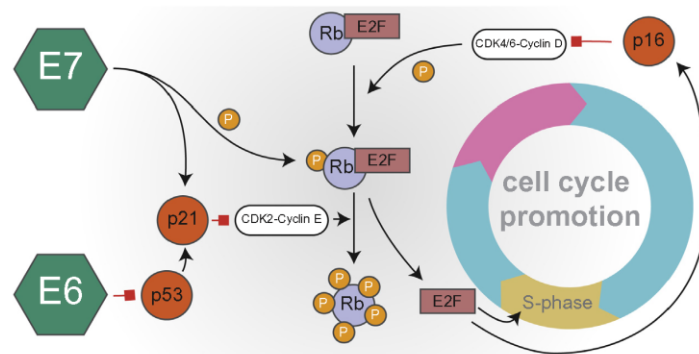


Figure 1. Main mechanisms of HPV-induced oncogenesis – Both E6 and E7 viral proteins can form specific complexes with cellular tumor suppressor gene products. The E7 protein binds and inactivates the retinoblastoma tumor suppressor Rb with a preference for the underphosphorylated, “active” form of Rb. The Rb family of proteins plays an essential role in controlling the cell cycle by governing the checkpoint to S phase. Underphosphorylated Rb binds to the E2F transcription factor forming an Rb-E2F complex, making E2F inaccessible for the transcription of genes associated with DNA synthesis. After the phosphorylation of Rb by cyclin-CDK complexes or Rb inactivation by E7 viral protein, E2F is released from the Rb-E2F repressor complex and can induce the transcription of S phase genes. Inactivation of Rb and inhibition of feedback loop mechanism lead to the overexpression of p16 protein. E7 viral oncoprotein can also interact with other cellular factors that control the cell cycle including the CDK inhibitor p21. Furthermore, HPV E6 proteins can bind to the p53 tumor suppressor protein and promote p53 degradation. Red arrows indicate inhibitory effect. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Table 1
Major differences between head and neck squamous cell carcinomas according to the human papillomavirus status.

	HPV-positive HNSCC	HPV-negative HNSCC
Risk factors	High-risk sexual practices	Cigarette and alcohol use
Tumor site	Lingual and palatine tonsils	Non-oropharyngeal sites
Histopathology	Basaloid, non-keratinizing, poorly differentiated	Keratinizing, moderately differentiated
p53 pathway disturbances	Degradation of wt p53 by E6	TP53 mutations, 17p LOH
Protein Rb pathway disturbances	Degradation of wt pRb by E7, p16 overexpression	p16 ^{INK4A} -promoter hypermethylation, 9p LOH
Cyclin D	Cyclin D gene amplified less frequently	Cyclin D gene is amplified frequently
TCAM-1	Upregulation	Non
14-3-3σ and RASSF1A	Low level of promoter methylation	Promoter hypermethylation
Relative responsiveness to chemoradiation	Better	Worse
Relative prognosis	Improved	Worse

HPV (human papillomavirus); LOH (loss of heterozygosity); pRb (retinoblastoma protein); wt (wild type). Adapted and extended from Pai et al. [11].

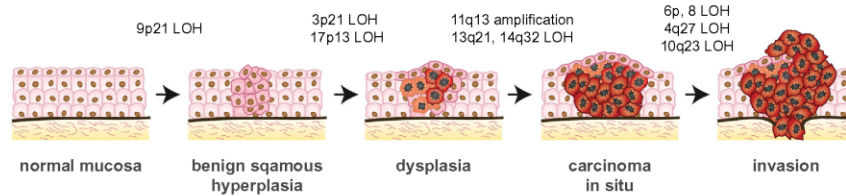


Figure 2. Genetic changes associated with the histological progression of HNSCC based on chromosomal material changes. LOH (loss of heterozygosity); Candidate tumor suppressors include p16 (9p21), p53 (17p13), RASSF1A (3p21), PTEN (10q23), and Rb (13q21). Candidate proto-oncogene includes cyclin D1 (11q13).

cells that are referred to as precursor fields [34,35]. Only a minority of the precursor fields might appear as clinically identifiable lesions, which show as either white or red mucosal areas (leukoplakia and erythroplakia). In such easy-to-diagnose precursor fields a tumor can develop; thus, these tumors are usually soon resected. However, the fields are not always resected entirely and malignant transformation of an unresected precursor field might cause a local relapse that is clonally related with the field and the primary tumor [36,37]. Recently, it became possible to visualize these fields using autofluorescence [38,39]. Furthermore, the development of local relapse was significantly associated with the low expression of keratin 4 and cornulin in the surgical margins of the index tumor [40]. The authors therefore propose using those genes to verify the resection margins. Ploidy studies of dysplastic leukoplakias demonstrated that most aneuploid lesions resulted in tumor occurrence, but only 60% of tetraploid lesions and only circa 3% of diploid lesions did the same [41]. Analogous studies on erythroplakias confirmed the potential of aneuploidy in predicting the SCC progression [42]. Furthermore, poorly differentiated tumors overexpress genes involved in cell adhesion, embryonic development, motility, differentiation, and extracellular matrix, whereas well-differentiated tumors overexpress genes involved in anti-apoptotic pathways, metabolism, and epithelial cell differentiation [43].

Loss of heterozygosity and chromosomal aberrations

Loss of heterozygosity (LOH) is an important marker of tumor progression and can cause inactivation of the tumor suppressor gene. Traditional methods of mapping LOH regions include the comparison of both tumor and patient-matched normal DNA samples. LOH studies for HNSCC show that the earliest alterations target genes located on chromosome locations 3p (RASSF1A), 9p21 (cyclin-dependent kinase inhibitors), and 17p13 (TP53) [44].

A loss of chromosomal region 9p21 is found in 70–80% of dysplastic lesions of the oral mucosa. At 9p21 two functionally and structurally different cell cycle regulators, p16 (INK4a) and p14 (ARF), encoded by the gene *INK4a/ARF* are located. 9p21 LOH and inactivation of the remaining alleles of *INK4a/ARF* by promoter

hypermethylation represent early and frequent events in the progression of HNSCC [35,45] together with the overexpression of EGFR, which rises with the increasing severity of dysplasia in pre-malignant lesions [46,47].

Chromosomal alterations which occur in connection with advanced grades of dysplasia and HNSCC include amplification of 11q13, PTEN (10q23.3) inactivation, and LOH at 13q21, 14q32, 6p, 8p, 4q27 [8,9,35,48]. The amplification of 11q13 was reported in one-third of HNSCC, where amplified cyclin D1 inflicts the cancer phenotype [49,50]. Genetic alterations in PTEN occur in 5–10% of HNSCC lesions and the loss of PTEN expression can be observed in approximately 30% of HNSCCs. The lack of PTEN expression could be an independent prognostic factor of poor clinical prognosis [51]. In sum, losses of heterozygosity were more frequently found in the histologically higher-grade lesions and in the lower-grade lesions when a high proliferation rate was present [48].

Protein markers of tumor progression

Several studies reported increased metallothionein (MT) protein levels in malignant tumors in the head and neck area [52–55]. Metallothioneins seem to support proliferation and anti-apoptotic activity and are considered to be involved in microenvironment remodelling [56]. Sochor et al. determined MT levels in tumor tissues of patients suffering from head and neck tumors using differential pulse voltammetry [54]. The highest MT level was determined in the tissues of oral tumors (170 ± 70 µg/g wet weight tissue, wwt) followed by tumors of hypopharynx (160 ± 70 µg/g wwt) and larynx (160 ± 70 µg/g wwt). The relatively lowest MT level was determined in oropharynx tumors (130 ± 50 µg/g wwt). In the study of Dutsch-Wicherek et al., tissue samples taken from patients with pharyngeal squamous cell carcinomas were analyzed. An increased MT immunoreactivity was observed in tissue samples from tonsillar squamous cell carcinomas in comparison to the reference group with chronic tonsillitis [56].

Furthermore, elevation of cyclooxygenase-2 (COX-2) was described in HNSCC at both mRNA and protein levels [57]. Increased expression of COX-2 resulted in angiogenesis promotion through an elevated level of prostaglandins E2 and VEGF, thus leading to

the promotion of tumor growth [58]. Angiogenesis is a crucial process in tumor growth and subsequent metastasis. Inter alia, oral cancer overexpressed 1 and 2 (ORAOV1 and 2) transmembrane proteins are involved in tumor angiogenesis and regulation of cell growth [59,60]. Overexpression of ORAOV1 as well as ORAOV2 was reported in HNSCC [60–62]. Another mechanism included in the tumorous angiogenesis lies in the intake and utilization of locally stored fibroblast growth factors (FGFs). The role of FGF binding protein (FGF-BP) in this mechanism is considered. Li et al. demonstrated a FGF-BP mRNA expression in primary HNSCC specimens and metastatic tumor specimens but not in adjacent control tissues [63]. Over and above, the supporting stroma of most epithelial cancers contains specialized fibroblasts, known as reactive tumor stromal fibroblasts or cancer-associated fibroblasts (CAFs). A relatively specific molecular marker of CAFs is the expression of fibroblast activation protein (FAP), which is a cell-surface protease [64]. The limited expression of FAP in normal tissues and benign epithelial tumors in contrast to its abundant expression in the stroma of many types of epithelial cancers show FAP as a promising marker of the malignant progression of cancer cells [65].

Growth factors and their receptors play an unarguably important role in HNSCC. EGFR, a member of the *cerbB* family of tyrosine kinase receptors, has been shown to be expressed in many tumors, including in up to 80% of HNSCC [66], where it was associated with poor patient outcomes [67]. The EGFR expression was namely associated with the higher proliferative index, advanced HNSCC tumor stage, and increased tumor angiogenesis [68]. While all members of the *cerbB* family were overexpressed in HNSCC, the overexpression of EGFR was usually the highest [68]. Potential biomarkers of HNSCC progression are listed in Table 2.

Tumor progression and miRNAs

Discoveries in the expression profiling of microRNA in head and neck oncology promise a great progress in the diagnosis, prognosis and therapy of HNSCC. Although the microRNAs are important regulatory factors in cancer development, our understanding of the role of miRNAs in the HNSCC oncogenesis remains unclear. Nevertheless, some alterations consistently identified in head and neck cancer, such as upregulation of miR-21, miR-31, miR-155, and

downregulation of miR-26b, miR-107, miR-133b, miR-138, and miR-139 were found [69,70].

Biomarkers of promoting metastases

Regardless of progress in the HNSCC treatment, the survival rate of five years after diagnosing advanced HNSCC remains insufficient, approximately 50%. One reason for high mortality associated with the late stage HNSCC is the inherent capability of tumor cells to go through locoregional invasion due to the presence of a rich lymphatic network and the overall high number of lymph nodes in the neck region [71]. Even in patients without the clinical evidence of lymph node involvement (NO), there is a high incidence of occult lymph node metastases, ranging from 10% to 50% [72]. The presence of lymph node metastases is significantly associated with the poor patient outcome [73]. The diagnosis of neck lymph node metastases is an essential requirement for clinical staging and treatment, and is now widely accepted as the most important factor in HNSCC prognosis [74–76]. HNSCC invasion and nodal metastases constitute a complicated process involving different signaling pathways and proteins; however, some possible markers of the metastatic process were discovered (see Table 3).

HNSCC is known to be associated with extensive chromosomal alterations. Numerous chromosome alterations associated with the propensity to metastasize were found (gains: 4q11–22, and Xq21–28; losses: 8p, 6p, 11q14–24, 4q26–28, 11q, and 17p11–12) [9,35,43]. Several genes associated with cell signaling (*BEX1*, *BEX2*, *ZNF6*, *NGFRAP1L1*, *GPRASP2*) are clustered just on chromosome Xq21–22, whose chromosome gain is associated with HNSCC metastasize [43]. This may be considered an explanation why the female sex is associated with the bad response to organ-sparing therapy and poor outcome [77].

Rickman et al. proposed four-gene model (*PSMD10*, *HSD17B12*, *FLOT2* and *KRT17*) as a predictor of metastases. HPV positive samples were eliminated from the study. The four-gene model was highly associated with the development of metastases (hazard ratio 6.5; 95% confidence interval 2.4–18.1). This four-gene model predicted the occurrence of metastases with 74% sensitivity and 78% specificity [43]. Furthermore, Rickman et al. found downregulation of genes that encode proteins involved in apoptosis (CASP1,

Table 2
Potential biomarkers in head and neck cancers.

Major classes	Member	Function
Cell-cycle regulation	<i>p16^{INK4A}</i>	A tumor-suppressor gene regulating senescence and cell-cycle progression [45]
	<i>TP53</i>	A tumor-suppressor gene regulating cell-cycle progression and cell survival [36]
Cell-cycle regulation	<i>PTEN</i>	A tumor-suppressor gene regulating signaling pathways controlling cell proliferation and apoptosis [51]
	<i>Rb</i>	A tumor-suppressor gene regulating cell-cycle progression and apoptosis [21,45]
	<i>Cyclin D1</i>	A proto-oncogene regulating cell-cycle progression [49]
Signal transduction	<i>Bmi-1</i>	Controls cell cycle and self-renewal of tissue stem cells [124]
	<i>EGFR</i>	A transmembrane TK that acts as a central transducer of multiple signaling pathways [77]
	<i>VEGF</i>	A transmembrane TK that promotes proliferation, migration, and survival of endothelial cells during tumor growth [125]
Secreted protein	<i>PIK3CA</i>	A gene encoding the p110 α subunit of phosphoinositide 3-kinase (PI3K) α [126,127]
	<i>FGF-BP</i>	Fibroblast growth factors binding protein [63]
	<i>FAP</i>	Protein secreted by cancer associated stroma [60]
Transmembrane protein	<i>TMEM16 (ORAOV2)</i>	Calcium-activated chloride channel [60]
	<i>ORAOV1</i>	A regulator of cell growth and tumor angiogenesis [59,61,62]
Transcription factors	<i>NFκB</i>	Proinflammatory transcription factor [84]
Prostaglandin metabolism	<i>Cox-2</i>	Catalyzes prostaglandin synthesis from arachidonic acid [57,128]
Metal ion homeostasis, oxidative stress	<i>MT</i>	Low-molecular weight proteins involved in heavy metal detoxification, essential metal ion homeostasis and cell protection against free radicals [53]
Oncoviruses	<i>EBV</i>	A causative agent for most nasopharyngeal carcinomas, plasma EBV DNA load is an independent prognostic factor [129,130]
	<i>HPV</i>	A causative agent for most oropharyngeal cancers [13]

EBV (Epstein-Barr virus); EGFR (Epidermal growth factor receptor); HPV (Human papillomavirus); Rb (Retinoblastoma); TK (Tyrosine kinase); TP53 (Tumor protein 53); FAP (Fibroblast activation protein); VEGF (Vascular endothelial growth factor); MT (Metallothionein); Cox-2 (Cyclooxygenase-2); Bmi-1 (B cell-specific Moloney murine leukemia virus integration site 1); ORAOV (Oral cancer overexpressed gene). Adapted and extended from [11].

Table 3
Biomarkers involved in promoting metastases.

Biomarker	Function	Expression	HPV status	References
CASP1	Apoptosis	↓ mRNA	HPV-negative	[43]
CCR7	Chemokine receptor	↑ mRNA, protein	NA	[131–133]
CD44	Cell–cell and cell–matrix adhesions	↑ mRNA, protein	NA	[134–136]
CEP55	Cell cycle regulation, cytokinesis	↑ mRNA, protein	NA	[137]
c-Met	Proliferation	↑ protein	NA	[138]
COL17A1	Cell interactions	↓ mRNA	HPV-negative	[43]
Cortactin	Cell motility and invasion	↑ mRNA, protein	NA	[139–142]
CXCR4	Chemokine receptor	↑ mRNA, protein	NA	[131,132,143,144]
DAPK3	Apoptosis	↓ mRNA	HPV-negative	[43]
DSG3	Cell-to–cell adhesion	↑ protein	NA	[145]
DST	Regulation of the cell cycle	↓ mRNA	HPV-negative	[43]
Hif-1 α	TF, hypoxia	↑ protein	NA	[146,147]
IL18	Apoptosis	↓ mRNA	HPV-negative	[43]
LRP6	Signaling	↑ mRNA	HPV-negative	[43]
MMP-2	ECM degradation	↑ mRNA, protein	NA	[137,148]
MMP-9	ECM degradation	↑ protein	NA	[134,136,149]
MYCN	Signaling	↑ mRNA	HPV-negative	[43]
NBS1	Cell cycle regulation, DNA double-strand break repair	↑ mRNA, protein	NA	[148,150]
NF κ B	Proinflammatory TF	↑ mRNA, protein	NA	[84,151,152]
PPP2R1B	Apoptosis	↓ mRNA	HPV-negative	[43]
RSK2	Cell cycle regulation, proliferation, apoptosis	↑ protein	NA	[153]
Snail	TF, regulator of EMT	↑ protein	NA	[146,148]
SPP1 (osteopontin)	Secreted phosphoprotein	↑ mRNA, protein	NA	[60,154]
Survivin	Inhibitor of apoptosis	↑ protein	NA	[155–157]
Twist	TF, regulator of EMT	↑ protein	NA	[132,146]
VEGF/R	Angiogenesis	↑ protein, mRNA	NA	[112,158–160]
14-3-3 σ	Cell cycle regulation	↓ mRNA, protein	NA	[31]

MMP (matrix metalloproteinase); ECM (extracellular matrix); TF (transcription factor); EMT (epithelial-mesenchymal transition); VEGF/R (Vascular endothelial growth factor/receptor); NA (not available).

DAPK3, IL18, PPP2R1B), negative regulation of the cell cycle (DST) and cell interactions (COL17A1), and upregulation of genes encoding proteins involved in signaling (MYCN, LRP6) in tumors which have developed metastases. Thereunto, overexpression of secreted phosphoprotein 1 (SPP1) correlated with lymph node metastasis and lymphatic invasion in Kashyap et al. [60].

Prognosis and survival markers

Many factors can influence the prognosis of HNSCC patients. The most important ones include the cancer type and location, stage of disease, and cancer grade. Other factors affecting prognosis include biological and genetic features of cancer cells, age of the patient, and general health condition and response to treatment.

HPV status is one of major factors affecting the prognosis. Specific molecular characteristics of HPV-positive tumors are discussed in the following chapter; in this chapter, the associations of HPV status with the prognosis are only mentioned. There are profound prospective and retrospective evidences that the subgroup of patients with HPV-positive oropharyngeal cancer has a more favorable prognosis as compared to patients with oropharynx cancer negative for HPV [19,78]. A meta-analysis of 23 studies analyzing the survival in 1747 HNSCC patients stratified for HPV status found a hazard ratio of 0.72 (95% CI, 0.5–1.0) for the overall survival in HPV-positive oropharyngeal HNSCC patients, and a hazard ratio of 0.51 (95% CI, 0.4–0.7) for the disease-free survival compared to HPV-negative patients [79]. Two recent studies suggest that patients with HPV-positive HNSCC have a lower risk of second primary cancers when compared to patients with HPV-negative tumors [80,81]. In these studies, the rate of second primary cancers in HPV-positive HNSCC patients was observed to range from 0 to 2.2% compared to 10.2–13% in HPV-negative patients. Moreover, HPV-positive HNSCC seems to be more radiosensitive than HPV-negative disease. It can be consequence of differences in p53 between the tumor types; although HPV-positive tumors have reduced levels of p53 due to E6-mediated degradation, the p53

protein is intact and not mutated, unlike the high percentage of HPV-negative tumors, which have mutated p53. Radiation-induced increases of p53 levels may be sufficient to provoke programmed cell death in the HPV-positive tumors in response to radiation-induced damage [82]. Furthermore, the presence of p53 mutations in surgical margins was found as an independent prognostic indicator for locoregional recurrence (relative risk = 7.1; $p = 0.021$; 95% confidence interval, 0.9–56) [36]. On the contrary, the absence of p53 mutations in surgical margins was significantly associated with no local and locoregional recurrence ($p = 0.027$ and $p = 0.028$, respectively).

For further, low EGFR and high p16 (which correlates with higher HPV titer) expressions were established as markers of good response to organ-sparing therapy, whereas high EGFR expression, combined low p53/high Bcl-xL expression, female sex, and smoking were associated with a poor outcome [10,77]. There was also some evidence of improved overall survival in patients with oropharyngeal squamous cell carcinoma with raised Bcl-2, amplification of 11q3 and loss of 16q genes, and low levels of c-met, Ki67, IMD, PLK, FHIT, nuclear survivin, or nuclear cyclin D1 [10]. On the other hand, the lack of PTEN expression and the loss of heterozygosity could be independent prognostic indicators of poor clinical prognosis [48,51] as well as elevation of mRNA and protein COX-2 levels [57,83].

An important role in HNSCC pathogenesis seems to be played by transcription factors such as NF- κ B. NF- κ B is a family of transcription factors composed of hetero- or homo-dimers from five different subunits, NF- κ B1, NF- κ B2, RELA, cREL and RELB. NF- κ Bs are transiently activated under physiological conditions in response to infection or injury, but these genes are aberrantly overactivated in cancers, promoting pathogenesis and therapeutic resistance. In HNSCC, a major role of NF- κ B in the regulation of tumorous transcriptome and proteome was appointed [84]. Human head and neck squamous cell carcinomas were among the first cancers for which a proof was gained for incorrect constitutive activation of NF- κ B [85]. Subsequent studies have shown that NF- κ B is acti-

vated in squamous dysplasias and carcinomas and correlate with the progression of dysplasia and decreased survival rate in patients with HNSCC [86]. Aberrant NF- κ B activation was detected in tobacco-associated as well as in viral-related HNSCC; these include EBV-related nasopharyngeal and HPV associated oropharyngeal carcinomas [85].

A key inhibitor of tumor suppressor p53 (iASPP) was found to be up-regulated in malignant conditions. Immunohistochemical staining indicated iASPP in both cytoplasm and nucleus. Importantly, the overexpression of cytoplasmic and nuclear iASPP was significantly associated with T, clinical stage, lymph node metastasis, and recurrence. Survival analysis demonstrated high iASPP expression in a significantly negative correlation with the disease-free survival and overall survival [87]. Coexpression of MMP7, MMP9, and MMP13 has also been associated with the poor outcome in esophageal squamous cell carcinoma ESCC [88].

Non-invasive biomarkers in HNSCC

Serum, plasma, and saliva contain a number of stable markers, whose differential expression patterns seem to be specific for certain diseases. Noninvasivity of these markers is very important for the acquisition of healthy controls for oncological case-control studies. Obtaining control healthy tissues from patients is not ideal, because of the genesis of unexpected alterations in the expression of selected mRNA, miRNA or proteins in a body burdened with neoplastic processes.

Salivary markers

The uppermost advantage of saliva as a diagnostic tool is the fact that it contains cells detached from the oral cavity and is in a direct contact with oral cancer lesions.

Hu et al. successfully confirmed five candidate biomarkers inclusive of myeloid related protein 14 (MRP14), Mac-2 binding protein (M2BP), profilin 1, CD59, and catalase on oral cancer patients and matched controls [89]. Furthermore, autoantibodies against the aberrantly expressed p53 were found in both saliva and serum of patients with oral cancer. P53 antibodies positivity strongly correlated with the poor treatment outcome in cancer patients [90–92]. Moreover, Sato et al. found higher interleukin-6 (IL-6) concentrations in saliva of patients with oral cancer than in controls [93]. Multivariate analysis revealed that postsurgery salivary IL-6 concentration was an independent risk factor for loco-regional recurrence in patients with oral squamous cell carcinoma (OSCC) ($p = 0.03$; relative risk, 0.14) [94,95]. Brailo et al. observed that salivary TNF- α levels and IL-6 levels were significantly higher in patients with oral leukoplakia in comparison with healthy controls [96]. In accordance, Rhodus et al. referred significantly increased salivary concentrations of IL-8, IL-1, IL-6 and TNF- α in the oral cancer group in comparison with the patients with dysplastic oral lesions and controls [97]. IL-8 was also detected at higher concentrations in the saliva ($p < 0.01$) of patients with OSCC compared with healthy controls ($p < 0.01$). These results were confirmed at both the mRNA and the protein levels [98]. Zhong et al. [103] found a 75% positive expression of telomerase in the saliva of OSCC patients. Using quantitative proteomics methods, higher levels of actin and myosin were also observed in the saliva of patients with malignant oral lesions in comparison to those with pre-malignant lesions. Sensitivity/specificity values for distinguishing between pre-malignant lesions and malignant lesions were 100%/75% ($p = 0.002$) for actin, and 67%/83% ($p < 0.00001$) for myosin in soluble saliva [99]. Salivary transferrin was also studied as a biomarker of early stage oral cancer detection. Increased salivary transferrin levels in patients with OSCC strongly correlated with

the tumor size and stage [100]. The tumor-specific mRNA in saliva could also be utilized as a biomarker for oral cancer. Elevated salivary mRNA for IL-1B, IL-8, dual specificity phosphatase 1 (DUSP1), ornithine decarboxylase antizyme 1 (OAZ1), H3 histone, family 3A (H3F3A), S100 calcium binding protein P (S100P), and spermidine/spermine N1-acetyltransferase (SAT) were clearly documented as oral cancer biomarkers [101,102].

Melanoma-associated antigen proteins (MAGE) suppress apoptosis and support proliferation therefore have a crucial role in carcinogenesis [103]. MAGE expression was detected in the sputa of HNSCC patients [104]. Since MAGE is not ordinarily expressed in normal tissues, except for the testis, and 5-year survival of pharyngeal cancer patients was lower in cases with MAGE-A expression, it could be considered as a promising marker for the detection of HNSCC [105–108].

With regard to salivary microRNAs, miR-31 was found to be elevated in HNSCC and could serve as a useful predictor for early detection and post-operative follow-up [109]. Furthermore, two miRNAs, miR-125a and miR-200a, were present at significantly lower levels ($p < 0.05$) in the saliva of patients with oral squamous cell carcinoma than in the saliva of control subjects [110].

Blood markers

There have been some studies of cytokines and angiogenesis factors as potential useful serum markers of disease progression, cancer recurrence and survival of patient with HNSCC [111]. For example, the serum levels of VEGF were significantly higher in patients with the advanced T stage (T3 or T4) ($p = 0.001$), lymph node metastases ($p < 0.001$) and advanced stages (stage III or IV; $p < 0.001$) [112,113]. Furthermore, mean serum concentrations of IL-8, hepatocyte growth factor (HGF), and growth regulated oncogene 1 (GRO-1) were increased in patients with HNSCC [114]. Serum concentrations of IL-6 were also significantly higher in patients compared with the levels detected in healthy individuals and subjects with oral premalignant lesions [98,114,115]. The serum IL-6 levels were especially high in patients with the higher pT status ($p < 0.001$), higher pathological stages ($p < 0.001$), positive bone invasion ($p < 0.001$), and higher tumor depths ($p = 0.005$). Patients with higher pre-treatment IL-6 levels (> 1.35 pg/mL, median level) had worse prognoses for 5-year overall survival and disease-specific survival despite the treatment [115].

Changes in the first post-treatment serum cytokine levels were correlated with response, progression, and survival. Post-treatment increases in IL-6 or HGF were observed in patients who had a relapse and inflammatory or infectious complications. Some relationship between the change in the pre-treatment and first post-treatment cytokine measurement with survival was detected for HGF, IL-8, IL-6, and VEGF. The association between longitudinal decreases in IL-6, L-8, VEGF, and HGF throughout the follow-up with survival was detected with a time-dependent Cox model ($p = 0.01, 0.07, 0.08, \text{ and } 0.05$, respectively) [114].

Furthermore, patients with HNSCCs had significantly higher serum MMP-3, -7, and -9 titers than controls ($p < 0.001$). The elevated MMP-3 and MMP-9, but not MMP-7, correlated with distant metastases and poor survival ($p < 0.05$) [116,117]. Kuropkat et al. showed a significant increase of MMP-8 in the serum of HNSCC patients [117], and Yen et al. reported MMP-10 and MMP-1 to be suitable markers for OSCC disclosure, with gingiva and margin as controls [118].

Chang et al. showed serum levels of C-reactive protein (CRP), matrix metalloproteases MMP-9, MMP-2, transforming growth factor-beta 1 (TGF-beta 1), IL-6, and E-selectin as having power of discrimination between leukoplakia, patients with untreated oral cavity squamous cell carcinoma, and age- and gender-matched healthy control groups with significant elevation trends

of those markers from control to OSCC. All examined markers decreased in relapse-free patients following the treatment. However, in patients with a relapse, IL-6, CRP, and serum amyloid A remained at elevated levels [119].

High levels of MMP-2 or MMP-9 were detected in the plasma of patients suffering from different kinds of cancer, including HNSCC [120].

Krejčova et al. analyzed MT levels in the blood of patients suffering from primary malignant tumor in the head and neck area. Tumor blood samples originated from patients with oropharyngeal cancer, laryngeal cancer, hypopharyngeal cancer, oral cavity cancer and rarely occurring nasal cavity and paranasal sinus cancers. Blood MT levels of healthy controls were lower than blood MT levels of oncological patients [55]. Up-regulation of miR-31, miR-10b, miR-24, miR-181, and miR-184 in the plasma of OSCC patients was also found [121].

Conclusions

Poor prognosis of HNSCC is mainly due to late disease presentation and lack of suitable biomarkers to detect the disease progression. Accordingly, biomarkers suitable for the early detection of HNSCC are sorely needed to improve patient outcomes. HNSCC evolve through a multistep process by the accumulation of genetic and phenotypic changes. Searching of biomarkers specific for high-risk tumors in early stages is therefore really essential. The application of molecular biologic techniques is promising in simplifying earlier detection and may generate protocols for screening of cancer patients. Molecular profiling may also help in the prediction of tumor behavior and responsiveness to therapy. The molecular pathways underlying tumorigenesis are ever better understood today, and therefore the number of possible biomarkers increases. More clinical studies, which will validate the sensitivity and specificity of these biomarkers in clinical settings are needed to translate these findings into potential strategies for early detection leading to improved patient outcomes.

While the detection of biomarkers and the targeted therapy for HNSCC have experienced a great progress, there are still significant facets of HNSCC that are not fully understood.

There is a 4% annual risk of HNSCC patients developing a second primary tumor [122]. These second primary tumors are thought to result from the “field cancerization” [11,123]. The next point of interest could be to identify biomarkers of field cancerization and to develop a target that would prevent disease recurrence or appearance of second primary tumors.

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Conflict of interest statement

None declared.

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1.4.2 Biomarkery klíčových procesů kancerogeneze – kapitola v knize

Kapitola v knize (str. 25) „Targeting Oral Cancer“ editovaná dr. Andrew Friblyem tematicky navazuje na předchozí práci¹¹ a rozšiřuje ji zejména v několika ohledech: Systematizace vychází z všeobecně uznávaného konceptu „Hallmarks of Cancer“¹², a obsahuje také potenciální biomarkery, dosud ověřené pouze na *in vitro* úrovni či na zvířecích modelech. V kapitole je věnována rozsáhlá pozornost tématu miRNA jako potenciálních biomarkerů spinocelulárních karcinomů.

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HNSCC Biomarkers Derived from Key Processes of Cancerogenesis

7

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Abstract

Head and neck squamous cell carcinoma (HNSCC) is one of the most frequent aggressive cancers in humans. Well-known risk factors include HPV infection, tobacco smoking, and alcohol consumption. HNSCC overall survival rate is one of the lowest among human malignancies. The poor prognosis of HNSCC often results from late-stage diagnosis, therapeutic resistance, high rates of recurrence, and frequent metastases to lymph nodes. To date, the TNM classification is still the best evaluation of disease progress; however, this method of staging does not pay attention to the molecular basis of tumorigenesis. An improvement in treatment efficacy and diagnostic capabilities will be realized through a better understanding of the pathogenesis and characteristics of HNSCC, a disease that has come to be characterized by confounding heterogeneity. This chapter is focused on molecular markers derived from key processes of cancerogenesis that are involved in metastasis, treatment resistance, avoidance of immune detection, inflammation, induction of angiogenesis, genome instability, dysregulation of cellular energetics, cell death, cancer stem cell biology, and rearrangement of tissues adjacent to the tumor. We will discuss biomarkers identified at different levels of cellular regulation (DNA, RNA, miRNA, and protein markers).

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7.1 Introduction

A biomarker is a readily measurable biological feature of an organism that reflects its specific physiological or pathophysiological state. Biomarkers can be utilized for detection of disease progression, assessment of remission, evaluation of therapy efficacy, and the detection of adverse drug responses. Clinical and pathological criteria are important for the management and prediction of patient outcome. For this discussion, it is important to consider that distinct variations in prognoses exist within patient cohorts, even in those with very similar clinicopathological characteristics, because tumorigenesis and tumor progression in head and neck cancers are driven by myriad signaling pathways. Disclosure of these pathways, and finding biomarkers involved in them, is thus highly important in the development of new useful diagnostic and therapeutic targets. Twelve key processes have been identified in head and neck squamous cell carcinoma (HNSCC) development [1, 2]:

1. Cell cycle disruptions (acquisition of autonomous proliferative signaling and avoiding of growth-suppressing mechanisms)
2. Avoiding of immune destruction
3. Replicative immortality
4. Induction of angiogenesis
5. Tumor promoting inflammation and oxidative stress
6. Invasion and metastasis creating
7. Genome instability
8. Cell death modifications
9. Dysregulation of cellular energetics
10. Pluripotency and cancer stem cells forming
11. Treatment resistance
12. Rearrangement of tumor-adjacent tissues and formation of tumor-favorable micro-environment

In this text, the main attention will be focused on biomarkers that play crucial roles in these 12 key processes.

7.2 Markers of Chronic Proliferation

Chronic dysregulated proliferation is probably the most essential characteristic of cancer cells and is widely regarded as a “hallmark” of malignancy. Normal tissues cautiously manage the production and release of growth-promoting factors, whereas cancer cells resist the cell cycle control signals and grow constantly.

7.2.1 Autonomous Proliferative Signaling

The acquisition of autonomous proliferative signaling occurs through the activation of growth-promoting proto-oncogenes into oncogenes [1]. When oncogenes are activated, cancer cells acquire the ability to proliferate without the need for exogenous growth factors. Epidermal growth factor receptor (EGFR/erbB1/HER1) is a proto-oncogene of great impact in HNSCC. The EGF receptor family signals through ligand binding (i.e., EGF, TGF- α , or amphiregulin) that induces receptor dimerization and autophosphorylation of the intracellular tyrosine kinase domain. EGFR ligands are often overexpressed in HNSCC [3]. EGFR phosphorylation mediates the activation of intracellular signaling pathways such as Ras/Raf/MAPK (mitogen-activated protein kinase) and PI3K/Akt (phosphatidylinositol 3-kinase/protein kinase B), which regulate cell proliferation and cell survival, respectively [4]. Forty-seven percent of HNSCC cases show at least one molecular alteration in the PI3K/Akt pathway, including *PI3KCA* and *AKT2* amplification, p110 α overexpression, and phosphatase and tensin homolog (PTEN) protein downregulation [5]. Loss of PTEN expression can be seen in approximately 30 % of HNSCCs and is considered to be an independent prognostic indicator of worse clinical outcomes [6]. *Ras* mutations occur only in 5–10 % of HNSCC and in about 35 % of tobacco-related oral cancers [7–9], but majority of HNSCCs display abnormally high *Ras* expression [10].

Overexpression and autocrine activation of EGFR were observed in approximately 90 % of HNSCC examined [3, 11] and were associated with a poor prognosis [12, 13]. Moreover, expression of EGFR increases with advancing dysplasia in premalignant head and neck lesions, indicating that EGFR alteration is an early oncogenic event [11, 14, 15]. Increased production of *TGF- α* and *EGFR* mRNA in the histologically normal tumor-adjacent mucosa of patients with HNSCC is a likely marker of tumor-induced pre-disposition to deregulated growth [11]. In tumor specimens from patients with HNSCC, *TGF- α* mRNA expression was increased 5-fold in 95 % of histologically normal tumor-adjacent tissues and 87.5 % of malignant regions. *EGFR* mRNA was elevated 29-fold in 91 % of histologically normal tumor-adjacent tissues and about 69-fold in 92 % of HNSCC tumors, in comparison with normal mucosa [11]. Activating point mutations in EGFR occur rarely in HNSCC [16], but the incidence differs between ethnic groups (0–4 % in whites and about 7 % in Asians) [17]. Despite the fact that kinase domain mutations of EGFR in HNSCC are rare, it could help to explain the lack of effect of EGFR inhibitors [16].

Targeted molecular therapy against EGFR has promising results as an adjuvant therapy in several solid tumors, including HNSCC. Selective compounds targeting the ligand-binding extracellular region of the EGFR (immunotoxins, monoclonal antibodies, and cytotoxic ligand-binding agents) and the intracellular tyrosine kinase region (various small molecule inhibitors) have been tested [18]. Nevertheless, only a small fraction of patients respond to EGFR inhibitors [18]. Even with successful EGFR inhibition, alternative signaling pathways can be activated through other cell-surface receptors including insulin-like growth factor receptor (IGF-1R) coupled with G protein, MET, or IL-6R/gp130 [19–22] (Fig. 7.1). Activation of IGF-1R can also be caused through the loss of its negative regulator (IGFBP3) [23, 24].

SET protein (I2PP2A or TAF1 β) is an inhibitor of PP2A and is responsible for the activation of Akt, c-Myc, and β -catenin pathways [25]. A recent

proteomic analysis of HNSCC samples identified that the SET protein is accumulated in oral tumors [26, 27]. SET has multiple proposed functions such as promoting Rac1-induced cell migration [28], chromatin remodeling [29], and sensing of oxidative stress that supports cell survival in HNSCC [27]. On the other hand, stable SET knockdown in HN12 cells stimulated epithelial-mesenchymal transition (EMT) and invasion [30] and caused an increase in the autophagic proteins Beclin-1, LC3B, and p62 [31]. Xi et al. found that the PP2A inhibitor cantharidin activated the unfolded protein response (UPR) and induced apoptosis in a panel of HNSCC cells and other cancers. Although cantharidin may exert its cytotoxic effect through additional mechanisms and the role of the UPR in cancerogenesis is not fully elucidated, it is possible that PP2A inhibition could have contradictory roles at different stages of HNSCC cancerogenesis [32].

STAT3 is another important proliferative and survival signal that is constitutively activated in HNSCC [33–35]. Constitutive activation of STAT3 plays an important role in many processes during cancerogenesis by modulating increased expression of cyclin D1, VEGF, bFGF, HIF-1 α , MMP-2, MMP-9, IP-10, and RANTES [33, 36, 37]. HNSCC patients express higher levels of STAT3 in both tumor and histologically normal adjacent tissues compared with epithelium of healthy control subjects. STAT3 protein levels were 9-fold higher in tumor-adjacent mucosa from cancer patients and 11-fold higher in HNSCC tumors compared with healthy controls [35]. STAT3 activation may be mediated by either EGFR or interleukin-6 (IL-6).

IL-6 was increased in the saliva of oral cancer patients (OSCC) [38] and was overexpressed in tumor tissues and sera of patients with HNSCC when compared with the healthy individuals [39]. Multivariate analysis revealed that postsurgery IL-6 concentrations in saliva were a risk factor for locoregional recurrence in patients with OSCC [40, 41]. Salivary IL-6 and TNF- α concentrations were notably higher in individuals with oral leukoplakia in comparison with the healthy subjects [42], and markedly higher levels

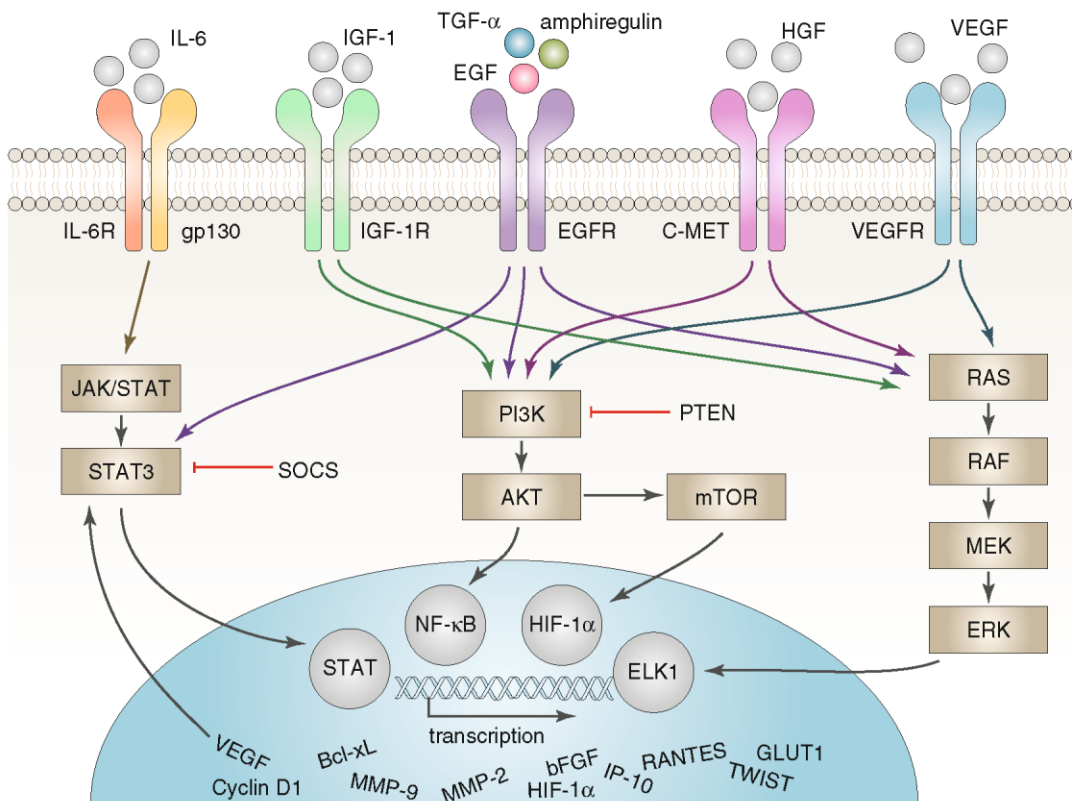


Fig. 7.1 EGFR signaling pathway and alternative signals. Even with successful EGFR inhibition, substitute signaling pathways can be activated by signaling through other cell-surface receptors including IGF-1R, IL-6R/gp130, c-MET, or VEGFR. Activation of STAT3 by IL-6R/gp130 involves phosphorylation of Janus-activated kinases (JAK), whereas EGFR activates STAT3 directly or

through the Ras/Raf/Mek/Erk pathway [46]. STAT3 may be negatively regulated by suppressors of cytokine signaling (SOCS). Constitutive activation of STAT3 plays a causative role in many processes in cancerogenesis through the overexpression of cyclin D1, VEGF, bFGF, HIF-1 α , MMP-2, MMP-9, IP-10, GLUT1, Twist, or RANTES

of IL-8, IL-6, IL-1, and TNF- α were found in OSCC patients' saliva when compared with subjects with dysplastic oral lesions and healthy controls [43]. Higher IL-6 serum levels were also observed in patients with OSCC compared to healthy controls, results that were confirmed at both the mRNA and the protein levels [44]. Serum IL-6 levels were also markedly higher in patients with higher TNM stage, higher pathological stages, positive bone invasion, and increased tumor depths. Patients with high pre-treatment levels of IL-6 (>1.35 pg/mL median level) had worse prognoses for 5-year overall and disease-specific survival [45].

Activation of STAT3 by IL-6R/gp130 involves phosphorylation of Janus-activated kinases (JAK) [19, 20], whereas EGFR activates STAT3 directly or through Ras/Raf/Mek/Erk signaling [46].

Conversely, STATs may be negatively regulated by suppressors of cytokine signaling (SOCS) [47]. Epigenetic silencing of SOCS1 and SOCS3 has been recently reported in HNSCC [48, 49]. SOCS3 expression is reduced early in tumor development, and the overexpression of SOCS3 in HNSCC cell lines diminished proliferation, migration, and invasion. The loss of SOCS3 function may be associated with reduced expression due to epigenetic silencing but also with abnormal subcellular localization impairing its function [50]. For these reasons, combined inhibition of the EGFR pathway and important intermediates in redundant crosstalk pathways might be necessary before an improvement of chemotherapeutic effect can be realized [21].

An excessive activation of proliferative pathways should hypothetically result in increased

proliferation of cancer cells; however, there appears to be a compromise between maximal mitogenic stimulation and avoidance of antiproliferative defenses such as senescence [1, 51, 52]. In accordance, there are several morphological and metabolic compartments in tumor tissue. The first is the tumor stroma with well-differentiated cancer cells that are nonproliferative and display lower numbers of mitochondria. Secondly, there are less differentiated cancer cells that are highly proliferative (higher Ki-67 expression) and mitochondria-rich [53]. The nonproliferative subgroup of tumor cells is probably positively selected by radiotherapy. In accordance, patients with HNSCC with low proliferation rate (Ki-67 $\leq 20\%$) have a worse response to radiotherapy than patients with high proliferative tumors [54]. High expression of Ki-67 and EGFR suggests that early lymph node metastasis and primary HNSCC tumors have rather high proliferative indexes and therefore, they should be sensitive to radiotherapy [55].

7.2.2 Disruption of Growth-Suppressing Mechanisms

Many tumor suppressors, operating through a variety of mechanisms, have been discovered due to their inactivation in various cancers. Important HNSCC tumor suppressors have also been identified through the effect of human papillomavirus (HPV) infection. Oncogenic HPV subtypes are able to transform epithelial tissue because of the action of the viral oncoproteins E6 and E7 [56]. In particular, the expression of the E6 and E7 leads to the inactivation of two important tumor suppressors, p53 and retinoblastoma protein (Rb). Viral E6 protein stimulates p53 degradation [56] and can also activate telomerase [57]. The E7 protein is able to bind pRb with a preference for the underphosphorylated “active” form of pRb. The Rb proteins play a key role in controlling the checkpoint between the G1 and the S phase of the cell cycle. E7 oncoprotein is also able to inactivate the cyclin-dependent kinase (CDK) inhibitor p21 [15]. HPV status also affects miRNA expression patterns in HNSCC. Certain miRNAs (miR-15a, miR-16, miR-195, miR-497 family, miR-143, miR-145, and miR-106–363 cluster) appear to be crucial for the HPV carcinogenesis [58].

Despite of reduced levels of p53 in HPV-associated tumors (E6-mediated degradation), p53 protein is not mutated and is functional. Radiation-induced p53 production may be sufficient to provoke apoptosis. In contrast, a high percentage of HPV-negative tumors have mutated p53 and create resistance to chemotherapy- and/or radiation-induced apoptosis [59]. Furthermore, promoters of tumor suppressors 14-3-3 σ and RASSF1A are hypermethylated, and there are cyclin D gene amplifications in HPV-negative HNSCC more frequently than in HPV-positive tumors [60–62]. Inactivation of 14-3-3 σ by promoter hypermethylation promotes metastasis in nasopharyngeal carcinoma [63]; based on the p16 and cyclin D1 expression, patients with downregulated p16 and overexpressed cyclin D1 had worse disease-free and overall survival [64].

Progression through the cell cycle is regulated by the coordinated activities of cyclins and cyclin-dependent kinases (CDK), which are regulated through the binding of CDK inhibitors. The CDK2AP1 (DOC-1) protein is a unique inhibitor of CDK2 and has an important growth-suppressing effect through association with a DNA polymerase α /primase complex [65, 66]. Additionally, CDK2AP1 can induce apoptosis [67, 68]; however, its expression is often reduced in HNSCC [69, 70] and intratumor reduction of CDK2AP1 expression is a negative prognostic marker in patients with surgically resected HNSCC [70]. On the other hand, induction of CDK2AP1 expression in the tumor stroma significantly reduced tumor cell growth and invasiveness [65].

7.2.2.1 TGF- β Signaling Pathway

Comprehensive evidence exists for the deregulation of the TGF- β signaling pathway in tumor formation and advanced disease progression [10, 71]. In normal epithelium, TGF- β suppresses tumor growth through antiproliferative and apoptosis-triggering effects. Nevertheless, as the tumor progresses, autocrine loops of TGF- β are established and tumor cells become resistant to antiproliferative stimuli [72]. Increased TGF- β 1 expression is seen in approximately 80 % of HNSCCs and is associated with disease progression and worse survival [8, 73]. TGF- β signaling can activate different proliferative non-Smad pathways including Jnk/p38/MAPK, Ras, Par6,

and PI3K/Akt [72, 74], which is in accordance with the fact that increased TGF- β 1 expression and activated phosphatidylinositol 3-kinase/Akt signaling are frequently observed as a consequence of disrupted canonical TGF- β signals [75]. Loss of chromosome 18q, a region encoding both Smad2 and Smad4, is common in HNSCC and loss of heterozygosity (LOH) at the Smad4 locus occurs in ~50 % of HNSCC [76, 77]. LOH affecting 18q has been linked to poor survival [78]. Smad4 was downregulated not only in HNSCC malignant lesions but also in normal adjacent mucosa. Furthermore, Smad4 (-/-)-deficient mice developed spontaneous HNSCC [79]. TGF- β promotes the assembly of a cell-surface receptor complex composed of type I (TGF β RI) and type II (TGF β RII) receptor serine/threonine kinases. Low expression of TGF β RII has been detected in a variety of tumors, including HNSCC, and rises as a consequence of genetic alterations or epigenetic silencing [80–82]. In sum, TGF β RII expression may be reduced in >70 % of HNSCCs [10] and correlates with diminished tumor differentiation and more aggressive behavior [83].

miR-211 promotes the progression of head and neck carcinomas directly by targeting TGF β RII [84]. Although genetic alteration of TGF β RI by mutation or deletion is not common in HNSCC, repression of TGF β RI expression *via* hypermethylation of its promoter has been reported [8, 85], and reduced TGF β RI expression was associated with increased invasion and metastasis in esophageal carcinoma [83]. TGF β RI mutations have been detected in 19 % of HNSCC metastases [86]; however, TGF β RI deletion does not initiate tumor formation in the oral epithelium, but instead allows progression to HNSCC after excessive Ras activation [10, 75]. Taken together, resistance to TGF- β -mediated growth inhibition may arise by different ways [87] *inter alia*: (1) due to activation of non-Smad pathways triggered by TGF- β , (2) due to mutations in the *Smad4* gene [88], (3) due to Smad4 decreased expression, (4) due to increased expression of inhibitory Smad7 [89], (5) due to decreased expression of TGF β RII or TGF β RI, or (6) due to mutations in the *TGF β RII* or *TGF β RI* genes.

7.2.2.2 Circadian Clock

Proliferation and the cell cycle regulation are also controlled by the circadian clock [90]. To date, at least nine mammalian core circadian clock genes (CCGs) have been identified: Period1 (*PER1*), Period2 (*PER2*), Period3 (*PER3*), *Clock*, Cryptochrome1 (*CRY1*), Cryptochrome2 (*CRY2*), *BMAL1*, Caseinkinase1E (*CK1E*), and Timeless (*TIM*). CCGs, and the proteins they encode, constitute the circadian oscillator and are responsible for circadian rhythms. Deregulation of the circadian clock may lead to excessive cell proliferation [91]. c-myc activation might be a possible mechanism, as its transcription is directly controlled by circadian regulators [92]. Many studies have reported altered expression of clock genes in tumors [93–96]. Mice deficient in circadian clock genes showed many phenotypic abnormalities, including increased cancer incidence following genotoxic stress [90, 92]. In *PER2* mutant mice, expression of proteins involved in tumor suppression and cell cycle regulation (Mdm-2, cyclin D1, cyclin A, Gadd45 α , and c-myc) is altered. All nine mentioned CCGs were markedly downregulated in the peripheral blood leukocytes of preoperative HNSCC patients. Restoration of *CLOCK* and *PER1* gene and protein expression were found in postsurgical patients with good prognosis unlike in patients that died within one year after surgery [97]. The expression of *CRY1*, *CRY2*, *BMAL1*, *PER1*, *PER2*, *PER3*, and *CK1E* genes exhibited significant downregulation in the tumor tissues. Downregulated expression of *CRY2*, *PER3*, and *BMAL1* was correlated with more advanced cancer stages. Downregulated *PER3* and upregulated *TIM* expression positively correlated with larger tumor size and lower expression of *PER3* correlated with deeper tumor invasion. Poor survival was related to lower expression of *PER1* and *PER3*. These results indicate a possible association of circadian clock genes with the pathogenesis and prognosis of HNSCC [98].

7.2.3 Avoiding of Contact Inhibition

In healthy tissues, cells grow inside defined areas and quit growing when they come into

contact with the dense extracellular matrix or other cells, a concept referred to as contact inhibition. Neurofibromatosis-2 protein (NF2 or merlin) plays a crucial role in the upkeep of contact inhibition. The growth inhibitory function of NF2 is dependent upon interaction with the cytoplasmic domain of CD44 [99], and numerous studies have indicated that NF2 suppresses tumor growth and metastasis [100, 101]. 22q12.2 is the known locus for the *NF2* gene and 22q is a frequent site of loss of heterozygosity (LOH) in HNSCC. Notably, allelic losses on 22q tended to occur especially in patients with stage 3 and 4 disease. Of particular interest is that laryngeal tumors had higher rates of 22q LOH than oral cavity or pharyngeal tumors [102]. The phosphorylated form of NF2 is growth promoting and predominantly appeared in the presence of hyaluronic acid-degrading enzymes (HAases); cells cultured without HAases contained mostly the growth inhibitory form of NF2 [103]. HAases have been found to transform the expression of CD44 isoforms, which could also play a significant role in tumor progression [104]. On the other hand, activity of the HAase Hyal-2 was dependent on the expression of CD44 in both living cells and enzymatic assays [105]. In accordance, salivary soluble CD44 concentration was markedly higher in HNSCC patients compared with healthy controls (1.09 ng/mL for controls and 7.85 ng/mL for HNSCC patients) [106, 107]. Similarly, HAase levels were elevated in HNSCC patients when compared to normal controls [108]. The presence of extremely high molecular weight hyaluronic acid (HMW-HA) and its binding to the CD44 receptor (together with poor HAase) activity played a significant role in the naked mole rat cancer resistance. Furthermore, enzymatic digestion of HMW-HA abrogated contact inhibition of cells [103] and cellular reactions turned on by HA are dependent on the HA polymer length. HMW-HA inhibits proliferation and has anti-inflammatory effects [109] while low molecular weight HAs induce proliferation and inflammation [110] (see Fig. 7.2 and Table 7.1).

7.3 Markers of Tumor Promotion Inflammation and Oxidative Stress

Inflammatory reactions accompanying early phases of neoplastic transformation create a favorable environment for cancer expansion. The accumulation of growth factors, cytokines, prostaglandins, free radicals, and matrix proteinases triggers pro-tumorigenic mechanisms and influences protein modification, DNA damage, and expression profile changes in genes and miRNAs. Together with the immunosuppressive impact of the adaptive immune response, and interactions between stroma and epithelium, these changes dramatically support tumor expansion [132]. Many tumors, including HNSCC, have been associated with constitutive inflammation and oxidative stress [132–134]. Several studies have reported that the synthesis of metallothionein (MT) was induced during oxidative stress [135–137], and two additional studies discovered increased MT protein levels in HNSCC tumors [138, 139]. The highest MT level was found in the tissues of oral tumors (170 ± 70 $\mu\text{g/g}$), whereas a relatively modest MT level was found in tumors of oropharynx (130 ± 50 $\mu\text{g/g}$) [139]. The blood level of MT in healthy controls was much lower than in HNSCC patients [140]. Furthermore, increased immunostaining of MT was also observed in the tissue samples from tonsillar squamous cell carcinoma in comparison with a chronic tonsillitis control group [141]. A positive association between MT protein staining and tumors (vs. healthy tissues) was demonstrated in HNSCC patients (odds ratio, OR, 9.95; 95 % CI 5.82–17.03) [142].

The transcription factor NRF2 manages a cellular response to electrophiles and oxidants by triggering expression of genes important for antioxidant defense. NRF2 is inhibited by Kelch-like ECH-associated protein 1 (KEAP1). Dysregulation of NRF2/KEAP1 pathway seems to be important prior to the formation of HNSCC [143, 144]. NRF2 expression was increased in 91.5 % of HNSCC tumors, and the expression

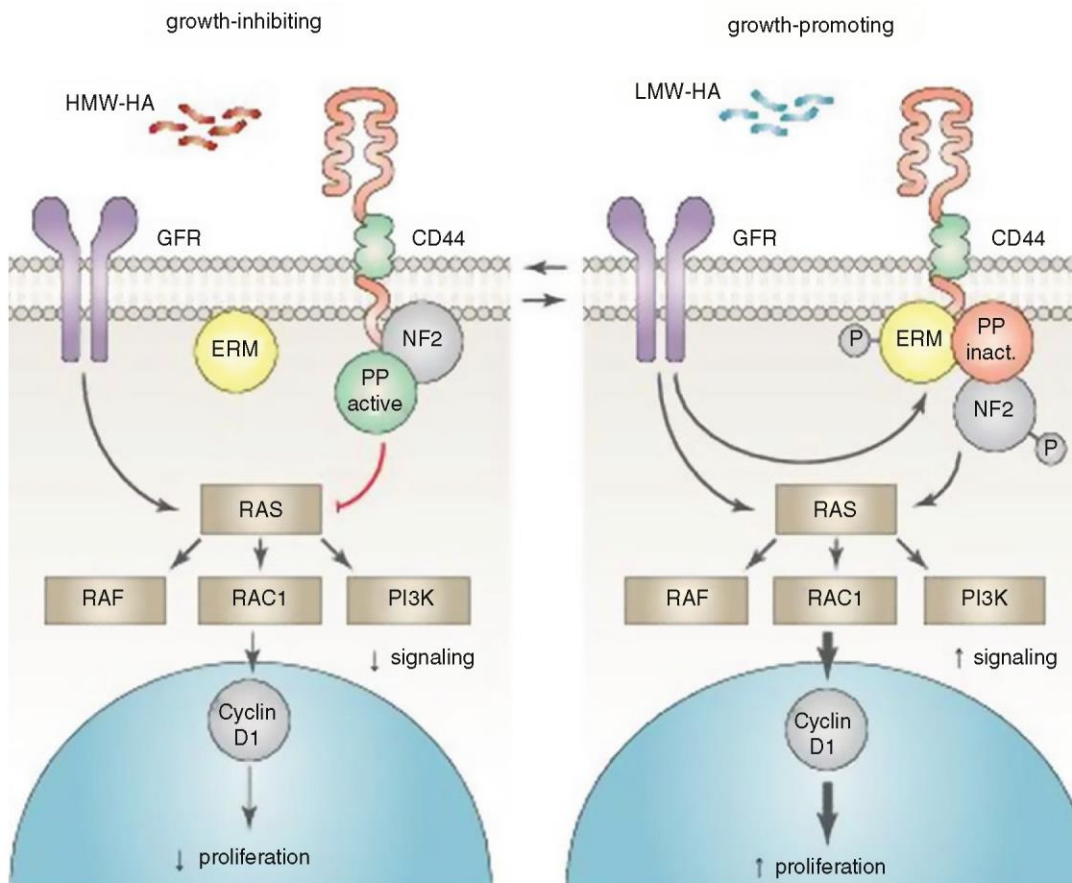


Fig. 7.2 Role of neurofibromin-2 protein and hyaluronic acid in contact inhibition. Growth factor receptor (*GFR*); protein phosphatase (*PP*) and ezrin-radixin-moesin (*ERM*) family of band 4.1 proteins. Additional components are likely associated with the CD44-bound complexes. Neurofibromin-2 protein (*NF2*) plays a crucial role in the upkeep of contact inhibition. The growth inhibitory function of *NF2* is dependent upon interaction with the

cytoplasmic domain of CD44. Furthermore, the phosphorylated (growth-promoting) form of *NF2* predominantly appears in the presence of hyaluronic acid-degrading enzymes. The presence of extremely high molecular weight hyaluronic acid (*HMW-HA*) and its binding to the CD44 receptor plays a significant role in the growth inhibition, while low molecular weight HAs induce proliferation and inflammation (Adapted from Pure et al. [110])

Table 7.1 Markers of chronic proliferation in HNSCC

Biomarker	Mechanism	Detected in	References
c-Met	↑ protein expression	Tumor tissue	[111]
CEP55	↑ mRNA and protein expression	Tumor tissue	[112]
CCGs	↓ mRNA and protein expression	Peripheral blood leukocytes, tumor tissue	[91, 97, 98]
CDK2AP1	↓ mRNA and protein expression	Tumor tissue	[69, 70]
CD44	↑ protein expression	Saliva	[106, 107]
Cyclin D ₁	↑ protein expression, gene amplification	Tumor tissue, HNSCC cell lines	[113–117]
DST	↓ mRNA expression	Tumor tissue	[118]
MAGE	↑ mRNA expression	Saliva	[119]

Table 7.1 (continued)

Biomarker	Mechanism	Detected in	References
MYCN	↑ mRNA expression	Tumor tissue	[118]
TGF- α	↑ mRNA and protein expression	Tumor tissue, HNSCC cell lines	[3, 11, 12]
TGF- β I	↑ protein expression	Plasma	[83]
RASSF1A	↓ mRNA and protein expression, LOH	Tumor tissue, HNSCC cell lines	[60–62]
14-3-3 σ	↓ mRNA and protein expression	Tumor tissue, HNSCC cell lines	[63]
EGFR	↑ mRNA and protein expression	Tumor tissue, HNSCC cell lines	[3, 11, 120]
HAase	↑ mRNA and protein expression	Saliva, HNSCC cell lines	[108]
Ras	↑ mRNA and protein expression, gain-of-function mutations	Tumor tissue	[7, 8, 10]
PI3KCA	↑ protein expression, gene amplification	Tumor tissue	[5, 121, 122]
IL-8	↑ protein expression	Saliva, serum	[39, 43]
p53	Inactivation, mutation, LOH	Tumor tissue, surgical margins	[56, 115, 123, 124]
NF2	↓ mRNA and protein expression, 22qLOH	NF2-deficient tumor-bearing mice, blood	[101, 102]
IL-6	↑ mRNA and protein expression	Saliva, serum, HNSCC cell lines, tumor tissue	[38–41, 44]
TNF- α	↑ protein expression	Saliva	[43]
PTEN	↓ protein expression, LOH	Tumor tissue	[5, 6, 125]
CDK4	↑ protein expression	Tumor tissue	[115]
pRb	↓ protein expression, inactivation, LOH	Tumor tissue	[56, 115, 126–128]
SET	↑ protein expression	Tumor tissue, HNSCC cell lines	[26, 27]
SMAD4	↓ protein expression, LOH, inactivation	HNSCC cell lines, tumor tissue, SMAD4-deficient tumor-bearing mice	[78, 79]
STAT3	↑ protein expression, activation	Tumor tissue	[35]
SOCS1 and SOCS3	↓ protein expression, mRNA expression, epigenetic silencing, abnormal subcellular localization	Tumor tissue, HNSCC cell lines	[48–50]
TGF β RI	↓ protein expression, epigenetic silencing	Tumor tissue	[8, 83, 85]
TGF β RII	↓ protein expression, epigenetic silencing, mutation	Tumor tissue, HNSCC cell lines	[10, 80–83]
VEGF	↑ protein expression	Tumor tissue, HNSCC cell lines, serum	[39]
AKT2	↑ protein expression, gene amplification	Tumor tissue	[5]
EBV	A causative agent for most nasopharyngeal carcinomas; plasma EBV DNA load is an independent prognostic factor	Tumor tissue, blood	[129, 130]
HPV	A causative agent for most oropharyngeal cancers	Tumor tissue, blood	[56, 60, 131]

Abbreviations: LOH loss of heterozygosity, EBV Epstein-Barr virus, HPV human papillomavirus, CCGs circadian clock genes

of the NRF2-regulated gene thioredoxin was increased in 75 % of tumors [145].

Nuclear factor-kappa B (NF- κ B) is a crucial factor in inflammatory and immune pathways that also acts as a tumor promoter [146]. In tumor cells, NF- κ B can be activated as a result of genetic alterations and can induce the expression of angiogenic factors, inflammatory cytokines, adhesion receptors, or inducible nitric oxide synthase (iNOS) and enzymes involved in the arachidonic acid metabolism such as cyclooxygenase-2 (COX-2). NF- κ B is also a major survival factor due to its ability to induce expression of Bcl-2 [134]. Long-standing inflammatory signals have been shown to induce an epigenetic switch from healthy cells to cancer through a positive feedback loop containing NF- κ B, Lin28, let-7, and IL-6 [147, 148]. STAT3, a transcription factor induced by IL-6 (discussed above, section 2.1), is able to activate miR-181b-1 and miR-21 that can trigger the epigenetic switch. MiR-181b-1 and miR-21 deactivate the tumor suppressors PTEN and CYLD, respectively, leading to elevated NF- κ B activity [147]. In HNSCC, a key role of NF- κ B in promoting alterations of the proteome and transcriptome has been recently established [149]; and studies have demonstrated NF- κ B activation in squamous dysplasia and in tobacco- and viral-associated HNSCC [150]. Increased nuclear levels of NF- κ B immunostaining highly correlated with progress of dysplasia and reduced survival in HNSCC patients [151].

Aberrant arachidonic acid (AA) metabolism, especially altered 5-lipoxygenase (5-LOX) and cyclooxygenase (COX), is often involved in HNSCC development [152]. COX-2 and 5-LOX enhance tumor cell proliferation and are proangiogenic due to the induction of FGF, VEGF, and MMPs [153–156]. Cyclooxygenases catalyze the production of prostaglandins (PGs). Elevation of COX-2 has been described at both the mRNA and protein level in HNSCC [157] and was associated with an adverse outcome [158]. Prostaglandin E2 (PgE2) is able to promote proliferation and may also inhibit apoptosis by increasing Bcl-2 expression [159]. Furthermore, PgE2 was shown to elevate the expression of angiogenic factors prior to

tumor cell invasion and metastasis [154]. Overexpression of inducible microsomal prostaglandin E synthase (mPGES) elevated the amount of PgE2 in HNSCC cells [160]. Patients with increased COX-2 and PgE2 tumor expression had significantly inferior overall survival [161]. Immunohistochemical experiments showed that an elevation in COX-2 expression was positively correlated with the amount of Foxp3+ regulatory T cells in the microenvironment of HNSCC, indicating that COX-2 enables the expansion of the regulatory T cells [162]. Increased expression of COX-2 resulted in elevated levels of PgE2 and VEGF and enhanced angiogenesis and tumor growth [153]. COX-2 inhibitors reduced invasion and viability of HNSCC cells by downregulating VEGF, MMP-2, and MMP-9 secretion [159, 163, 164]. Taken together, inflammatory mediators such as ROS, metabolites of arachidonic acid, and cytokines contribute to tumor progression by inducing oncogenic mutations, resistance to apoptosis, adaptive responses, and changes in the microenvironment, including induction of angiogenesis.

7.4 Avoiding Immune Destruction

The immune system acts as an important barrier against tumorigenesis and tumor progression.

The unaffected immune system can eliminate cancer cells, but the effectiveness of immune antitumor defense is often suppressed by the tumor microenvironment. There is significant evidence that reactivation of an antitumor immune response can cause tumor regression and have a favorable clinical impact [165]. Tumor cells generate high levels of proinflammatory agents (such as ROS); nevertheless, the tumor microenvironment sides with immune suppressors rather than with immune effectors [165]. For example, soluble phosphatidylserine (sPS) released from tumor cells is able to interact with the PS receptor on macrophages to trigger an anti-inflammatory response and immune escape [166]. The tumor immune infiltrate is also shifted toward an anti-inflammatory status and

mode of immunosuppression, due to the expression of surface molecules such as PD-L1 and reduced expression of molecules that present antigens [167].

7.4.1 Immune Cells

HNSCC patients typically display significant immunosuppression. They have elevated Treg (CD4+ CD25+ FoxP3+ regulatory T cells) and increased progenitor CD34+ cells that are able to suppress CD4+ helper T cells and CD8+ cytotoxic T cells at the primary tumor area. Treg presence was associated with a poor prognosis [168–170]. Treg can inhibit T-cell reactivity and help to expand the level of inhibitory cells by converting CD4+/CD25- cells into inhibitory CD4+/CD25+/FoxP3+ cells [171]. It also appears that an enrichment in circulating Treg is a long-lasting immune modification that is not overturned by cancer therapy [172]. Cancer patients often display an increased neutrophil/lymphocyte ratio, a low neutrophil and CD14 (high) CD16+ monocyte activation state, and an elevated CD4/CD8 ratio, which has been related to poor survival. In contrast, a high percentage of CD98+ Th cells appeared to be associated with a better outcome [173].

Natural killer (NK) cells are crucial for the management of immune response and removal of faulty cells with downregulation of MHC I molecules. In HNSCC, NK cells appear to be strongly defective. The habitual downregulation of MHC I molecules provides the tumor cells invisibility to T-cell-mediated immunity; NK cells seemingly evolved as an evolutionary response to the downregulation of MHC I molecules [174]. Nevertheless, recent studies have demonstrated that tumor cells are able to internalize NK cells. Survey from long-term real-time imaging experiments indicates that almost all of the NK cells (>95 %) die within 24 h after internalization [175]. Furthermore, internalization of T cells by melanoma cells provides a survival advantage to the tumor cells under conditions of starvation [176]. Internalization of NK cells into tumor cells requires the actin cytoskeletal regulator (ezrin) and leads to programmed cell-

in-cell death of NK cells [175]. Patients with tumors expressing high ezrin levels had shorter disease-free survival than those with low expression [177]. NK cells can be stimulated by double-stranded RNA through Toll-like receptor 3 (TLR3). TLR3 was expressed on the cell surface of naive NK cells but was quickly internalized due to the action of the HNSCC microenvironment. This TLR3 internalization presents other possible immune evasion mechanism in HNSCC [178].

7.4.2 Surface Molecules

Host immune recognition is avoided in HNSCC by several mechanisms, including reduced expression of MHC classes I and II, and upregulation of programmed cell death ligand-1 (PD-L1) and FasL [179–182]. The expression of MHC class I molecules on the cell surface is necessary for the presentation of peptide antigens to cytotoxic CD8+ T lymphocytes. Approximately 50 % of HNSCC cases demonstrated a loss of class I HLA molecules that could be correlated with regional lymph node metastasis [181]. Downregulation of class I major histocompatibility complex (MHC) molecules shields tumor cells from immune recognition. In addition, upregulation of FasL leads to apoptosis of T cells. Recent studies have shown that plasma of patients with oral cancer contains FasL+ membranous vesicles having the capacity to induce T-cell death [183, 184]. The transfer of activated T cells after blocking PD-L1 with a neutralizing antibody (10B5) delayed the development of HNSCC and resulted in enhanced survival, compared with the transfer of T cells alone [167].

7.4.3 Immune Regulators

One of the mechanisms by which HNSCC tumors can weaken host immune reactions is through the alteration of the cytokine environment at the tumor area. Recent studies have shown that alteration of cytokine immune responses can promote growth and metastasis [185]. The production of cytokines such as IL-6 and IL-10 by tumor cells

supports a Th2 response that is accompanied by a substantially weakened antitumor defense [184, 186]. As a consequence of the shift from the Th1 to the Th2 type of T-cell cytokine response, HNSCC tumors produce elevated levels of immunosuppressive agents such as TGF- β that can directly inhibit cytotoxic T cells and NK cell-mediated immunity [187] and contribute to the recruitment of immunosuppressive M2 macrophages and myeloid-derived suppressor cells (MDSCs) [185, 188, 189]. In summary, TGF- β mutes antitumor immunity while promoting tumor-supporting inflammation.

Granulocyte-macrophage colony-stimulating factor (GM-CSF) and PgE2 usually have proinflammatory effects that promote the maturation of neutrophils and macrophages in the initial phases of inflammation. They are also secreted by the HNSCC tumor cells to favor angiogenesis, growth, and immune response avoidance [39]. Tumor-produced GM-CSF has been found to stimulate MDSC maturation and recruitment, and increased levels of GM-CSF are related to an adverse prognosis in HNSCC patients [169, 190]. Elevated levels of PgE2 are associated with angiogenesis and invasiveness in aggressive primary tumors [191] and PgE2 expression is associated with decreased CD8+ T-cell numbers and increased immune suppressor cells at the tumor area. PgE2 can induce the secretion of IL-10 and repress the secretion of cytokines by CD4+ T cells directly, and PgE2 seems to be crucial for tumor-associated immunosuppression [184–186]. Monocyte chemotactic protein 1 (MCP-1), produced by tumor cells, attracts IL-10- and TGF- β -secreting M2 macrophages leading to increased immunosuppression [192]. The maturation of dendritic cells in patients with HNSCC seems to be impaired by tumor-produced VEGF [181, 193, 194]. Furthermore, within HNSCC tissue, plasmacytoid dendritic cells have been shown to be defective in their capacity to produce interferon alpha (IFN- α), a cytokine that is important for antitumor reactivity [195]. A significant decrease in serum IL-13, IFN- γ , and MIP-1 β levels, and a significant increase of serum inducible protein-10 (IP-10), in HNSCC patients was demonstrated, regardless of primary tumor

site [196]. Neutrophil/lymphocyte ratio and serum concentrations of IL-8, CCL4 (MIP-1 β), and CCL5 (RANTES) were significantly higher in the peripheral blood of HNSCC patients than controls [197].

Main HNSCC protective mechanisms against immune reactivity are shown in Table 7.2.

7.5 Markers of Tumorous Angiogenesis

Angiogenesis is an essential process for tumor progression, outgrowth, and dispersion. Vascular endothelial growth factor A (VEGF-A) is a well-characterized agent that can induce angiogenesis. VEGF-A expression can be upregulated by hypoxia, through the action of hypoxia-inducible factor 1 alpha (HIF-1 α), and by oncogene signaling through EGFR, Raf, MEK, and PI3K signals [198, 199]. von Hippel-Lindau (*VHL*) is a tumor suppressor gene located on chromosome 3p that is involved in a multiprotein complex that regulates oxygen-dependent degradation of HIF-1 α [199]. Loss of heterozygosity (LOH) within *VHL* was found at a high frequency (45.5 %) in tongue squamous cell carcinoma patients [200]. Loss of *VHL* function decreases HIF-1 α proteolysis under aerobic conditions and thereby elevates expression of many HIF-1 α downstream genes [201]. Furthermore, osteopontin (OPN) expression correlates negatively with *VHL* expression, suggesting OPN connection with tumor hypoxia. Accordingly, OPN expression was associated with HNSCC tumor progression [202, 203]. Overexpression of VEGF (in tumor tissue or in serum of HNSCC patients) as a result of hypoxia or oncogene signaling was correlated with resistance to cytotoxic treatment, worse prognosis, and advanced disease [204–208]. In a meta-analysis of 1002 HNSCC patients, VEGF was significantly associated with decreased survival [209].

There are many other factors playing important role in angiogenesis, including platelet-derived growth factor (PDGF), epidermal growth factor (EGF), hepatocyte growth factor (HGF), prostaglandins, TGF- β , COX-2, and IL-6 [10, 36, 73, 153, 154,

Table 7.2 HNSCC protective mechanisms against immune reactivity

Mechanism	Biomarker	Consequence	References
Escape from immune recognition	Loss of MHC	↓ presentation of tumor antigens	[179–181]
	↑ PD-L1 expression	↑ T-cell apoptosis	[167, 182]
	↑ FasL expression	↑ T-cell apoptosis <i>via</i> Fas-FasL	[183, 184]
	↑ ezrin expression	↑ internalization of NK cells, NK cell apoptosis	[175]
	Internalized TLR3	NK cell impairment	[178]
Modulation of the cytokine response	↑ PgE2, COX-2	Decreased levels of CD8+ T cells; expansion of the regulatory T cells (Treg)	[162, 184–186, 191]
	↑ TGF-β	Inhibition of cytotoxic T cells and NKs; MDSCs and M2-skewed macrophages recruitment	[185, 187–189]
	↑ IL-10	M2-skewed macrophage activation and promotion of a Th2 response	[184, 186]
	↑ VEGF	Impaired maturation of dendritic cells	[181, 193, 194]
	↑ GM-CSF	MDSCs recruitment	[39]
	↓ IFN-α and IFN-γ	↓ antitumor reactivity	[195, 196]
Induction of immune suppressor cells	MDSCs recruitment	Suppression of T-cell proliferation and activity	[185, 188, 189]
	M2-skewed macrophages recruitment	Downregulation of T-cell activity and secretion of high levels of growth factors, angiogenic factors, and matrix remodeling enzymes	[192]
	Treg (CD4+ CD25+ FoxP3+ regulatory T cells) expansion	Suppression of CD8+ T cell- and CD4+ helper T cell-mediated immunity	[168–170]
	CD34+ progenitor cell expansion	Suppression of CD8+ T-cell- and CD4+ helper T-cell-mediated immunity	[168–170]

Abbreviations: MHC major histocompatibility complex, GM-CSF granulocyte-macrophage colony-stimulating factor, MDSCs myeloid-derived suppressor cells, TLR3 Toll-like receptor 3

210]. Oral cancer overexpressed 1 and 2 (ORAOV1 and 2) are transmembrane proteins involved in tumor angiogenesis and regulation of cell growth *via* VEGF induction [211, 212]. Overexpression of ORAOV1 as well as ORAOV2 was reported in HNSCC [212, 213]. Loss of transforming growth factor-beta type II receptor (TGFβRII) is commonly observed in human HNSCC and leads to increased inflammation and angiogenesis [10].

Fibroblast growth factors (FGF) provide another angiogenic signaling mechanism in the tumor microenvironment. Basic fibroblast growth factor (bFGF) is a potent inducer of angiogenesis

and is usually found immobilized on the extracellular matrix within the tumor milieu. Expression of the secreted binding protein for fibroblast growth factors (FGF-BP) appears to be a mechanism by which immobilized FGF can be activated [214, 215]. FGF-BP is produced extensively by primary HNSCC tumors and some metastatic cells but not in normal adult mucosa [216].

Hasina et al. studied the expression of cytokines FGF-2, VEGF, IL-8, and HGF in HNSCC, dysplastic, and healthy samples and demonstrated different ways by which particular tumors can provoke angiogenesis [217]. The highest

expression of all studied cytokines was in HNSCC tissues, and two distinct tumor clusters were identified with regard to angiogenesis. One group of tumors had significantly increased microvessel density with high levels of FGF-2 and VEGF and relatively low levels of IL-8 and HGF. The second group of tumors displayed lower microvessel density and expressed low levels of FGF-2 and VEGF and higher levels of IL-8 and HGF. This might have some relevant therapeutic consequences, because it is not rational to inhibit angiogenesis by targeting molecules that are poorly expressed in treated type of tumor.

NF- κ B is also vastly important for angiogenesis in tumors. Some NF- κ B downstream genes are powerful angiogenic agents, such as VEGF, IL-8, and COX-2 [134, 218]. Thrombospondin-1 (TSP-1) was recently identified as a target gene of Ras-modulated Myc activity, which represses TSP-1 expression and increases tumor angiogenesis. In HNSCC patients, TSP-1 was found to be downregulated [219, 220].

Thus far, clinically tested antiangiogenic therapies have unfortunately demonstrated very disappointing effectiveness, primarily in regard to overall survival. Several studies have reported that drugs targeting VEGF can suppress the growth of primary tumors but may also promote tumor metastasis [221, 222]. Furthermore, anti-VEGF agents induced the production of several cytokines (granulocyte colony-stimulating factor, placental growth factor, osteopontin, IL-6, erythropoietin) that may promote VEGF-independent angiogenesis and metastasis [223]. Another possible unwanted side effect of VEGF inhibitors is suppression of pericytes on tumor vessels. As a result, leaky and immature vessels facilitate the entry of tumor cells and consequent metastatic spread [224].

7.6 Genomic Instability and HNSCC Progression

Progression of HNSCC is usually associated with vast chromosomal abnormalities and subsequent alterations in gene expression. Recently, the presence of more subtle expression regulators such as microRNAs was discovered.

7.6.1 MicroRNA (miRNA)

Many dysregulated miRNAs have been identified that contribute to HNSCC progression. Microarray analysis disclosed ten miRNAs that could differentiate malignant head and neck cancer samples from paired normal tissues; seven miRNAs (miR-29b-1*, miR-181a, miR-181a-2*, miR-181b, miR-221*, miR-744, and miR-1271) were upregulated in cancer samples while three miRNAs (miR-95, miR-101, and miR-141) were downregulated [225]. Some miRNAs seem to be related to the progression of carcinoma or premalignant lesion in head and neck malignancies. miR-21, miR-181b, and miR-345 expression were positively correlated with increased lesion severity [226] and miR-137 and miR-193a were reported to be tumor suppressors that were often epigenetically silenced during oral carcinogenesis [227]. With the exception of only a few miRNAs, all reported HNSCC miRNA studies to date have shared little in common; thus, the exact importance/contribution of particular miRNA species remains to be elucidated. Upregulation of miR-21, miR-31, miR-10b, miR-181, and miR-221 and downregulation of miR-133a/b, miR-138, miR-139, miR-375, and miR-200c are, however, trends that have been consistently identified in HNSCC [228–232]. Increased expression of miR-21, miR-18a, and miR-221 and decreased miR-375 and the ratio of miR-221/miR-375 expression were found to have a high specificity and sensitivity for diagnosis of HNSCC [233].

7.6.2 Chromosomal Aberrations

Chromosomal rearrangements resulting in very complex karyotypes are often found in HNSCC; in fact, more than 70 % of tumors are aneuploid [234]. Several genes associated with the DNA damage response pathway are frequently altered in HNSCC, including *BRCA1*, *BRCA2*, *FANCD2*, and *FANCG*. Other cancer-related genes linked to hereditary cancer syndromes such as *VHL*, *MLH1*, *XPC*, *RBI*, [235], and recently discovered germline alterations in *RAD51C*, are also

known to be mutated in HNSCC [236]. SMAD 4 deficiency (loss of chromosome 18q) is a common mutation that suppresses Fanc-/Brca-mediated DNA repair and increases genomic instability [8]; and recent findings suggest that interactions with tobacco and polymorphisms in *XRCC1* and *XRCC2* could increase the risk of HNSCC [237–239]. Furthermore, viral E7 protein is able to disrupt centrosome duplication causing abnormal mitosis, aneuploidy, and increased genomic instability [240]. NK cell internalization is another mechanism that results in aneuploidy. It is hypothesized that NK cell internalization might facilitate the development of aneuploidy in tumor cells by interfering with cell division resulting in disturbed chromosome segregation and/or cytokinesis [175]. To date, more than 30 sites of significant chromosomal aberrations and 15 frequently mutated genes including *TP53*, *CDKN2A*, *HRAS*, *PIK3CA*, *NFE2L*, and *NOTCH1* have been discovered in HNSCC patients. Most of these genetic alterations occur also in lung squamous cell carcinoma [144, 241]. Genomic regions most frequently amplified (>35 %) were located on 3q (candidate genes *AIS* (p40/p73L), *SSR3*, *CCLN1*, *hTERC*, *SKIL*, *ECT2*, *PIK3CA*, *SST*, *TP63*, and *TFRC*) [242, 243]), 5p, 8q (candidate gene *Myc*), 9q, 11q (candidate gene *CCND1*), 20q, and 7p (candidate gene *EGFR* on 7p12); 7p and 3q amplification occurs in early tumor development [234, 235, 244]. Regions most frequently deleted

(>40 %) involved chromosomes 3p (*FHIT* gene maps to 3p14.2, *VHL* to 3p25, *RASSF1A* to 3p21), 8p, 9p (candidate genes *p16/CDKN2A*), 13q, and 18q [235, 244]. The loss of 18q is associated with a worse clinical outcome [245]. Rearrangements that often occur in higher grades of dysplasia and SCC include *PTEN* (10q23.3) inactivation, 11q13 amplification, and LOH at 6p, 8p, 13q21, 14q32, and 4q27 [246–249]. Amplification of 11q13 was found in about one-third of HNSCC, with amplified cyclin D1 playing crucial role in promoting the cancer phenotype [250, 251]. Gain on chromosome 8q22.3, the location of *YWHAZ* (14-3-3 zeta), is found in 30–40 % HNSCC cases [252]. LOH analyses suggest that initial alterations target crucial genes located on chromosomes 3p (*RASSF1A* and *VHL*), 9p21 (cyclin-dependent kinase inhibitors), and 17p13 (*TP53*) [200, 253] (Fig. 7.3). LOH of the 9p21 region occurs in 70 % of dysplastic lesions in oral tissues simultaneously with promoter hypermethylation; subsequent inactivations of the remaining alleles of *14^{ARF}* and *p16* are crucial events in HNSCC progression [126, 248].

Some studies revealed that distinct chromosomal aberrations can be connected with clinical parameters such as losses at 11p13-p14, 10q23-q26, 11q14-24, 17q11, 8p, 8q, and 6p and gains at 4q11-22 and Xq12-28 that are significant for metastatic carcinoma [118, 234, 248, 249]. Some genes involved in cell signaling (*NGFRAP1L1*,

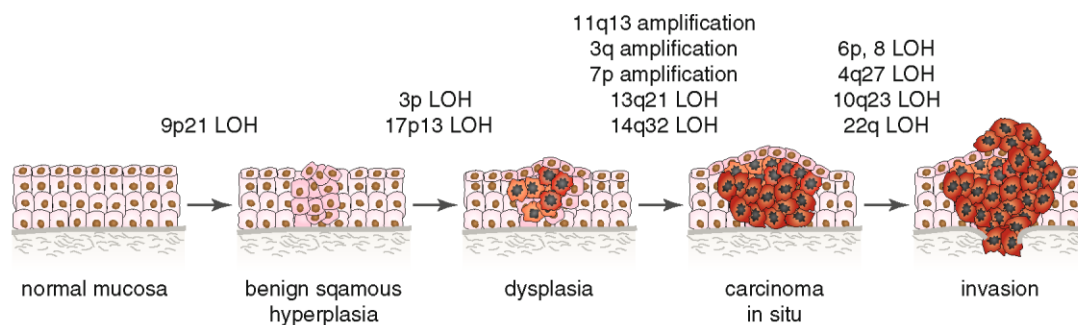


Fig. 7.3 Genetic changes associated with the histologic progression of HNSCC based on changes of chromosomal material. Loss of heterozygosity (*LOH*); candidate tumor suppressor genes include p16 (9p21), p53 (17p13), *RASSF1A* (3p21), *FHIT* (3p14.2), *VHL* (3p25), *PTEN*

(10q23), *NF2* (22q12.2), and *Rb* (13q21). Candidate proto-oncogenes include *CCND1* (11q13), *CCLN1* (3q25.31), *EGFR* (7p12), *PIK3CA* (3q26.31-26.32), *AIS* (3q), and *ORAOV1* (11q13) (Adapted from Polanska et al. [15])

GPRASP2, *BEX1*, *BEX2*, and *ZNF6*) reside on chromosome Xq21–22, whose amplification is associated with HNSCC metastasis [118]. This could possibly help explain why the female sex is associated with a bad response to organ-sparing therapy and poor outcome [120]. Furthermore, LOH affecting 18q, 22q, and 3p25 and amplification at 14q23–q24.2 (*HIF-1 α* gene) and chromosomal gains on 1q and 16q have already been linked to poor survival [78, 102, 200, 245, 254, 255].

7.6.3 HNSCC Subtypes

With regard to the presence of known genetic alterations occurring in tumors, HNSCC can be divided into four subtypes, which will be discussed below [144].

7.6.3.1 Classical Subtype (CL)

Comprising ~18 % of HNSCC, CL is also found in lung squamous cell cancer and is associated with alterations of the oxidative stress genes *KEAP1*, *CUL3*, and *NFE2L2* [143, 144, 256]. This subtype manifests genomic alterations typical for SCC (deletion of 9p and 3p, amplification of 3q, and gene amplification of *CCND1* and *EGFR*). The CL subtype is also associated with heavy smoking and exhibits elevated expression of genes induced by cigarette smoke exposure, including *NFE2L2*, *GPX2*, and *AKR1C1/3* [144]. In particular, *TP53* mutation, *CDKN2A* loss of function, and larynx subsite co-occurred in most classical subtype tumors [256].

7.6.3.2 Atypical Subtype (AT)

About 24 % of HNSCC is AT. This subtype demonstrates no amplification of *EGFR*, is HPV-positive, and expresses high levels of PI3 kinase (*PIK3CA*); the name atypical was used due to the lack of *EGFR* amplification or 9p deletion. AT tumors show a clear HPV+ signature represented by increased expression of transcription factors such as *RPA2*, *CDKN2A* (p16), and *LIG1* [144, 257]. Additionally, the atypical subtype is characterized by an occurrence of activating mutations in *PIK3CA* [256].

7.6.3.3 Mesenchymal Subtype (MS)

MS accounts for ~27 % of HNSCC and mostly contains mutations in *FGFR1* and *FGFR2*. The name mesenchymal was chosen because of the significant expression of epithelial to mesenchymal transition (EMT) markers (*VIM*, *DES*, *Twist1*, and the growth factor *HGF*) observed in these tumors [144, 258]. MS tumors typically have alterations in innate immunity genes, *inter alia* high expression of CD56 (NK cell marker) [256].

7.6.3.4 Basal Subtype (BA)

Thirty-one percent of HNSCC are of the BA subtype, which appear similar to basal epithelial cells in airways for which they were named. BA cells have a characteristic gene expression pattern inclusive of higher expression of *EGFR*, *COL17A1*, and the transcription factor TP63. An inactivation of *NOTCH1* with intact oxidative stress pathways and rather rare alterations of chromosome 3q are typical for the BA subtype. Moreover, the basal subtype included tumors with co-mutations of the *HRAS* and *CASP8* and with 11q13 and 11q22 co-amplification [256].

The CL and AT subtypes demonstrate higher expression of *ALDH1* and *SOX2* compared to MS and BA. BA seems to express less *SOX2* than healthy tonsil tissue [256]. Both of these genes have been hypothesized to be cancer stem cell markers due to their role in self-renewal and pluripotency [259, 260]. The BA subtype expressed significantly more *TP63* than any other subtype. Patients in the HPV-negative AT subgroup generally have the most adverse outcome [144].

7.7 Replicative Immortality and Stemness

An intrinsic cellular mechanism allows normal cells to divide only a finite number of times and blocks cell division beyond a certain limit. Limited telomerase activity usually results in telomere erosion and activation of proliferative barriers (senescence and crisis/apoptosis) [1]. The ability of cancer to escape these barriers is due to several mechanisms; reversible polyploidy [2], CDK1 or survivin overexpression, p53 inactivity,

and telomerase activation have been well characterized [1, 261–263]. Telomerase activation can be caused *inter alia* by the HPV-16 viral E6 protein [57]. It has been hypothesized that telomeres are involved in unrestricted proliferation by preventing erosion of the chromosomes [263]. In accordance, the levels of telomerase activity in HNSCC tissues are significantly higher than those in the normal tissues [264] and expression of telomerase in saliva of the oral cancer patients was also increased [265]. Furthermore, Mao et al. detected telomerase activity in 100 % of examined HNSCC cell lines, in 90 % of invasive tumors, in 100 % of dysplastic lesions, and in 100 % of examined hyperplastic lesions, whereas no normal tissues or hyperkeratotic lesions had detectable telomerase activity [266]. Multivariate analysis revealed that overall stage, tumor depth, and telomerase activity were independent variables associated with poor survival [267]. Telomerase expression in peripheral blood mononuclear cells (PBMCs) was correlated with N stage and the prognosis of HNSCC patients [204]. At the RNA level, miR-21, let-7, miR-107, miR-200c, and miR-138 appear to have complex roles

in the modulation of immortality, stemness, and epithelial-mesenchymal transition (EMT) in HNSCC [230]. HNSCC tumor cells are morphologically and functionally heterogeneous which implies they arise from specific progenitor cells and not merely from the clonal expansion of a single mutated cell with telomerase activation [268]. Substantial evidence has shown that a small subpopulation of cancer stem cells (CSC) is responsible for tumor initiation and progression. CSCs are described as a small percentage of cells in a tumor that are able to self-renew and produce daughter cells. CSCs are associated with a specific state of differentiation (e.g., mesenchymal features) [269, 270]. EMT is a process by which a polarized epithelial cell develops a mesenchymal phenotype, and it appears to be involved in the process leading to the acquisition of stemness by epithelial tumor cells [271]. CSCs are highly important for disease prognosis due to their crucial role in recurrence and metastasis. Possible biomarkers associated with CSCs in HNSCC are summarized in Table 7.3. All mentioned stemness markers are usually associated with bad prognosis.

Table 7.3 Biomarkers of stemness

Biomarker	Mechanism	Detected in	References
ABCG2	↑ mRNA and protein expression	CSC-like subpopulation of tumor cells	[272]
ALDH1	↑ protein expression	CSC-like subpopulation of tumor cells	[273, 274]
CD44	↑ mRNA and protein expression	CSC-like subpopulation of tumor cells, peripheral blood	[275–279]
Oct4	↑ mRNA and protein expression	Tumor tissue, HNSCC cell lines	[55, 280]
Sox	↑ mRNA expression	CSC-like subpopulation of tumor cells	[269]
Nanog	↑ mRNA expression	CSC-like subpopulation of tumor cells	[269]
CD133	↑ protein expression	CSC-like subpopulation of tumor cells	[281–283]
Telomerase	↑ protein expression, activation	CSC-like subpopulation of tumor cells	[2]
HMGA2	↑ mRNA and protein expression	Tumor tissue, CSC-like subpopulation of tumor cells	[268]
ZEB1/2	↑ expression	Tumor tissue, CSC-like subpopulation of tumor cells	[283]
ADAM17	↑ expression	Tumor tissue, HNSCC cell line	[284, 285]
BMI-1	↑ mRNA and protein expression	Tumor tissue, CSC-like subpopulation of tumor cells	[268, 286–288]
c-Met	↑ protein expression	Tumor tissue, CSC-like subpopulation of tumor cells	[111, 289]

(continued)

Table 7.3 (continued)

Biomarker	Mechanism	Detected in	References
miR-200c	↓ expression	Tumor tissue, CSC-like subpopulation of tumor cells	[230, 288]
Beta-catenin	↑ protein expression	Primary HNSCC cell line, CSC-like subpopulation of tumor cells	[280]
GRP78	↑ protein expression	Tumor tissue, CSC-like subpopulation of tumor cells	[290]
LGR5 (also known as GPR49)	↑ mRNA and protein expression	Tumor tissue, HNSCC cell lines	[291]
STAT3	↑ mRNA and protein expression	Tumor tissue, mouse embryonic stem cells, epiblast stem cells	[35, 292, 293]
S100A4	↑ mRNA and protein expression	Tumor tissue, CSC-like subpopulation of tumor cells, HNSCC cell line	[294]

7.8 Invasion and Metastasis

Many studies have investigated variances in expression profiles of primary and metastatic HNSCC and discovered that specific gene expression changes are required for metastasis [295]. One of the key processes facilitating metastasis is EMT, the process by which a polarized epithelial cell develops a mesenchymal phenotype. EMT is associated with elevation of invasiveness, recurrence, and a worse prognosis in many cancers, including HNSCC. During EMT, low E-cadherin expression and increased vimentin are common [271, 296]. E-cadherin transcriptional repressors such as Snail (*SNAI1*), Slug (*SNAI2*), ZEB-1/2, SIP-1, E12/E47 [297, 298], and Twist [299] have traditionally been implicated in promoting EMT. Slug was extremely elevated in the HNSCC cells in response to hypoxia [300]. Smad2 loss can increase EMT; the absence of Smad2 can increase Smad3/4 binding at the Snail promoter thereby increasing Snail transcription and triggering EMT [8]. Finally, Bmi-1 also regulates Snail expression and promotes metastasis [301].

Dysregulation of miRNAs has also been reported during EMT and metastasis. For example, Twist can activate the expression of miR-10b [302] promoting migration and invasion [230, 303], an association between cancer metastasis and miR-211 expression in OSCC is known, and miR-211 expression was increased in tumors with vascular invasion and correlated with poor

prognosis [84, 304]. Overexpression of miR-181 was associated with vascular invasion, lymph node metastasis, and worsened survival rates. Ectopic expression of miR-181 promoted invasion and cell migration but did not apparently promote nor support anchorage-independent growth of OSCC cells. Increased miR-181 levels were found in both tumor tissues and plasma [305]. Oncogenic miR-31 was found to increase HIF1- α expression and tumorigenicity in HNSCC [306]. Reduced miR-138 expression was found in highly metastatic cells. Renewed expression of miR-138 reduced invasion, caused cell cycle arrest, and promoted apoptosis [307, 308]. MiR-138 has been reported to modulate invasivity and migration through targeting the Ras homolog gene family, particularly RhoC and Rho-associated kinase ROCK2. Restricted miR-138 expression may cause elongated, spindle-shape cell morphology, enhance cell stress fiber production, and increase cell migration and invasion [309]. miR-34c is another suppressor of metastasis that targets c-Met to inhibit cell growth and invasivity [310]. miR-203 inhibits HNSCC lung metastasis by repression of prometastatic factors affecting extracellular matrix remodeling (SPARC), cytoskeletal dynamics (LASP1), and cellular metabolism (NUAK1). Expression of miR-203 and its signaling network corresponds with HNSCC overall survival [311]. Results also indicated that stem renewal factor Bmi-1 is a direct target of inhibition for

miR-203 [312]. Furthermore, transfected miR-133a, miR-133b, and miR-145 (tumor suppressor miRNAs) inhibited cell proliferation and cell invasion in esophageal squamous cell carcinoma (ESCC) cells. miRNAs with binding sequences in the 3'UTR of fascin homolog 1 (FSCN1, actin-binding protein) decreased oncogenic FSCN1 expression [313]. Renewed expression of miR-141 and miR-200c significantly decreased the migration potential of HNSCC cells [314]. Salivary miR-31 is elevated in OSCC and may serve as a useful predictor for early detection and postoperative follow-up [315], while miR-200a and miR-125a are significantly under-expressed in the saliva of OSCC patients

[316]. In plasma, miR-31 [317], miR-10b [318], miR-24 [319], miR-181 [305], and miR-184 [320] are upregulated in OSCC patients.

EMT appears to be involved in the process leading to CSC's occurrence; hence, the biomarkers associated with stemness (Table 7.3) can also serve as metastasis markers. For example, elevated expression of c-Met supports invasion and metastasis of oral tongue carcinoma [321, 322]. Other crucial metastasis markers are shown in Table 7.4. In high-grade HNSCC, dysregulation of E-cadherin and NF- κ B signaling can be found together with loss of tight and adherens junctions. Subsequent transformation of cells to a spindle shape enables movement through the basal

Table 7.4 Biomarkers involved in metastasis

Biomarker	Function	Expression	Detected in	References
ALDH1A3	Aldehyde dehydrogenase	↑ mRNA, protein	Tumor tissue, HNSCC cell lines	[55]
CASP1	Apoptosis	↓ mRNA	Tumor tissue	[118]
CAV-1	Signal transduction, vesicular transport, and cell receptor localization	↑ mRNA, protein	Tumor tissue, HNSCC cell lines	[55, 325]
CCR7	Chemokine receptor	↑ mRNA, protein	Tumor tissue, HNSCC cell lines	[326–328]
c-FLIP	Procaspase-8-like regulator of death ligand-induced apoptosis	↑ mRNA, protein	Tumor tissue	[329]
CEP55	Cell cycle regulation, cytokinesis	↑ mRNA, protein	Tumor tissue	[112]
HSulf-1	Modulation of heparin-binding growth factor signaling	↓ mRNA	HNSCC cell lines	[330]
COL17A1	Cell interactions	↓ mRNA	Tumor tissue	[118]
Cortactin	Cell motility and invasion	↑ mRNA, protein	Tumor tissue	[331–334]
CXCR4	Chemokine receptor	↑ mRNA, protein	Tumor tissue, HNSCC cell lines	[326, 327, 335, 336]
DAPK3	Apoptosis	↓ mRNA	Tumor tissue	[118]
DDR2	Receptor tyrosine kinase	↑ mRNA, protein	Tumor tissue, HNSCC cell lines	[337]
DSG3	Cell-to-cell adhesion	↑ protein	Tumor tissue, lymph nodes, HNSCC cell lines	[338]
DST	Regulation of the cell cycle	↓ mRNA	Tumor tissue	[118]
Hif-1 α	TF, hypoxia	↑ protein	Tumor tissue, HNSCC cell lines	[299, 339]
IL18	Apoptosis	↓ mRNA	Tumor tissue	[118]
LRP6	Signaling	↑ mRNA	Tumor tissue	[118]
Nuclear LKB1	Serine/threonine kinase	↓ nuclear protein	Tumor tissue	[340]

(continued)

Table 7.4 (continued)

Biomarker	Function	Expression	Detected in	References
LOX	Lysyl oxidase family, important modulators of the ECM	↑ mRNA, protein	Tumor tissue	[341]
MMP-2	ECM degradation	↑ mRNA, protein	Serum, HNSCC cell line, tumor tissue	[112, 342, 343]
MMP-3	ECM degradation	↑ mRNA, protein	Serum	[344]
MMP-7	ECM degradation	↑ mRNA, protein	Tumor tissue, HNSCC cell lines	[55]
MMP-9	ECM degradation	↑ protein	Serum, tumor tissue	[278, 343–346]
MYCN	Signaling	↑ mRNA	Tumor tissue	[118]
NBS1	Cell cycle regulation, DNA double-strand break repair	↑ mRNA, protein	Tumor tissue, HNSCC cell line	[342, 347]
NF-κB	Proinflammatory TF	↑ mRNA, protein	Tumor tissue, HNSCC cell lines	[149, 323, 324, 348]
Notch1	Single-pass transmembrane receptor	↑ protein	Tumor tissue	[349]
PPP2R1B	Apoptosis	↓ mRNA	Tumor tissue	[118]
RAC1	Rho family of Ras-related guanosine triphosphate (GTP)-binding proteins	↑ protein	HNSCC cell lines	[350]
RSK2	Cell cycle regulation, proliferation, apoptosis	↑ protein	Tumor tissue, HNSCC cell lines	[351]
Smad2	Mediates TGFβ signaling	↓ mRNA, protein	Tumor tissue	[8, 352, 353]
Snail	TF, regulator of EMT	↑ protein	Tumor tissue, HNSCC cell line, CSC-like subpopulation of tumor cells	[8, 299, 301, 342]
Survivin	Inhibitor of apoptosis	↑ protein	Tumor tissue, HNSCC cell lines	[262, 354, 355]
Twist	TF, regulator of EMT	↑ protein	Tumor tissue	[299, 327]
TLR-4	Interleukin-1R receptor family	↑ mRNA, protein	Tumor tissue, HNSCC cell lines	[55, 356]
TRIM-29	TF	↑ mRNA, protein	Tumor tissue, HNSCC cell lines	[55]
VEGF/R	Angiogenesis	↑ protein, mRNA	Serum, tumor tissue, HNSCC cell lines	[204, 207, 345, 349, 357]
miR10b, miR211, miR181, miR31	Gene expression regulators	↑	Plasma, HNSCC cell lines, tumor tissue	[84, 230, 303–306, 358]
miR138, miR34c, miR203, miR200c, miR141	Gene expression regulators	↓	HNSCC cell lines, tumor tissue	[307, 308, 310, 311, 314]

Adapted and extended from Polanska et al. [15]

MMP matrix metalloproteinase, *ECM* extracellular matrix, *TF* transcription factor, *EMT* epithelial-mesenchymal transition

membrane and promotes metastasis [323, 324]. Masood et al. showed that ALDH1A3, CAV-1, MMP-7, OCT-4, TRIM-29, and TLR-4 proteins have elevated expression in HNSCC cells with severe metastatic phenotypes suggesting that these proteins could have a crucial importance in

the metastatic potential of HNSCC cells [55]. Rickman et al. compiled a list of metastasis predictor genes in a four-gene model (*FLOT2*, *HSD17B12*, *KRT17*, and *PSMD10*) that excluded HPV-positive samples. The four-gene model was highly predictive for expansion of metastasis (hazard ratio 6.5; 95 % confidence interval 2.4–18.1) and predicted occurrence of metastasis with 74 % sensitivity and 78 % specificity [118]. Furthermore, it was reported that tumors with developed metastases had decreased expression of genes associated with apoptosis (*PPP2R1B*, *CASP1*, *IL18*, *DAPK3*), negative cell cycle regulation (*DST*) and cell interactions (*COL17A1*), and increased expression of signaling genes such as *MYCN* and *LRP6*.

7.9 Cell Death Modifications

Impairment of apoptosis is an important contributor to the development of HNSCC. HNSCC cells evolve many survival mechanisms that allow malignant cells to evade apoptosis. One of these mechanisms is loss of function of TP53 tumor suppressor. TP53 is a crucial damage sensor that triggers apoptosis [1]. Alternatively, tumors may evade apoptosis by elevated expression of anti-apoptotic agents (Bcl-2, Bcl-xL) [359] and survival signals (survivin [262, 354, 355]), by downregulation of proapoptotic factors (Bax, Bim, Puma) [360], or by mechanisms associated with cell cycle disruption. In particular, Bcl-xL and Bcl-2 overexpression strongly contribute to the development of HNSCC [361]; they inhibit apoptosis through prevention of cytochrome c release from mitochondria which impedes formation of the apoptosome, caspase activation, and cell death. NF- κ B is also a major survival factor able to upregulate many survival proteins including Bcl-2 [134, 149, 323, 324, 348, 362]. Activation of the Wnt/ β -catenin signaling pathway inhibits mitochondria-mediated apoptosis in HNSCC [363]. MiR-21 was found to target PTEN, programmed cell death 4 gene (*PDCD4*), and tropomyosin 1 (*TPM1*) to inhibit apoptosis, stimulate transformation, and enhance colony formation [364–366]. Recent

studies demonstrated that miR-21 is also a key player in the STAT3 anti-apoptotic signaling pathway [147].

Anoikis is a type of programmed cell death that liquidates anchorage-dependent cells when they become detached from the extracellular matrix (ECM); however, the detached cancer cells are often resistant to chemotherapy or other cytotoxic and inflammatory stresses and do not undergo anoikis. Anoikis is caused by many factors including insufficient energy production. Osteopontin is able to upregulate energy metabolism. Interaction between osteopontin-a, which elevates the cellular glucose level, and osteopontin-c, which exploits this glucose to ensure energy production, is important to overcome conditions that lead to anoikis [367]. Resistance to anoikis is a critical step in metastasis as cancer cells are better able to survive during invasion and intrusion into the blood and lymphatic system [368]. Wnt-/ β -catenin-mediated inhibition of apoptosis and anoikis is dependent on the death receptor signaling pathway [363]. Mutation of *TP53* can also participate in resistance to anoikis. For example, serine substitution of proline at codon 151 of *TP53* is a gain-of-function mutation leading to anoikis resistance and tumor progression in HNSCC [369]. Furthermore, growth factors secreted by endothelial cells (e.g., VEGF) could protect HNSCC against anoikis [370]. 14-3-3 zeta [252], Bcl-x(L) [361], Mcl-1 [371], CAV-1 [55, 325], and c-FLIP [329] also repress anoikis in HNSCC cells. Bit1 is downregulated in metastatic cancer [368]. Other well-known key players in HNSCC such as IGF-1R, EGFR, MAPK, RAS, NF- κ B, Twist, RAC1, and TGF- β have been shown to suppress anoikis [372, 373].

Autophagy is an important physiological process that is usually very low during homeostasis that can be induced by cellular stress [374]. The induction of autophagy together with blocked apoptosis in hypoxic areas allows survival of tumor cells and promotes an aggressive phenotype in immunocompetent murine HNSCC models. On the other hand, autophagy defects are associated with neurodegeneration, liver failure, aging, and some types of cancer [375]. The role of autophagy in cancerogenesis is complicated

and probably context-dependent. On one hand, failure to induce autophagy promotes cancer initiation due to persistent oxidative stress, DNA damage, and inflammation. On the other hand, a high level of autophagy facilitates survival of cancer cells under starvation and probably contributes to treatment resistance [376–378]. Schaaf et al. demonstrated that ULK1, unfolded protein response (UPR), and hypoxia-regulated genes are associated with autophagy induction, hypoxia tolerance, and a worse clinical outcome in HNSCC patients [379]. ULK1 triggers autophagy by phosphorylating Beclin-1 and activating VPS34 lipid kinase [380].

7.10 Biomarkers of Treatment Response

Many biomarkers associated with drug resistance that are common in cancer stem cells, EMT, and resistance to cell death were discussed earlier. Chemical inhibition of the Ras-related guanosine triphosphate (GTP)-binding protein Rac1, which controls the organization of the actin cytoskeleton thereby regulating cell adhesion, polarity, motility, and EMT, resulted in significant improvement of HNSCC sensitivity to ionizing radiation and cisplatin [373]. Treatment-resistant HNSCC were reported to be CD44 high/EGFR low [381]. Furthermore, many conventional therapies fail because they utilize apoptotic mechanisms that require intact p53 signaling [382] or that can be inhibited by anti-apoptotic proteins such as Bcl-xL or Bcl-2 [383, 384]. Bcl-xL expression was correlated with resistance to radiotherapy (regardless of p53 status) [385, 386] and with resistance to chemotherapeutic agents such as cisplatin, bleomycin, vincristine, doxorubicin, and etoposide [385]. High Bcl-2 expression inhibits cisplatin-induced apoptosis and predicts poor response to cisplatin therapy in advanced OSCC [387]. In univariate and in multivariate analyses of tumor biopsy specimens, the simultaneous detection of bcl-2 protein overexpression and p53 gene mutation was associated with greater risk of locoregional failure and worse 5-year survival in HNSCC patients treated by radiotherapy [384]. Downregulation of miR-

296 could significantly decrease the expression of Bcl-2, P-glycoprotein, and MDR1 and upregulate the expression of Bax [388].

Hypoxia in tumors modulates release of many angiogenic factors and cytokines and is associated with resistance to radiotherapy, treatment failure, and worse prognosis in HNSCC patients. Toustrup et al. developed a hypoxia classifier based on the expression of 15 genes [389]. The expression of miR-210 in head and neck cancer was in line with other approaches for assessing hypoxia and was correlated with locoregional disease recurrence and poor overall survival [390]. Hypoxia is also involved in creation of polyploid giant cancer cells that are important contributors to resistance [2, 391]. Moreover, the interaction between HIF- α /MIF and NF- κ B/IL-6 axes plays an important role in the hypoxia-induced accumulation of MDSCs and tumor growth in HNSCC [392].

Excision repair cross complementation group 1 (ERCC1) is a crucial element in repairing of interstrand DNA cross-links. Accordingly, ERCC1 may cause resistance to mitomycin C (MMC) and platinum chemotherapeutics and might be used to predict a reduced therapeutic response [393, 394]. ERCC1+ cells exhibit elevated chemoresistance and appeared to be radiosensitive and less hypoxic [395]. High ERCC1 expression was associated with better overall survival rates in HNSCC. Nevertheless, patients with oropharynx SCC presenting with high ERCC1 expression remain disease-free and have increased survival rates when compared to non-oropharyngeal squamous cell carcinoma patients with high ERCC1 expression despite treatment modalities and HPV status [396, 397].

Oral cancer overexpressed 1 (ORAOV1) seems to be a crucial oncogene in OSCC. The ORAOV1 gene is often amplified in esophageal SCC and is reported to enhance tumorigenicity and tumor growth and, together with pyrroline-5-carboxylate reductase (PYCR), is associated with resistance to oxidative stress-generating therapies [398]. Moreover, the antioxidant capacity of lactate may also contribute to radioresistance in malignant tumors [399].

Subpopulations of Hoechst 33342 dye-resistant cells termed “side population” (SP) cells manifest

stem cell characteristics [272]. Hoechst 33342 dye and also many kinds of cytostatics are exported out of the cell by the ATP-binding cassette (ABC) family of proteins including MRP1, MDR1, ABCG2, and ABCB5 [401, 402]. SP cells from OSCC are more chemoresistant, are more tumorigenic, and show self-renewal characteristics [272]. Finally, ABCC2/G2 inhibition in HNSCC cells with MK571 sodium salt hydrate markedly enhanced cisplatin sensitivity [400]. It was also suggested that the expression of ABCG2 is predictive for tumorous transformation of oral leukoplakia [401]. Likewise, elevated ABCB5 expression correlated with OSCC recurrence and progression [402]. ABCG2 and podoplanin protein expression may be useful markers of erythroplakia progression; 91 % of erythroplakic areas showing immunohistochemical positivity for both markers in the suprabasal layer of the oral epithelium became cancerous [403].

Cells in the tumor microenvironment (TME) have also been shown to induce tumor chemoresistance: (1) by the local release of cytokines that promote tumor growth and survival, (2) through specific interactions between tumor cells and ECM, (3) by oncologic trogocytosis, (4) by the transformation of primary cancer cells into cancer stem cells, and (5) by the creation of unique niches of tumor cells that provide survival advantages to the tumor following anticancer drug exposure or hypoxia (including upregulation of growth factors production by the stromal cells) [404].

7.11 Deregulation of Cellular Energetics

HNSCC cellular profiles have demonstrated many differences in the concentration of many metabolites, including lactate, fumarate, aspartate, alanine, phenylalanine, glycine, isoleucine, tyrosine, valine, taurine, NAD⁺, acetate, creatine, glutamine, UDP-sugars, myoinositol glutathione, and AMP/ADP [405]. As glucose is the main fuel required for ATP production in HNSCC cells [406], glutamine and glutaminolysis appear to be a major carbon source [405]. The glutamate/glutamine ratio was increased in HNSCC cells compared to normal human oral

keratinocytes, indicating elevated glutaminolysis [405]. Glucose transporter GLUT1 expression was associated with poor survival and increased tumor growth [407].

Many *in vitro* metabolic studies have indicated that HNSCC cells are glycolytic with increased L-lactate production (Warburg effect). Recent *in vivo* studies demonstrated that metabolic diversity and metabolic symbiosis occur in HNSCC (see Fig. 7.4) [53]. The idea of a symbiotic relationship within the tumor microenvironment is supported by the fact that the Warburg effect exists in stromal cells rather than in cancer cells themselves. Cancer cells are able to consume lactate generated by stromal cells [408–411]. Accordingly, increased lactate levels in HNSCC tumors are associated with the occurrence of metastases [412, 413]. Curry et al. reported that MCT1 and MCT4 are functional biomarkers of metabolic symbiosis in HNSCC [53]. MCT4 transports L-lactate and ketone bodies out of cells and may also serve as a biomarker of oxidative stress [414–416]. On the contrary, MCT1 transporter imports ketone bodies and L-lactate into the cell. High MCT1 expression causes an increase in oxidative phosphorylation (OXPHOS) and decreases glycolysis [411, 417]. The expression of MCT4 is triggered during hypoxia and oxidative stress through the activation of HIF-1 α [418]. Significant MCT4 immunostaining was discovered in a vast majority of cancer-associated fibroblasts (CAFs) and differentiated carcinoma cells and was absent in adjacent normal stromal fibroblasts [53].

Interestingly, the cell proliferative index of cancer cells (determined by Ki-67 expression) was highly correlated with OXPHOS and MCT1 expression ($p < 0.001$). According to the metabolic characteristics, three distinct tumor areas were disclosed: (1) mitochondrial-rich and highly proliferative cancer cells (MCT1+/low MCT4/Ki-67+/TOMM20+/COX+), (2) mitochondrial-poor and nonproliferative cancer cells (MCT1-/MCT4+/Ki-67-/TOMM20-/COX-), and (3) mitochondrial-poor and nonproliferative stromal cells (MCT1-/MCT4+/Ki-67-/TOMM20-/COX-) [53]. Nonproliferative populations (Ki-67-/MCT4+)

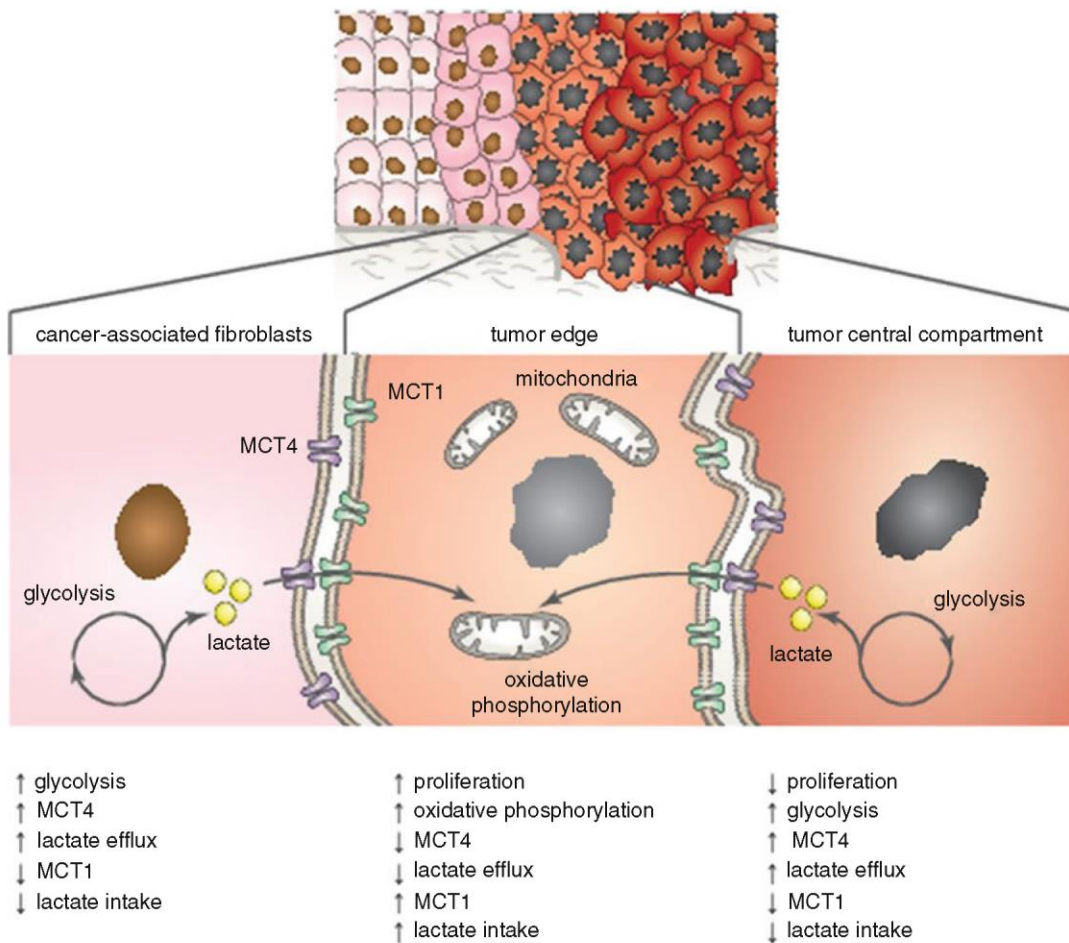


Fig. 7.4 Metabolic symbiosis in HNSCC tumor tissue. Highly proliferative cancer cells rely on oxidative phosphorylation and are mitochondrial-rich with high expression of MCT1. MCT1 transporter imports ketone bodies and L-lactate into the cell. Cancer-associated fibroblasts

(CAFs) and nonproliferative cancer cells rely on glycolysis and are mitochondrial-poor with high expression of MCT4. MCT4 transports L-lactate and ketone bodies out of cells. Cancer cells are then able to consume lactate generated by stromal cells (Adapted from Curry et al. [53])

have also been shown to influence clinical outcome, possibly due to the ability to provide an energy source for highly proliferative subpopulations of cancer cells. MCT4 positivity in low-proliferative epithelial cancer cells was associated with poor clinical outcome (tumor recurrence; $p < 0.0001$). Similarly, MCT4 positivity was a specific marker for CAFs ($p < 0.001$) and was predictive for higher tumor stages ($p < 0.03$) [53].

7.12 Tumor Microenvironment

Tumors are not merely huddles of malignant cells but are in fact complicated well-organized complex tissue networks able to recruit many other cell types. Cooperation between tumor cells and nonmalignant cells creates the tumor microenvironment (TME). The nonmalignant TME cells play a complex role and promote all stages of

tumorigenesis [1]. The TME in HNSCC includes many different cell types such as carcinoma-associated fibroblasts (CAFs), normal fibroblasts, myofibroblasts, endothelial cells (and their precursors), smooth muscle cells, pericytes, and immune cells [419]. Key TME cells and their properties are summarized in Table 7.5.

CAFs are critical elements of the TME participating in invasion, proliferation, and metastasis. These cells originate in normal fibroblasts located in close proximity to the tumor or in circulating mesenchymal stem cells [420]. Mutation of *TP53* in tumor cells is associated with increased CAF migration to the TME, while intact p53 protein inhibits migration [421]. Oral

CAFs exhibit cytokeratins, MMP-2, vimentin, and α -SMA–positive staining, whereas normal oral fibroblasts express only vimentin [159]. Marsh et al. highlighted the limited prognostic value of TNM staging and suggested that an SMA-positive stroma rich for myofibroblasts might be a powerful marker for the prediction of OSCC mortality [422]. CAFs express high levels of MCT4, which is a marker of glycolytic metabolism, oxidative stress, and L-lactate and ketone bodies export from the cell [53].

Proinflammatory factors known to be chemoattractants for macrophages and neutrophils are significantly upregulated in CAFs (e.g., CXCL-1, CXCL-5, and CCL-2) [423–425].

Table 7.5 Key cells of the HNSCC tissue microenvironment

Cell type	Markers	Secreted factors	References
Cancer-associated fibroblasts	α -SMA, integrin α 6, FAP, tenascin-C, desmin, NG2, POSTN, PDGFR α/β , palladin, podoplanin, MCT4+, MCT1-	HGF, FGF-2, IGF-2, CCL-2, CXCL-1, CXCL-5, CXCL-12, TGF- β , MMP-2, MMP-9, VEGF, PDGF, type IV collagen, Col15-binding integrins, tenascin-C and tenascin-W, PGE-2	[53, 420, 422, 425–427, 441–444] [440]
Regulatory T cells	CD4+CD25+FoxP3+	IL-10, IL-12, TGF- β	[184, 445]
Cytotoxic T cells	CD8+, TCR, Fas, PD-1	Perforin, granzymes, granulysin	[184, 445]
Th2 suppressor cells	CD4+	IL-4, IL-6, IL-10	[184, 445]
CD34 ⁺ progenitor cells	CD34+	TGF- β	[184, 445]
MDSCs	CD11b+ Gr1+ CD31+	IL-10, arginase, ROS, RNS, VEGF	[188, 392, 446]
Th17 T helper cells	CD4+, CD161 downregulation, CCR6, IL-23R, ROR2C	IL-17A, IL-17F, IL-21, IL-22, IL-26	[447, 448]
Tumor-associated macrophages (M2)		IL-10, IL-6, PDGF, TGF- β , MIF, EGF, CSF-1, MMP-9, CXCL-2, CXCL-8, VEGF, ROS, RNS, PGEs, CCL-17, CCL-18, CCL-22	[134, 436, 437, 449–451]
Tumor-associated neutrophils		MMP-9, VEGF, HGF, elastase, ROS, PGEs	[450–452]
Endothelial cells		IL-6, CXCL1, CXCL8, endothelins	[159, 430]

Adapted and extended from Curry et al. [419]

Abbreviations: ROS reactive oxygen species, RNS reactive nitrogen species, MIF macrophage migration inhibitory factor

CAF-produced FGF-2 and VEGF then boost the process of angiogenesis. Known factors that stimulate tumor cell proliferation (SDF-1/CXCL-12, insulin-like growth factor-2 (IGF-2), and HGF) are also secreted by CAFs. Moreover, CAFs enhance tumor invasion through the secretion of tenascin-C, tenascin-W, HGF, and MMPs along with TGF- β [420, 426]. CAFs and cancer cells can produce sphingosine-1-phosphate (S1P) and prostaglandin E2 (generated by COX-2 activation) that can cause chemoresistance and can enhance cell survival through the PI3K-Akt/PKB pathway [427, 428]. Taken together, CAFs can promote tumor progression in several aspects through their ability to produce elevated levels of various growth factors, cytokines, ECM proteins, and MMPs [429].

The ability of cancer cells to produce angiogenic factors to create and maintain tumor vasculature has been well described; and the important influence of factors produced by endothelial cells on the tumor cells, in this regard, is also known. Gene expression analysis demonstrated that EGF, IL-6, and IL-8 are upregulated in cocultures of endothelial cells and HNSCC [430]. Inactivation of EGF, IL-6, or IL-8 secreted by endothelial cells (with antibodies or gene silencing) blocked phosphorylation of Akt, ERK, and STAT3 in tumor cells. Bcl-2 signaling is known to trigger the production of EGF, IL-6, and IL-8, which could be a mechanism for the upregulation of these cytokines in tumor-affected endothelial cells [430]. Bcl-2 gene expression is remarkably higher in the tumor-associated endothelial cells of HNSCC patients compared to endothelial cells derived from the normal oral mucosa [431]. Zeng et al. reported that Jagged1 (the Notch ligand), induced *via* growth factors and mitogen-activating protein kinase, caused Notch activation in endothelial cells adjacent to HNSCC and promoted capillary-like sprout formation [432]. Mast cells, also known as mastocytes, are granulocytes that directly cooperate with endothelial cells to stimulate angiogenesis [433]. Enhanced mastocyte numbers during HNSCC progression correlated with angiogenesis [434, 435].

Macrophages are usually the most common immune cell population in the TME. Recruitment

of circulating monocyte precursors triggers their differentiation into tumor-associated macrophages (TAM). The CCL-2 chemokine produced by tumor cells may be an important TAM chemoattractant along with other molecules such as TGF, VEGF, PDGF, and macrophage colony-stimulating factor (M-CSF) [134]. The phenotype of TAM resembles M2 cells with pro-tumor functions such as inhibition of Th1 adaptive immunity, production of growth and survival factors (e.g., EGF, IL-6, and IL-8), the secretion of angiogenic factors (e.g., VEGF, PDGF, TGF), and a variety of chemokines. TAM can also induce the degradation and remodeling of ECM, *via* the expression of MMPs, and suppress Th1 adaptive immune responses through the production of immunosuppressive mediators (e.g., IL-10, PGE2, TGF) and chemokines capable of recruiting Th2 cells (e.g., CCL-17, CCL-18, and CCL-22) [436, 437]. Taken together, TAM plays an important role in support of tissue remodeling, tumor progression, and angiogenesis, while suppressing Th1-type adaptive immunity [438].

Tumor cells are challenging opponents. The high level of genetic instability in cancer has proven to be a difficult barrier to overcome with specifically targeted therapies. Tremendous redundancy of the key pathways driving tumorigenesis is another challenge that we have not been able to conquer and, with single target therapies, probably never will. It is necessary to achieve complex knowledge of tumor biology in order to create more effective therapies. If we are not able to hit tumor cells directly, therapies might need to be focused to eliminate malignant collaborators or to interrupt communication between tumor cells and their host cells in the TME. CAFs appear to be genetically stable and might provide reliable targets for immunotherapy [439]. Activated CAFs express some specific molecules such as alpha-smooth muscle actin (α -SMA), fibroblast activated protein (FAP), tenascin-C (TN-C), periostin (POSTN), neuron-glia antigen2 (NG2), PDGFR α/β , podoplanin, and plectin [440]. TME-directed therapies can also target proteins and networks that mediate crosstalk within tumor stroma. For example, targeting Galectin-1 in CAFs inhibited OSCC metastasis

by downregulating MCP-1/CCL2 expression [425]. A more comprehensive approach such as this, in which the complexity of malignancy is taken into account, could produce promising results and provide more effective treatments.

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1.4.3 Role zinku a mědi u HNSCC – review a meta-analýza

Všeobecný trend identifikovat prognosticky významné molekuly se typicky zaměřuje na studium na úrovni genomiky či proteomiky. K odlišnostem nádorových a nenádorových buněk patří ale také alterace metabolismu¹² – typicky vznikající jako konsekvence selekčního tlaku hypoxie v nádorovém ložisku. Diagnostický potenciál je proto možné spatřovat také na úrovni metabolomiky či metalomiky.

Kovy jsou typickými kofaktory širokého spektra enzymů. Od sedmdesátých let dvacátého století vzrostl počet studií poukazujících na fakt, že zejména zinek a měď jsou významnými regulačními mechanismy fyziologických procesů. Ve studiích zejména posledních let je poukázáno na fakt, že tyto kovy zasahují do významných „hallmarks“ – do regulace apoptózy, genové exprese, diferenciaci buněk. Změny v hladinách těchto kovů jsou pak spojeny s rozvojem patologií vč. nádorových^{95 96}.

V meta-analýzách Gumulec *et al.* zaměřujících se na studium hladin zinku⁹⁷ a zinek-vázacího proteinu metalothioneinu⁴² bylo prokázáno, že u spinocelulárních a ovariálních nádorů dochází k signifikantnímu nárůstu exprese zinek-vázacího metalothioneinu v nádorové tkáni a naopak k poklesu této molekuly u hepatocelulárních karcinomů. Signifikantní pokles koncentrace zinečnatých iontů byl prokázán nejen v prostatické nádorové tkáni, ale také u hepatocelulárních nádorů, nemalobuněčných karcinomů plic a u nádorů štítné žlázy. Zajímavým zjištěním práce byl fakt, že u karcinomů prsu byl prokázán signifikantní vzestup koncentrace zinku oproti kontrole v nádorové tkáni.

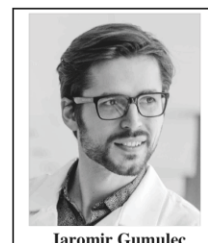
Na tato review navazuje práce Ressenrova *et al.*⁹⁸ na str. 72, zaměřující se výhradně na diagnostický a terapeutický potenciál těchto kovů u HNSCC. Vyjma zinku je v práci fokusováno také na měď a její transportní mechanismy. Bylo zjištěno, že v séru pacientů s HNSCC dochází k signifikantnímu snížení hladiny zinku a ke zvýšení koncentrace mědi a měď-vázacího ceruloplazminu. Naopak, v nádorové tkáni bylo zjištěno signifikantní zvýšení jak zinku (a zinek-vázacího metalothioneinu), tak mědi (viz obr. na str. 83).

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Zinc and Copper Homeostasis in Head and Neck Cancer: Review and Meta-Analysis

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Abstract: Metals are known for playing essential roles in human physiology. Copper and zinc are trace elements closely dependent on one another and are involved in cell proliferation, growth, gene expression, apoptosis and other processes. Their homeostasis is crucial and tightly controlled by a resourceful system of transporters and transport proteins which deliver copper and zinc ions to their target sites. Abnormal zinc and copper homeostasis can be seen in a number of malignancies and also in head and neck cancer. Imbalance in this homeostasis is observed as an elevation or decrease of copper and zinc ions in serum or tissue levels in patients with cancer. In head and neck cancer these altered levels stand out from those of other malignancies which makes them an object of interest and therefore zinc and copper ions might be a good target for further research of head and neck cancer development and progression. This review aims to summarize the physiological roles of copper and zinc, its binding and transport mechanisms, and based on those, its role in head and neck cancer. To provide stronger evidence, dysregulation of levels is analysed by a meta-analytical approach.

Keywords: Zinc, copper, ceruloplasmin, metallothionein, cancer, head and neck tumour, metallomics, meta-analysis.

1. INTRODUCTION

Cancer is a major public health problem and after heart diseases the second leading cause of death. The most common cancers include lung, breast, prostate and head and neck. While a lot of attention is focused on the first three neoplasia, tumours of head and neck are discussed relatively less frequently. Every year, 650,000 patients are newly diagnosed with this cancer and 350,000 of these patients die every year due to this disease. That makes head and neck cancer the sixth most common type of cancer worldwide [1]. In the USA in 2015 there were expected to be 45,780 new cases and 8,650 deaths of patients with cancer of the oral cavity and pharynx [2]. The attention these tumours should attract is based on a very poor prognosis - the five year survival outcomes of patients are approx. 40–50 %.

The vast majority of head and neck cancers are squamous cell carcinomas. Anatomically, cancers of head and neck may arise from the epithelium of any part of the upper aerodigestive tract including the oral cavity, nasal cavity, nasopharynx, oropharynx, hypopharynx, larynx and paranasal sinuses. Alcohol consumption, tobacco smoke [3] and also infection with human papillomavirus (HPV) [4] have a role in the pathogenesis of head and neck cancer.

Early detection is key for the successful therapy of any malignancy and this is also applicable for tumours of the head and neck. However, alternative diagnostic techniques and novel anticancer agents are needed. Diagnosis of this type of cancer has several limitations. There are numerous tumour markers for the most common types of cancers – prostate (prostate-specific antigen PSA), breast (CA15-3, CA27.29, BRCA, HER2, oestrogen receptor ER, CEA and others), lung (EGFR, cytokeratin fragments 21–1, ALK and others). However, no such easy-to-detect substance has been

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described for head and neck cancer. Therefore it is necessary to dig deeper into the molecular mechanisms of cancer and focus on mechanisms that can be targeted by specific therapies.

Since the Seventies, numerous pieces of research have pointed out the fact that various metals – notably zinc and copper – are important regulators of physiological and pathological processes. However, the expansion of molecular biology has tended to downplay the role of metallomics, but in recent years it has been pointed out that metals are involved in crucial processes such as apoptosis, gene expression or cell differentiation. Consequently it is not surprising that an alteration of the metal-level-regulators may lead to the development of various diseases, including cancers.

Recently, it has been found that the levels of zinc ions and zinc-binding protein metallothionein differ in head and neck tumours compared to other epithelial malignancies – prostate, breast, gynaecological, lung and others [5, 6].

This review aims to summarize the physiological roles of copper and zinc and based on those, the role of these ions in head and neck cancer will be outlined, as well as possible diagnostic and therapeutic implications for this disease.

2. PHYSIOLOGICAL ROLE OF ZINC AND COPPER

Up to 10 % of human proteome encodes zinc-binding proteins (ca. 2800 proteins). [7]. Zinc plays an essential role in catalysis and gene expression, it is also involved in apoptosis, signalization, regulation of cell differentiation, proliferation [8, 9] and is also a fundamental element for the immune system [10]. Zinc plays an essential catalytic function in hundreds of enzymes from all classes of enzymes [11]. Zinc finger proteins are involved in the initiation of transcription and gene expression and are typical for having zinc finger transcription motifs which interact with DNA, RNA or proteins [8, 12].

Copper is also an essential component in gene expression, cellular respiration, free radical defence, synthesis of melanin pigment, connective tissue biosynthesis, erythropoiesis and cellular iron metabolism and also affects enzyme activity as an allosteric component of catalytic centres of several cuproenzymes. Copper plays a role in oxidation–reduction reactions due to its ability to cycle between oxidised, Cu(II), and reduced, Cu(I) states [13, 14].

Zinc and copper are trace elements heavily dependent on each other. In this chapter, physiological mechanisms will be described, ranging from copper and zinc uptake to transport to the target sites in the body.

2.1. Dietary Requirements, Daily Intake and Toxic Levels

Zinc and copper are both trace elements required for survival, thus deficiency or overload has fatal consequences. It is therefore necessary to maintain dietary requirements to avoid these conditions which can lead to various types of diseases including cancer.

2.1.1. Zinc

USDA reports the daily reference intake to be 11 mg/day and 8 mg/day for males and females, respectively. Although the majority of the Western population is without signs of deficiency [15], rare cases of zinc deficiency illustrates the role of this transition metal: it leads to growth retardation, sexual and skeletal maturation delay, behavioural changes [16] and dysregulation of immunity functions [17]. On a cellular level, it can have a pro-apoptotic effect [18] and upregulates p53 expression [19]. However, low intracellular zinc leads to increased oxidative DNA damage and reduces the ability of p53, NF- κ B, and activator protein 1 (AP1) to bind to consensus DNA sequences [20]. Zinc deficiency might be connected with the development of cancer [19]. However, not only zinc deficiency, but also zinc overload can be connected with an increased risk of neoplastic formation [21]. These consequences are discussed in a later part of this text. Upper limits of zinc intake were established as 35 and 45 mg/day for female and male, respectively. High zinc intakes can also cause dysregulation of other trace elements' metabolisms, for example copper deficiency [16].

2.1.2. Copper

The average normative requirement for healthy adults is 0.9 mg/day [16]. Similarly to zinc and according to numerous studies, copper deficiency is a rather marginal problem [13, 22]. It manifests with haematologic abnormalities such as anaemia and neutropenia [23, 24]. High zinc and iron intakes can result in copper deficiency [23, 25, 26]. Patients with Menkes disease have systemic copper deficiency [27] resulting from inherited mutations in the gene encoding Menkes copper-translocating P-type ATPase – ATP7A [28]. Patients with Wilson's disease accumulate copper in tissues, especially in the liver [29]. This is due to a mutation in copper-translocating P-type ATPase – ATP7B

[28]. P-type ATPases are described in a later part of this text.

The upper limit to the safe range of population means the intake of copper for adults has been set at 12 mg/day for men and 10 mg/day for women [16]. The richest dietary sources of copper are considered to be viscera – especially liver, shellfish, nuts, seeds and grains. The major source of excessive copper is mostly from drinking water [30]. On the other hand, fruit, vegetables and dairy products tend to have a small amount of copper [13, 31]. The amount of copper in foods depends on the soils on which the food is grown and the contamination before and after the food reaches the market place [31].

2.2. Intestinal Absorption

Copper(I) and zinc(II) received in the diet are transported through the enterocytes to the portal circulation with the cooperation of various metal-binding proteins and transporters. Here we describe the mechanism of copper(I) and zinc(II) uptake from the lumen of the intestine through the membrane of the enterocytes.

2.2.1. Zinc

Exogenous zinc(II) is absorbed *via* the small intestine. The highest rate of zinc(II) absorption is in the jejunum compared to the other parts of the small intestine [32]. A negative regulatory mechanism exists: increased dietary zinc(II) uptake causes decreased zinc(II) absorption and increased excretion and vice versa [33]. In addition, intestinal absorption is stimulated with the presence of glucose [32].

Because zinc(II) cannot pass through membranes freely, special transport proteins evolved. These transporters belong to the ZIP and ZnT families and are discussed in a subsequent chapter of this review. Although the majority of these transporters are localised on all cells, some are mentioned here due to their particular role in enterocytes.

The major transporter mediating the transfer of zinc(II) to enterocytes is ZIP4. It is a protein located on the apical side of enterocytes (see Fig. 1) [34, 35]. In the case of zinc(II) deficiency, the gene expression of this transporter elevates accordingly [34]. Another zinc(II) transporter ZIP5 has an antagonistic function, compared to ZIP4 (see Fig. 1). Although this transporter is expressed ubiquitously, in the intestine it is located on the basolateral surface of the enterocytes and contrary to ZIP4 is degraded during periods of dietary zinc(II) deficiency [36, 37]. Along with the knowl-

edge of the antagonistic roles of ZIP4 and ZIP5, these proteins might play an important role in the control of zinc(II) homeostasis, presumably by removing zinc(II) from the blood in periods when the dietary zinc(II) intake is abundant [36]. In this way the above-mentioned autoregulatory intake mechanism is maintained.

ZnT-1 transporter is also localized on the basolateral surface of enterocytes [38]. It controls zinc(II) efflux from the enterocytes into the circulation (see Fig. 1) [39]. Zinc(II) supplementation in rats led to the elevated expression of ZnT-1 protein and elevated levels of ZnT-1 mRNA [38].

Zinc(II) is ubiquitously localised in all the tissues and fluids of the human body. The total amount of zinc(II) in a healthy human body is approximately 2 g [16]. Most of the zinc(II) can be found in muscle and bone tissue. The highest concentrations of zinc(II) in humans can be found in the liver, kidney, pancreas, eye, prostate, prostatic secretions and sperm [9, 33].

2.2.2. Copper

After digestion of food in the stomach and duodenum, copper is absorbed in the small intestine [13]. The gastrointestinal system of humans is able to absorb 30–40 % of copper from the diet [40]. When dietary copper intake is high, more copper is absorbed, but copper uptake is less efficient. When the dietary copper intake is low, a higher percentage of copper is absorbed and this metal is absorbed more efficiently [41]. As previously mentioned, high zinc intake can result to copper deficiency. This is due to an antagonistic effect of zinc on copper absorption. [42, 43].

Metallothionein (MT) is an important zinc binding protein which has however a higher binding affinity to copper than to zinc [44, 45]. Larger zinc intakes induce MT synthesis, therefore if MT is present in excess it binds copper to a significant extent. This complex of metallothionein and copper is then excreted which in turn can lead to copper deficiency when this happens for a long period of time [46, 47].

Similarly to zinc, copper is also transported to cells by special transporters. Copper(II) is present in oxidized form in the intestinal lumen. Metalloreductases from the STEAP metalloredutase family (genes *STEAP1*, *STEAP2*, *STEAP3*) reduce copper(II) to copper(I) state. This reduced form is required for transport through the membrane. STEAP proteins also stimulate copper(I) uptake into the cells *in vitro* [48].

Transport of copper(I) to the intestinal cells happens *via* Ctr1 transporter (see Fig. 1) [49].

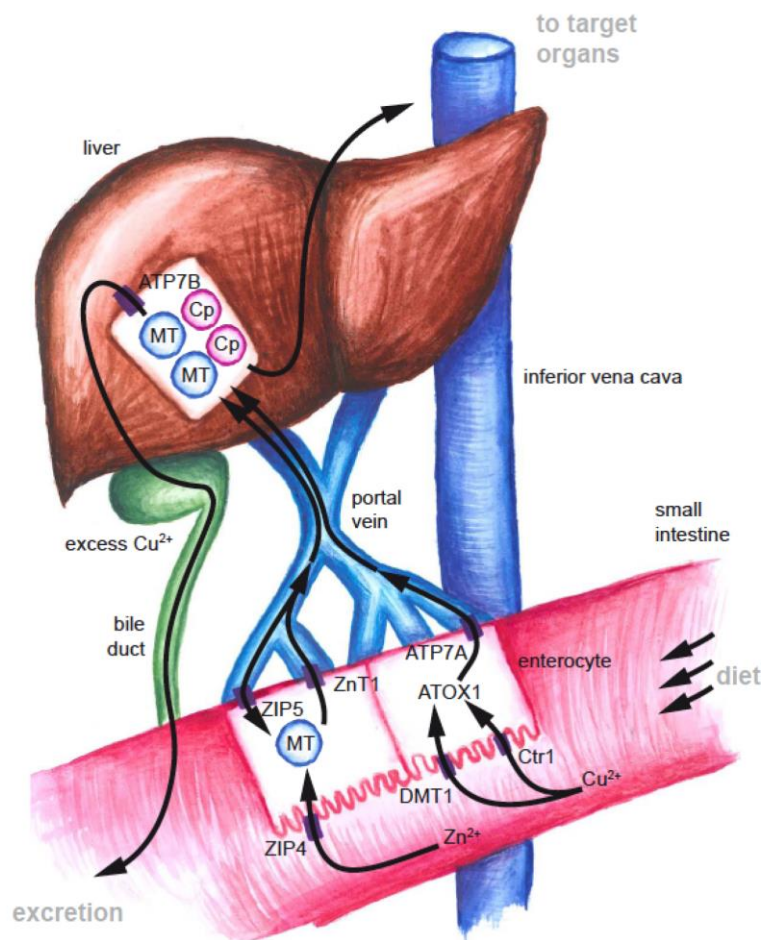


Fig. (1). Copper and Zinc homeostasis in body. Zinc(II) from the diet enters the enterocytes *via* ZIP4 transporter. Within the cell binds to the Metallothionein (MT) – mobile pool and to the Zn-binding proteins- immobile pool. This intracellular zinc binding might be applicable to every cell in the body, not only to the enterocytes. Zinc efflux from the enterocytes to the portal circulation is provided by the ZnT1 transporter. ZIP5 transporter is responsible for zinc influx from the blood to the cell. Copper(I) enters the enterocytes *via* DMT1 and Ctr1 transporters (Steap reductase is not shown). Within the enterocytes binds to Atox1 which delivers copper to the Golgi network to ATP7A. ATP7A translocates from the Golgi to the basolateral site of the cell and promotes copper(I) efflux to the portal circulation. In the plasma, zinc(II) binds to the α 2-macroglobulin and albumin. Albumin delivers zinc(II) to the liver from which is distributed to the target organs. Copper(II) in the plasma is present in oxidised form and carried by albumin and transcuprein to the liver where copper(I) incorporates to the ceruloplasmin (Cp). Ceruloplasmin is then transported by circulation to the target organs. Excess copper(I) is secreted into the bile by ATP7B translocation to the apical site of hepatocytes and excreted from the body.

Another type of copper(I) uptake pathway in enterocytes is *via* DMT1 (known also as DCT1 and Nramp2) metal transporter (see Fig. 1). DMT1 transporter is located on the apical side of the intestinal cells. It's the only transporter responsible for iron absorption from the lumen of the intestine. Apart from iron, this transporter is capable of the transport of other

divalent ions e.g. Cd^{2+} , Mn^{2+} , but it has been discovered that DMT1 is also a physiologically relevant copper(I) transporter. According to Arredondo at least 50 % of copper(I) transport is *via* DMT1 transporter. Also uptake competition between iron and copper(I) is observed; iron uptake is depressed when extracellular copper is elevated and *vice versa* [50, 51].

Johnson *et al.* found out that men had significantly lower copper absorption (64 %) than women (71 %), but this difference disappeared at the age of 60 and older. The body of a healthy 70 kg human adult contains 80–120 mg of copper [16, 31, 52] and according to Linder, there is 10 mg of copper in the liver, 8.8 mg in brain and 6 mg in blood [53].

2.3. Serum Transport

After delivery to the bloodstream, free zinc(II) and copper(II) ions are maintained in the pico- to nanomolar range, because of its reactivity the majority is bound to special carriers delivering ions to the target sites of the human body. Due to differential mechanisms involved in serum transport, zinc and copper are discussed in separate chapters.

2.3.1. Zinc

Plasma concentration of zinc(II) ranges from 12–16 μM [17]. However, zinc(II) plasma levels are significantly higher in the morning than in the afternoon. Zinc(II) plasma levels of men are significantly higher than in women, but this varies with age. Mean serum zinc(II) levels in men change from low in childhood, increase during adolescence and progressively decrease in the older adult. In females, this trend is much less pronounced [54, 55].

The major zinc(II) binding protein in plasma is albumin which binds 60 % of plasma zinc(II).

Albumin is synthesized in the liver, then released to plasma where it acts like a carrier for poorly water-soluble molecules, metal ions and other compounds. Albumin is important for the maintenance of the oncotic pressure and is an important antioxidant [56]. Decrease of zinc(II) concentrations in plasma in the elderly population might be due to an increase in oxidative stress in ageing people when zinc ions are being displaced from albumin [57, 58]. In terms of albumin binding, other conditions such as inflammation play a distinctive role in zinc(II) depletion [58].

Following albumin, 30 % of plasma zinc(II) is bound by α_2 -macroglobulin and the remaining 10 % is bound to transferrin [59]. After absorption of zinc(II) from the intestine into the circulation, albumin delivers zinc(II) to the liver and then it is transported to the target tissues (see Fig. 1) [60].

2.3.2. Copper

Plasma copper(II) is oxidized. Plasma or serum copper(II) in healthy individuals is in the range 0.8–1.2

$\mu\text{g/ml}$ [16]. Its levels increase with age attaining a maximum in the 60's and then slowly decreasing [31].

Following absorption, copper(II) is transported in serum *via* albumin and transcuprein [61] to the liver where it is incorporated into liver synthesized cuproenzyme ceruloplasmin which ultimately carries six copper atoms strongly coupled in the molecule. Ceruloplasmin is then secreted to plasma (see Fig. 1) [62–64]. Ceruloplasmin is a multifunctional major copper-containing protein, binding more than 95 % of total serum copper(II) [16, 31].

The remaining amount of copper(II) is bound to albumin and to a lesser extent, amino acids [31]. Apart from copper(II)-transport abilities, [65] ceruloplasmin also acts as ferroxidase, accordingly it also plays an important role in the metabolism of iron [66, 67]. Ceruloplasmin is an antioxidant because of its superoxide radicals-scavenging activity [68] and is also an acute phase reactant so its plasma levels are elevated during pregnancy, infections and inflammation [68, 69]. Ceruloplasmin is also involved in tissue angiogenesis [70] and coagulation [71].

2.4. Cellular Transport and Intracellular Buffering

In a similar way to plasma, free zinc(II) and copper(I) ions are maintained in very small amounts in cytosol. This is due to transporters and transport proteins within the cells which can be considered as a “shuttle service” due to its ability to provide influx, intracellular delivery and efflux of copper(I) and zinc(II) depending on the needs of the individual cell.

2.4.1. Zinc

Zinc(II) is transported through the membranes *via* zinc transporters. There are two zinc-transporter families – ZIP (SLC39) family, responsible for the influx of zinc(II) to the cytoplasm from the outside of the cell or from the organelle lumen and ZnT (SLC30) family reducing the cytoplasmic zinc(II) by efflux from the cytoplasm to the outside of the cell or to the organelles [72, 73]. Both of these families are widespread, from prokaryotic to eukaryotic organisms, including bacteria, fungi, plants and mammals [74–77]. Although they have different numbers of transmembrane domains, both ZIPs and ZnTs share similar characteristics in a long loop region with histidine rich motifs. It's supposed that these motifs might be liable for binding of the metal during transport [72, 76]. Members of both of these transporter families are described in a number of reviews [77–79].

ZIP transporters were first described in *Saccharomyces cerevisiae* [80]. ZIP transporters have 14 members in humans [74, 77]. It is assumed that ZIP transporters have eight transmembrane domains [72, 76]. ZnT transporters were first described by Nies and Silver [81], have 10 members in humans [82] and can be divided into three subgroups, but only II and III are in mammals [74]. It is assumed that most of the transporters from the ZnT family have six transmembrane domains [38, 83]. ZIP and ZnT transporters play an important role in various diseases including cancer; these mechanisms are discussed in a subsequent chapter.

The majority, 99 %, of total body zinc(II) is localized intracellularly and bound or associated with proteins and for that reason free intracellular zinc(II) is very low – approx. < 0.01 % [9, 84] and forms a non-protein bound zinc pool. 90 % of total zinc(II) in intracellular compartment binds tightly to the large number of zinc-binding metalloproteins, metalloenzymes and nucleoproteins where zinc(II) is a structural component or cofactor. This tightly bound zinc(II) forms an immobile, nonreactive zinc pool [8, 85]. The rest, about 10 % of total intracellular zinc(II) binds loosely to zinc ligands, such as ZnHistidine, ZnCysteine, ZnAspartate, ZnGlutamate, ZnCitrate and mainly to metallothionein. This fraction of zinc(II) binding proteins acts as a mobile, reactive zinc(II) pool (see Fig. 1) [85] and forms a buffering system which protects zinc(II) from unlimited and unspecific binding with “wrong” metalloproteins. Without this buffering system, many metalloproteins would be dysfunctional and potentially cause cell death [86]. Nevertheless, according to the Maret study, to date there is still a lack of evidence regarding the clear chemical definition or molecular basis of terms such as: labile, mobile, rapidly exchanging/highly exchangeable or loosely bound zinc. Consequently this author proposes to designate the pool as “free zinc” without dependence on the coordination environment of the zinc ions to proteins [87].

Metallothionein (MT) – the major mammalian endogenous cysteine-rich zinc-binding metalloprotein – binds three divalent metal ions with its β -domain and four divalent metal ions with α -domain [88]. MT buffers zinc(II) in cytosol and thus contributes to the maintenance of very low free zinc(II) levels [84]. It transfers zinc(II) to enzymes and this distribution is possible in both directions – from enzymes to thionein (the apoform of metallothionein) and from metallothionein to the apoenzymes [89]. It has been demonstrated that MT also protects cells against oxidative stress by quenching radicals and the expression of metallothionein is in-

creased by ROS and oxidative stress [90-92]. When there is an excess of zinc(II) within the cell, MT (and also efflux transporter ZnT-1) expression is induced by zinc(II) itself which binds to the metal regulatory transcription factor 1 (MTF-1) [93]. MTF-1 then translocates to the nucleus where it binds to metal responsive elements of metallothionein and ZnT-1 promoters and starts gene expression [94].

2.4.2. Copper

Copper(I) transport into the cell is provided by a family of Ctr transporters [95]. In a human genome, genes SLC31A1/CTR1 and SLC31A2/CTR2 are encoding proteins Ctr1 and Ctr2. Both genes are located in 9q31/32. [96, 97]. Members of Ctr family transporters are structurally conserved from yeast to mammals [98].

Ctr1 and Ctr2 have copper(I) and cis-platin as predominant substrates [97, 99], their expression is age dependent [100] and are expressed in all tissues and organs, with the highest expression in liver [96].

Ctr1 transporter is responsible for high-affinity copper(I) uptake (see Fig. 2) and was first described in *Saccharomyces cerevisiae* in 1994 [95]. The physiological role of copper(I) transporter Ctr1 was studied in mice with inactivated CTR1 genes by targeted mutagenesis. In mice with homozygote deletion of CTR1 (with complete deficiency for Ctr1), early embryonic lethality was observed and mice died *in utero* in mid-gestation, suggesting that Ctr1 copper transporter plays an important role in mammalian embryonic development [101]. Ctr1 is also important for copper(I) uptake in the brain [101] and spleen [102]. Overexpression of Ctr1 leads to enhanced copper(I) uptake in mice [103, 104].

Localization of Ctr1 transporter depends on the cell type. Some carcinoma cell lines (HeLa, A549, H441, HepG2) have Ctr1 located in cytoplasmic vesicular compartments around the nucleus [105]. Localization of Ctr1 transporter in the polarized epithelial cells of the intestine is still unclear and results of various studies are contradictory. Expression of Ctr1 occurs mainly near the apical membrane [106, 107]. Ctr1 in enterocytes is essential not only for dietary copper(I) uptake and delivery to peripheral tissues, but is essential for prevention of copper(I) hyperaccumulation in intestinal epithelial cells. This was demonstrated in CTR1 knockout mice [49]. Another study detected Ctr1 transporters at the basolateral surface of enterocytes suggesting that it mediates the uptake of copper from the bloodstream to the enterocytes [51].

Another member of the Ctr family is Ctr2 transporter. Ctr2 transporter is a low-affinity copper(I) transporter and is localized in endosomes, lysosomes [108] and in plasma membrane [109]. However its function in copper(I) uptake to the cells is poorly understood.

Free copper(I) within the cells is toxic and this happens particularly when there is excess copper(I) in the organism. In that case free copper(I) starts to accumulate, reacts and makes highly reactive hydroxyl radicals which are the most powerful oxidizing radicals that can appear in biological systems and can damage lipids, proteins and nucleic acids [110-112]. However, similarly to zinc(II), there is less than 0.01 % of free cellular copper(I) in the cell cytoplasm [113] because only bounded copper(I) is found within cells. Inappropriate copper(I) binding with other cellular components must also be prevented. For that reason, a highly specialized system of copper(I) chaperons exists inside the cells. Chaperons deliver copper(I) to intracellular targets [114-116]. There are three recently discovered main copper-delivery pathways connected with chaperons – Copper transport protein ATOX1 (Atox1), Copper chaperon for superoxide dismutase (CCS) and Cytochrome c oxidase copper chaperone (Cox17). Here their functions are briefly described.

Copper transport protein ATOX1 (Atox1) (gene - *ATOX1*) [117] is a human homologous copper chaperone first discovered in *saccharomyces cerevisiae* as metal homeostasis factor ATX1 (gene - *ATX1*) [118]. When copper(I) crosses the plasma membrane, it binds to Atox1 chaperon and is delivered to membrane-bound copper-transporting P-type ATPases in trans-Golgi network [114, 117]. P-type ATPases accept copper(I) from Atox1 and transport it through the membrane into the lumen of Golgi where copper(I) incorporates into various cuproenzymes, and is then delivered to the secretory pathway [119, 120]. P-type ATPases are located in trans-Golgi membrane and their main role is to supply copper(I) to cuproenzymes when there is an excess of copper(I), P-type ATPases can translocate to the basolateral (ATP7A) or apical (ATP7B) membrane of the cell where they induce copper(I) excretion to avoid its accumulation [119, 121].

In different organs, different types of P-type ATPases occur. Copper-transporting ATPase1 (ATP7A) (gene - *ATP7A*) is expressed in most of the tissues of human body but not in the liver. It is suggested that after copper(I) crosses the membrane of enterocytes and Atox1 delivers copper(I) to the Golgi, ATP7A is responsible for copper(I) transports to the portal circulation [122] by translocating from the Golgi

to the basolateral surface of the cells (see Fig. 1) [123]. Copper-transporting ATPase2 (ATP7B) (gene- *ATP7B*) type is expressed primarily in the liver but it can also be found in the placenta and kidney [124]. After copper(I) from the portal circulation crosses the membrane of hepatocytes in liver, it binds to Atox1 in cytosol. Atox1 delivers copper(I) to ATP7B. ATP7B translocates from Golgi to the vesicular transport system and delivers excess copper(I) for secretion into the biliary canaliculi at the apical surface of the cells (see Fig. 2 and 2) [30]. Atox1 also delivers copper(I) to the liver for synthesis of cuproenzyme ceruloplasmin (see Fig. 2) [120].

Copper chaperon for superoxide dismutase (CCS) (gene - *CCS*) delivers copper(I) to Cu/Zn Superoxide dismutase (SOD1) (Gene - *SOD1*) which scavenges superoxide anions (see Fig. 2) [125, 126]. CCS chaperon consist of three protein domains. Domains I and III contain metal binding motif. The central domain II is strongly homologous to its target SOD1, interacts with SOD1 and secures the enzyme during copper(I) insertion. SOD1 is normally homodimer, but forms heterodimer with CCS during copper(I) transfer [127-130]. SOD1 is localized primarily in the cytosol [131], but can be found in the intramembrane space of mitochondria together with its CCS chaperon (see Fig. 2). SOD1 enters the intramembrane mitochondrial space immature and is converted to an active holoenzyme inside the mitochondria [132] where it protects mitochondria from oxidative damage [133, 134]. Even though a CCS chaperon is necessary for SOD1 activation in humans, activation of SOD1 without copper(I) CCS chaperon was observed in yeast [135, 136].

Another copper(I) chaperon is Cytochrome c oxidase copper chaperone (Cox17) (gene- *COX17*) that delivers copper(I) to the mitochondria where is needed for cytochrome c oxidase (Gene - *CCO*) [137]. Cytochrome c oxidase is a large transmembrane protein complex localized in the inner membrane of mitochondria and acts as a dimer [138]. Cytochrome c oxidase is known as Complex IV – the last enzyme of the eukaryotic respiratory chain, where it catalyses the reduction of molecular oxygen to water [139-141]. Mitochondrial genome encodes three structural subunits from a total of 13 subunits. Cytochrome c oxidase subunit 1 Cox1 (gene - *COX1*) and cytochrome c oxidase subunit 2 Cox2 (gene - *COX2*) are encoded by mitochondrial genome. Subunit Cox1 contains copper(I) binding centre CuB and Cox2 subunit contain copper(I) binding centre CuA [142]. In these binding centres copper insertion occurs [137, 143, 144].

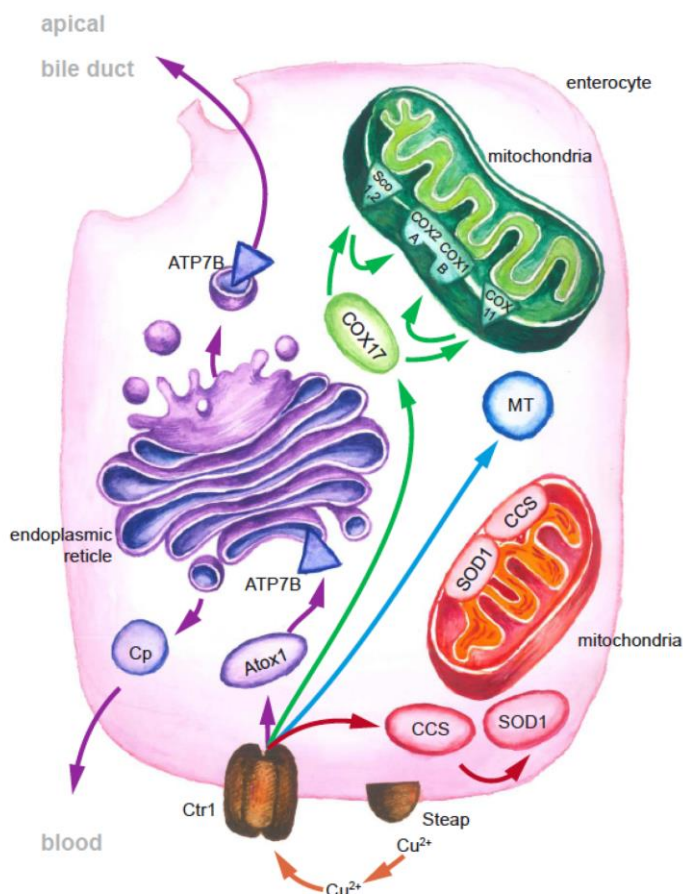


Fig. (2). Copper delivery pathways in hepatocyte in liver. Copper(II) from portal circulation is reduced to copper(I) by Steap reductase and imported to the hepatocyte *via* Ctr1 transporter. **Purple pathway** – copper(I) binds to Atox1 chaperon which delivers copper(I) to the ATP7B in trans-Golgi network. Copper(I) is then incorporated to the ceruloplasmin (Cp) which exits the cell to the blood plasma. When there is excess of copper, ATP7B translocates to the vesicular transport system and delivers copper(I) for secretion to the apical site of hepatocyte. **Green pathway** – copper(I) is distributed by chaperon Cox17 to cytochrome c oxidase in the inner membrane of mitochondria. Cox17 transmit copper(I) to Sco1 and Sco2. Those insert copper(I) to the CuA (A) binding centre in the Cox2 subunit of cytochrome c oxidase. Cox17 also transmits copper(I) to Cox11 which delivers copper(I) to the CuB (B) binding centre in the Cox2 subunit of cytochrome c oxidase. **Blue pathway** – metallothionein (MT) binds copper(I) when copper(I) resources are low and acts as a copper(I) reserve for chaperones. **Red pathway** – CCS chaperon delivers copper(I) for Cu/Zn Superoxid dismutase (SOD1) which is localized in cytosol or in the intramembrane space of mitochondria together with CCS.

In mammals, a large array of accessory proteins is involved in the insertion of copper(I) to the copper(I) sites of cytochrome c-oxidase. After crossing the membrane of the cell, copper(I) binds to the Cox17 chaperon that delivers copper(I) to the Sco1, Sco2 and Cox11 mitochondrial inner membrane proteins [137, 145]. Sco1 and Sco2 cooperate in the mediation of transfer of copper(I) to the Cox2 subunit and thus to its binding centre CuA [143, 146]. Cox11 is delivering copper(I) to the Cox1 subunit and its copper(I) centre

CuB (see Fig. 2) [144]. In yeast, cells lacking Cox17 gene have a respiratory deficiency (Cytochrome oxidase deficiency) on account of the inability to create a functional cytochrome oxidase complex. However overexpression of protein Sco1 was able to suppress mutations in Cox17, it was also found that cells lacking Sco1 have respiratory deficiency [137]. In contrast to yeast, in humans, both Sco1 and Sco2 are necessary for the mitochondrial respiratory chain.

Metallothionein also plays a role in copper(I) homeostasis and might be considered a copper(I) chaperon although in particular it responds to zinc(II) status [147]. MT binds excess copper(I) in the liver in Wilson's disease [148] but in healthy humans MT does not frequently bind copper(I). However in the case of copper(I) deficiency when copper(I) resources in the liver are low, MT starts to bind copper(I) and acts as a copper(I) reserve and provides copper(I) to chaperones (see Fig. 2) [149].

3. ZINC AND COPPER IN HEAD AND NECK CANCER

The previous chapter outlined an important role of both zinc and copper ions. As a consequence of dysregulation of those physiological mechanisms, changes in serum and/or tumour tissues occur immediately. Pathophysiological consequences of the dysregulation in neck spinocellular cancers will be discussed in association with altered levels of these metals and their binding proteins.

3.1. Serum/Tissue Levels Dysregulation

Zinc and copper are involved in many essential biological processes. Dysregulation of these processes is also connected with cancer, therefore zinc and copper mechanisms and dysregulation in zinc and copper levels in plasma or cancerous tissue are the subject of many studies. It has been revealed that in patients with a combination of low zinc and high copper levels the risk of cancer mortality is increased significantly [150]. This link between mortality and the combination of low zinc and high copper levels is however not yet fully explained. Several hypotheses have been published to date; The significant role of immune system dysregulation together with oxidative damage is anticipated. Low zinc levels are associated with a depression of immune function [151], which in turn through cytokine modulation promote inflammation, angiogenesis and metastasis [152]. High copper levels, on the other hand, are associated with oxidative damage and may promote inflammatory processes [153].

A number of other studies refer to dysregulated serum and tissue levels of copper and zinc in various types of cancer. Here follows a summary of recent studies describing altered levels of copper and zinc as well as metallothionein and ceruloplasmin in patient's sera or tumour tissue. To facilitate this a meta-analytical approach was used instead of referencing simple mentions of metal levels.

3.1.1. Meta-Analysis Methodology

The following databases were searched for relevant studies: Web of Science (Science citation index expanded 1945 to March 2016), Pubmed (Medline 1968 to March 2016) search engines and in cited bibliographies of cited references. The following keywords were used (oral OR pharyn* OR "head and neck" OR laryn*) AND (epithel* tissue / serum OR plasma OR blood) AND (cancer OR neoplas* OR tumour OR tumour) AND (zinc OR zn / copper OR Cu / metallothionein OR MT OR MT1 OR MT2 / ceruloplasmin OR Cp). Data in the following formats were accepted to the meta-analysis: means, standard deviations and sample size, or means sample size and p value, or sample size, p value and effect direction in case of continuous data and frequency of staining positivity/negativity in tumour and non-tumour tissue in case of dichotomous data. Only full text articles were included. The following criteria were used for exclusion: (1) Studies with less than 15 participants, (2) no histological definition, (3) no full text, (4) conference proceedings and abstracts, (5) poor/limited definition of controls. If similar data was found in more than one study, the study with more extensive data set was included. The eligibility of the studies was assessed by two authors (J.G. and A.R.). Standardized mean difference with 95 % confidence intervals was used as a point estimate. To assess heterogeneity across studies, Higgins I^2 , describing the percentage of variability in point estimates was calculated. When significant heterogeneity was observed ($I^2 \geq 60$), a random effects model was used. When the number of studies within a subgroup was higher than 4, publication bias was evaluated using funnel plots and two-sided Egger's test. Funnel plots whose Egger's test $p < 0.05$ are asymmetric. Comprehensive Meta-analysis version 2 (Biostat, Englewood, NJ, USA) was used for analysis.

A total 41 studies were included in the final analysis after the elimination of duplicates and articles not satisfying the selection criteria. The set of 41 studies includes 1212 blood samples of patients with tumours and 1324 healthy individuals' blood, 469 tissue samples of tumours and 205 tissue samples of non-tumour tissue. In the largest study, 215 patients were involved. On the other hand, the smallest study involved 15 patients. The year of the studies varied between 1979 to 2015.

3.1.2. Zinc Serum Levels

Lower serum zinc levels are observed in chronic inflammatory or infectious diseases. This is due to serum zinc redistribution into the liver during the acute phase

response caused by elevated production of proinflammatory cytokines.

Zinc serum decrease is observed in most types of malignancies. A total 13 studies were identified with a total 501 cancer cases and 697 control subjects. 11 studies demonstrated a significant decrease of serum zinc [154-164] and two studies demonstrated no significant difference [165] and elevation [166]. Due to a significant level of heterogeneity, $I^2 = 97.68\%$, a random effects model was used. Using this model, a significantly lower serum zinc was observed in cancer patients, standardized mean difference -2.30, 95 % confidence interval, CI, = -3.54 to -1.47 (Fig. 3, Table 1). Publication bias was detected using Egger's test with p value 0.01 (two-tailed).

It was observed that serum zinc levels are lower in those patients who do not respond to therapy and die within 12 months, when compared to those who survive [161].

3.1.3. Zinc Tissue Levels

There were four studies identified regarding tissue zinc levels in patients with head and neck neoplasms [154, 167-169]. A total 68 cancer cases and 54 healthy individuals were included. All four studies presented a significant elevation in cases compared to controls, so significant difference was determined, standardized mean difference 3.97 (95 % CI 1.72 to 6.22) using a random effects model (Fig. 4, Table 1). No publication

bias was observed using Egger's two tailed test ($p = 0.17$). No connection between tissue zinc levels and stage of the tumour was found in the studies.

3.1.4. Copper Serum Levels

There were ten studies included regarding copper levels in serum including 524 cancer cases and 520 healthy individuals [154, 155, 157, 159, 163-166, 170]. A significant serum copper elevation was determined by all the studies and using a random effects model with a positive standardized mean difference 2.36 (95 % CI 1.62 to 3.10) (Fig. 3). Using Egger's two tailed test, a publication bias was observed with $p = 0.04$.

Garofalo *et al.* found a correlation between tumour staging and copper serum levels, which were significantly higher in advanced stages of head and neck cancer [171]. Mali *et al.* investigated the correlation between serum copper levels and response to radiotherapy in patients with cancer, 71 % with head and neck squamous cell carcinoma. Patients had elevated copper serum levels (compared to controls) that decreased during radiotherapy. Nevertheless, the patient's post-treatment copper levels were higher than those of healthy controls, but nearly normal in those with complete response to the treatment [170]. The same effect of copper decline in serum during and after radiotherapy was also observed in nasopharyngeal carcinoma [172].

Table 1. Summary of metal levels and major metal-binding proteins in cancer cases and healthy individuals displayed as a standardized difference in means. CI, confidence interval.

Biological material	Detected compound	Effect size and 95% confidence interval			Heterogeneity			Model used
		Number Studies	Std. diff. in means (95% CI)	P-value	Q-value	P-value	I^2	
Serum/Plasma								
	Zinc	13	-2.5 (-3.54 to -1.47)	< 0.001	516.61	< 0.001	97.68	random effects
	Copper	10	2.36 (1.62 to 3.10)	< 0.001	228.51	< 0.001	95.62	random effects
	Ceruloplasmin	5	1.86 (0.38 to 3.33)	0.014	127.57	< 0.001	96.86	random effects
	Albumin	2	-0.62 (-1.86 to 0.62)	0.328	13.64	< 0.001	92.67	random effects
Tissue								
	Zinc	4	3.97 (1.72 to 6.22)	0.001	36.93	< 0.001	91.88	random effects
	Copper	2	2.08 (1.16 to 3.00)	< 0.001	2.61	0.106	61.67	random effects
	Metallothionein	6	1.60 (0.99 to 2.21)	< 0.001	39.19	< 0.001	82.14	random effects

3.1.5. Copper Tissue Levels

Only two studies were identified with a total of 55 cases and 30 controls [154, 169]. The random effects model revealed a significantly higher copper level in tumorous tissues compared to non-tumour counterparts (standardized mean difference 2.08, 95 % confidence interval 1.16 to 3.00. However, no correlation between the grade of malignity and copper tissue levels was found [169].

3.1.6. Ceruloplasmin Serum Levels

The copper serum fraction which is not bound to ceruloplasmin is lower than 1.3 $\mu\text{mol/l}$ of copper [173]. Therefore an increase in serum copper levels can be also detected by detecting the serum concentration of ceruloplasmin [174], its major binding protein.

A total of five studies regarding serum ceruloplasmin level in cases and controls was determined. With a total of 187 cases and 167 controls [159, 175-178], no significant difference was determined in two publications [176, 177] while the remaining ones showed a significant increase. Due to significant heterogeneity, $I^2 = 96.86\%$, a random effect model was used and revealed a significant elevation of this transport protein, standardized mean difference 1.86, 95 % CI = 0.38 to 3.33. With regard to publication bias, none was determined using Egger's test, $P = 0.07$.

In a long term follow-up study, patients with recurrent laryngeal cancer had a significantly higher ceruloplasmin serum level after and even before radiotherapy than patients without recurrent cancer. Patients who died within 5 years of having radiotherapy had a significantly higher ceruloplasmin level than those who lived more than 5 years [177]. In patients with nasopharyngeal carcinoma the serum level of ceruloplasmin was up to fifty fold higher compared to control subjects [179]. Ceruloplasmin was also found to be significantly increased in the serum of patients with oral cancer in both sexes [159].

3.1.7. Ceruloplasmin Tissue Levels

No data for meta-analytical approach could be used since there is only one study revealing ceruloplasmin expression in head and neck cancerous tissue.

Lokamani *et al.* demonstrated that ceruloplasmin is not expressed in normal squamous epithelium at all, suggesting that ceruloplasmin provides iron, necessary for proliferation of tumour cells, by tumour cells synthesizing ceruloplasmin by themselves [180]. The essential role of ceruloplasmin in regulation of cellular iron is demonstrated in patients with the hereditary dis-

ease aceruloplasminemia [181]. Strongly enhanced expression of ceruloplasmin was also observed in malignant areas of nasopharyngeal lesions compared to normal nasopharyngeal tissue [179]. Also, a study on human breast and colon cancer cell lines showed a three fold higher gene expression of ceruloplasmin in comparison to normal rat liver [182].

3.1.8. Metallothionein Serum Levels

Due to the fact that MT is an intracellular-binding protein, there is a lack of studies focusing on this issue. According to Sochor *et al.* study, the highest levels of MT were determined in the oral squamous cancers while the lowest MT level was determined in tumours of the oropharynx. No associations were observed between MT levels and tumour stage and grade [183].

3.1.9. Metallothionein Tissue Levels

A total 246 cancer cases and 141 controls were identified in six studies [184-189]. Apart from a Sundelin *et al.* study, a significant elevation of this metal-binding protein was determined. A significantly higher metallothionein was accordingly determined using random effects model meta-analysis (standardized mean difference 1.60, 95 % CI = 0.99 to 2.21), see Fig. (4). No publication bias was revealed in this subanalysis, Eggers' test $P = 0.81$. We also took into account stage and grade of the tumour and no significant correlation with MT expression was found [5].

3.1.10. Albumin

Higher pre-treatment albumin serum levels are connected with better survival of cancer patients in various types of cancer [190]. This was also confirmed by a study of patients suffering from nasopharyngeal cancer where patients with albumin lower than 43.0 g/L had a worse overall survival prognosis. Poor survival prognosis was also confirmed when pretreatment albumin to globulin ratio is low [191]. Low albumin serum levels were also suggested as a negative prognostic marker of higher risk of bad radiation therapy outcome in patients with laryngeal and pharyngeal carcinoma [192]. In surgical patients with head and neck cancer, low preoperative albumin in serum was a prediction of an elevated risk of wound infection and also poorer survival [193].

3.1.11. Summary on altered level dysregulation

Both copper tissue and copper serum levels are elevated in head and neck cancer. Apart from head and neck tumours, dysregulations of these ions and their buffering mechanisms were observed in almost all types of cancer, not just in head and neck cancer.

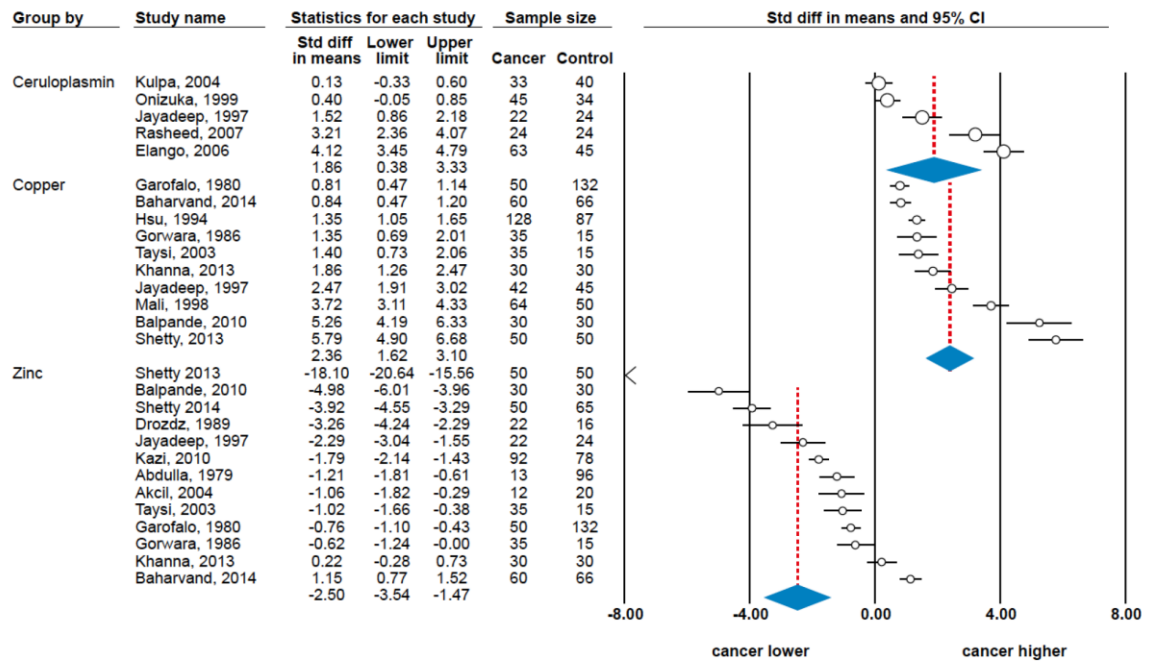


Fig. (3). Forrest plot showing associations between zinc, copper and ceruloplasmin level in sera of patients with spinocellular head and neck cancers (tumours *versus* healthy controls). Forrest plot displayed as standardized mean difference between cases and controls and 95 % confidence intervals. CI, 95 % confidence interval.

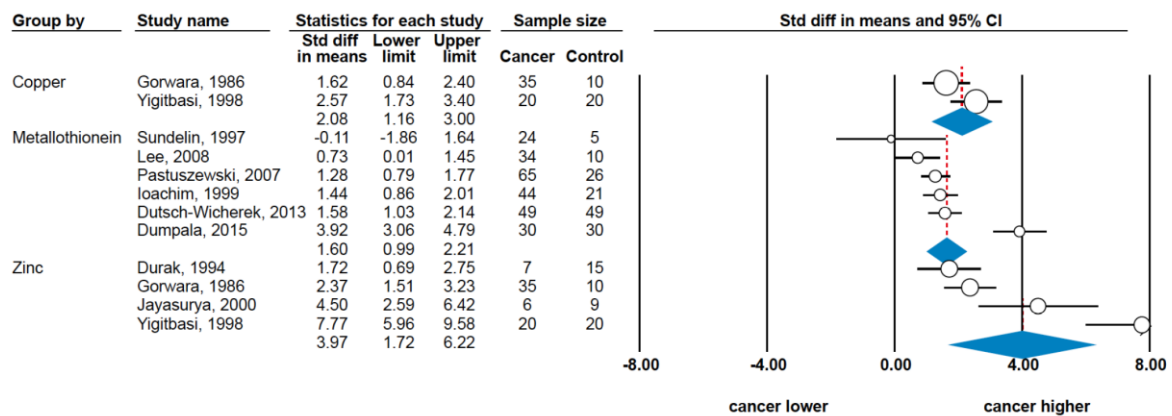


Fig. (4). Forrest plot showing associations between zinc, copper and metallothionein level in tumour tissues of patients with spinocellular head and neck cancers (tumorous vs. non-tumour tissues). Forrest plot displayed as standardized mean difference between cases and controls and 95 % confidence intervals. CI, 95 % confidence interval.

Levels of ceruloplasmin, as the major copper binding protein, are significantly elevated in all types of tumours even in head and neck tumours [159, 194, 195] suggesting that levels of ceruloplasmin might not depend on the organ localization of tumour but on the malignant process itself [196]. Many studies suggest ceruloplasmin as a good diagnostic marker in cancer, thus its levels are significantly increased in malignant

tumours and the degree of elevation mostly correlates with tumour staging [194-198]. However further research is needed to know if patients with advanced tumours have an increase of ceruloplasmin serum levels early enough to be applicable as a clinical indicator [194]. We might suggest this also for copper, since ceruloplasmin is its major binding protein and copper levels are elevated with ceruloplasmin levels as well.

Zinc serum levels tend to be decreased in all head and neck carcinomas, but there is no strong evidence of an outstanding role of zinc in serum in head and neck carcinoma when compared to other types of carcinomas. Therefore levels of zinc in serum do not show sufficient specificity to be potentially used as a biomarker just for malignancies in the head and neck region, but might be a good biomarker for pointing out the general inflammatory process possibly connected with development of malignancy in the human body, notably in relation to the age of patients. The incidence of most types of cancer including head and neck is increasing with the age of the patients. Low zinc and high copper plasma levels are considered a biomarker of ageing and all-cause mortality of people with advanced age [55, 58, 199, 200]. Buntzel *et al.* study suggests that zinc serum level is an important marker for the definitive palliative situation of patients suffering with head and neck cancer, whereas the rapidly decreasing serum zinc concentration was related to the terminal situation in these patients as they had an extremely low concentration of zinc in serum 4 last weeks before their death [201].

Unexpectedly, zinc tissue levels are highly elevated. We found out that this makes head and neck tumours notable when compared to other carcinomas from different regions of the body. However, when looking at studies investigating tissue zinc levels in different types of cancers, this pattern of zinc increase can be also seen in breast and colorectal carcinomas, but not in other carcinomas [5]. Inasmuch as head and neck carcinoma together with breast and colorectal carcinomas are the only examples with elevated zinc in tissues, we suggest that the mechanisms of the relation of zinc ions in malignant processes might be similar in these three tumours. There are an insufficient amount of studies investigating pathological mechanisms during cancer development and their relation with zinc, copper or their binding proteins in head and neck cancer patients or cell lines. However there are a good amount of studies revealing these mechanisms in colorectal and breast carcinomas. Consequently, because of the lack of head and neck tumour-related studies, colorectal and breast tumours will be included. It was previously mentioned that breast cancer, colorectal cancer and head and neck cancer mechanisms of deregulation might be similar to each other, because all three have increased zinc in tissue, in the case of MT the situation is different. MT expression in breast and colorectal cancer tissue is significantly lower compared to healthy tissues according to meta-analytical approach by Gumulec *et al.* [5]. On

the contrary, MT expression in head and neck cancer tissue is significantly higher (see Fig. 4). MT and its possible connection with carcinogenesis will be discussed later.

With regard to albumin, it has been revealed that poor nutritional status and inflammation suppress synthesis of albumin [202], anti-cancer treatment and also mechanisms in the tumour itself often lead to malnutrition in patients [203]. Albumin levels might be used as an independent predictor of cancer patients' survival as malnutrition and inflammation might play a role in the progression of cancer [190, 191]. However these findings are not specific for head and neck cancer, although they might also be useful as a pretreatment predictor of survival in this disease. Albumin carries various compounds and thus its predictive value is limited due to such low specificity. Rather it can serve as a general marker of nutritional status and inflammation that is present in cancer patients

3.2. Pathophysiological Consequences of Dysregulation in Head and Neck Cancer

This chapter's structure is based on well-known pathological states related to multistep cancer development, described by Hanahan and Weingerg [204]. Alteration of tissue and serum zinc and copper might be related to these mechanisms, alteration of these metal levels might also be events which can induce or help malignant process or might be an outgrowth of the malignant process itself. The data describing pathological mechanisms which are related to zinc, copper, ceruloplasmin and metallothionein in head and neck malignancies are described here. As mentioned in the previous chapter, there is not a sufficient amount of studies revealing these mechanisms in head and neck cancer. Given that head and neck, breast and colorectal carcinomas are the only carcinomas with raised tissue zinc levels, there might be similar mechanism in these three tumours. Therefore studies describing these mechanisms in colorectal tumours and breast carcinomas are also included.

Cellular zinc homeostasis is controlled by a zinc-buffering system, a mobile-reactive zinc pool. Cellular zinc accumulation is dependent upon zinc-transporters (ZIP and ZnT). Dysregulation of zinc levels can result from insufficient dietary intake or pathological expression of zinc-transporters or zinc binding proteins or dysregulation of zinc-dependent pathways. Therefore homeostasis imbalance plays an important role in many diseases including cancer.

3.2.1. Cell Proliferation and Growth

Extracellular zinc treatment of HT29 colorectal cancer cells activated MAPK-Mitogen activated protein kinases (Raf-1, MEK and ERK). ERK pathway is known for stimulating proliferation [205], but can also inhibit cell growth by induction of negative cell cycle regulators such as p21 [206, 207]. Adding 100 μM ZnCl_2 into medium caused ERK prolonged activation in HT29 cells and was related with induction and nuclear localization of negative cell cycle regulator p21^{Cip/WAF1} and also cell cycle regulator cyclin D1 induction. However, negatively regulated growth was observed in this situation [208, 209]. Zinc had an anti-proliferative effect on colorectal cancer cells not by apoptosis, but by G1 cell cycle arrest [208]. Adding 10 μM of ZnCl_2 resulted in the induction of cyclin D1 which promoted growth and proliferation of HT29 cells [209]. Differential regulation of growth by extracellular zinc treatment in colorectal cancer cells is mediated by receptor-mediated signal transduction because no elevation in intracellular zinc level was observed [209]. These data confirm involvement of zinc in growth and proliferation and suggest that zinc might be used to control the growth of colorectal cancer cells [208]. There are currently no data about ZIP or ZnT transporters involvement in colorectal cancer.

3.2.2. Apoptosis

Zinc is involved in regulatory functions of apoptosis. The mechanism of apoptosis is well described in a number of excellent reviews [210-214]. A link between zinc and its effect on apoptosis has been well described in many studies, however apoptotic pathways associated with the role of zinc are cell-specific and not fully elucidated. Zinc can have an anti-apoptotic effect [215], but on the other hand in many mammalian cell types is an apoptotic inducer [216, 217], as well as exposure to low zinc levels causing apoptosis [18] or the same effect being caused by exposure to high zinc levels [218, 219]. Zinc also has an important regulatory role in apoptotic pathway by influencing the caspase apoptotic regulators. Intracellular zinc chelation induces caspase-3 activity directing cell to the apoptosis, suggesting that zinc has an anti-apoptotic effect by maintaining caspase-3 inactive [215, 220, 221].

The apoptotic pathway is regulated by anti-apoptotic Bcl-2 and pro-apoptotic Bax mitochondrial membrane protein. Zinc supplementation and elevation in intracellular zinc level of apoptotic cells results in increased Bcl-2/Bax ratio which leads to suppression of apoptosis [222]. In human neuronal precursor cells,

zinc deficiency induces a cascade of events beginning with activation of p53 and translocation of phosphorylated p53 to mitochondria, increase of pro-apoptotic Bax mitochondrial protein and regulation of caspase-mediated apoptotic mechanism by mitochondrial p53 [223]. These findings point out the enormous importance of cellular zinc homeostasis and fatal consequences of its dysregulation.

Overexpression of ZIP4 transporter has been investigated in pancreatic cancer cells where it is responsible for resistance to apoptosis induced by zinc deficiency and also that ZIP4 knockdown induced apoptosis [224].

ZIP9 transport protein expression is elevated in breast cancer. Treatment of MDA-MB-468 breast cancer cells with testosterone resulted in elevated intracellular free zinc, induction of apoptosis with up-regulated pro-apoptotic Bax genes, p53 and c-Jun N-terminal kinases which were blocked by zinc chelation. This suggests that ZIP9 is important in the mediation of apoptosis promoted by testosterone [225].

It is assumed that metallothionein (MT) protects cancer cells against apoptosis and supports the cancer cells proliferation [168, 226]. Meta-analysis investigating the association of MT immunohistochemical staining and tumour presence found positive association of this staining in tumours compared to healthy tissues in head and neck cancer [5]. Jayasurya studied the relationship between tissue zinc levels in nasopharyngeal carcinoma and MT expression in these tissues. It was found that increased zinc tissue levels correlates with increased MT immunostaining and both are found to be mainly in the nucleus of cancer cells [168, 227]. These levels are related to increased proliferation of cancer cells. This can be due to the capability of zinc to block apoptosis and thus allowing cells to proliferate more actively leading to tumour progression [227], or due to the capability of MT to protect cells from entering the apoptosis [168].

It was also revealed that ceruloplasmin interacts with NF- κ B transcription factor in cervical squamous cell carcinoma [180]. It's known that NF- κ B is involved in apoptosis suppression [228] and in oncogenesis [229]. However it was demonstrated that NF- κ B can act both ways in apoptosis (suppresses and promotes) [230]. Blockage of NF- κ B leads to enhanced apoptosis and unexpectedly – spontaneous development of squamous cell carcinoma in mouse skin [231] and even in normal human epidermal tissue [232].

A study of Korean patients discovered that expression of NF- κ B significantly correlates with TNM stag-

ing, recurrence and lymph node metastasis of oral squamous cell carcinoma tissues considering NF- κ B as a oncogene in OSCC [233]. The same upregulation of NF- κ B with progression of lesion of oral squamous cell carcinoma was also observed in other studies [234, 235]. It seems that when NF- κ B activates with development of head and neck squamous cell carcinoma, apoptosis is enhanced. This apoptosis enhancement is, on the other hand, reduced by Ras protein [232]. The mechanism between ceruloplasmin and activation of NF- κ B interaction is still unknown and calls for further investigation [180].

3.2.3. Angiogenesis

Patients with Wilson's disease typically have a low level of ceruloplasmin in serum [236]. Copper accumulates in tissues of patients with Wilson's disease. Patients suffering from Wilson's disease were experimentally treated with the anticopper drug ammonium tetrathiomolybdate which prevents copper absorption into the bloodstream. This treatment has proved to be highly effective [237-240]. Tetrathiomolybdate acts through creating a complex with copper and serum albumin, restricting cellular uptake of copper and suppressing inflammatory cytokines [241].

It's known, that copper and copper containing molecules act as a necessary cofactor in angiogenesis (formation of new blood vessels) [242] and angiogenesis is essential for progressive tumour growth and metastasis [243, 244]. It was also found that tumour differentiation and metastatic ability correlates with copper levels in tumour tissue [245, 246]. Brewer *et al.* invented an anticopper, antiangiogenic approach for the treatment of mostly metastatic cancer using tetrathiomolybdate (previously used at treatment of WD) as an anticopper drug, suggesting that reducing copper levels inhibits angiogenesis in tumours. Human trials with tetrathiomolybdate had varying results in metastatic solid tumours [247], metastatic colorectal cancer [248], hormone-refractory prostate cancer [249], advanced kidney cancer [250] and oesophageal cancer [251]. In summation we can consider these trials beneficial. Copper depleting angiogenic therapies is further reviewed by Nasulewicz *et al.* [252].

3.2.4. Invasivity and Metastasis

In nasopharyngeal cancer, downregulation of ZIP4 might elevate the radiosensitivity in those cells. Also, a positive correlation between ZIP4 expression and tumour stage was observed as well as the fact that ZIP4 promotes cells migration and forming metastasis sug-

gesting that ZIP4 might be a good predictive biomarker for tumour metastasis in nasopharyngeal carcinoma and cancer therapy target [253].

Members of the ZIP zinc transporters family are involved in a number of cancers [254]. However there is insufficient quantity of findings regarding zinc-dependent mechanisms and ZIP transporters-related mechanisms in head and neck cancer, only ZIP4 in nasopharyngeal carcinoma as mentioned above.

Oestrogen-regulated ZIP6 transporter in breast cells is known for its role in cancer growth and metastasis [255-257]. ZIP6 is expressed more in non-adherent cells resistant to anoikis characterized by active proliferating even after detachment. This is the first step in the process of forming metastasis [257]. ZIP10 transporter is related to higher invasiveness and lymph node metastasis in breast cancer samples and breast cancer lines [258].

High glucose levels in the human breast cancer cell line MCF-7 induced migration of cells which was inhibited by zinc chelation or ZIP6 and ZIP10 knock-down [259] suggesting that zinc transported *via* ZIP6 and ZIP10 has a crucial role in migratory activity induced by high glucose stimulation [259]. A similar outcome was found when using MDA-MB-231 and MDA-MB-435S cells where their migratory activity was inhibited by ZIP10 knockdown or zinc chelation. These data suggest that ZIP10 might be used as a marker for the breast cancer metastatic phenotype and potential target for new therapeutic approaches [258].

According to Nexprot, microarray ZIP6 and ZIP10 mRNA is expressed also in the region of head and neck (mouth, nasopharynx). Further research of ZIP6 and ZIP10 transporters in head and neck cancer is needed to find out if the role of these ZIP transporters in head and neck cancer is similar to their mechanisms in breast cancer. However as ZIP6 transporter is oestrogen regulated it is not expected to play such a significant role in head and neck cancer as it plays in breast carcinoma.

Elevated immunoexpression of MT increases with higher grades of oral squamous cell carcinoma [189] and with tumour invasiveness, with highest MT expression in the nucleus in metastatic cells in the lymph node [260]. Increased expression levels of MT (detected electrochemically) in oral tumours, hypopharynx and larynx tumours with relation to the metastatic activity were observed by Sochor *et al.* [261]. Elevated MT expression is associated with a poor prognosis of these patients [189, 262, 263].

3.2.5. Resistance

Metallothionein overexpression also correlates with resistance to cisplatin in squamous cell carcinoma of the oesophagus [264]. MT-negative tumours respond better to chemoradiation therapy using cisplatin in oesophageal squamous cell carcinoma than MT-positive tumours [265-267]. MT overexpression could cause chemoresistance to cytotoxic therapy due to chelation of platinum-based drugs and indirect influence on p53 zinc-dependent activities [268, 269].

Copper transport proteins are known for playing a crucial role in the mechanism of cisplatin resistance [270]. Ctr1 copper transporter is involved in cisplatin uptake and its deletion leads to enhanced cisplatin resistance [99]. Upon exposure to a higher amount of copper, CTR1 goes through endocytosis and is degraded. Higher expression levels of CTR1 are linked to better response to cisplatin and thus better prognosis, possibly due to increased uptake of cisplatin [271-273]. It has also been shown, that in the presence of copper ions, DNA can be more sensitive to damage by radiation [274]. Nevertheless, the impact of copper in the presence of oxygen can be contradictory. One part is a sensitizing effect caused by the interplay of copper(II) ions, H₂O₂, and superoxide radicals. The second part is a possible protective effect resulting from scavenging of water-derived radicals (H[•], e⁻_{aq}, and OH[•]) [275]. Atox1 have been reported to interact with cisplatin and it is considered to be involved in cisplatin resistance [276-279]. Evidence suggests that ATP7A and ATP7B also play a role in platinum-based drug resistance [270, 280, 281]. Overexpression of ATP7B is related to cisplatin resistance in oral squamous cell lines [282]. Martin *et al.* found copper-dependent activation of hypoxia-inducible factor (HIF-1) [283]. Overexpression of HIF-1 is commonly found in head and neck cancer in association with resistance to therapy [284].

3.2.6. Summary of Dysregulation Aspects

In summary, elevated expression of ZIP transporters in various types of carcinomas is related to tumour invasiveness, resistance to anoikis, increased resistance to apoptosis, enhanced proliferation and growth. Knockdowns of ZIP transporters confirm these facts as they induce apoptosis and inhibit migratory activity. Zinc itself can play contradictory roles depending on the types of cells and amount of zinc involved in regulatory mechanisms. When zinc tissue levels are elevated, expression of MT also tends to increase. MT, when highly expressed, is related to the same pattern of processes as overexpressed ZIPs. MT overexpression is

connected with higher proliferation and metastatic activity of tumour cells and poor prognosis of patients.

Copper is elevated in malignant tissue where it plays a significant role in angiogenesis and tumour progression. This is demonstrated by copper chelating which facilitates a decrease in tumour angiogenesis in patients with various types of cancer. Copper serum level decrease can also be considered evidence that copper is an important element for tumour progression. It is proved that copper transport proteins have their share of resistance to cisplatin with copper/cisplatin transporter Ctr1 in the leading position. Malignant lesions provide several times higher expression of ceruloplasmin, than normal tissue. It indicates that ceruloplasmin is involved in apoptotic regulation through NF-κB.

3.3. Proposed Therapeutic Implications

Zinc deficiency, hand in hand with immune dysfunctions are often present in patients with head and neck cancer (HNSCC). Moreover, zinc deficiency has been associated with an increased tumour size and the overall stage in this type of cancer [285]. Dietary zinc has also been shown to modulate COX-2 expression (some results suggest a correlation between COX-2 expression and overall survival of HNSCC patients [286, 287]) and lingual and oesophageal carcinogenesis [288]. HNSCC is frequently accompanied by relatively low serum zinc levels implying that zinc supplements may be beneficial in HNSCC patients to improve their treatment outcome. Nevertheless, results of zinc studies are very often contradictory. Patients with head and neck cancer undergoing radiotherapy may experience various radiation-induced side effects of this treatment as the radiation can damage healthy tissues around the irradiated area. It has been observed, that zinc supplementation with zinc sulphate during radiotherapy can prevent taste abnormalities in these patients [289, 290]. Reduction and delay of development of radiation-induced oropharyngeal mucositis in head and neck cancer is another beneficial effect of zinc supplementation [291-293]. Although there is a study with results showing that zinc supplementation does not prevent mucositis [294]. A double blind placebo controlled clinical study found that patients with advanced nasopharyngeal carcinoma have improved overall survival when using zinc supplementation during radiotherapy and chemotherapy [295]. On the other hand, there is a contradictory study, where zinc sulphate administration during radiation therapy of HNSCC patients showed no elevation in survival of patients or increase in T lym-

phocyte subpopulations [296]. Nevertheless, zinc sulphate administration can be beneficial as a supplementary agent for immunomodulatory strategies [297]. Furthermore, low dose photocatalytic therapy of HNSCC with zinc oxide nanoparticles (ZnO-NPs, 0.2 µg/ml) could strengthen the anti-cancer effect of chemotherapeutic agents such as paclitaxel and cisplatin [298]. Although some cytotoxic properties of ZnO-NPs were demonstrated in non-malignant cells, it concerned concentrations above 10 µg/ml [299]. Tumour cells could be eliminated effectively at 0.2 µg/ml, whereas toxic actions in oral mucosa were found at 20 µg/ml. Moreover, Hanley *et al.* demonstrated significantly higher ZnO NPs-mediated toxicity (~28–35 times) in cancer cells and cancer associated T-cells than in their non-malignant opposites [300].

Biological functions of metallothionein (MT) proteins include reaction to oxidative stress and regulating of cell proliferation, differentiation and cell death. Downregulation of MT with anti-sense oligonucleotide in human nasopharyngeal carcinoma cells resulted in a decrease in cell viability and proliferation [301]. This phenomenon was also observed in other types of cancer [268]. As previously mentioned, MT overexpression is linked with chemoresistance to cytotoxic therapy. In contrast, restriction of MT expression could increase the cytotoxic effect of chemotherapeutics [268, 269]. MT-negative tumours respond better to chemoradiation therapy using cisplatin in squamous cell carcinomas than MT-positive tumours [265–267]. Therefore these findings can lead us to the consideration of MT as a prognostic biomarker in patients with squamous cell carcinoma [265]. However, more studies are needed in this field.

The pro-tumorigenic effects of MT could be partially mediated by zinc chelation, inasmuch as zinc deficiency was associated with inflammatory processes and cancer progression [302] and forced overexpression of MT by lentiviral transduction decreased bioavailable levels of zinc [269].

Copper is an essential cofactor for the function of many enzymes promoting angiogenesis [303]. Increased serum copper concentrations have been observed in nearly 40 % of the HNSCC patients [241, 283, 284]. Therefore, copper suppression may be of some benefit for therapy of HNSCC. Accordingly, copper chelation using tetrathiomolybdate (TM) was efficient in preclinical and animal models as an anti-angiogenic agent [304]. Furthermore, TM suppresses growth of squamous cell carcinoma and tumour vascularisation as well as metastasis by decreasing cancer

cell motility, invasiveness and by supporting anoikis [303, 305, 306]. Nevertheless, reaching an adequate copper deficiency may take several months. TM monotherapy would then probably not be beneficial in patients with advanced and rapidly progressive disease. TM therapy together with chemotherapy or radiation has been well tolerated in animal models [307].

Oncolytic herpes simplex viruses (oHSV) are genetically engineered to replicate in tumour tissue and evade infection and replication in non-transformed cells [308, 309]. Treatment of HNSCC with oHSV is a promising type of therapy with great clinical potential [310, 311]. Nevertheless, *in vitro* and *in vivo* assays disclosed that physiological levels of copper (18–20 µmol/l [153]) inhibit oHSV therapeutic efficacy due to disrupted oHSV infectivity and replication [241]. Copper chelator ATN-224 (choline tetrathiomolybdate; a second-generation analog of TM) significantly enhanced the serum stability of oHSV RAMBO (an oHSV with the antiangiogenic Vstat120) and allowed their systemic delivery in HNSCC tumour xenografts. Moreover, the combination of ATN-224 and RAMBO strongly inhibited lung metastases of HNSCC in mice [241]. ATN-224 alone suppresses superoxide dismutase 1 (SOD1) in tumour and endothelial cells. The inhibition of SOD1 results in decreased endothelial cell proliferation and downturn of angiogenesis. Inhibition of SOD1 in tumour cells leads to the apoptosis triggering [312]. Uptake of the important anticancer drug cisplatin is mediated by the copper transporter Ctr1. As previously mentioned, Ctr1 transporter goes through endocytosis when exposed to higher amounts of copper. These results suggest that cisplatin uptake can be modulated by copper chelating [313].

CONCLUSION

Zinc and copper are clearly involved in many regulatory mechanisms in the human body. Adequate cellular zinc and copper levels deeply influence cellular metabolism and have a large role in the initiation, progression and possible prevention of cancer. There are some hints suggesting that zinc deficiency can cause DNA damage and the triggering of malignant transformation. Changes in expression of zinc and copper transporters due to tumorigenesis have also been observed. The outcome of deregulated zinc and copper homeostasis is detectable as an alteration of plasma, serum, and tissue copper and zinc levels. Plasma and serum zinc is usually decreased and the copper/zinc ratio is higher in HNSCC patients compared to healthy controls. Inasmuch as copper is an essential player in angiogenesis,

copper chelation may cause some benefits for therapy of HNSCC. Some evidence also suggests that cisplatin uptake through copper transporter CTR1 can be modulated by copper chelating and higher expression of CTR1 is related to a better prognosis of patients undergoing cisplatin chemotherapy. Unlike copper, zinc can play contradictory roles in tumorigenesis depending on the type of cancer cells and particular step of tumorigenesis. A protective role of zinc seems to manifest itself rather at the initial stages of tumorigenesis. Zinc is important for stabilization and activation of tumour suppressor p53 and is also involved in apoptosis due to activation of some caspases. On the other hand, zinc is essential for cell proliferation and is underlying in the rapid dividing of cancer cells. Considering the information above, zinc and copper ions and their transporters or binding molecules might be promising targets of further cancer research. Deeper insight into zinc and copper physiology would help to understand the mechanisms which can lead to tumours arising.

CONFLICT OF INTEREST

The authors confirm that this article content has no conflict of interest.

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2 Hypotézy práce

Cílem práce je odpovědět na níže uvedené hypotézy:

- Mikroprostředí nádoru ovlivňuje progresi nádoru a toto je využitelné i diagnosticky – změny v transkriptomu nenádorové – k nádoru přilehlé tkáni mají predikční/prognostické využití
- Prognostický význam má nejen změna transkriptomu nádorové tkáně, ale také změna v epigenetických mechanismech.
- V nádoru existují různé buněčné subpopulace s různou schopností proliferovat, migrovat, či založit metastázy a s různou schopností podporovat nádorovou tkáň v růstu.
- S nádorem asociované fibroblasty ovlivňují odezvu nádorové tkáně na radioterapii

3 Metodické přístupy

Mikroprostředí bylo studováno víceúrovňově – na úrovni analýzy séra, tkáňových vzorků, ale také na úrovni jednotlivých subpopulací buněk získaných z nádorových vzorků pacientů. Bylo využito metod analýzy genové exprese, metod studia buněčné migrace, invazivity, buněčného růstu a byla analyzována míra reparace dvouřetězcových zlomů DNA. Zde jsou metody sumarizovány a uvedeny odkazy na konkrétní články zmiňující metodiku detailněji.

Nábor a definice pacientů, zpracování tkáňových vzorků a analýza genové exprese pomocí kvantitativní real-time PCR a následná statistická analýza je popsána v práci Raudenska *et al.*⁹⁹, metodika dostupná na str. 104. Zpracování vzorků nádorové tkáně pro analýzu miRNA a následná analýza přežití (Log rank test, Kaplan Meierova analýza) je dostupná v práci Hudcova *et al.*¹⁰⁰, metodika je dostupná na str. 124. Metody elektrochemické detekce miRNA jsou popsány v práci Hudcova *et al.*¹⁰¹ na str. 132. Metody zpracování vzorků séra pro následnou detekci proteinu EGFR pomocí ELISA jsou popsány v práci Polanska *et al.*, metodika je dostupná na str. 141. Metody detekce HPV16 a HPV18 (detekce pomocí PCR a sekvenování) jsou popsány v práci Polanska *et al.*¹⁰², metodika dostupná na str. 148. Zpracování biotických vzorků pro přípravu primokultur *in vitro* a jejich následná separace pomocí paramagnetických částic s protilátkami specifickými proti CD44 a CD90 je dostupná v práci Svobodova *et al.*¹⁰³, metodika dostupná na str. 158. Metody ověření subpopulací fluorescenční mikroskopií, analýza buněčného růstu, migrace pomocí wound-healing assaye, real-time analýza migrace a invazivity, analýza cytotoxicity pomocí MTT testu bioinformatická analýza pro studium transkriptomu je dostupná tamtéž. Analýza tvorby dvouřetězcových zlomů, resp. jejich reparace pomocí mikroskopické analýzy jaderných fóků γ H2AX/53BP1 je popsána v práci Falk *et al.*¹⁰⁴, metodika dostupná na str. 176.

4 Výsledky (komentovaný soubor publikací)

V této části jsou formou komentovaného souboru publikací shrnuty poznatky autora v oblasti onkologie hlavy a krku publikované výhradně v časopisech s impakt faktorem. Z důvodu logické návaznosti je dána přednost integraci vlastních článků přímo do textu práce a nikoli až na její konec v podobě přílohy. Každé publikované studii předchází její shrnutí, je uvedena v návaznost na dílo předchozí a návaznosti na další výzkum.

4.1 Molekulární markery HNSCC na úrovni mRNA

V práci Raudenska *et al.*⁹⁹ bylo cílem na tkáňové úrovni popsat asociace exprese genů s klinicko-patologickými vlastnostmi pacientů (charakteristika viz tabulka na str. 105). Byla provedena analýza exprese následujících genů: *EGF*, *EGFR*, *MKI67*, *BCL2*, *BAX*, *FOS*, *JUN*, *TP53*, *VEGF*, *FLTI*, *MMP2*, *MMP9*, *MT1A*, a *MT2A*; jejich popis a role viz kapitola Prognostické biomarkery na str. 7. Do této studie bylo zahrnuto celkem 94 tkáňových vzorků histologicky ověřeného spinocelulárního karcinomu hlavy a krku.

Tkáň přilehlá nádoru, jinak histologicky „normální“ je v mnohých studiích považována za referenční. Nicméně tato tkáň může podporovat růst vlastní nádorové tkáně, např. prostřednictvím fibroblastů asociovaných s nádorem¹⁰⁵. Toto je v práci zohledněno – ve studii jsou proto kontrolní tkáně dvě – přilehlá (tj. tkáň stejného pacienta, „adjacent“, 31 pacientů) a tkáň pacientů po tonsilektomii („tonsillectomy“, 10 pacientů).

Nejprve byly sledovány rozdíly mezi těmito kontrolními tkáněmi. Byly prokázány signifikantní rozdíly u těchto genů – vyšší exprese *JUN*, *EGF*, *MT2A* a *BAX* a výrazné zvýšení poměru *BAX/BCL2* v nádoru přilehlé tkáni oproti vzorkům tonsilektomie. Přítomnost nádoru tedy ovlivňuje přilehlou tkáň tím, že v ní zvyšuje míru apoptózy. Vzhledem k významným rozdílům byla v dalších analýzách exprese těchto genů posuzována zvláště u obou kontrolních skupin, zatímco u zbylých genů toto nebylo zohledněno. Nejvýznamnější rozdíly v expresi byly pozorovány u genu pro metalothionein 2A, *MT2A* (viz obrázek na str. 106). Nejvyšší míra exprese tohoto genu byla pozorována v nádorové tkáni a naopak nejmenší míra v zánětlivě změněné tkáni tonsilektomií.

V rámci této studie byla pozornost také zaměřena na spojitosti mezi tkáňovou expresí výše uvedených genů a klinicko-patologickými metrikami pacientů. Co se týče stagingu TNM, nebyla prokázána spojitost mezi stádiem T a expresí žádného genu. Bylo nicméně zjištěno, že tkáň

přílehlá k nádoru také vykazuje změny v expresních profilech v závislosti na stage tumoru, a zasažením uzlin. Bylo zjištěno, že přílehlé (nenádorové) tkáně pacientů se zasažením mízních uzlin vykazovaly snížení exprese genů *BAX*, *EGFR*, *FLT1* a *MMP2*; s pozitivitou uzlin souvisela i vyšší exprese *BCL2*.

Aby bylo možné robustněji uchopit vzájemné interakce mezi sledovanými geny a tím více proniknout do patogeneze choroby, byla provedena analýza hlavních komponent, vícerozměrná statistická metoda umožňující komplexní výsledky zredukovat do několika jednoduchých závěrů. Nejvýznamnější posun v rámci expresního vzorce byl nalezen u *EGFR* a *MKI67* mezi tkání nádorovou a přílehlou k nádoru oproti tkáni pacientů indikovaných k tonzilektomii. Pro nádorovou tkáň byl typický posun v expresi *JUN*, *BCL2* a *MMP9*. Pro tkáň přiléhající k nádoru byl charakteristický posun v expresi *JUN* a *FLT1* a pro tonzilektomie bylo typické odlišné uspořádání *EGF*, *MT2A* a *MT1A* v rámci expresního vzorce (viz obrázek na str. 108).

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RESEARCH ARTICLE

Prognostic significance of the tumour-adjacent tissue in head and neck cancers

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Abstract Even with significant advances in operative skills and adjuvant therapies, the overall survival of patients suffering with head and neck squamous cancers (HNSCC) is unsatisfactory. Accordingly, no clinically useful prognostic biomarkers have been found yet for HNSCC. Many studies analysed the expression of potential markers in tumour tissues compared to adjacent tissues. Nevertheless, due to the sharing of the same microenvironment, adjacent tissues show molecular similarity to tumour tissues. Thus, gene expression patterns of 94 HNSCC tumorous tissues were compared with 31 adjacent tissues and with 10 tonsillectomy specimens of non-cancer individuals. The genes analysed at RNA level using quantitative RT-PCR and correlated with clinico-pathological conditions were as follows: *EGF*, *EGFR*, *MKI67*, *BCL2*, *BAX*,

FOS, *JUN*, *TP53*, *VEGF*, *FLT1*, *MMP2*, *MMP9*, *MT1A* and *MT2A*. The elevated *MT2A*, *BAX*, *EGF* and *JUN* expression was associated with the influence of tumour cells on the rearrangement of healthy tissues, as well as a significant shift in the *BAX/BCL2* ratio. Our investigation also indicated that adjacent tissues play an important role in cancerogenesis by releasing several tumour-supporting factors such as *EGF*. A gradual increase in the metallothionein expression, from the lowest one in tonsillectomy samples to the highest ones in tumour samples, suggests that MT expression might be tissue reaction to the presence of tumour cells. The results of this study confirmed the significance of metallothionein in tumorigenesis and gave evidences for its use as a potential HNSCC biomarker. Furthermore, this study highlighted the importance of histologically normal tumour-adjacent tissue in prediction of HNSCC progress.

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Keywords Head and neck neoplasms · Biological markers · Prognosis · Tumour microenvironment · Metallothionein · Matrix metalloproteinase 9 · Gene expression

Introduction

Tumours of the head and neck, which affect nasal cavity and paranasal sinuses, oral cavity and the upper aerodigestive tract, are aggressive malignant tumours arising from the epithelial layer of mucosal linings. The most frequent histological type of head and neck cancer is squamous cell carcinoma (HNSCC) [1]. Even with significant advances in operative skills and adjuvant therapies, the overall survival of HNSCC patients has not been improved significantly. The 5-year survival rate of patients diagnosed with an advanced stage of HNSCC does not exceed 50 % [1, 2]. Besides the poor outcome, HNSCC has an undesirable impact on the patient's life

quality due to its anatomic localization. About a half of patients with the advanced disease (stage III and IV) show recurrence (90 % within the first 2 years) [3, 4]. Nowadays, main prognostic variables of HNSCC are considered the tumour size and location, the presence of lymph node and distant metastasis [5]. Clinical and pathological criteria are important for the management and prediction of patients' outcomes. However, there is still a considerable variation in the prognosis within a group of patients sharing the same clinicopathological characteristics. No clinically useful prognostic biomarkers similar to the *HER2* or oestrogen receptor in breast cancers [6], nor PSA in prostate [7] have been found yet for HNSCC [8]. Accordingly, an assessment of the mRNA expressing patterns of particular tumours should be useful.

Many studies have analysed the expression of selected markers in the tumour tissue compared to healthy adjacent tissues by way of histological survey. Nevertheless, due to the sharing of the same microenvironment, adjacent tissues show a molecular similarity to tumour tissues [9], and thus can hardly be considered an ideal "control" tissue. Some cytokines and growth factors involved in the neoplastic transformation process are also produced by histologically normal epithelial cells and other cell types surrounding the tumour [10]. On the other hand, paracrine effects of tumour-secreted growth factors could distort the normal tissue homeostasis by inducing inflammatory responses or angiogenesis [11]. Thus, there is a great interest in the characterisation of signal links between the tumour and the adjacent tissues.

This study is focused on molecular markers and their potentially important role for the development of cancer: (1) acquisition of autonomous proliferative signalling (EGF/EGFR); (2) proliferative activity of tumour cells (Ki-67); (3) cell cycle and cell death modifications (Bcl-2, Bax, cFos, cJun, p53); (4) angiogenesis (VEGF/FLT1); (5) metastatic potential (MMP-2, MMP-9); and (6) oxidative stress response (MT1A, MT2A).

The aim is to confirm, that the gene expression patterns of "normal" adjacent epithelial cells are also predictive for the HNSCC progression. Thus, the gene expression patterns of 94 HNSCC tumorous tissues were compared with (a) 31 adjacent tissues and (b) with 10 tonsillectomy specimens of non-cancer individuals.

Material and methods

Preparation of tissue samples

The study was conducted in accord with the Helsinki Declaration of 1964 and all subsequent revisions thereof. It was approved by the ethical committee of St. Anne's Faculty Hospital, Brno, Czech Republic. All surgical tissue samples were obtained from HNSCC patients after they signed the informed

consent. Histologically verified primary HNSCC carcinoma tissues (T), histologically verified matched noncancerous adjacent tissues obtained from incision adjacent to the tumour site (A) and available tissues from noncancerous tonsillectomy (Tons) were collected. The tissue material harvested at surgery was placed into RNAlater Solution for RNA stabilisation and storage (Ambion, Carlsbad, CA, USA). The material was maintained cold, and RNA was isolated within 24 h.

RNA isolation and reverse transcription

TriPure Isolation Reagent (Roche, Basel, Switzerland) was used for RNA isolation. The isolated RNA was used for cDNA synthesis. RNA (1000 ng) was transcribed using the transcriptor first strand cDNA synthesis kit (Roche, Switzerland), which was applied according to manufacturer's instructions. cDNA (20 μ l) prepared from total RNA was diluted with RNase-free water to 100 μ l, and the amount of 5 μ l was directly analysed by using the LightCycler®480 II System (Roche, Basel, Switzerland).

Quantitative real-time polymerase chain reaction

qRT-PCR was performed using TaqMan gene expression assays with the LightCycler®480 II System (Roche, Basel, Switzerland), and the amplified DNA was analysed by the comparative Ct method using β -actin as an endogenous control. Primer and probe sets for *ACTB* (assay ID Hs99999903_m1), *MT2A* (Hs02379661_g1), *MT1A* (Hs00831826_s1), *TP53* (Hs01034249_m1), *BAX* (Hs00180269_m1), *BCL2* (Hs00608023_m1), *VEGFA* (Hs00900055_m1), *FLT1* (Hs01052961_m1), *MMP2* (Hs01548727_m1), *MMP9* (Hs00234579_m1), *FOS* (Hs00170630_m1), *JUN* (Hs00277190_s1), *MKI67* (Hs00606991_m1), *EGF* (Hs01099999_m1) and *EGFR* (Hs01076078_m1) were selected from the TaqMan gene expression assays (Life Technologies, USA). qRT-PCR was performed under the following amplification conditions: total volume 20 μ l, initial incubation at 50 °C/2 min followed by denaturation at 95 °C/10 min, then 45 cycles at 95 °C/15 s and at 60 °C/1 min.

Data analysis

Log-transformed gene expression data were analysed using multivariate one-way and factorial ANOVA with the subsequent use of planned comparisons (contrast analysis). Pairs of tumour and tumour-adjacent tissues were analysed using the paired *t* test. Relations of continuous variables were analysed using Pearson's correlations and the principal component analysis. Unless noted otherwise, *p* level <0.05 was considered

significant. Software Statistica 12 (StatSoft, Tulsa, OK, USA) was used for analysis.

Results

Clinico-pathological characterisation of HNSCC patients

In this study, in total 94 biopsy samples of tumours from patients with histologically verified spinocellular carcinoma and comprehensive patient history were used. The location of these tumours was as follows: larynx (24 samples), oropharynx (35 patients), hypopharynx (14 samples), oral cavity (8 samples) and other locations (13 patients). Gene expression of the selected genes was compared with the following two control groups: first, matched tumour-adjacent histologically normal tissue (31 samples), and second, tonsillectomy samples of otherwise healthy individuals (10 samples).

In the next step, the effect of clinico-pathological conditions of patients on the expression of the selected genes was analysed. Brief description of the cases and control is shown in Table 1. In the following text, these three tissue types are consistently abbreviated as follows: T for tumorous tissue, A for adjacent “control” tissue of the same patient and Tons for tonsillectomy samples from patients without any cancer disease.

Gene expression pattern in cases and controls

The expression analysis of mRNA was performed on all three tissue samples to characterise the expression profile of selected genes in the particular tissue types. Multivariate test revealed a significant effect of the tissue type on the gene expression pattern ($F(30, 236)=2.87, p<0.001$).

In accordance with the aim of this study, the suitability of tumour-adjacent samples as controls was assessed, and the expression in these tissues was compared with the tonsillectomy samples. *MT2A* (fivefold change; 95 % CI 1.09–18.26, $p=0.04$), *BAX* (threefold change; 95 % CI 1.08–10.74, $p=0.04$), *EGF* (12-fold change 95 % CI 1.42–93.13, $p=0.02$) and *JUN* (fivefold change; 95 % CI 1.52–18.04, $p=0.01$) expression was higher in the tumour-adjacent tissues (A) compared to the tonsillectomy (Tons) samples. Significant was also the difference in *BAX/BCL2* ratio between A and Tons, which was 33-fold higher in A (95 % CI 1.30–812.83, $p=0.03$). Due to this fact, that the expression of these genes between two “control” tissues was differential, all consequent gene expression analyses of these genes were related to both control groups separately, while the remaining genes were compared to two types of “control” groups together.

Most profound differences in the gene expression were observed in *MT2A* gene (Fig. 1); the highest expression of

Table 1 Characteristics of the samples of patients and controls

Factor	Group	N	Age (min-max)
Group	Tumour	94	64 (25–89)
	Tonsillectomy	10	42 (21–64)
	Tumour-adjacent	31	62 (44–87)
Gender	Male	113	64 (21–89)
	Female	22	56 (21–76)
Smoking habit	Yes	52	63 (44–79)
	No	31	68 (49–89)
	Not specified	52	59 (21–87)
Tumour grade	1	4	61 (53–68)
	2	63	64 (44–89)
	3	27	62 (47–76)
Tumour stage	T1–2	40	64 (44–89)
	T3–4	54	64 (47–87)
Node positivity	No	39	67 (44–89)
	Yes	55	63 (47–77)
Meta positivity	No	89	65 (44–89)
	Yes	5	61 (55–71)

Note that tumour staging and grading refer to the first “tumour” group of patients only

Online resource 1: Table of effects of clinico-pathological status on the gene expression. Results of multivariate ANOVA are followed by planned comparisons. Results of multivariate ANOVA displayed in the column “factor”, groups that are compared with each other, are shown in the column “level of factor”. Displayed as a point estimate and 95 % confidence intervals (CI)

MT2A was found in tumour tissues (T) and the lowest in tonsillectomies (Tons).

MT2A (12-fold change; 95 % CI 3.32–43.74, $p=0.001$) and *BAX* (threefold change; 95 % CI 1.08–8.78, $p=0.04$) expression was higher in tumour tissues (T) as compared to Tons. When comparing the remaining genes expression in tumour tissues (T) versus A and Tons in one group, *EGFR* expression was 11-fold higher (95 % CI 1.89–65.07, $p=0.01$), *MMP2* expression was sevenfold higher (95 % CI 1.33–37.97, $p=0.02$), and *MMP9* expression was 11-fold higher (95 % CI 1.54–78.87, $p=0.02$) in the tumour tissue. Furthermore, *MMP9* was three times more expressed in T as compared with A (threefold change; 95 % CI 1.15–8.05, $p=0.03$). Conversely, *EGF* was expressed less in T than in A (0.3-fold change; 95 % CI 0.09–1.01, $p=0.05$).

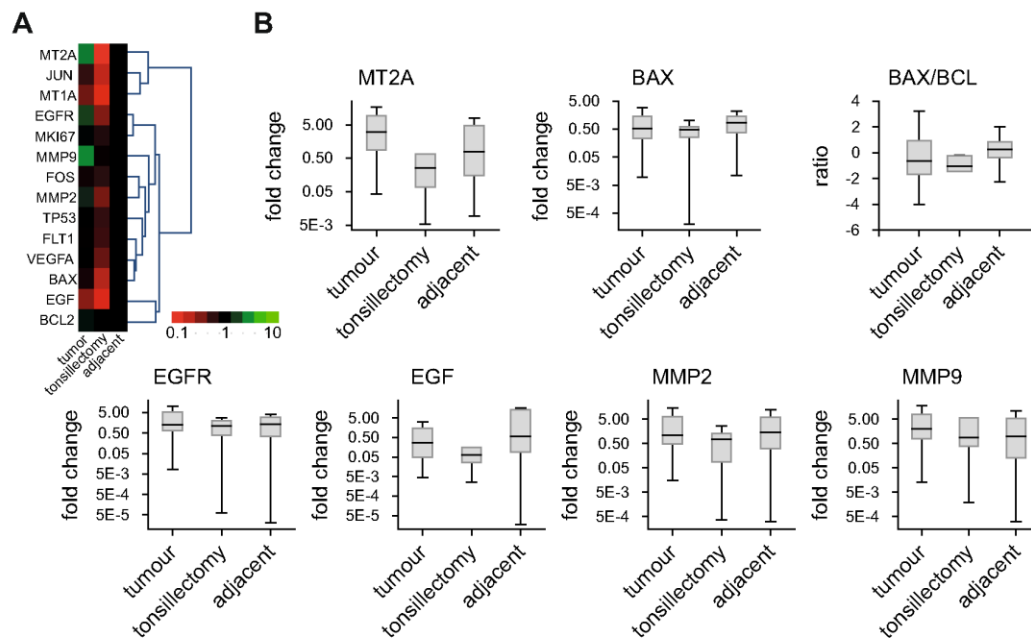


Fig. 1 Gene expression analysis in tumorous tissue, tumour-adjacent tissue (first control) and tonsillectomy tissue (second control). **a** Heatmap of all studied genes clustered by co-expression pattern.

b Box plots of genes showing significant differences across groups according to multivariate ANOVA. Displayed as median, *box* shows second–third quartile, *whiskers* show non-outlier range

Furthermore, mRNA expression for the tumour samples and the matched adjacent tissues ($T=31$, $A=31$) were analysed using the paired t test analysis. Two of more differentially expressed genes were *MMP9* ($p=0.001$) and *VEGFA* ($p=0.01$), which were both more expressed in tumour tissues.

Gene expression and tumour staging

Consequently, the effect of tumour staging on the expression of the above-mentioned genes was analysed. In accordance with the fact, that tumour-adjacent tissues are often involved in the development and progression of the tumour, the effect of tumour staging was not only related to the expression in the tumorous tissue, but also to the expression in the tumour-adjacent tissue samples.

First, the effect of TNM T staging was analysed. No significant association between the selected gene expressions and TNM T staging was determined, either in the tumour or in the tumour-adjacent tissue.

Subsequently, the effect of node positivity (TNM N >1 vs. 0) was analysed. Ninety-four tumour tissue samples (N positive=55; N negative=39) and 31 tumour-adjacent tissue samples (N positive=17; N negative=14) were involved in the analysis. Planned comparisons revealed a significantly higher gene expression of *BCL2* and a lower *BAX/BCL2* ratio in the tumour tissue (T) of patients with positive nodes (twofold higher, 95 % CI 1.13–5.15, $p=0.02$ for *BCL2* and 0.11-times lower, 95 % CI

0.02–0.53, $p=0.006$ for *BAX/BCL2*) compared to the tumour tissue samples of patients with negative nodes. Furthermore, a lower gene expression of *BAX* in the tumour-adjacent tissue (A) of patients with positive nodes (0.3-fold lower, 95 % CI 0.1–0.96, $p=0.04$) was revealed. *EGFR*, *FLT1*, and *MMP2* expression was also significantly lower in the tumour-adjacent tissue (A) of patients with positive nodes (0.18-times lower, 95 % CI 0.04–0.84, $p=0.03$ for *EGFR*; 0.24-times lower, 95 % CI 0.06–0.98, $p=0.05$ for *FLT1*; and 0.17-times lower, 95 % CI 0.04–0.74, $p=0.02$ for *MMP2*). See Fig. 2 and Online resource 1.

The effect of the presence of distant metastases was not analysed due to a small set of M-positive patients (94 tumour tissue samples; M positive=5; M negative=89 and 31 tumour-adjacent tissue samples; M positive=4; M negative=27).

Gene expression and histological grading

Ninety-four tumour tissue samples ($gr1=4$; $gr2=63$; $gr3=27$) and 31 tumour-adjacent tissue samples ($gr1=3$; $gr2=23$; $gr3=5$) were involved in the analysis. The expression of *MT2A* and *MT1A* was significantly associated with the tumour grade (3 vs. 2 grades; impact of tumour and adjacent tissues assessed together). Higher expression was identically found in 3 grade tumours (36-fold change; 95 % CI 3.87–329.1 at $p=0.0002$ for *MT2A* and 34-fold change; 95 % CI 1.42–792.06 at $p=0.03$ for *MT1A*, respectively). Then, A and T tissues were evaluated separately. The expression of *BCL2*, *MT2A* and

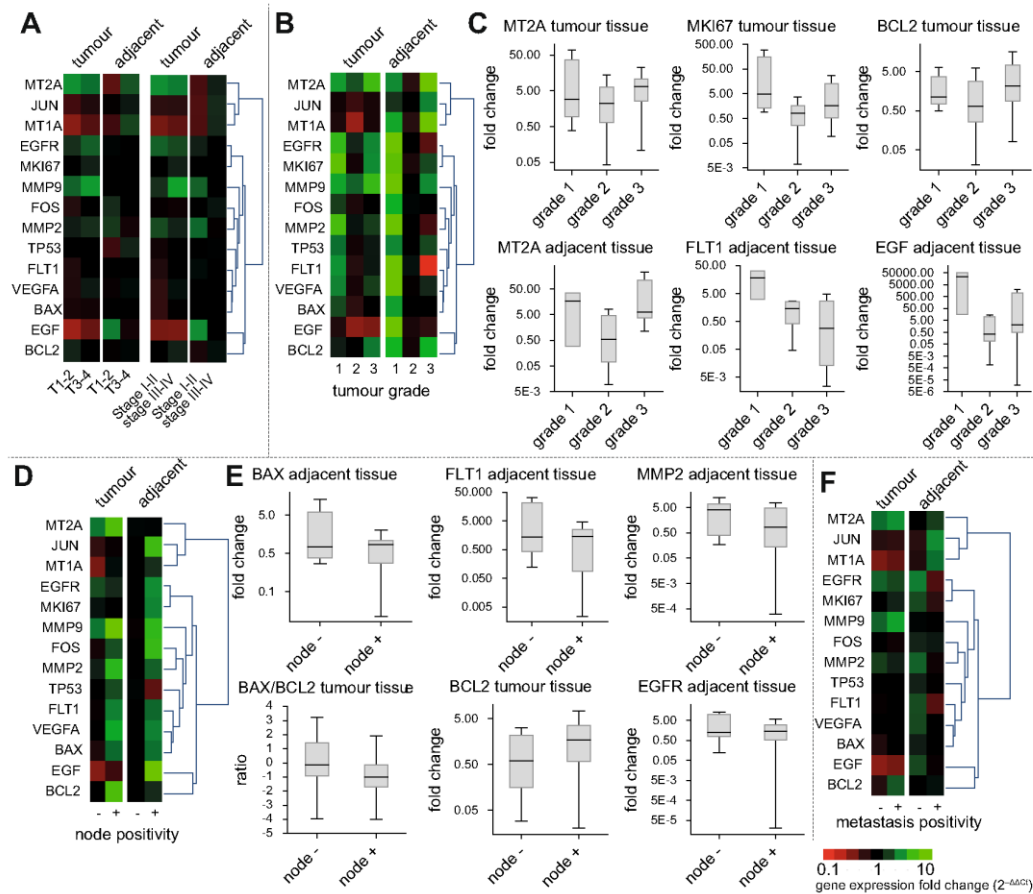


Fig. 2 Gene expression analysis in tumorous tissues and tumour-adjacent tissues according to tumour staging and grading. **a** Heatmap showing the gene expression pattern according to tumour staging and TNM staging. None of the selected genes demonstrated significant difference according to the staging. **b** Heatmap showing the effect of tumour grade. **c** Genes showing significant trends based on grading. **d** Effect of lymph node positivity. **e** Presence of metastatic

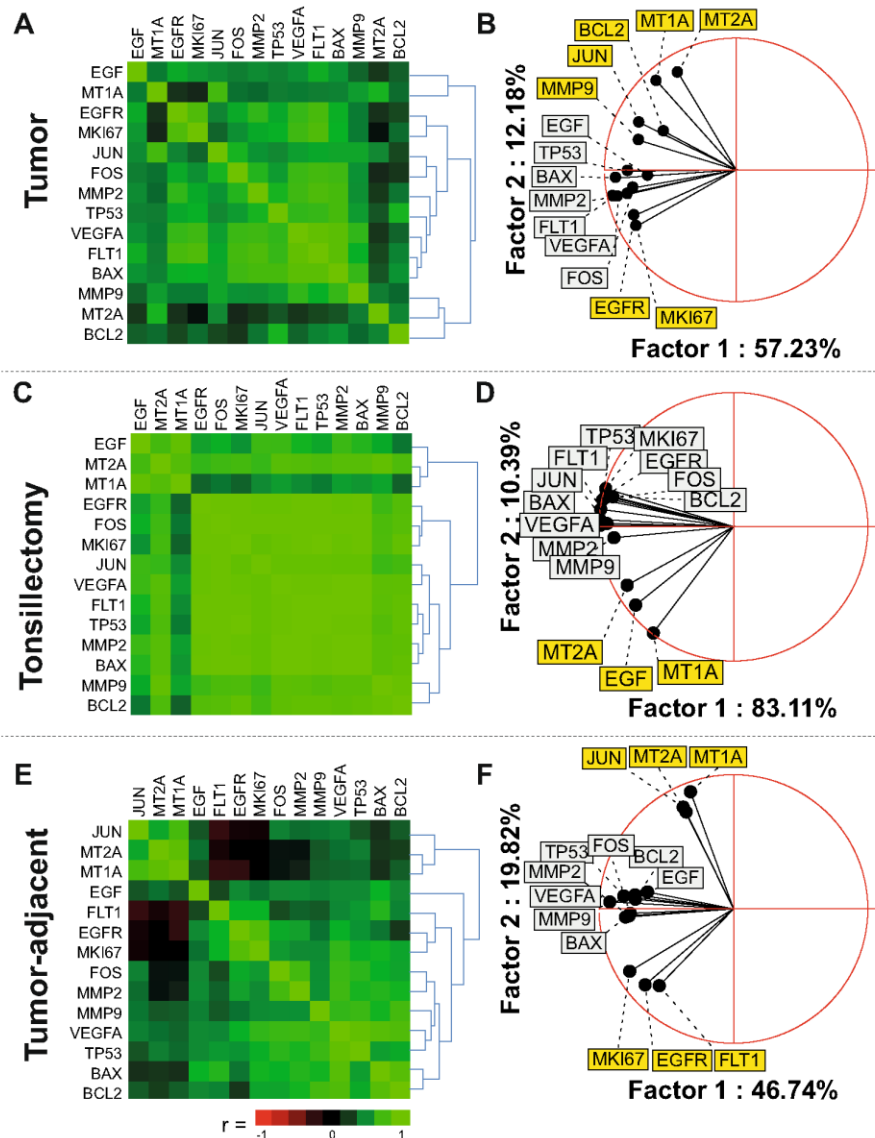
dissemination on the gene expression in primary tumours and tumour-adjacent tissues. Displayed only genes showing significant trends based on node positivity. **f** Heatmap showing the effect of metastatic dissemination and the expression of MMP9, the only gene showing significant trend. Displayed as median, box shows second–third quartile, whiskers show non-outlier range

MKI67 was higher in the 3 grade tumour tissue T (3 vs. 2 grade patients); (fourfold change, 95 % CI 1.66–8.41 at $p=0.002$ for *BCL2*; threefold change, 95 % CI 1.22–7.07 at $p=0.02$ for *MT2A*; and fourfold change, 95 % CI 1.3–11.71 at $p=0.02$ for *MKI67*, respectively). The expression of *MT2A* was higher too (12-fold change, 95 % CI 1.58–93.47 at $p=0.02$) in 3 grade tumour-adjacent tissue A (3 vs. 2 grade patients). Lower *BAX/BCL2* ratio was found in the 3 grade tumour-adjacent tissue (A) compared to the 2 grade tumour-adjacent tissue (0.02-times lower, 95 % CI 0–0.91, $p=0.05$). Furthermore, the expression of *FLT1* and *EGF* was associated with the tumour grade (3 vs. 1+2 grade patients) in adjacent tissues. Lower *FLT1* and *EGF* expression was found in the 3 grade patients’ tumour-adjacent tissue ($p=0.002$ and $p=0.04$, respectively). See Fig. 2 and Online resource 1.

Co-expression patterns of the genes

The previous analyses did not sufficiently highlight trends and relationships in the expression profiles of particular genes in the specific tissues (T, A or Tons). Therefore, correlations between gene expressions were performed; tumour tissues (T), adjacent tissues (A) and tonsillectomy (Tons) samples were analysed separately (Fig. 3a, c, e). In all studied tissues, some consistently occurring correlations were found. Strong positive correlations with $r>0.50$ at $p<0.05$ were identified between *MKI67* and the following genes: *EGFR*, *VEGFA*, *FLT1*, *BAX*, *TP53*, *MMP2*, *MMP9* and *FOS*. *TP53* correlated positively at equally strong correlation coefficient with *EGFR*, *MMP2*, *MMP9*, *FLT1*, *VEGFA*, *FOS*, *BAX* and *BCL2*. *EGFR* correlated with *MKI67*, *TP53*, *FLT1*, *MMP2*, *VEGFA*, *FOS*

Fig. 3 Gene co-expression pattern in tumour tissue, tonsillectomy tissue and tumour-adjacent tissue. **a, c, e** Heatmap of Pearson correlation coefficients. **b, d, f** Principal component analysis showing projection of variables (genes) on the factor plane of the first two factors. Thus, 69.41, 93.50 and 66.56 % of the total variance sum of variance of factors 1 and 2) of gene expression data is displayed using this two-factor analysis for tumorous, tonsillectomy, and tumour-adjacent tissue, respectively. Note that genes pointing to the different location than the “major cluster” are specific for this particular tissue type. For details, see the Results section



and *BAX*. Apoptosis regulator *BAX* correlated with *TP53*, *EGFR*, *BCL2*, *MKI67*, *MMP2* and *9*, *FOS* and *FLT1*. Expression of matrix metalloprotease 2 correlated with *FOS*, *VEGFA*, *MMP9*, *EGF* and *BCL2*, *MT1A* correlated positively with *JUN* and *MT2A*, and *FLT1* correlated with *VEGFA*.

On the other hand, some correlations were typical just for one kind of studied tissue. Strong correlations with $r > 0.70$ at $p < 0.05$ between gene expressions found exclusively in the tonsillectomy samples were as follows: *MT2A* and *BCL2*, *MKI67* and *BCL2*, *EGFR* and *BCL2*, *FLT1* and *BCL2*, *JUN* and *BCL2*, *MT2A* and the following genes: *BAX*, *TP53*, *MKI67*, *FOS*, *VEGFA*, *FLT1*, *MMP2*, *EGFR*, and *TP53* and *JUN* correlated with *MKI67*.

Co-expressions of mRNAs occurring only in the tonsillectomy and tumour samples and missing or weak in the tumour-adjacent tissues were as follows: *FLT1* correlated positively with *MMP2*, *MT1A* and *JUN*; *FOS* correlated with *MT1A*; and *JUN* correlated with *EGFR*.

Co-expressions of mRNAs occurring strongly in the tonsillectomy (Tons) and tumour-adjacent tissues (A) samples and weak in the tumour tissues (T) were as follows: *BCL2* correlated with *MMP9*, *VEGFA* and *FOS*.

Nevertheless, the correlation analysis did not make it possible for us to interpret complex multidimensional relationships between the genes’ expression—so called “expression patterns” of tissues. Therefore, the principal component

analysis was used. The component analysis allowed us to detect the structure in relationships between gene expressions, and thus helped us reveal characteristic gene expression patterns for the respective tissues (Fig. 3b, d, f). To illustrate the model of specific tissue, two-factor analysis was chosen, which illustrates 69.41, 93.50 and 66.56 % of the total variance of gene expression data for tumorous, tonsillectomy and tumour-adjacent tissues, respectively (sum of total variance for each factors). Based on this analysis, the majority of genes are clustered together. In contrast, genes that are not included in this cluster (their vector points in another direction) are characteristic for these tissues. The most significant shift of expression within the expression patterns was found for *EGFR* and *MKI67* in case of the distinction of patients samples (T and A) from histologically normal (Tons) tissues; characteristic shift of *JUN*, *BCL2* and *MMP9* in case of the tumour tissue (T); characteristic shift of *JUN* and *FLT1* in case of the tumour-adjacent tissue (A); and specific shift of *EGF*, *MT2A* and *MT1A* in case of the Tons samples.

Discussion

Due to the large inter-individual variability in mRNA expressions, it is highly unlikely to find a single marker suitable for HNSCC prediction and prognosis. Therefore, our study was focused on the expression analysis of a panel of molecular markers that present key functions related to the neoplastic transformation-acquisition of autonomous proliferative signalling, proliferative activity of tumour cells, cell cycle and cell death modifications, angiogenesis, metastatic potential and oxidative stress response. The rationale of the use of the two types of control tissues (tonsillectomy group or tumour-adjacent tissues) is to overcome the limitations of each. Tonsillectomy tissues were used as an example of the inflamed, yet not transformed tissue. Tumour-adjacent tissues seem to be more similar to tumour tissues in many ways. *MT2A* and *BAX* expression was higher in the tumour-adjacent tissues compared to the tonsillectomy samples, which was simultaneously observed in the tumour tissues compared to the tonsillectomy samples. The most significant shift of expression within the expression patterns was found for *MT2A*, *MT1A*, *EGF*, *EGFR* and *MKI67* in case of the distinction of patients samples (T and A) from histologically normal (Tons) tissues (see Fig. 3), suggesting a significant role of these genes in characteristics of the respective tissues. Significant was also the difference in *BAX/BCL2* ratio between A and Tons, which was 33-fold higher in A. This could refer to remodelling of adjacent tissues which is often accompanied by apoptosis [12]. Differences between tumour-adjacent tissues and tumour tissue involved differentially expressed *MMP9*, *MT2A*, and *VEGFA*, which were more expressed in tumour tissues and *EGF*, which was more expressed in A.

Many tumours, including HNSCC, have been associated with permanent inflammation and oxidative stress [13, 14]. In a number of experiments, the synthesis of metallothionein (MT) was shown to be induced during oxidative stress [15, 16] and indeed numerous studies discovered increased MT protein levels in HNSCC tumours [17–19], comprehensively summarised in Gumulec et al. review [20]. Furthermore, results of Inoue et al. indicate that MT protects against inflammation via suppression of IL-1 expression and through its anti-oxidative potential [21, 22]. Accordingly, *MT2A* expression level was significantly higher in the HNSCC tumour tissue samples than in those of the tonsillectomy group or tumour-adjacent tissues.

Although the patients undergoing tonsillectomy were younger than the HNSCC patients, differences in the expression should still be significant, because the metallothionein expression shows rather an age-related decrease [23]. In this study, the higher expression of *MT1A* and *MT2A* was significantly associated with a higher tumour grade, suggesting an important role of MT expression in cancerogenesis. Dutsch-Wicherek et al. revealed that MT expression was significantly higher in the tumour-adjacent tissue than in the cancer tissue in cases with the presence of lymph node metastases. In general, the adjacent tissues seem to respond to the presence of tumour by the expression of MT [24], which makes this tissue type (together with other reasons) inappropriate to consider as healthy control. This fact can also be supported by the finding that fibroblasts exhibited high vimentin and MT immunoreactivity levels in the tumour microenvironment [19], which could be reason for the higher expression of MT in the tumour-adjacent tissues. Furthermore, the rate of MT protein expression in macrophages and fibroblasts in the tumour microenvironment appears to be an indicator of microenvironment remodelling associated with the local progression of cancer [25]. Higher MT expression was detected in the primary tumour itself than in its derived lymph node metastases [26]. Co-expression of *MT2A* with *MKI67*, *TP53*, *BAX*, *FLT1* and *EGFR* specific for tonsillectomy tissues indicates that oxidative stress and DNA damage triggers differential MT2-associated response in tonsillectomy and tumorous tissues. In tonsillectomies, the MT expression is associated with proliferation and apoptosis [27], whereas in HNSCC patients, the MT expression is associated with different mechanisms, such as preventing oxidative stress.

cJUN NH2-terminal kinase (JNK) is a member of the mitogen-activated protein kinases (MAPK) involved in non-canonical Wnt signalling and planar cell polarity. It is activated in response to growth factors, inhibition of DNA and protein synthesis, environmental stress and inflammatory cytokines, all of which regulate cell proliferation, differentiation and apoptosis [28]. *JUN* expression was higher in adjacent tissues compared to Tons. Co-expression of *JUN* and the proliferation marker *MKI67* was present only in the tonsillectomy

samples. This implies a key role of *JUN* in the cell proliferation in non-transformed tissue, and on the other hand, not so important role of *JUN* in proliferation triggering in the tumour-adjacent tissues. Li et al. demonstrated that the expression of *JUN* in fibroblasts in the tumour microenvironment can promote the secretion of IGF-1. They also showed IGF-1 acting as a paracrine molecule that stimulates epithelial cell proliferation [29, 30]. IGF-1 receptor (IGF-1R) activation was also found to be able to suppress anoikis and hence to promote the development of metastases [31, 32].

The higher expression of *MKI67* in the tumour tissue was significantly associated with a higher tumour grade (3 vs. 2) in our study. Couture et al. have previously found that patients with low-proliferation OSCC ($Ki-67 \leq 20\%$) have a worse response to radiotherapy than patients with highly proliferative tumours [33]. Notably, cell proliferation in cancer cells (determined by Ki-67 immuno-staining) was significantly correlated with the oxidative metabolism in mitochondria (OXPHOS) and with the consumption of mitochondrial fuels [34].

Furthermore, the elevated expression of *BCL2* in the HNSCC tumour tissue was also significantly associated with node positivity and a higher grade. Accordingly, a lower *BAX/BCL2* ratio was also found in the adjacent tissue of grade 3 tumours as compared with grade 2, which implies the persistent important role of evading apoptosis in advanced HNSCC tumours. The simultaneous detection of *BCL2* protein over-expression and p53-gene mutation in a tumour biopsy specimen was associated with the worse survival of HNSCC patients treated by radiotherapy [35]. It was also suggested that *BCL2* works as an antioxidant by preventing ROS production and precluding cellular damage caused by lipid peroxidation. Moreover, *BCL2* plays a role in redistributing GSH to specific cellular compartments [36, 37]. In addition to its anti-apoptotic property, *BCL2* was also found to enhance the invasivity and migration of some cancer cell lines by altering the level of matrix metalloproteinases (MMPs) and their inhibitors [38, 39]. The over-expression of *BCL2* resulted in eminent elevation of MMP-2 expression and secretion, as well as in the increase of its activity [40]. Accordingly, a significant correlation between the expression of *MMP2* and *BCL2* in all studied tissue types was observed in this study.

Many studies revealed that gelatinases (MMP-2, MMP-9) are over-expressed in HNSCC [41–46]. A degradation of the basement membrane is an important initial step for invasion and metastasis formation. Main component of the basement membrane is type IV collagen; disintegration of this extracellular matrix protein is mediated by MMPs, namely by MMP-2 and MMP-9. Accordingly, these MMPs are tightly associated with the malignant potential of tumour cells [47]. MMP-9 and MMP-2 expression was significantly elevated in malignant tissues as compared with adjacent normal tissues and tonsillectomies assessed in one group. In consistence with other

experimental results [48, 49], MMP9 is overproduced by HNSCC cells themselves, rather than by cancer-associated fibroblasts. Thus, MMP-9 appears to be a promising molecule for targeted cancer therapy [50]. Nevertheless, a possible anti-tumour activity of MMP9 (*inter alia*, production of anti-angiogenic molecules) and the interference with physiological MMP9 functions must be taken into account [51].

When the expression for the tumour samples and the matched adjacent tissue samples were analysed using a paired *t* test analysis, two of more differentially expressed genes were *MMP9* and *VEGFA*, both more expressed in the tumour tissues. *VEGFA* expression was not significantly associated with the tumour size, histological grade or presence of metastases in HNSCC patients in our study; nevertheless, in the study published by Parikh et al.⁵⁰, strong *VEGFA* immuno-staining was found as an independent unfavourable prognostic factor in the tumour specimens of laryngeal cancer. Several studies have shown that *VEGF*-targeted drugs suppress the growth of primary tumours, but on the other hand may promote tumour metastases [52, 53]. *VEGFA* is produced by cancer cells as well as by stromal cells and manifests strong angiogenic actions by binding to its receptors, one of which is *FLT1* (fms-related tyrosine kinase 1). Immunohistochemistry of HNSCC tissue samples showed *FLT1* and *VEGFA* to be co-expressed [54], which is in accordance with the significant correlation between *FLT1* and *VEGFA* gene expression found in all studied types of tissues in our analysis. The weakest *FLT1* and *VEGFA* correlation was recorded in the tumour-adjacent tissues, which might imply, that some portion of produced *VEGFA* mRNA could be used for the tumour and not for the adjacent tissues themselves [55]. It was shown that in addition to its pro-angiogenic functions, *VEGF* directly influences proliferation, migration and epithelial to mesenchymal transition of tumour cells, which mediates switch to an invasive phenotype [56]. Interestingly, the expression of *FLT1* in the tumour-adjacent tissues was significantly negatively associated with the tumour grade and lymph node-positive status (N+). A lower *FLT1* expression was identically found in 3 grade and N+ patients. Moreover, a lower expression of *EGFR* in the tumour-adjacent tissues of N+ patients was found. In conjunction with the fact that *EGF* was expressed less in T than in A, existence of grow supporting mechanisms of tumour cells by tumour-adjacent tissues can be assumed. It was also found that *EGF* gradients could direct invasion into surrounding tissues [57]. Van Limbergen et al. observed that *FLT1* increased expression in the tumour tissue as compared with the normal epithelium [54], but we found no significant differences between *FLT1* expression in tumour, tumour-adjacent tissues or tonsillectomies.

A higher expression of *EGFR* in tumour tissues compared to A and Tons tissues was found, too. Over-expression and autocrine activation of *EGFR* was found in approximately 90 % of HNSCC, this over-expression being associated with

the poor prognosis, independent of therapy [58–61]. Slightly lower expression levels of *EGF* were found in 3 grade tumour-adjacent tissues, which imply that *EGF* plays a significant role in the early stages of oral carcinogenesis. Once a carcinoma has developed, the role of *EGF* appears to become less defined [62].

Conclusion

Accumulation of alterations in expression patterns is an important event for the transformation from normal to cancer tissue in the multistep carcinogenesis. Histopathologically healthy tumour-adjacent tissue might be considered as a cancerisation field, which is typified by genetic changes required for the development of cancer. In our study, the elevated *MT2A*, *BAX*, *EGF*, and *JUN* expression was associated with the influence of tumour cells on the rearrangement of healthy tissues, as well as a significant shift in *BAX/BCL2* ratio. Moreover, expression patterns of tumour-adjacent cells seem to be helpful in defining tumour progression or in predicting of node positivity. Our investigation also demonstrated that adjacent tissues play an important role in cancerogenesis by releasing several tumour-supporting factors such as *EGF*. A gradual increase in the metallothionein expression, from the lowest in tonsillectomy samples to the highest in tumour samples, suggests that MT expression might be a tissue response to the presence of tumour cells. Many examined trends in expression and expression patterns were statistically non-significant due to patients' heterogeneity and cellular heterogeneity. However, the inclusion of all cell types in the evaluated tissues made it possible for us to assess the strongest effects of expression patterns on pathogenesis of HNSCC.

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Conflicts of interest None.

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4.2 Prognostická role c-Met: meta-analýza

V předchozí práci Raudenska *et al.*⁹⁹ byly studovány změny v tkáňové expresi genů mezi kontrolami a pacienty s nádory, jejich prognostický potenciál studován nebyl. Ve spojitosti se spinocelulárními karcinomy je jednou z široce diskutovaných molekul tyrozinkinázový receptor pro hepatocytární růstový faktor, c-Met^{106,107}. Jeho fyziologická úloha je spojena s klíčovými procesy během embryogeneze a hojení ran, jmenovitě např. schopnost migrujících buněk odloučit se od extracelulární matrix, uniknout anoikis a zůstat v nově vznikající tkáni²². Ve spojitosti s progresí nádorů byla tato molekula spjata s fenotypem nádorových kmenových buněk a s mechanismem rezistence oproti inhibitorům EGFR novými terapeutiky, např. cetuximabem¹⁰⁸.

I když existuje spektrum prací asociujících tento tyrozinkinázový receptor s nepříznivou prognózou, jejich výsledky se liší a neexistuje meta-analýza, která by toto zjištění podpořila silnější statistickou metodou^{109,110}.

V této práci bylo formou meta-analýzy shrnuto 17 prací s celkem 1724 pacienty studující asociaci přežití pacientů s overexpresí c-Met. Bylo zjištěno, že overexprese je signifikantně korelována s nepříznivým celkovým přežitím (hazard ratio (HR) = 2.19, 95 % interval spolehlivosti (CI) = 1.55-3.10) a přežitím bez relapsu (HR = 1.64, 95% CI = 1.24-2.17, viz obrázek na str. 117). Pomocí této meta-analýzy bylo také zjištěno, že overexprese cMet je také asociována s pozitivitou uzlin, odds ratio (OR) = 1.76, 95 % CI = 1.26-2.45. Na základě změn v expresi c-Met je tedy možné predikovat nepříznivou prognózu pacientů.

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OPEN Prognostic role of c-Met in head and neck squamous cell cancer tissues: a meta-analysis

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This meta-analysis aims to evaluate the effects of high c-Met levels in head and neck squamous cell carcinomas (HNSCC) on survival and clinicopathological features. Publications concerned with the clinical significance of c-Met protein expression in HNSCC were identified from the Scopus and Web of Science database searches. To elucidate the relationship between c-Met expression and clinical outcomes, a meta-analysis of the selected articles was conducted. Seventeen publications involving a total of 1724 patients met the inclusion criteria. c-Met overexpression was significantly correlated with poor overall survival (hazard ratio (HR) = 2.19, 95% confidence interval (CI) = 1.55–3.10). c-Met immunohistochemical staining positivity was also associated with worse relapse-free survival (HR = 1.64, 95% CI = 1.24–2.17) and presence of regional lymph node metastases (odds ratio (OR) = 1.76, 95% CI = 1.26–2.45). High levels of c-Met expression in HNSCC predict unfavorable prognosis associated with common clinicopathological features.

c-Met is a transmembrane tyrosine kinase receptor for hepatocyte growth factor (HGF) also named scatter factor (SF). Its physiological function is connected to key processes in tissue embryogenesis and wound healing such as the ability of migratory cells to detach from the extracellular matrix, to elude anoikis and finally to settle in newly forming or damaged tissue¹. By hijacking these pathways through c-Met activation, tumors increase their potential for invasive growth and metastasis².

Head and neck squamous cell carcinoma (HNSCC) is the prominent histological type of head and neck cancer causing more than 200,000 deaths each year³. The currently most used prognostic system is the TNM classification which relies on gross pathological features of the tumor and has inherent limitations in its ability to predict the aggressiveness of the neoplasm⁴.

To address these issues, several biomarkers are currently under investigation as potential prognostic factors in HNSCC⁵. c-Met has recently gained significant attention due to it is being associated with the cancer stem cell population⁶ and also acting as a key component of resistance mechanism against epidermal growth factor receptor (EGFR) inhibition by novel therapeutics such as cetuximab⁷.

Owing to its importance in cancer progression, aberrant c-Met signalling has been studied specifically in the context of HNSCC mainly by identifying *MET* gene mutations and copy number alterations or by assessing c-Met overexpression⁸. While some studies have linked c-Met dysregulation to worse prognosis in HNSCC patients^{9–11}, others have not found any significant correlation^{12,13}. The aim of this meta-analysis is, therefore, to aid investigators and physicians in evaluating the role of c-Met as a robust prognostic factor in head and neck squamous cell carcinoma.

Results

Identification and characteristics of relevant studies. The literature selection process of the eligible studies is presented in Fig. 1. The set of 17 final articles included 1724 patients with 101 tissue samples per study on average. The date of publication ranged from 2001 to 2017 with a median of the year 2011. A total of 8 studies were proceeded in Asia, 6 in Europe and 3 in the USA. All studies analyzed c-Met expression levels with immunohistochemical methods (IHC), which was chosen as the main and the only determinant of c-Met expression level in this meta-analysis. Several papers also investigated *MET* gene amplification status as a prognostic factor but the scarcity and heterogeneity of the data prevented a reasonable statistical analysis^{11,12,14}. Only studies explicitly

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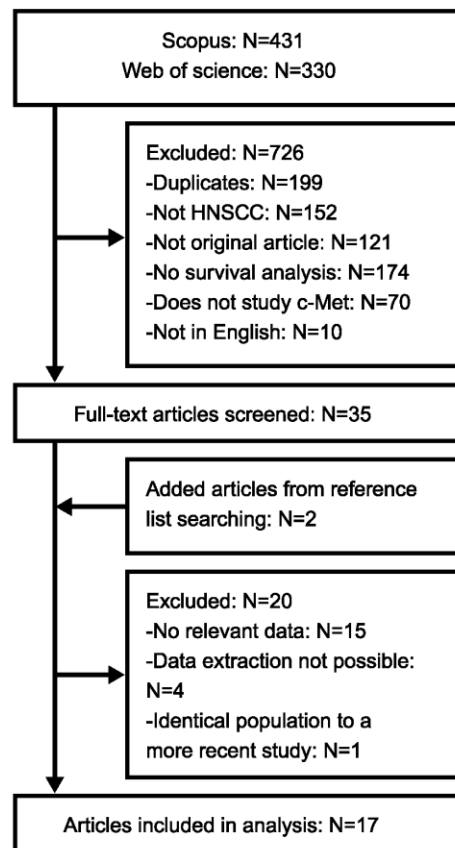


Figure 1. Flow chart of the article selection process.

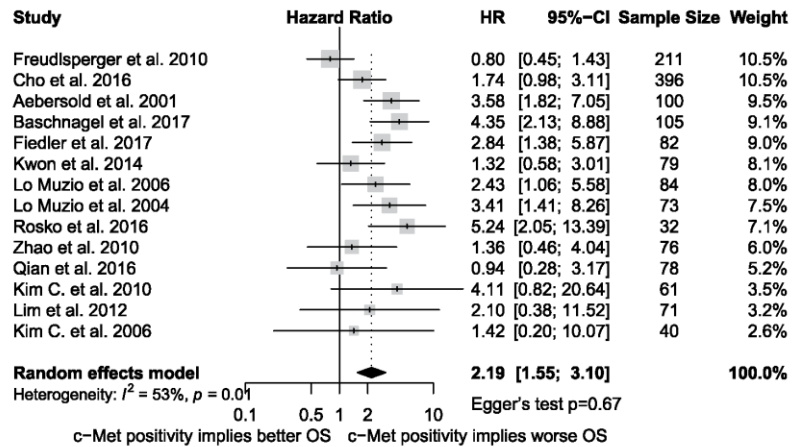
including squamous cell cancer were included. While 4 studies in total have explored c-Met expression levels in all various sites of the head and neck, 9 studies have focused only on the oral cavity, 3 only on oropharyngeal tumors and lastly, a single study included solely hypopharyngeal lesions. A subgroup analysis dissecting differences among these locations was not feasible due to lack of data on the association between clinical outcomes and the tumor site.

Fourteen studies explored the prognostic significance of c-Met in overall survival, while only 11 investigated relapse-free survival (i.e. disease-free survival). Association of several clinicopathological parameters with c-Met expression level was also studied by some of the selected papers. A single study only analyzed c-Met staining positivity and clinicopathological parameters but not patient survival¹⁵. Specifically, 12 articles evaluated N tumor staging, 10 also assessed T tumor staging and/or prognostic clinical staging, 9 studied tumor differentiation level (i.e. histological grading), 6 investigated the presence of distant metastases, 4 articles measured locoregional failure occurrences and finally, 2 papers provided p16 positivity values in high and low c-Met expression groups. Supplementary information presents all the studies included in the meta-analysis in detail.

Relationship of c-Met expression with overall and relapse-free survival. Meta-analysis of 14 applicable studies showed that positive staining for c-Met was associated with lower OS ($HR = 2.19$, 95% $CI = 1.55-3.10$; Fig. 2a)^{9,10,12-14,16-24}. A moderate measure of heterogeneity was observed among these studies ($I^2 = 53\%$, $p_h = 0.01$). Combined data from a total of 11 studies also demonstrated a relationship between positive c-Met staining and poor RFS ($HR = 1.64$, 95% $CI = 1.24-2.17$; Fig. 2b) without significant interstudy heterogeneity ($I^2 = 20\%$, $p_h = 0.25$)^{9,10,12,14,18,19,21-23,25,26}.

Additional analysis of the 9 studies with stricter cutoff criteria revealed association of high c-Met expression and poorer OS ($HR = 2.15$, 95% $CI = 1.44-3.21$; Fig. 3a)^{10,12,14,16,18,20-23} without significant heterogeneity ($I^2 = 27\%$, $p_h = 0.20$). Seven of these studies also evaluated the impact of c-Met overexpression on worse RFS. Statistically significant association was found after combining the data ($HR = 1.58$, 95% $CI = 1.12-2.22$; Fig. 3b)^{10,12,14,18,21-23}.

a Overall survival



b Relapse-free survival

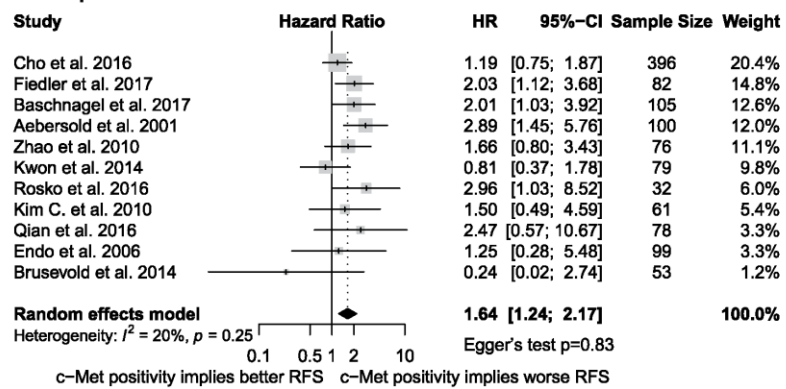


Figure 2. Forest plot of the association between c-Met staining positivity and OS (a) and between c-Met staining positivity and RFS (b). HR, hazard ratio; CI, confidence interval.

All studies were separated into groups based on the location where they were conducted (Asia vs. Europe and USA), sample size (equal to or greater than 100 vs. less than 100) and the ratio of c-Met positive to c-Met negative samples (equal to or greater than 50% vs. less than 50%). In all studied subgroups, c-Met staining positivity was associated with both poorer OS and RFS, except for the "Asian" subgroup which had a significant relationship only between c-Met staining positivity and worse OS but not between c-Met staining positivity and poorer RFS (Table 1).

c-Met expression and clinicopathological features. c-Met staining positivity was significantly correlated with regional lymph nodes metastasis ($N0$ vs. $N1 + N2 + N3$; $OR = 1.86$, 95% $CI = 1.14-3.03$; Table 2). No significant relationship was found between c-Met staining positivity and advanced T stage ($T1 + T2$ vs. $T3 + T4$; $OR = 1.26$, 95% $CI = 0.86-1.86$), presence of distant metastasis ($OR = 1.96$, 95% $CI = 0.88-4.37$), locoregional failure ($OR = 2.48$, 95% $CI = 0.97-6.35$), poor tumor differentiation ($G1 + G2$ vs. $G3$; $OR = 0.82$, 95% $CI = 0.46-1.43$) or condensed TNM stage ($I + II$ vs. $III + IV$, $OR = 1.70$, 95% $CI = 0.90-3.19$). Also, no correlation was found between high levels of c-Met and p16 positivity ($OR = 0.65$, 95% $CI = 0.30-1.43$), albeit only 2 studies investigated this parameter. Results of this analysis are presented in Table 2.

Publication bias. Publication bias was analyzed for OS, RFS and all subgroup analyses larger than 5 studies. Upon visual inspection of Begg's funnel plots, no obvious asymmetry was noted (Supplementary information). Further investigation by Egger's test also did not provide evidence for publication bias in any of the aforementioned parameters (Table 1, Figs 2 and 3).

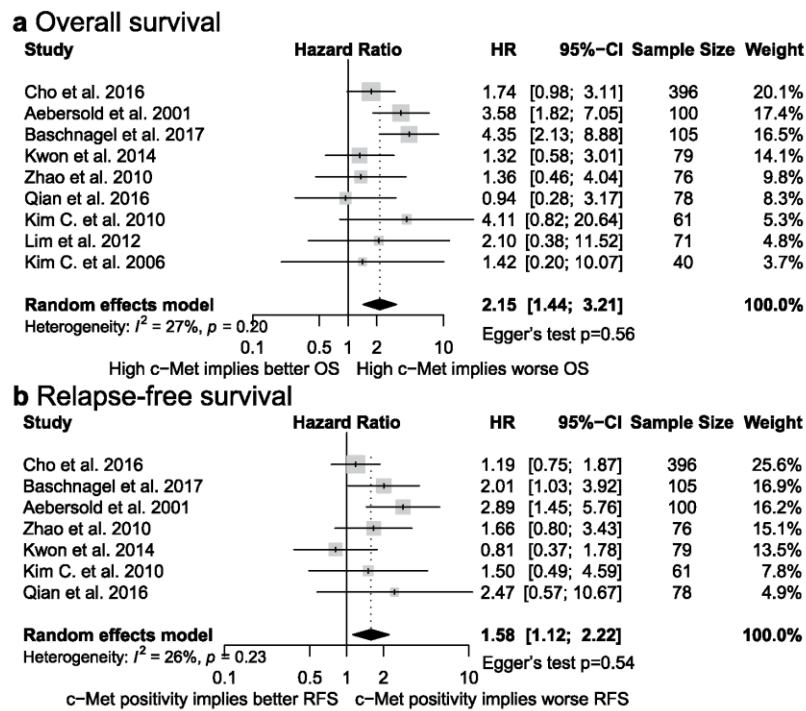


Figure 3. Forest plot of the association between c-Met expression and OS (a) and between c-Met expression and RFS (b) in studies with stricter cutoff criteria. HR, hazard ratio; CI, confidence interval.

Discussion

Identification of prognostic markers enables stratification of patients into high-risk groups for whom a specific therapy could be necessary. In this meta-analysis, a significant relationship between high c-Met expression level and poor overall survival in the context of head and neck squamous cell carcinoma has been demonstrated.

Even though immunohistochemistry was used in all the included studies, a primary obstacle in interpreting and combining the outcome data was the diversity of cutoff definitions for the high and low expression patient groups used by the authors. In some studies, the chosen criteria have taken into account all the possible immunohistochemical factors and at the same time have been strict enough to warrant the usage of the term high/low c-Met expression. In other included articles, the criteria have not been clear or stringent enough and thus the separated patient groups can only be recognized as c-Met staining positive or negative, while the c-Met staining positive group could have expression levels ranging from fairly low, through moderate to high. To improve the interpretability of the results, overall and relapse-free survival values were calculated for all of the studies taken together and then separately for the high/low expression studies with stricter criteria. With all the studies combined, c-Met staining positivity was associated with poorer overall and relapse-free survival. When considering only the subset of studies with stricter cutoff criteria, high c-Met expression was correlated with worse overall survival but not with poorer relapse-free survival. This discrepancy could have arisen due to reduced statistical power when analyzing only a subset of all the possible studies. A unified method of scoring tissue samples for c-Met overexpression would greatly improve the comparability of future studies. A possible candidate for this role is a scoring method used in three of the eight high/low expression studies, the only one employed by more than a single group of authors.

Subgroup analysis showed that neither sample size, c-Met positive to c-Met negative ratio, nor the stratification of patients by Asian and Western countries have influenced the statistical significance of the association of c-Met staining positivity with poor overall survival. This was also true for the relationship between c-Met staining positivity and relapse-free survival, except for the Asian subgroup, where an insignificant hazard ratio was calculated, perhaps due to a higher measure of survival data extraction from Kaplan-Meier curves and therefore introduced error in the Asian subgroup.

The biological role of c-Met provides a possible explanation for its association with poor prognosis. Aberrant HGF/c-Met signalling has been previously shown to suppress E-cadherin expression and on the other hand, increase expression of matrix metalloproteinases thus inducing a migratory phenotype in the affected cells^{27,28}. Indeed, in the present meta-analysis, a relationship between c-Met positivity and regional lymph node metastases was observed. Furthermore, a previously published study has shown c-Met-positive head and neck cancer cells

Factor	Subgroup	Sample characteristics		Survival statistics		Heterogeneity		Publication bias
		Studies N	Patients N	HR (95% CI)	p_{hr}	I^2 (%)	p_h	Egger's test p
OS								
	Overall	14	1488	2.19 (1.55–3.10)	<0.01	53	0.01	0.67
	Asia	6	723	1.67 (1.12–2.49)	0.01	0	0.88	0.54
	EU/USA	8	765	2.50 (1.54–4.07)	<0.01	70	<0.01	0.51
	Large Sample	4	812	2.12 (1.00–4.51)	0.05	83	<0.01	NA
	Small Sample	10	676	2.31 (1.63–3.28)	<0.01	6	0.39	0.60
	High c-Met positivity	8	721	1.73 (1.04–2.90)	0.04	51	0.05	0.65
	Low c-Met positivity	6	767	2.72 (1.80–4.13)	<0.01	44	0.11	0.35
RFS								
	Overall	11	1161	1.64 (1.24–2.17)	<0.01	20	0.25	0.83
	Asia	5	711	1.21 (0.88–1.68)	0.24	0	0.76	0.86
	EU/USA	6	450	2.22 (1.58–3.12)	<0.01	5	0.51	0.31
	Large Sample	3	601	1.81 (1.06–3.08)	0.03	59	0.09	NA
	Small Sample	8	560	1.57 (1.11–2.24)	0.01	8	0.37	0.45
	High c-Met positivity	5	368	1.98 (1.29–3.03)	<0.01	13	0.33	0.20
	Low c-Met positivity	6	793	1.52 (1.07–2.15)	0.02	26	0.24	0.7

Table 1. Subgroup analysis of the studies reporting the association of c-Met positivity and overall survival (OS) or relapse-free survival (RFS). HR, hazard ratio; CI, confidence interval; p_{hr} , random effects model p values for hazard ratios; p_h , test of heterogeneity p value; NA: not available.

Clinicopathological feature	Studies N	Sample size	OR (95% CI)	I^2 (%)	p_h	Egger's test p
T3/T4	10	1230	1.26 (0.86–1.86)	65	0.020	0.21
N ⁺	12	1371	1.86 (1.14–3.03)	65	0.01	0.22
Distant metastasis	6	675	1.96 (0.88–4.37)	6	0.38	0.57
Stage III/IV	10	883	1.70 (0.90–3.19)	65	<0.01	0.69
Poor differentiation	9	679	0.82 (0.46–1.43)	42	0.09	0.43
Locoregional failure	4	282	2.48 (0.97–6.35)	59	0.06	NA
p16 ⁺	2	475	0.65 (0.30–1.43)	58	0.12	NA

Table 2. association between c-Met staining positivity and clinicopathological features. OR: odds ratio; p_h : test of heterogeneity p value; NA: not available.

to possess cancer stem cell properties and to contribute to chemo- and radiotherapy resistance⁶. HPV positivity of head and neck tumors is usually accompanied by p16 positivity and can have very different outcomes when compared to HPV negative cancers²⁹. A relationship between c-Met positivity and p16 positivity could be in part responsible for these varying outcomes. However, no statistically significant correlation was found in our study.

There are several limitations which must be kept in mind when interpreting the results of this meta-analysis. First, usage of different antibodies against c-Met among studies contributes to ambiguity. Second, the number of patients in each study is generally small, thus reducing the power and precision of subgroup analyses. Lastly, only manuscripts published in English were considered for inclusion in this study, ignoring potential high-quality articles in other languages.

In summary, this meta-analysis shows that c-Met overexpression is an indicator of poor overall survival in head and neck squamous cancer patients, while c-Met immunohistochemical staining positivity is associated with worse overall and relapse-free survival as well as higher rates of regional lymph node metastases. These findings can help expand the application of c-Met as a prognostic marker in the clinical setting.

Methods

Search strategy and selection criteria. A systematic search of the published literature was conducted in the Scopus and Web of Science databases using the following terms: (c-met OR “kinase MET” OR “MET kinase” OR HGFR OR SFR OR “scatter factor receptor” OR “hepatocyte growth factor receptor”) AND (hnscc OR (“head and neck” AND cancer) OR ((*phary* OR ling* OR oral OR laryn*)) AND (cancer OR tumo* OR carcinoma*)). Titles and abstracts of the resulting studies were reviewed to remove unrelated papers. The remaining part of the studies was read in full with the reference lists scanned for relevant articles.

In order to be selected, a study had to meet the following inclusion criteria: (1) was published in English; (2) measures c-Met protein level in human head and neck tumors; (3) provides survival data such as hazard ratio (HR) of overall survival (OS) or relapse-free survival (RFS) or corresponding Kaplan-Meier curves or provides data correlating c-Met protein level and clinicopathological features such as tumor TNM staging. When multiple

articles included the same population of patients, only the most recently published paper was considered. The eligibility of the studies for meta-analysis was evaluated by two authors (V.V. and J.G.).

Data extraction. The following characteristics were extracted from each eligible study: (1) age and sex of the patient population; (2) follow-up length; (3) number of cases in c-Met positive and negative groups; (4) TNM staging, clinical staging, histological grades and anatomical site of the tumor samples; (5) c-Met positivity definition and cutoff values; (6) HRs with 95% CIs for OS and RFS or corresponding Kaplan-Meier curves. Preferentially, values calculated with multivariate (i.e. multivariable) Cox proportional hazard model were used in the meta-analysis. If these were not available, results from univariate Cox regression or estimates from Kaplan-Meier survival curves were utilized instead. Calculation of HR from Kaplan-Meier curves was conducted according to Tierney *et al.*³⁰. Extracted data are available at Supplementary dataset 1.

Cutoff definitions for high c-Met expression. Due to no clear and standardized criteria for stratification of patients into high- and low-expression groups based on c-Met IHC results, authors of the included studies employed a wide variety of scoring algorithms to distinguish these two patient groups. Of the 17 included studies, only 9 have evaluated both staining intensity along with the relative number of cells stained and also had stringent enough cutoff criteria to truly separate patients into high- and low-expression groups^{10,12,14,16,18,20–23}. Of these 9 articles, three have used a completely identical cutoff definition^{16,21,31}. The 8 remaining papers have based their cutoff values only on either weak staining intensity or a relatively small proportion of stained cells without explicitly mentioning any consideration was given to the strength of the staining^{9,13,15,17,19,24–26}.

Based on this, the 9 selected studies with stricter cutoff criteria have also been analyzed separately as c-Met high expression studies while all articles taken together are further termed c-Met positivity studies (Supplementary information).

Statistical analysis. The expression of c-Met was determined as high or low, according to the definition and cutoff values of each study. To evaluate the effect of association between c-Met expression and OS or RFS, the hazard ratio of patients with low c-Met expression vs. patients with high c-Met expression was used. HR > 1 implied worse prognosis for the patients with high levels of c-Met expression.

All analyses were carried out using the R packages meta (version 4.8–4, <https://CRAN.R-project.org/package=meta>) and metafor (version 2.0-0, <https://CRAN.R-project.org/package=metafor>). Because each included study was done on a different population and using varying c-Met expression cutoff values, a random effects model was used to combine the data. Heterogeneity among studies was evaluated by the restricted maximum-likelihood (REML) method. Publication bias was first assessed graphically by Begg's funnel plot analysis and consequently using Egger's test with significant publication bias defined as $p < 0.05$. Only parameters with at least 5 applicable studies were analyzed for publication bias.

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Author Contributions

Study concept: M.M., J.G.; study design: M.M., M.R., V.V., data acquisition: V.V.; data analysis and interpretation: V.V., J.G.; manuscript preparation: V.V., M.R.; manuscript review and editing: M.R.

Additional Information

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4.3 Molekulární markery HNSCC na úrovni miRNA

Práce Raudenska *et al.*⁹⁹ se zabývala analýzou potenciálních tkáňových biomarkerů na úrovni mRNA. Translace (těchto RNA) do proteinů je ovlivněna řadou procesů, mj. epigenetickými mechanismy – pomocí miRNA. Následující práce Hudcova *et al.* se zaměřuje na studium právě těch miRNA ovlivňujících expresi genů over/under-exprimovaných v nádorové tkáni, jak ukázáno ve studii⁹⁹. K predikci bylo využito dat z literatury¹¹¹ a databáze Targetscan (<http://www.targetscan.org>). Důraz byl kladen zejména na gen metalothionein a jeho regulační molekuly, např. transkripční faktor MTF1.

V této práci (str. 123) byla ověřována exprese miR-29c-3p, miR-34a-5p, miR200b-5p a miR-375 v nádorové tkáni a ve tkáni přilehlé k nádoru. Signifikantní rozdíly v expresi byly nalezeny u miR-29c-3p a miR-375. Obě zmiňované miRNA byly více exprimované ve tkáni přiléhající k nádoru v porovnání s tkání nádorovou, což odpovídá jejich předpokládané roli nádorového supresoru (viz obrázek na str. 126).

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Expression profiles of miR-29c, miR-200b and miR-375 in tumour and tumour-adjacent tissues of head and neck cancers

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Abstract Altered expression of microRNAs (miRNAs) has been shown in many types of malignancies including the head and neck squamous cell carcinoma (HNSCC). Although there are many new and innovative approaches in the treatment of HNSCC, a clear marker of this disease is still missing. Three candidate miRNAs (miR-29c-3p, miR-200b-5p and miR-375-3p) were studied in connection with HNSCC using quantitative real-time PCR expression levels in 42 tissue samples of HNSCC patients and histologically normal tumour-adjacent tissue samples of these patients. Primary HNSCC carcinoma tissues can be distinguished from histologically normal-matched noncancerous tumour-adjacent tissues based on hsa-miR-375-3p expression (sensitivity 87.5 %, specificity 65 %). Additionally, a significant decrease of hsa-miR-200b-5p expression was revealed in tumour-adjacent tissue samples of patients with node positivity. Lower expression of hsa-miR-200b-5p and hsa-miR-29c-3p in HNSCC tumour tissue was

associated with higher tumour grade. Consequently, survival analysis was performed. Lower expression of hsa-miR-29c-3p in tumour-adjacent tissue was associated with worse overall and disease-specific survivals. Lower expression of miR-29c-3p in tumourous tissue was associated with worse relapse-free survival. hsa-miR-375-3p seems to be a relatively promising diagnostic marker in HNSCC but is not suitable for prognosis of patients. Furthermore, this study highlighted the importance of histologically normal tumour-adjacent tissue in HNSCC progress (significant decrease of hsa-miR-200b-5p expression in tumour-adjacent tissue of patients with node positivity and low expression of hsa-miR-29c-3p in HNSCC tumour-adjacent tissue associated with worse prognosis).

Keywords Head and neck neoplasms · Carcinoma, squamous cell of head and neck · MicroRNAs · Biomarkers, tumour · Survival · Proportional hazards models

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Introduction

Head and neck squamous cell carcinoma (HNSCC) is the sixth most common type of cancer [1]. Despite new approaches in treatment of HNSCC, the overall 5-year survival rate for patients with HNSCC is only 50 % mostly because of the high rate of recurrences and advanced stage of disease by diagnosis [2]. Epidemiology of HNSCC has changed during the past 30 years; formerly, HNSCC was most commonly seen in older adults with a history of alcohol and tobacco use, and now it can be seen in younger adults in their 40s and 50s [3]. Thus, biomarkers with specific indications for diagnosis, prognosis and prediction of therapeutic response are desperately needed.

Many studies proved that the aberrations in the microRNA (miRNA) expression are tightly connected with pathogenesis of human cancers, including HNSCC [4, 5]. miRNAs are small RNA molecules (20–22 nucleotides) unable to encode proteins but managing significant catalytic, structural and post-transcriptional regulatory functions. They regulate target molecule by binding to target messenger RNA (mRNA) and inhibit protein translation or induce degradation of mRNA [6]. In this study, we focused on expression profiles of miR-29c-3p, miR-200b-5p and miR-375-3p. With detailed 5p arm and 3p arm and sequence presentation, we could achieve more reproducible results. The arm annotation is quite often lacking in other studies.

miR-29c belongs to the miR-29 family and is deregulated in many different types of cancer including nasopharyngeal carcinomas. miR-29c habitually has tumour-suppressive effect in those cancers [7–11]. The 3p arm of the miR-29 precursor is a prevailing product (miR-29c or miR-29c-3p), although the 5p arm (miR-29c* or miR-29c-5p) also objectively exists [12]. miR-200b-5p is a key regulator of epithelial-mesenchymal transition (EMT) involved in cancer metastasis and chemoresistance [13]. Furthermore, RNA-sequencing analysis revealed that enhanced expression of pri-miR-200b resulted in increased expression of both miR-200b-3p and miR-200b-5p. miR-200b-5p was not expressed in triple negative breast cancer cell lines with EMT features [14]. Hui et al. revealed that the downregulation of miR-375, presented in 91 % of HNSCC, would result in enhanced proliferation, deregulated growth and nonfunctional apoptosis [15]. Jung et al. also disclosed the miR-375-mediated suppression of multiple oncogenic pathways in HPV-associated carcinogenesis [16]. Downregulation of miR-375 may be a potential marker of metastasis occurrence and poor outcome in HNSCC [17].

In this study we focused on the evaluation of three miRNAs with supposed tumour-suppressive effect (miR-29c-3p, miR-200b-5p and miR-375-3p) as diagnostic and prognostic markers of HNSCC.

Materials and methods

Sample preparation

All procedures performed in this study were approved by the ethical committee of St. Anne's Faculty Hospital, Brno, Czech Republic, and were in accordance with the 1964 Helsinki Declaration and its later amendments or comparable ethical standards. All surgical tissue samples were obtained from HNSCC patients after they signed the informed consent. Histologically verified primary HNSCC carcinoma tissues (T) and matched non-cancerous adjacent tissues (A) were collected. The tissue material

harvested at surgery was placed into RNAlater solution for RNA stabilization and storage (Ambion, USA). The material was maintained cold, and RNA was isolated within 24 h.

Total RNA extraction, quantitative real-time PCR

We obtained total RNA from samples using TRIzol reagent (Invitrogen, UK). RNA concentrations and purity were determined by a NanoDrop spectrophotometer (Thermo Fisher Scientific, Wilmington, DE, USA). An optical density ratio at 260:280 nm was calculated to evaluate protein contamination of RNA. In addition to the ratio at 260:280 nm, measurements were taken also at 280 and 230 nm. Our 260:280 values were between 1.84 and 2.08. The A260/A230 ratio was greater than 1.5 in all samples. According to manufacturer's instructions, 10 ng of isolated RNA was transcribed using the TaqMan® miRNA reverse transcription kit (Applied Biosystems, USA), and 1.33 µl of the transcribed miRNA was used directly in the quantitative real-time PCR reaction. The primer and probe sets were selected from TaqMan miRNA expression assays hsa-miR-29c (assay ID: 000587), hsa-miR-200b (assay ID: 002274) and hsa-miR-375 (assay ID: 000564) (Applied Biosystems, USA). The amplified DNA was analysed by the comparative Ct method using RNU44 (assay ID: 001094) as an endogenous control. The qRT-PCR was performed under the following amplification conditions: total volume 20 µl, initial denaturation 95 °C/10 min, then 45 cycles 95 °C/15 s, 60 °C/1 min with the 7500 real-time PCR system (Applied Biosystems, USA). Sequence of studied miRNAs is shown in Table 1.

Data normalization and statistical analysis

Log-transformed miRNA expression data were analysed using a paired test (tumour versus tumour-adjacent tissue) and using one factor ANOVA. Survival analysis was analysed using Cox proportional hazard regression with miRNA expression levels as covariates. Receiver-operator curves (ROC), cutoff, sensitivity and specificity were calculated using Cutoff Finder (<http://molpath.charite.de/cutoff>) according to [18]. Unless noted otherwise, *p* level < 0.05 was considered significant. Software Statistica 12 (StatSoft, Tulsa, OK, USA) was used for analysis.

Table 1 Sequence of studied miRNAs

miRNA	Sequence
hsa-miR-375-3p	UUUGUUCGUUCGGCUCGCGUGA
hsa-miR-29c-3p	UAGCACCAUUUGAAAUCGGUUA
hsa-miR-200b-5p	CAUCUUACUGGGCAGCAUUGGA

Results

Clinico-pathological characterization of HNSCC patients

In this study, in total, 42 biopsy samples of tumours from male patients with histologically verified spinocellular carcinoma and comprehensive patient history were used. Only patients fulfilling following criteria were included: descriptors of the tumour are present (histology, tumour staging, grading) and patients with no current or previous malignancy. Therapeutic strategy was not taken into account. Sampling was performed before the therapy begun (either chemo-, radiotherapy or surgery). Age of patients and HPV status was not taken into account. Tumour-adjacent tissue was verified histologically. Expression of the selected miRNAs in tumour tissue was compared with the control group consisting of matched tumour-adjacent histologically normal tissue (39 samples). Brief description of the cases is shown in Table 2. In the next step, the effect of clinico-pathological conditions of patients on the expression of the selected miRNAs was analysed.

miRNA expression pattern in tumour and tumour-adjacent tissues

The expression analysis of miRNA was performed to characterize the expression profile of selected miRNAs in the particular

Table 2 Characteristics of the samples of patients and controls. Note that tumour staging and grading refer to the first “tumour” group of patients only

Factor	Group	Number	Age (min–max)
Group	Tumour	42	63 (47–87)
	Tumour-adjacent	39	62 (47–87)
Gender	Male	42	63 (47–87)
Tumour grade	High	35	64 (47–87)
	Low	4	65 (56–79)
Tumour stage	T1–2	18	62 (47–83)
	T3–4	22	65 (47–87)
Node positivity	No	15	64 (51–83)
	Yes	20	64 (47–87)
Meta positivity	No	31	64 (47–87)
	Yes	4	61 (55–71)

tissue type. A multivariate test revealed a significant effect of the tissue type on the miRNA expression pattern ($F(6, 144) = 3.07, p = 0.007$).

In accordance with the aim of this study, the expression of selected miRNAs in tumour tissue and histologically normal tumour-adjacent samples was assessed. miRNA expression for the tumour samples and the matched adjacent tissues (tumour = 39, adjacent = 39) were analysed using the paired *t* test analysis.

hsa-miR-375-3p and hsa-miR-29c-3p were both more expressed in tumour-adjacent tissues (11.59-fold higher expression, $p = 0.0001$ and 2.63-fold higher, $p = 0.048$ for miR-375 and -29c, respectively). No statistically significant change in expression of hsa-miR-200b-5p between adjacent and tumour tissues was found.

ROC (receiver-operator curves) analysis identified a sensitivity 87.5 % (95 % CI 94.5–73.9), specificity 65 % (95 % CI 77.9–49.5) and area under curve (AUC) = 0.74 for hsa-miR-375-3p and sensitivity 59.0 % (95 % CI 72.9–43.4), specificity 69.2 % (95 % CI 81.4–53.6) and AUC = 0.62 for hsa-miR-29c-3p (see Fig. 1).

miRNA expression and tumour staging

Consequently, the effect of tumour staging on the expression of the above-mentioned miRNAs was analysed. Tumour-adjacent tissues are involved in the development and progression of the tumour; therefore, the effect of tumour staging was not only related to the expression in HNSCC tumourous tissue but also to the expression in tumour-adjacent tissue samples. First, the effect of TNM T staging was analysed (T1–2 versus T3–4). Thirty-five tumour tissue samples (T1–2 = 15, T3–4 = 20) and 34 tumour-adjacent tissue samples (T1–2 = 15, T3–4 = 19) were involved in the analysis. No significant association between the selected miRNA expression and T stage was determined either in the tumour or in the tumour-adjacent tissue. Subsequently, the effect of node positivity was analysed. Thirty-five tumour tissue samples (N positive = 20, N negative = 15) and 33 tumour-adjacent tissue samples (N positive = 19, N negative = 14) were involved in the analysis. A significant decrease of hsa-miR-200b-5p expression was revealed in tumour-adjacent tissue samples of patients with node positivity (0.17-fold expression, 95 % CI 0.03–0.87; $p = 0.035$). In the next step, the effect of the presence of distant metastases was analysed. Thirty-five tumour tissue samples (M positive = 4, M negative = 31) and 33 tumour-adjacent tissue samples (M positive = 4, M negative = 29) were included in the analysis. No significant association between distant metastasis and the selected miRNA expression was determined either in the tumour or in the tumour-adjacent tissue.

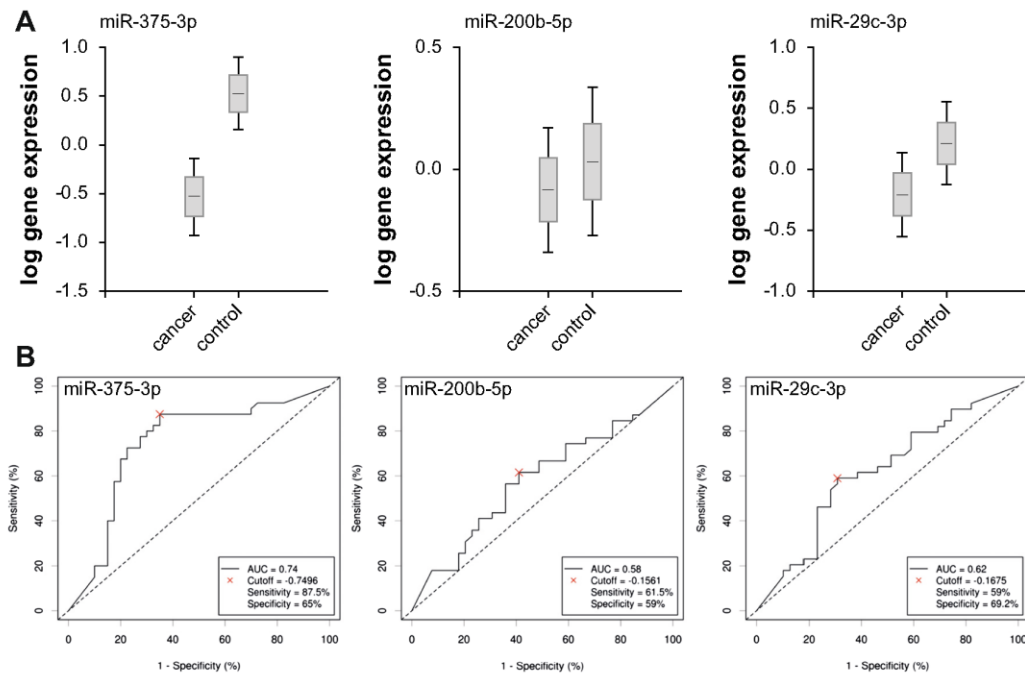


Fig. 1 Tissue gene expression of microRNAs. **a** Expression in tumour and tumour-adjacent (control) tissues. Displayed as logarithm of gene expression, mean \pm 1 and 2 SE. Asterisks indicate difference significant at $p < 0.05$ in paired test. **b** Area under curves, sensitivity and specificity for particular miRNAs

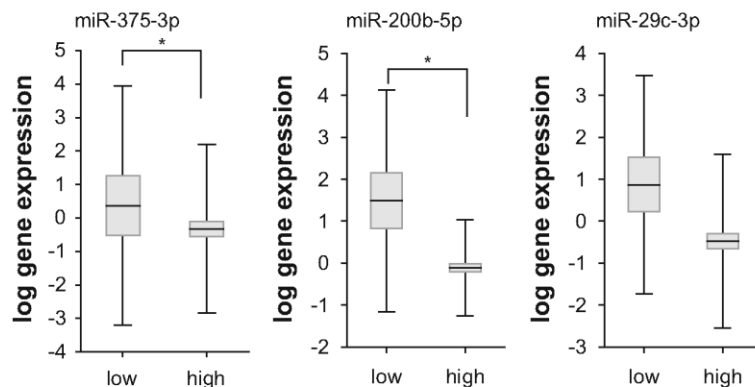
Gene expression and histological grading

Thirty-four tumour tissue samples (high grade = 30, low grade = 4) and 32 tumour-adjacent tissue samples (high grade = 29, low grade = 3) were involved in the analysis. Low expression of hsa-miR-200b-5p (0.03-fold change, 95 % CI 0.001–0.15; $p = 0.0001$) and hsa-miR-29c-3p (0.05-fold change, 95 % CI 0.001–0.63; $p = 0.023$) in tumour tissue was significantly associated with higher tumour grade (see Fig. 2).

Association between miRNA expression and disease-free and overall survivals

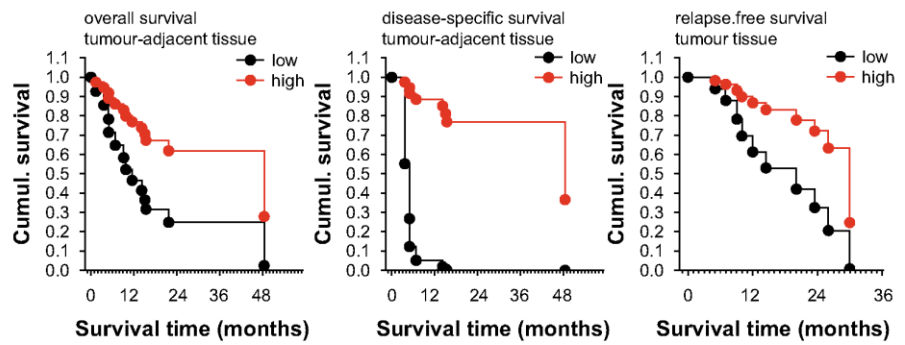
The prognostic value of miR-29c-3p, miR-200b-5p and miR-375-3p expressions on overall, disease-specific and recurrence-free survivals was studied by Cox proportional hazard regression. Similar to previous chapters, the hazard was calculated for the miRNA expression in tumour and tumour-adjacent tissues separately. Survival analysis showed a significant effect of

Fig. 2 Gene expression of miRNAs in tumour tissue in relation to tumour grade. Displayed as logarithmic gene expression, mean \pm 1 and 2 SE. High grade indicates grade > 2



Tumor Biol.

Fig. 3 Cox proportional hazard model for overall, disease-specific and recurrence-free survivals for miR-29c. miRNA expression as a continuous predictor. Note that overall and disease-specific survivals are displayed for tumour-adjacent tissue and relapse free for tumour tissue expressions. For detailed results see Table 3



miR-29c-3p on overall and disease-specific survivals in tumour-adjacent tissue (hazard ratio, HR = 0.27, 95 % CI = 0.01 to 0.85 and 0.07, 95 % CI = 0.01 to 0.59, respectively) (Fig. 3). In addition, there was a significant effect of miR-29c-3p on recurrence-free survival in tumour tissues (HR = 0.31, 95 % CI = 0.10 to 0.91). miR-200b and 375 were not associated with a hazard in overall, disease-specific and recurrence-free survivals. For details, see Table 3.

Discussion

MicroRNAs (miRNAs) are important regulators of gene expression. Downregulation of tumour suppressor miRNAs or overexpression of particular onco-miRNAs is involved in pathogenesis of human cancers and cause tumourigenesis in mouse models [19].

In this study, we focused on the expression profiles of presumed tumour suppressor miRNAs (miR-29c-3p, miR-200b-5p

Table 3 Cox proportional hazard model for overall survival, disease-specific survival and recurrence-free survival. Displayed for tumour tissue and tumour-adjacent tissue miRNA expressions separately

Outcome type	Tissue	miRNA	Hazard ratio (95 % CI)	p value
Overall survival	Tumour	miR-200b	1.00 (0.42 to 2.38)	0.993
		miR-375	1.32 (0.76 to 2.27)	0.321
		miR-29c	0.89 (0.47 to 1.70)	0.732
	Tumour-adjacent	miR-200b	0.84 (0.30 to 2.40)	0.755
		miR-375	2.17 (0.83 to 5.65)	0.113
		miR-29c	0.27 (0.01 to 0.85)	0.024
Disease-specific survival	Tumour	miR-200b	1.25 (0.51 to 3.08)	0.63
		miR-375	1.45 (0.74 to 2.81)	0.278
		miR-29c	0.80 (0.37 to 1.75)	0.573
	Tumour-adjacent	miR-200b	0.44 (0.04 to 4.23)	0.488
		miR-375	4.48 (0.90 to 22.33)	0.067
		miR-29c	0.07 (0.01 to 0.59)	0.014
Recurrence-free survival	Tumour	miR-200b	0.91 (0.14 to 5.23)	0.924
		miR-375	1.77 (0.67 to 468)	0.248
		miR-29c	0.31 (0.10 to 0.91)	0.034
	Tumour-adjacent	miR-200b	0.09 (0.00 to 7.77)	0.290
		miR-375	3.09 (0.10 to 96.39)	0.521
		miR-29c	1.14 (0.06 to 21.08)	0.928

CI confidence interval

and miR-375-3p) in HNSCC. Many of the miRNAs have gender-related expression levels, and oestrogen-dependent miRNA regulation is also well known [20, 21]. For these reasons, only male HNSCC patients were included into analysis. hsa-miR-375-3p and hsa-miR-29c-3p were both less expressed in tumour tissues. ROC (receiver-operator curves) analysis identified a sensitivity 87.5 %, specificity 65 % and AUC = 0.74 for hsa-miR-375-3p and sensitivity 59.0 %, specificity 69.2 % and AUC = 0.62 for hsa-miR-29c-3p. No statistically significant change in expression of hsa-miR-200b-5p between tumour-adjacent and tumour tissues was found. Downregulation of tumour suppressor miR-375 could lead to uncontrolled cancerous inhibitor of protein phosphatase 2A (CIP2A) expression and strengthened stability of MYC oncogene, which contributes to the promotion of tumourous phenotypes, such as increased proliferation, colony formation, migration and invasion [22]. Furthermore, it was shown that common anti-cancer drugs such as doxorubicin, 5-fluorouracil, trichostatin A or etoposide reactivated miR-375 and its primary transcript pri-miR-375 expression in tongue cancer cells [23]. Lower hsa-miR-29c-3p expression in tumour tissue is also in accordance with other studies [11, 24–26]. Missing significant difference in hsa-miR-200b-5p expression between tumour-adjacent tissues and HNSCC tumour tissues could be caused by the presence of activated cancer-associated fibroblasts (CAFs) in tumour-adjacent tissue. Tang et al. revealed that miR-200s are generally downregulated not only in breast cancer tissues but also in activated CAFs. Fibroblasts with downregulated miR-200s displayed accelerated migration and invasion [27]. In accordance, a decrease of hsa-miR-200b-5p expression in tumour-adjacent tissue samples was associated with node positivity in HNSCC patients. Low expression of hsa-miR-200b-5p and hsa-miR-29c-3p in tumour tissue was also significantly associated with higher tumour grade. Using the survival analysis, it revealed a significant effect of hsa-miR-29c-3p expression in tumour-adjacent tissue on the overall and disease-specific survivals and in tumour tissue on recurrence-free survival (higher expression of this miRNA was associated with better prognosis). Nevertheless, no significant effect of hsa-miR-375-3p or hsa-miR-200b-5p expressions was demonstrated using the survival analysis. It could be due to context-dependent effects of miRNAs. They are tumour suppressive in context of pro-tumourigenic pathways, but they also can confer a resistance to the chemotherapy [28]. For example, artificial overexpression of miR-375 in cervical cancer cells decreased paclitaxel sensitivity in vitro and also in vivo [29]. miR-141 and miR-200 expressions may lead to two counteracting effects: resistance to platinum compounds on the one hand but increased sensitivity to paclitaxel on the other [28, 30]. The negative correlation between hsa-miR-200b-5p and hsa-miR-200b-3p

expressions and cisplatin sensitivity was revealed also in NCI60 platform by CellMiner (<http://discover.nci.nih.gov/cellminer/>).

Conclusions

In conclusion, hsa-miR-375-3p seems to be a relatively promising diagnostic marker inasmuch as primary HNSCC carcinoma tissues can be distinguished from histologically normal-matched noncancerous tumour-adjacent tissues based on these miRNA expressions. A close relationship was found between tumour miRNA expression profiles and circulating miRNAs [31, 32]. Consequently, hsa-miR-375-3p could be a promising target of further research of diagnostic markers in circulation.

Furthermore, this study highlighted the importance of histologically normal tumour-adjacent tissue in HNSCC progress, inasmuch as a significant decrease of hsa-miR-200b-5p expression was revealed in the tumour-adjacent tissue samples of patients with node positivity, and low expression of hsa-miR-29c-3p in HNSCC tumour-adjacent tissue was significantly associated with worse prognosis.

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Compliance with ethical standards

Conflicts of interest None

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4.4 Elektrochemická detekce miRNA, hladina v séru

V porovnání s molekulárně-biologickými metodikami pro detekci miRNA, které jsou založeny na hybridizaci (qRT-PCR, Northern blot, ISH), jsou voltametrie a CD spektroskopie mnohem rychlejší (v řádu minut). Přinášejí také informace o sekundární struktuře, které mohou mít další zajímavá využití. Elektrochemické metody mohou být neobvyklým a novým přístupem při detekci a analýze miRNA. V naší studii jsme vybrali tři miRNA (miR-23a, miR-34a a miR-320a), které jsou deregulovány v nádorech hlavy a krku a pokusili jsme se je charakterizovat a detekovat pomocí elektrochemických metod. V této studii byly provedeny voltametrické a spektrální analýzy za účelem lepšího poznání struktury miRNA. Pro základní srovnání spekter nukleotidů miRNA byly výsledky doplněny o uracilové DNA (DNA (U)), které mají stejné oligonukleotidové sekvence jako miRNA. Účinkem nahrazení ribózy za deoxyribózu v oligonukleotidu byla prokázána strukturální diverzita. V první části jsme se zabývali eliminací guaninového píku a charakterizací jednotlivých miRNA podle obsahu guaninu (viz obrázek na str. 135). Z výsledků je zřejmé, že podle guaninového píku lze rozlišit od sebe všechny tři studované miRNA.

V tomto metodickém článku byla také provedena analýza exprese sérových hladin výše zmíněných miRNA na testovací kohortě 48 pacientů. U všech ze sledovaných bylo popsáno signifikantní zvýšení hladin oproti kontrolní skupině (obr. na str. 134).

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Novel biophysical determination of miRNAs related to prostate and head and neck cancers

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Abstract In this study we have chosen a new approach and characterized three miRNAs (miR-23a, miR-34a and miR-320a) related to prostate cancer and head and neck cancer by spectral (circular dichroic and UV-absorption spectra) and electrochemical (voltammetry at graphite and mercury electrodes) methods. The spectral and voltammetric results, reflecting different nucleotide sequences of miRNAs, were complemented by the results of DNAs(U) having the same oligonucleotide sequences as miRNAs. The effect of the substitution of ribose for deoxyribose was shown and structural diversity was confirmed. The stability of RNA and DNA(U) was studied using CD and UV-absorption spectroscopy and melting points were calculated. MiRNA-320a with the highest content of guanine provided the highest melting point. With respect to the rapid progress of miRNA electrochemical sensors, our results will be useful for the research and development

of sensitive, portable and time-efficient miRNA sensors, which will be able to diagnose cancer and other diseases.

Keywords miRNA · Circular dichroism · Electrochemistry · Prostate cancer · Head and neck cancer

Introduction

MicroRNAs (miRNAs) are small, non-coding ribonucleic acids (ncRNAs) that regulate gene expression at the post-transcriptional level (Mirnezami et al. 2009). The biogenesis of miRNAs is a multilevel process involving many enzymes and proteins; hence, its regulation is quite different from the previously described regulators of gene expression. Within the canonical model of biogenesis, genes for miRNAs contain their own promoters and are transcribed by RNA polymerase II into primary transcripts (pri-miRNA) (Bartel 2007). The effect of miRNAs is most often based on binding to the untranslated region (3'UTR) of the target mRNA, which causes the degradation (or inhibition) of the target mRNA and can be seen in many cellular processes such as proliferation, differentiation, apoptosis, metastases, angiogenesis and the immune response (Mattie et al. 2006; Scapoli et al. 2010). Recently, many studies have shown that miR-23a, miR-34a and miR-320a are associated with prostate and head and neck cancer (HNC) (Ayaz et al. 2013; Hsieh et al. 2013; Kumar et al. 2012; Mattie et al. 2006; Scapoli et al. 2010; Yamamura et al. 2012). Prostate cancer (PCa) is the second most frequently diagnosed cancer and the second leading cause of cancer death in males in developed countries (Jemal et al. 2011). HNC, which includes head and neck squamous cell carcinoma—cancers of the oral cavity, larynx, oropharynx and hypopharynx, is

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the sixth most common cancer malignancy in the world (Warnakulasuriya 2009). Important risk factors include frequent consumption of alcoholic beverages and tobacco (Zygianni et al. 2011) and human papillomavirus infections (HPV) (Marur et al. 2010). As compared with the healthy subjects, the expression level of miR-23a is reduced in PCa, whereas in colorectal adenocarcinoma, the level is increased and miR-23a supports the migration and invasiveness of these tumor cells (Mattie et al. 2006). MiR-23a, with 12 other miRNAs, is significantly over-expressed in oral squamous cell carcinoma (Scapoli et al. 2010). In another study it was found that 1'-acetoxychavicol acetate (ACA) may have anticancer effects against human HNC through the downregulation of miR-23a, which can repress tumor suppressor PTEN (Wang et al. 2013). Recent studies have pointed to the role of miR-34a as a tumor suppressor in many types of cancer including PCa, gastric cancer, cervical cancer, hepatocellular carcinoma and HNC (Cao et al. 2014; Chakraborty et al. 2014; Kumar et al. 2012; Liang et al. 2013; Liu et al. 2011; Ribeiro and Sousa 2014; Yamamura et al. 2012; Yang et al. 2014). The enforced expression of miR-34a in bulk or purified CD44+ PCa cells inhibited clonogenic expansion, tumor regeneration and metastasis (Liu et al. 2011). MiR-34a regulates the levels of PCa protooncogene c-MYC, which activates genes stimulating proliferation and is frequently deregulated in tumors. MiR-34a also inhibits cell proliferation in vitro (cell line PC-3) and in vivo, and it promotes apoptosis in PCa (Yamamura et al. 2012). MiR-34a plays an important role in the p53 protein pathway, the position of which is unique for cancer malignancy in almost all types of tumors. P53 activates the expression of miR-34a and regulates a feedback loop when miR-34a inhibits SIRT1, resulting in acetylation and p53 activation (Liang et al. 2013). Significant downregulation of miR-34a was observed in HNC patients as well as in HNC cell lines (Kumar et al. 2012). The regulatory axis of p53/miR-34a has also proved to be very important in controlling Bax-dependent apoptosis in non-small-cell lung carcinoma cells (Chakraborty et al. 2014). Mir-320a regulates the expression of PFKm (phosphofructokinase), which is a glycolytic enzyme of muscle type involved in the regulation of glycolysis—and it is known that cancer cells have a modified metabolism and increased aerobic glycolysis, which is why this miRNA is so important for cancer research (Tang et al. 2012). In PCa, the expression of miR-320a is significantly reduced; miR-320a inhibits the expression of catenin by annealing to the 3' UTR region of mRNA and suppresses the stem cell-like characteristics for PCa. This means that miR-320a is a key negative regulator in prostate tumor-initiating cells (Hsieh et al. 2013). In laryngeal squamous cell carcinoma, which is the second most

common HNC worldwide, the significantly upregulated miR-320a may thus serve as a biomarker of this disease (Ayaz et al. 2013).

Typical strategies for studying miRNAs are northern blotting (NB), qRT-PCR, in situ hybridization (ISH) and microarrays technologies (Hamidi-Asl et al. 2013). These techniques are well known and described, including possibilities for their modifications, which may be using locked nucleic acids (LNAs) in NB (Varallyay et al. 2008), ISH (Oberosterer et al. 2007) or qRT-PCR (Raymond et al. 2005). Nanomaterial-based assays have become popular; their use is sensitive, specific and often label-based or requiring a hybridization step. Modifications of this technique can use electroanalytic tags (Gao and Yang 2006), magnetic bead-based bioassay (Bettazzi et al. 2013) or a bioassay based on the ruthenium oxide nanoparticle-initiated deposition of an insulating film (Peng and Gao 2011). There are already many techniques conducive to using electrochemistry to detect miRNA (de Planell-Saguer and Rodicio 2011; Hamidi-Asl et al. 2013) and also spectroscopy (Driskell and Tripp 2010). Deamination of cytosine to produce uracil is a common and potentially mutagenic lesion in the genomic DNA (Larson et al. 2008). Uracil in DNA results from the spontaneous or enzymatic deamination of cytosine, resulting in mutagenic U:G mispairs (Sousa et al. 2007).

In this study we used for the first time the linear sweep voltammetry and elimination voltammetric procedure in connection with the pencil graphite electrode and circular dichroism (CD) for the determination of the miRNA structures and their changes. For the comparison of DNA and RNA, we used the DNA backbone with the base of uracil in which the presence of mutagenic cells has been demonstrated.

Materials and methods

RNA isolation and reverse transcription

Total RNA was obtained from the sera of patients with PCa (at St. Anne's University Hospital Brno). The study was conducted in line with the principles of the Declaration of Helsinki and was approved by the Ethics Committee of the Faculty of Medicine, Masaryk University Brno. In total, we used 38 serum samples from patients and 10 serum samples from healthy males. Isolation from the patients' sera was performed by using the commercial High Pure miRNA isolation kit (Roche, Switzerland). According to manufacturer's instructions, 10 ng of isolated miRNA was transcribed using the TaqMan[®] microRNA reverse transcription kit (Applied Biosystems, USA), and 1.33 μ l of transcribed miRNA was used directly in the real-time PCR reaction.

Quantitative real-time polymerase chain reaction (qRT-PCR)

The qRT-PCR was performed in triplicate using the TaqMan gene expression assay system with the 7500 real-time PCR system (Applied Biosystems, USA). The primer and probe sets were selected from TaqMan miRNA expression assays: hsa-miRNA-23a (assay ID: 000399), hsa-miRNA-34a (assay ID: 000426) and hsa-miR-320a (assay ID: 002277). The qRT-PCR was performed under the following amplification conditions: total volume 20 μ l, initial denaturation 95 °C/10 min, then 45 cycles 95 °C/15 s, 60 °C/1 min. The amplified DNA was analyzed using the CT (cycle threshold) values of cycle expression for each miRNA. SW Excel 2007 (Microsoft, USA) was used to arrange the data set. SW Statistica 9.1 (StatSoft, USA) was used to perform the statistical analysis and chart construction.

Biophysical analysis

MiRNAs synthetic oligonucleotides (hsa-miR-23a: AUCAC AUUGCCAGGGAUUUC, hsa-miR-34a: UGGCAGUGU CUUAGCUGGUUGU and hsa-miR-320a: AAAAGCUGG GUUGAGAGGGCGA) were purchased from Sigma-Aldrich (MO, USA) and used for analysis by LSV or CV (linear sweep voltammetry or cyclic voltammetry), CD and UV-absorption measurements. Lyophilized oligonucleotides were dissolved in milliQ-H₂O to the millimolar concentration according to nucleosides. To compare the CD and UV-absorption spectra of the individual miRNAs, we used the same number and order of bases carried on DNA backbone as in miRNAs. The synthetic oligonucleotides of miRNAs with the DNA backbone (DNA(U)) were purchased from Sigma-Aldrich (MO, USA) as well and dissolved to an equal extent as miRNAs.

Linear sweep voltammetry (LSV) and cyclic voltammetry (CV)

Oxidation and reduction signals were recorded on the pencil graphite electrode (PeGE) and on the hanging mercury drop electrode (HMDE) in aqueous buffered solutions. Experimental conditions were as follows: the concentration of the oligonucleotides was 1×10^{-4} M; phosphate-acetate buffer (pH 4.55–7.47); room temperature (23 °C). The electrochemical analyzer AUTOLAB PGSTAT 20 (EcoChemie, Utrecht, The Netherlands) linked with the VA Stand 663 and controlled by the GPES Manager 4.9 software was connected to a set of three electrodes: PeGE (0.5 HB Tombow, Japan) with an effective area of 8.5 mm² or HMDE with an effective area of 0.4 mm² as a working electrode, Ag/AgCl/3 M KCl as a reference electrode and Pt wire as an auxiliary electrode. LSV or CV parameters corresponded to

the scan rate: 100, 200, 400 mV/s; potential range: from 0 to –1.8 V (HMDE) and from 0 to 1.2 V (PeGE); accumulation time $t_a = 30$ s and $t_a = 0$ s for HMDE and PeGE, respectively; potential step: 2 mV. For a further data treatment, the potential-current values were exported to Excel. We also tried to analyze miRNAs on PeGE using square-wave voltammetry (SWV) and differential pulse voltammetry (DPV).

Elimination voltammetric procedure (EVP)

The essence of the elimination voltammetric procedure (EVP) (formerly known as elimination voltammetry with linear scan—EVLS) consists in the transformation of voltammetric currents into functions that are capable of eliminating some unwanted current components (e.g., diffusion, capacitive or kinetic). The transformation resulting in the elimination function is based on the different dependence of a current component on the scan rate (polarization speed). Elimination coefficients of the elimination function can be calculated by using the software developed by the authors of this article. There is a possibility to eliminate capacitive kinetic or diffusion currents as well as their combinations so we can eliminate selected current components concurrently. EVP improves voltammetric signals and not only increases the sensitivity of voltammetric methods, but also improves the separation of overlapping signals. Such signals can be, for example, overlapped reduction signals of adenine and cytosine on HMDE (Trnkova et al. 2003, 2006) or oxidation signals purine derivatives on graphite electrodes (Navratil et al. 2014). The form of the function eliminating the capacitive and kinetic current components and conserving the diffusion current component for scan rates of 200, 400 and 600 mV/s is as follows:

$$f(I) = -14.674I_{200 \text{ mV/s}} + 29.350I_{400 \text{ mV/s}} - 14.670I_{600 \text{ mV/s}}$$

Circular dichroism (CD) and UV-absorption spectroscopy

CD and UV-absorption measurements were taken using the Jasco 815 dichrograph (Tokyo, Japan) in 0.1-cm path-length quartz Hellma cells placed in a thermostatic holder (23 °C). The concentration of miRNAs was 1×10^{-4} M (in nucleosides); phosphate-acetate buffer (pH 4.55–7.47). For CD spectra, the scan rate was 100 nm/min, averaging four measurements. CD signals are expressed as a difference $\Delta\epsilon$ in molar absorption of the left- and right-handed circularly polarized light.

Results and discussion

To determine the function of biologically important molecules responsibly, it is necessary to investigate their structure

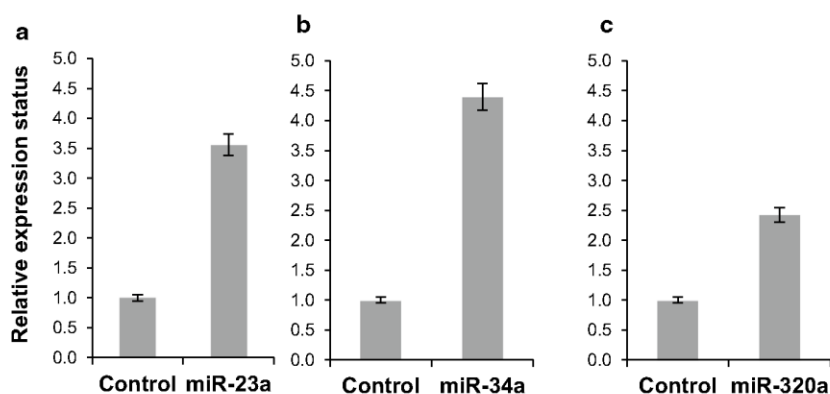


Fig. 1 Express status of miRNAs in patients' sera as compared with healthy controls. Comparison of the expression status of miRNAs (miR-23a, miR-320a and miR-34a) from the sera of patients with those of healthy controls using qRT-PCR. MiR-23a was expressed 3.5

times more in the patients' sera compared to the healthy controls (a); for miR-320a it was almost 2.5 times more (c). In the healthy controls, miR-34a was observed in a very small amount, while in the patients' sera, this miRNA showed good expression (b)

in dependence on eligible parameters, resulting in a correct understanding of the structure-function relationship. It could be an example of miRNAs, which have a very important regulatory function at the transcriptional level. We do not focus solely on the primary miRNA structures, but also on the secondary structures, whose influence on the behavior of RNA has a crucial role. De-coiled and more exposed structures of miRNA are more amenable to external effects such as mRNA annealing, creating complexes with proteins, hybridizing with the complementary structures and—for our purpose—annealing to the electrode surface. The secondary structure of miRNA was studied in the context of the primary transcript (pri-miRNA) and the precursor of miRNA (pre-miRNA) only (Diederichs and Haber 2006), but the description of the secondary structure of mature miRNA has never been studied by using the electrochemical and spectral methods such as voltammetry and CD spectroscopy. Compared to molecular-biological methods (qRT-PCR, NB, ISH), where the measurements are time-consuming, taking hours, voltammetry and CD spectroscopy are inexpensive, accessible and not time consuming (on an order of minutes).

Figure 1 shows the expression status of all three studied miRNAs—miR-23a, miR-34a and miR-320a—in the context of comparing healthy controls with PCa patients. The expression of the studied miRNAs in the patients with PCa is in larger quantities than in the healthy controls (by more than two times and in the following order: 34a > 23a > 320a); this is why we chose them for this study. From a biological point of view, these miRNAs are of interest to us and can be used as a suitable target for study using electrochemical and spectral methods. PCR methods inform about the primary structure level of miRNAs. To learn about their secondary structure, we need other approaches applying electro- and spectral analysis.

The direct oxidation of adenine and guanine in the nucleotide chains on bare carbon electrodes showed a poor response with a slow electron transfer, and the oxidation of adenine required a relatively higher overpotential than that of guanine. We investigated the guanine oxidation signals of miRNAs on PeGE by means of voltammetry in connection with our elimination procedure. A comparison of the oxidation peaks for all three miRNAs, measured by LSV using PeGE and evaluated by EPV is shown in Fig. 2a. Guanine peaks are situated around a potential of 1.1 V (vs. Ag/AgCl/3 M KCl). For the EPV analysis, three polarization rates (200, 400 and 600 mV/s) were used and a corresponding elimination function was calculated. The content of guanine in Fig. 2b corresponds with the peak height of each miRNA. In miR-320a with the highest content of guanine (45.5 % of the total), the highest peak was observed. The smallest peak was exhibited by miR-23a in which the content of four guanines was the lowest of all miRNAs.

To determine differences between the RNA and DNA skeletons, electrochemical (LSV analysis on HMDE) and spectroscopic (CD spectroscopy) analyses were carried out. Using electrochemistry (CV on a mercury electrode), we demonstrated that the RNA fragments form a single peak corresponding to the oxidative peak for guanine (Studnickova et al. 1989), while DNA(U) forms two peaks in the same region of potentials (Fig. 3a, b). The oxidation guanine signal (G peak) is known especially from the studies of oligodeoxynucleotides. It corresponds to the oxidation of the reduced product of guanine (7,8-dihydroguanine) proceeding at very negative potentials (more than -1.6 V) (Trnkova et al. 1980). The prerequisite for obtaining an oxidative signal is the accessibility of guanine residues of the oligonucleotide chain to the electrode surface. The dual oxidation guanine peak is a characteristic feature of

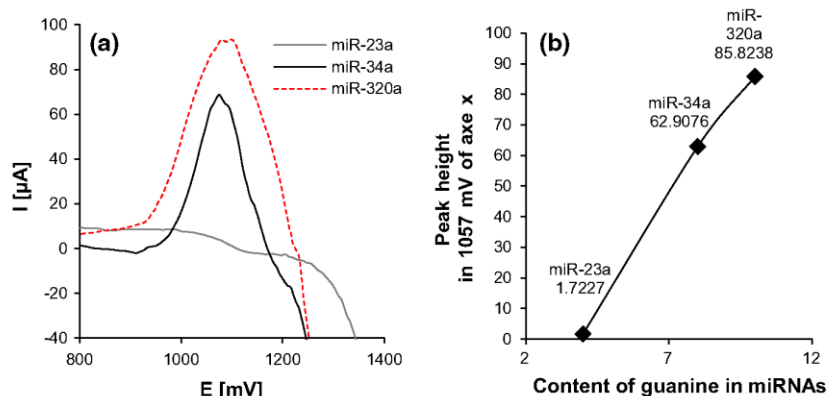


Fig. 2 Elimination voltammetric guanine peaks on the PeGE and dependence of these peak heights on the content of guanine. Comparison of guanine peaks (EPV from LSV as current-potential dependence) for three miRNAs (miR-23a, miR-34a, miR-320a) (1×10^{-4} M) peak (a) in phosphate-acetate buffer (pH 5.0), scan rate:

200,400,600 mV/s; activation of PeGE surface at potential -1.4 V (time 60 s); pre-treatment of PeGE by miRNA at potential 0 V (time 10 min). Peak height increases with the content of guanine in each miRNA (b)—measured under the same conditions as in (a)

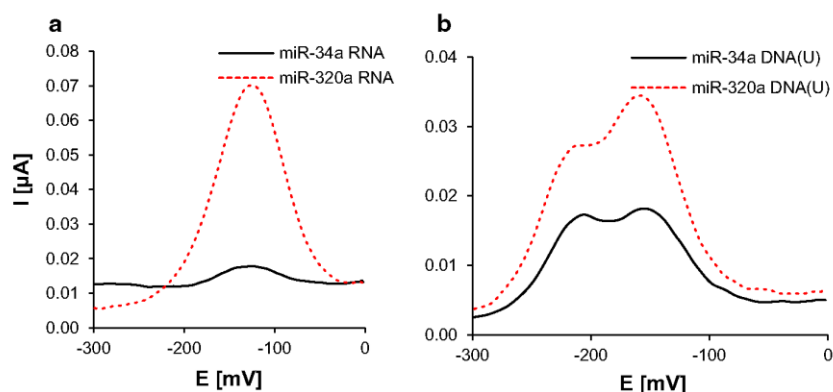


Fig. 3 Comparison of the guanine peak for RNA and DNA(U) backbone structures of two miRNAs using the mercury electrode. LSV as current-potential dependence for two forms of backbone structures—RNA and DNA(U) measured on miRNAs (1×10^{-4} M) in the phosphate-acetate buffer (pH 5.0), scan rate: 400 mV/s; accumula-

tion potential -0.1 V and accumulation time 60 s. LSV curves were measured on a hanging mercury drop electrode. It is evident that the RNA structure of the sugar-phosphate backbone forms only one peak (a), while the DNA(U) structure forms two oxidative peaks for guanine (b)

DNA fragments. In the same buffer solutions, RNA is able to provide only one guanine oxidation peak. The difference can be attributed to the DNA structure conditioned by ribose and deoxyribose and to the different interaction of sugar with the nucleotide backbone.

To characterize miRNAs and DNA(U) structures and to obtain more information about their secondary structure, we also used CD and UV-absorption spectroscopy. This study focuses on a basic characteristic of miRNA, so we used higher concentrations of synthetic oligonucleotides (10^{-4} M) than the relevant concentration of miRNA in biological samples (10^{-8} M). The signals obtained from the

CD spectra shown in Fig. 4a, b, c indicate clearly that the CD spectra reflect significant differences in the secondary structures of the miRNAs. The CD spectrum for miR-23a (Fig. 4a) shows a huge positive peak at 270 nm, a shallow negative peak at 240 nm and specifically the presence of a negative peak at 210 nm, which are characteristic signs for the A-form arrangement of the RNA. The DNA fragment of the same sequence yields a CD spectrum with small amplitudes corresponding to that of a sequentially heterogeneous non-perfectly ordered B-DNA form. The MiR-34a spectrum (Fig. 4b) has a positive shoulder around 240–250 nm (at the places where the classical A-form has

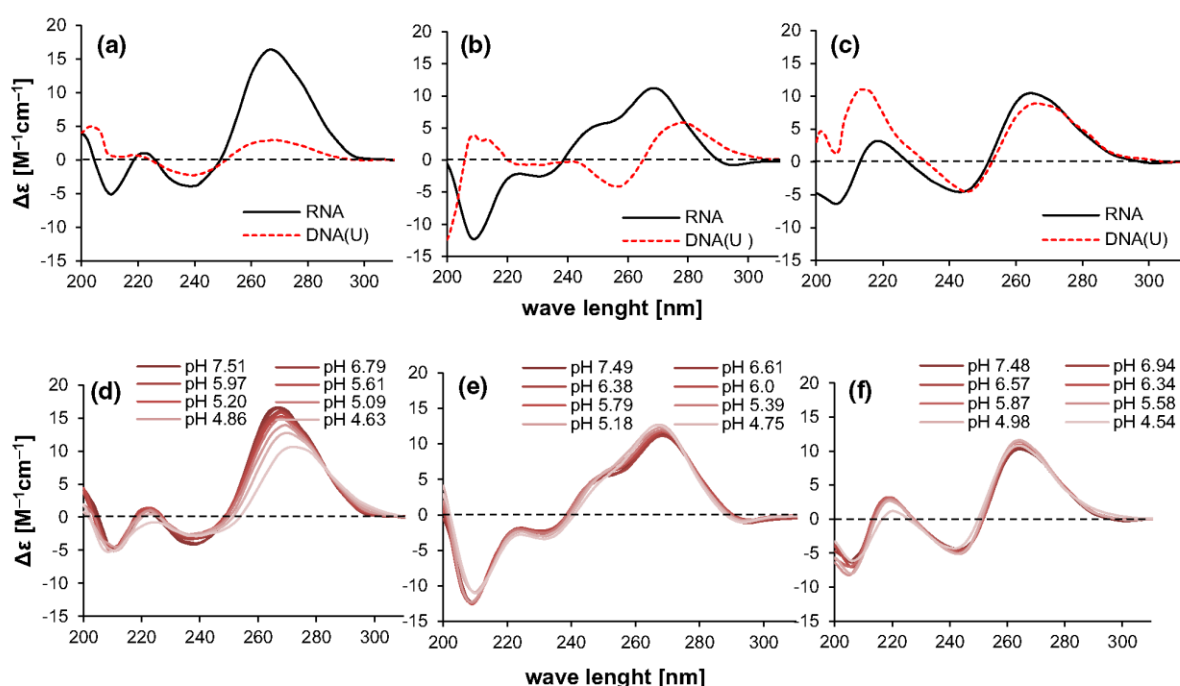


Fig. 4 CD spectra of miRNA and DNA-U. *Upper figures* CD spectra of miRNAs (**a** miR-23a, **b** miR-34a, **c** miR-320a—black lines) and DNA(U) (red lines) in phosphate-acetate buffer (pH 5.0). *Bottom fig-*

ures CD spectra of miRNAs (**d** miR-23a, **e** miR-34a, **f** miR-320a)—dependence on pH (the phosphate-acetate buffer). The concentration of miRNAs was 1×10^{-4} M

a shallow and rather negative minimum), which may result from the presence of non-standard pairing, such as G-U. The CD spectrum of the DNA analog corresponds to the standard B-form; only the positive CD peak is somewhat higher, and the whole spectrum is slightly shifted to longer wavelengths probably also because of the presence of the atypical U-base in the DNA structure. The CD spectrum of miR-320a (Fig. 4c) corresponds to the A-form RNA. Interestingly, its DNA analog provides nearly the same spectrum; only the minimum at 210 nm does not acquire negative values. The A-like spectrum of DNA could be a consequence of the probable A-U pairing enabled by the sequence. CD spectra of miRNAs do not distinctly change with changes of pH values (Fig. 4d, e, f) within a range of pH 7.5–4.5, where the A-form remains stable.

In this work, the stability of miRNA and DNA(U) structures was examined and quantified using UV absorption temperature dependences from which melting points (T_m^o) were determined for both miRNA and DNA(U) (Table 1). The big difference in melting points ΔT_m^o (14.5 °C) between RNA and DNA(U) for miR-34a might be due to the generation of several hairpin secondary structures according to its primary structure. In comparison, for miR-320a the difference in ΔT_m^o is only 3 °C (and indicates significantly higher stability of the secondary structure related

Table 1 Comparison of melting points between RNA and DNA(U) forms of miRNA oligonucleotides

Melting points measured by UV-absorption	
RNA backbone of miRNA	
miR-23a	25.7 °C
miR-34a	32.0 °C
miR-320a	49.5 °C
DNA backbone of DNA(U)	
miR-23a	33.3 °C
miR-34a	46.5 °C
miR-320a	52.5 °C

to more hydrogen bonds in this molecule of miRNA). Differences between RNA and DNA with uracil are evident; however, it can be generally concluded that miRNA has a lower melting point than the corresponding DNA(U).

Conclusions

Detection of miRNAs using electrochemical and spectral methods has become popular and useful. However, this still does not make possible the perfect characterization

of an ideal biomarker, which requires specificity, sensitivity, a low price, simplicity and reproducibility. This work attempts a combination of several techniques in the context of miRNA research that has not been used yet. PCR methodology focuses on a study with the help of the primary structure, which is the most common way to study miRNAs. As it is known that changes in the secondary structure are common in RNA, we decided to take advantage of this knowledge and to expand upon the subject. The difference between the DNA and RNA backbones has been demonstrated by LSV and CD. Different methods have been developed for the analysis of miRNA so far, but the combination of LSV, CD and UV-absorption methods using the information about the secondary structure for the characterization of miRNA has not been published yet. We expect that our novel approach will contribute to the development of a new label-free detection system for miRNA.

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Conflict of interest The authors declare they have no competing interests as defined by the European Biophysics Journal or other interests that might be perceived to influence the results and discussion reported in this paper.

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4.5 Molekulární markery HNSCC na úrovni proteinu

Další nadějně HNSCC markery a rovněž markery vytipované na základě předchozích experimentů byly ověřeny i na proteinové úrovni ve vzorcích krve. Markery stanovované z krve jsou méně invazivní než tkáňové biopsie a odběr vzorků se může opakovat, což umožňuje sledovat v reálném čase progresi onemocnění a odpověď na léčbu. Jako první byla ověřena hladina EGFR v plazmě HNSCC pacientů a kontrol. Jako kontrola byla využita skupina jedinců bez medikace a bez diagnostikovaných chorob a diabetická skupina (diabetes typu 2; T2DM). Charakterizace pacientů a kontrol je uvedena v tabulce na str. 143. Jako první byla vyhodnocována asociace mezi plazmatickými hladinami EGFR a výskytem HNSCC. Hladina plazmatického EGFR byla u pacientů s HNSCC významně vyšší než u obou typů kontrol se senzitivitou 76,09 % a specifitou 67,27 %; viz obr. na str. 142. Mezi oběma typy kontrol nebyla nalezena statisticky významná změna v hladinách plazmatického EGFR.

Dalším stanovovaným parametrem byla asociace plazmatických hladin EGFR a klinicko-patologických charakteristik HNSCC pacientů. Nebyla nalezena žádná signifikantní asociace mezi hladinami EGFR a HPV infekcí stádiem či diferenciací nádoru, zasažením mízních uzlin či výskytem vzdálených metastáz. Vliv HPV infekce a T2DM byl však na hranici statistické významnosti (a je blíže zkoumán v následující práci Polanska *et al.* na str. 147).

Dalším studovaným parametrem byla délka přežívání pacientů. Prognostický význam plazmatických hladin EGFR byl posuzován pomocí Kaplan-Meierovy analýzy přežití a Coxova modelu proporcionálních rizik, nebyla ale prokázána asociace mezi hladinami EGFR a přežíváním pacientů (viz obr. na str. 143).

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Evaluation of EGFR as a prognostic and diagnostic marker for head and neck squamous cell carcinoma patients

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Abstract. Approximately 90% of all head and neck tumors are squamous cell carcinomas. The overall survival of patients with head and neck squamous cell carcinoma (HNSCC) is low ($\leq 50\%$). A non-invasive marker of disease progression is sorely required. The present study focused on the plasmatic levels of epidermal growth factor receptor (EGFR) in HNSCC patients (N=92) compared with healthy (N=29) and diabetic [type 2 diabetes mellitus (T2DM); N=26] controls. Enzyme-linked immunosorbent assay using antibodies against the extracellular region of EGFR (L25-S645) was performed. No significant changes were observed between diabetic and healthy controls. However, there were significantly higher EGFR plasma levels in HNSCC patients compared with both control groups (P=0.001 and 0.005, respectively). Receiver operating characteristic curve analysis identified a sensitivity of 76.09%, a specificity of 67.27% and an area under curve of 0.727 for this comparison. No significant association was observed between EGFR plasma levels and tumor stage, tumor grade, lymph node or distant metastasis occurrence, smoking habit or hypertension. However, the presence of human papillomavirus infection and T2DM in HNSCC patients had borderline effect on the plasma EGFR levels. Survival analysis

revealed no significant influence of plasmatic EGFR levels on the overall and disease-specific survival of HNSCC patients. In conclusion, EGFR plasma levels appear to be a relatively promising diagnostic, but poor prognostic, HNSCC marker.

Introduction

Epidermal growth factor receptor (EGFR), also known as ErbB1, is a 170-kDa transmembrane glycoprotein belonging to the ErbB/human epidermal growth factor receptor family of receptor tyrosine kinases (1-3). EGFR is composed of an extracellular highly glycosylated ligand-binding domain (ECD) comprising amino acids 1-621, a hydrophobic transmembrane domain (amino acids 622-644) and an intracellular domain with tyrosine kinase activity for signal transduction (amino acids 645-1,186) (Fig. 1) (1-3). Upon binding of a ligand-like amphiregulin, EGF or transforming growth factor α (TGF α) undergoes a conformational change by homo-dimerization or hetero-dimerization with another member of the erbB family, followed by auto-phosphorylation (4,5). This results in tyrosine kinase activation and triggering of signaling cascades. Activation of EGFR leads to the activation of intracellular signaling pathways that regulate cell proliferation, invasion, angiogenesis and metastasis (4,5). EGFR has been selected as a target of anticancer treatments due to its critical roles in cell survival and proliferation (6). EGFR is a strong prognostic marker in head and neck, ovarian and cervical cancer (7-9). EGFR expression has been associated with a higher proliferative index, advanced tumor stage and increased tumor angiogenesis in HNSCC (9). Overexpression of EGFR and TGF α significantly predicted a shorter disease-free and overall survival (9). EGFR activation also resulted into increased cell invasiveness and motility (10) via the induction of epithelial-to-mesenchymal transition (11,12). Furthermore, EGFR can interact with the receptor cluster of differentiation 44, resulting in a migratory cell phenotype (13). In addition to membrane-bound EGFR, tumor

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Key words: spinocellular cancer, biomarker, EGFR, head and neck tumors, plasma, diagnosis

cells express soluble EGFR proteins that can be produced by alternative messenger (m)RNA splicing events, aberrant translocation or disintegration of circulating tumour cells (14,15). Another 110-kDa soluble EGFR isoform, termed proteolytic isoform-soluble (PI-s)EGFR, is disengaged by proteolytic cleavage partially caused by metalloproteases (16,17). Sanderson *et al* (18) have also reported two soluble isoforms of EGFR (150 and 100-kDa) within exosomes.

The present study focused on plasmatic EGFR levels of HNSCC patients, which were analyzed by enzyme-linked immunosorbent assay (ELISA) using anti-EGFR antibodies raised against the L25-S645 region of full-length EGFR. Notably, information about binding sites of ELISA antibodies are often not provided in the literature, despite that it could be very important for interpretation of the results obtained. Blood markers are less invasive than tissue biopsies, and sample collection can be repeated, which enables real-time monitoring of disease progression and treatment response in patients. As a control group, a gender- and age-matched healthy cohort, and a gender- and age-matched cohort of patients with type 2 diabetes mellitus (T2DM), were used. The T2DM group was included because a proportion of the present HNSCC patients also exhibited T2DM, and certain studies have shown that diabetes suppresses the expression of EGFR (19). Since EGFR is affected by both female estrogen receptors (20,21) and male androgen receptors (22), EGFR may be a potential mediator of gender-related differences in HNSCC. Based on these facts, female HNSCC patients were excluded from the current study.

Materials and methods

Samples preparation. The present study was approved by the ethical committee of St. Anne's Faculty Hospital (Brno, Czech Republic). All surgical tissue samples were obtained from male HNSCC patients treated at St. Anne's Faculty Hospital between April 2013 and June 2015 upon providing informed consent. Histologically verified primary HNSCC carcinoma tissues were collected (92 samples). The tissue material harvested at surgery was placed into RNeasy lysis solution for RNA stabilization and storage (Ambion; Thermo Fisher Scientific, Inc., Waltham, MA, USA). The material was maintained cold, and RNA was isolated within 24 h. Additional information about the patients and controls is presented in Table I.

Blood samples from HNSCC patients and healthy (N=29) and T2DM (N=26) controls were obtained by venipuncture, and 5 ml was placed into an S-Monovette® 4.9 ml, K3EDTA test-tube (Sarstedt AG & Co., Nümbrecht, Germany) for plasma preparation. The blood samples were centrifuged at 1,200 x g at 4°C for 10 min within 60 min of collection. Plasma was aliquoted and stored at -80°C until analysis.

ELISA analysis. Plasma levels of EGFR were determined with a commercial ELISA kit (RayBiotech, Inc., Norcross, GA, USA) according to the manufacturer's protocol. The ELISA was designed to detect human EGFR in plasma or serum with a detection limit of 4 pg/ml, a 10% intra-assay variability and a 12% inter-assay variability, as described in the manufacturer's instructions. For the assay, plasma samples were diluted 100-fold, and evaluated with anti-EGFR antibodies raised against the L25-S645 region of EGFR.

Table I. Characterization of patients and controls.

Group	Factor	Number of cases	Age, years (range)
HNSCC patients		92	62.90 (44-89)
	TNM T1-2	39	62.42 (44-89)
	TNM T3-4	52	63.17 (47-87)
	TNM N0	42	65.04 (44-89)
	TNM N1	49	61.26 (44-77)
	TNM M0	86	62.86 (44-89)
	TNM M1	5	62.12 (55-71)
	Grade 1	6	63.46 (53-79)
	Grade 2	50	63.26 (44-89)
	Grade 3	32	61.49 (47-75)
	Non-smoker	28	66.99 (46-89)
Smoker	59	62.01 (44-78)	
Healthy controls		29	64.38 (54-69)
Diabetic controls		26	56.73 (50-83)

TNM, tumor-node-metastasis; HNSCC, head and neck squamous cell carcinoma.

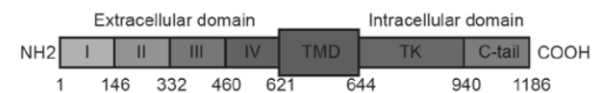


Figure 1. Schematic representation of EGFR protein (170-kDa). EGFR is composed of an extracellular highly glycosylated ligand-binding domain (comprising amino acids 1-621; exons 1-15), a hydrophobic transmembrane domain (amino acids 622-644; exons 15-17) and an intracellular domain with tyrosine kinase activity for signal transduction (amino acids 645-1186; exons 18-28). I-IV denote subdomains of the extracellular domain. The figure was adapted from Albitar *et al* (1). TMD, transmembrane domain; TK, tyrosine kinase; EGFR, epidermal growth factor receptor.

RNA isolation and reverse transcription (RT). TriPure Isolation reagent (Roche Diagnostics, Basel, Switzerland) was used for RNA isolation. The isolated RNA was used for complementary (c)DNA synthesis. RNA (1,000 ng) was reverse transcribed using Transcriptor First Strand cDNA Synthesis kit (Roche Diagnostics) according to manufacturer's protocol. The cDNA (20 µl) prepared from total RNA was diluted with RNase-free water to 100 µl, and 5 µl cDNA was directly analyzed using the LightCycler® 480 II System (Roche Diagnostics).

RT-quantitative polymerase chain reaction (qPCR). RT-qPCR was performed using TaqMan® Gene Expression Assays (Life Technologies; Thermo Fisher Scientific, Inc.) with the LightCycler® 480 II System, and the amplified DNA was analyzed by the comparative $\Delta\Delta Cq$ calculation (23) using β -actin as an endogenous control. The primer and probe sets for β -actin (Hs99999903_m1), metallothionein (MT)2 (Hs02379661_g1), MT1 (Hs00831826_s1), tumor protein p53 (TP53) (Hs01034249_m1), B-cell lymphoma (BCL)-2 associated X protein (BAX) (Hs00180269_m1), BCL-2 (Hs00608023_m1), vascular endothelial growth factor A

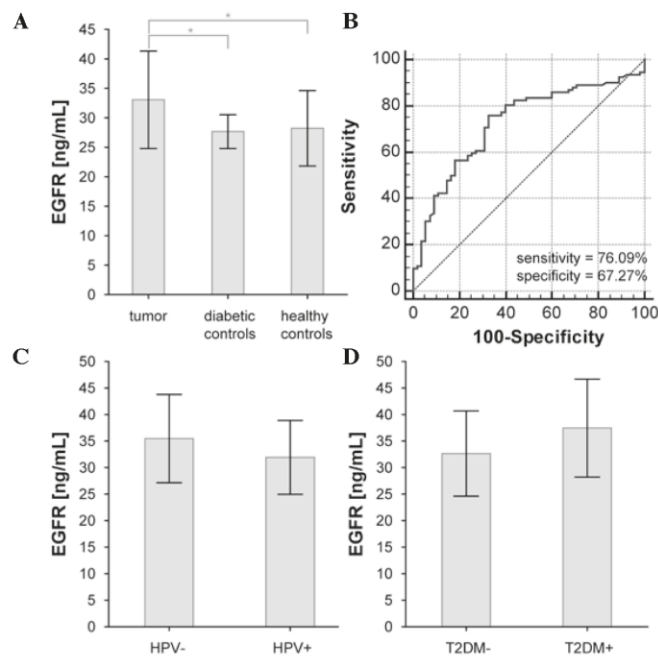


Figure 2. Plasmatic EGFR levels. (A) Plasma EGFR levels in head and neck squamous cell carcinoma patients and in two control groups (* $P < 0.05$ vs. controls). (B) Receiver operating characteristic curve analysis indicating the sensitivity and specificity of plasma EGFR detection. (C) Plasma EGFR levels in human papillomavirus-positive and -negative patients. (D) Plasma EGFR levels and type 2 diabetes mellitus status in patients. EGFR, epidermal growth factor receptor; T2DM, type 2 diabetes mellitus; HPV, human papillomavirus.

(VEGFA) (Hs00900055_m1), fms-related tyrosine kinase 1 (FLT1) (Hs01052961_m1), matrix metalloproteinase 2 (MMP2) (Hs01548727_m1), MMP9 (Hs00234579_m1), proto-oncogene c-Fos (FOS) (Hs00170630_m1), c-Jun (JUN) (Hs00277190_s1), marker of proliferation Ki-67 (MKI67) (Hs00606991_m1), EGF (Hs01099999_m1) and EGFR (Hs01076078_m1) were selected from TaqMan® Gene Expression Assays. RT-qPCR was performed under the following amplification conditions in a total volume of 20 μ l (5 μ l cDNA, 10 μ l TaqMan® Gene Expression Master Mix, 4 μ l molecular-grade water and 1 μ l TaqMan Gene Expression Assay): Initial incubation, 50°C for 2 min, followed by denaturation at 95°C for 10 min and then 45 cycles of 95°C for 15 sec and 60°C for 1 min.

Human papillomavirus (HPV) detection. The 142 bp-long sequence of the conservative major capsid protein L1 gene were amplified using general primers, GP5 and GP6, for non-specific identification of HPV-positive subjects. The PCR mixture from New England BioLabs, Inc. (Ipswich, MA, USA) contained PCR buffer (10 mM Tris HCl, pH 8.3, 50 mM KCl and 2.5 mM MgCl₂), 0.05 mM of each deoxynucleotide, and 0.05 mM of GP5 (5'-TTTGTACTGTGGTAGATAC-3') and GP6 (5'-GAAAAATAAACTGTAAATCA-3') primers. The DNA amplification was performed during 40 cycles that included a denaturation step at 94°C for 30 sec, annealing at 45°C for 30 sec and extension at 72°C for 30 sec.

As internal quality control of the isolated DNA, the β -actin gene (600 bp) was amplified (forward primer 5'-CCTGAA CCCTAAGGCCAAC-3' and reverse primer 5'-GCAATG

CCTGGGTACATGGT-3'). Each PCR product was analyzed using electrophoresis on 1% agarose gels stained with ethidium bromide.

Data analysis. Differences between the two groups were calculated using the *t*-test. Survival analysis was conducted using Cox proportional hazard regression analysis with plasma EGFR levels as covariates. Receiver operating characteristic (ROC) curves were calculated using the DeLong methodology. Subsequently, Kaplan-Meier analysis was used with continuous data being divided into two groups as follows: Low expression (<mean values) and high expression (\geq mean values) groups. The associations between the continuous variables were analyzed using Pearson's correlations. Unless noted otherwise, $P < 0.05$ was considered to indicate a statistically significant difference. Software STATISTICA 12 (StatSoft, Inc., Tulsa, OK, USA) and MedCalc 15.8 (MedCalc Software bvba, Ostend, Belgium) were used for analysis.

Results

Association between plasma levels of EGFR and HNSCC occurrence. No significant changes in EGFR plasma levels were observed between diabetic and healthy controls ($P = 0.690$). However, there was a significant difference between EGFR plasma levels in HNSCC patients and in both control groups ($P = 0.001$ and 0.005 , respectively) (Fig. 2A and Table II). If both control groups were assessed together, the statistical significance was $P = 0.0001$. ROC curve analysis identified a sensitivity of 76.09%, a specificity of 67.27% and an area

Table II. Plasma levels of EGFR and clinical characteristics in HNSCC patients.

Factor	Status (number of cases)	EGFR levels, ng/ml mean \pm standard deviation	P-value
Cases vs. controls	HNSCC patients (92)	33.1 \pm 8.3	-
	Healthy controls (29)	28.2 \pm 6.4	0.001
	Diabetic controls (26)	27.7 \pm 2.9	0.005
Smoking	Yes (59)	31.8 \pm 7.7	0.150
	No (28)	34.5 \pm 8.5	-
Hypertension	Yes (28)	34.4 \pm 8.8	0.380
	No (58)	32.7 \pm 8.0	-
Diabetes mellitus	Yes (10)	37.4 \pm 9.2	0.085
	No (76)	32.6 \pm 8.0	-
TNM T-staging	T1-2 (39)	32.5 \pm 8.1	0.580
	T3-4 (52)	33.4 \pm 8.5	-
TNM N-staging	N+ (49)	33.7 \pm 8.2	0.430
	N- (42)	32.3 \pm 8.5	-
TNM M-staging	M+ (5)	31.3 \pm 8.1	0.640
	M- (86)	33.1 \pm 8.3	-
Tumor grade	High (82)	32.8 \pm 8.1	0.270
	Low (6)	36.8 \pm 12.3	-
HPV status	HPV+ (49)	32.0 \pm 7.0	0.084
	HPV- (18)	35.5 \pm 8.3	-

EGFR, epidermal growth factor receptor; HNSCC, head and neck squamous cell carcinoma; TNM, tumor-node-metastasis; HPV, human papillomavirus.

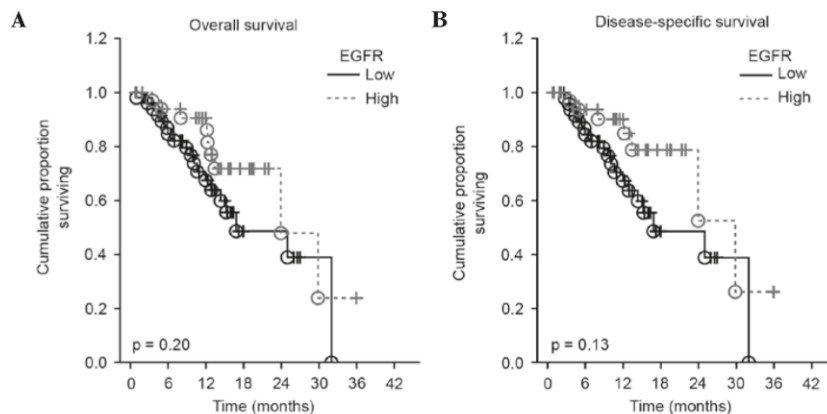


Figure 3. Survival analysis. (A) Kaplan-Meier overall survival analysis. (B) Disease-specific survival analysis. High/low EGFR indicate EGFR values above/below the mean EGFR values. P-value was calculated by Cox proportional hazard regression analysis. EGFR, epidermal growth factor receptor.

under the curve (AUC) of 0.727 for this comparison (Fig. 2B). Additional information about the patients and controls is contained in Table I.

Correlation between tumor gene expression and EGFR plasma levels. Correlations between plasma EGFR levels and expression of genes in tumor tissues of HNSCC patients were examined. There was no significant correlation between plasma EGFR levels and tumor tissue EGFR mRNA

expression, and only a weak negative correlation with MMP9 mRNA was observed ($r=-0.21$, $P=0.040$). Gene expression analyses of EGF, EGFR, MKI67, BCL-2, BAX, FOS, JUN, TP53, VEGFA, FLT1, MMP2, MMP9, MT1A and MT2A genes in HNSCC tumor tissue compared with tumor adjacent tissue and tonsillectomies have been published elsewhere (24).

Association between plasma levels of EGFR and clinico-pathological characteristics. By examining the associations

between plasma EGFR levels and clinicopathological characteristics of HNSCC patients, no significant association was identified for smoking habit, T2DM, hypertension, HPV infection, tumor stage, tumor grade, or lymph node or distant metastasis occurrence. However, the presence of HPV infection and T2DM in HNSCC patients had a borderline effect on the plasma EGFR levels (Table II and Fig. 2C and D).

Association between plasma levels of EGFR and disease-free and overall survival. The prognostic value of EGFR plasma levels on overall and disease-free survival was studied by Cox proportional hazard regression analysis and Kaplan-Meier curves. Survival analysis revealed no significant influence of plasmatic EGFR levels on overall or disease-specific survival in the present cohort of HNSCC patients [hazard ratio (HR)=0.97; 95% confidence interval (CI)=0.92-1.01; P=0.200, and HR=0.96; 95 CI=0.91-1.01; P=0.130 for overall and disease-free survival, respectively] (Fig. 3).

Discussion

Numerous studies have shown that EGFR is overexpressed in HNSCC tumor tissue, but only few studies focused on soluble EGFR levels (24-26). There are contradictory studies on soluble EGFR levels, which could be either decreased or elevated in cancer patients compared with a healthy cohort. For example, Partanen *et al* reported that patients with asbestosis-induced lung cancer have elevated serum soluble EGFR ECD levels (27). Increased soluble EGFR ECD levels were also reported in the urine of patients with squamous cell carcinomas of the lung, head and neck (28), whereas patients with ovarian cancer had decreased levels of serum p110 EGFR compared with the normal population (29). In the present study, ELISA using antibodies against the L25-S645 region of EGFR was used to measure the levels of EGFR in plasma samples of HNSCC patients. Female patients were excluded from the present study due to possible gender-specific EGFR interactions with estrogen or androgen receptors (20-22). Significantly higher EGFR plasma levels were detected in HNSCC patients compared with the healthy cohort and the diabetic control group (P=0.001 and 0.005, respectively). This finding is in accordance with that of Perez-Torres *et al* (17), who suggested that the mechanism of proteolytic cleavage of EGFR and shedding of PI-sEGFR into the plasma may be activated in malignant cells that overexpress the full-length receptor. The cleavage of EGFR probably occurs in the transmembrane domain between G625 and M626 (17). In HNSCC patients, EGFR expression is supposed to be higher in tumor tissues compared with tonsillectomy samples and tumor-adjacent tissues (24). Furthermore, the release of two soluble EGFR isoforms within the exosomes is activated by EGF (17), which is highly produced by HNSCC tumor-adjacent tissues (24).

No significant changes in EGFR plasma levels were observed between diabetic and healthy controls, which is not in accordance with the Vairaktaris *et al* hypothesis that diabetes suppresses the expression of EGFR (19). However, a slight change on the borderline of statistical significance was observed between HNSCC patients with or without diabetes (P=0.085), while HNSCC patients with diabetes tended to have higher EGFR plasma levels. Borderline changes in EGFR

plasma levels were also noticed between the HPV-positive and HPV-negative groups of HNSCC patients (slightly higher levels of plasmatic EGFR were detected in the HPV-negative cohort), although these changes were not significant.

Survival analysis revealed no significant influence of the plasmatic EGFR levels on overall and disease-specific survival in the present cohort of HNSCC patients. By contrast, Ye *et al* demonstrated that non-small-cell lung cancer patients with lower plasma EGFR concentrations (<27.24 ng/ml) had a significantly shorter overall survival compared with patients who had higher plasma EGFR concentrations (\geq 27.24 ng/ml) (18.2 vs. 33.4 months, P=0.021) (30).

In conclusion, EGFR plasma levels appear to be a relatively promising diagnostic, but poor prognostic, HNSCC marker. However, further studies are required to determine the clinical value of plasmatic EGFR levels in HNSCC patients. The next important step in soluble EGFR research should be a precise distinction between 110-kDa PI-sEGFR originating from full-length EGFR protein cleavage and EGFR isoforms originating from alternative splicing of EGFR gene transcripts. These EGFR isoforms could readily have slightly different functions. For example, 110-kDa PI-sEGFR originating from full-length EGFR protein cleavage could reflect the presence of malignant cells that overexpress the full-length receptor (17) or a necrotic disintegration of tumor cells. Such form of soluble EGFR was probably originally involved in a proliferative signaling pathway, and could be marker of poor prognosis, while the soluble EGFR isoform originating from alternative splicing was probably not an activator of these proliferative signaling pathways due to the missing intracellular domain, and could exhibit a high affinity binding for EGF, which should result in decreased proliferative signaling and better prognosis.

Acknowledgements

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4.6 Biologická a klinická charakterizace HPV⁺ a HPV⁻ tumorů

V 80. letech dvacátého století byl odhalen vliv lidského papillomaviru (HPV) v incidenci HNSCC ⁵⁶. HPV infekce je zřejmě časnou či dokonce iniciační událostí při patogenezi těchto nádorů. Expresí virových proteinů E6 a E7 vede k inaktivaci klíčových nádorových supresorů TP53, retinoblastomového proteinu (RB1), P107, P130 a P21 a tím zvyšuje syntézu DNA a expresi genů požadovaných pro přechod z G1 do S fáze.

Cílem následující¹⁰² studie Polanska *et al.* (str. 147) bylo popsat, jak tkáňová exprese genů souvisejících s buněčným cyklem, buněčnou smrtí, angiogenezí aj. souvisí s přítomností HPV. Do této studie bylo zahrnuto 74 vzorků nádorové tkáně histologicky ověřeného HNSCC, 16 pacientů bylo HPV16-pozitivních (21,6 %), 9 bylo HPV18-pozitivních (12,2 %), 27 bylo infikováno jiným HPV subtypem (36,5 %) a 22 bylo HPV-negativních (29,7 %), popis pacientů viz str. 149. Ve studované kohortě HNSCC pacientů nebyla nalezena žádná asociace mezi výskytem HPV⁺ HNSCC a kouřením, věkem, diferenciací a stádiem nádoru. Dále byl studován vliv HPV-infekce na expresi těchto genů: *EGF*, *EGFR*, *MKI67*, *BCL2*, *BAX*, *FOS*, *JUN*, *TP53*, *VEGF*, *FLTI*, *MMP2*, *MMP9*, *MT1A* a *MT2A*; (viz tabulka na str. 150). U HPV-negativních nádorů byla pozorována vyšší exprese genů *MT2A*, *MMP9*, *FLTI*, *VEGFA* a *POU5F*; viz obrázek na str. 150. Na tomto trendu se v případě genu *MT2A* nejvýznamněji podílela infekce virem HPV18. Dále byla prokázána silná pozitivní korelace s $r > 0,50$ specifická pouze pro HPV-negativní nádory mezi *MT1A* a *MKI67*, a *MMP2* a *FLTI*. Expresí *MT2A* pozitivně korelovala s *BAX*, *MMP2*, *MMP9*, *FLTI*, *VEGFA*, *FOS* a *JUN*.

Lepší celkové přežití bylo identifikováno u skupiny HPV⁺ pacientů s vyšší expresí *TP53* a *BAX* a nižší expresí *BCL2* (viz obr. na str. 152). Naopak, vysoká exprese *MMP9* byla spojená s horším přežíváním u HPV⁺ i HPV⁻ HNSCC pacientů; 70 % pacientů s nízkou expresí *MMP9* v nádorové tkáni bylo naživu ještě po dvou letech od diagnózy onemocnění, zatímco ve skupině s vysokou či střední expresí to bylo pouze 30 %.

Polanska H, Heger Z, Gumulec J, *et al.* Effect of HPV on tumor expression levels of the most commonly used markers in HNSCC. *Tumor Biology*. 2016;37(6):7193-7201.



ORIGINAL ARTICLE

Effect of HPV on tumor expression levels of the most commonly used markers in HNSCC

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Abstract Approximately 90 % of head and neck cancers are squamous cell carcinomas (HNSCC), and the overall 5-year survival rate is not higher than 50 %. There is much evidence that human papillomavirus (HPV) infection may influence the expression of commonly studied HNSCC markers. Our study was focused on the possible HPV-specificity of molecular markers that could be key players in important steps of cancerogenesis (*MKI67*, *EGF*, *EGFR*, *BCL-2*, *BAX*, *FOS*, *JUN*, *TP53*, *MT1A*, *MT2A*, *VEGFA*, *FLT1*, *MMP2*, *MMP9*, and *POU5F*). qRT-PCR analysis of these selected genes was performed on 74 biopsy samples of tumors from patients with histologically verified HNSCC (22 HPV–, 52 HPV+). Kaplan-Meier analysis was done to determine the relevance of these selected markers for HNSCC prognosis. In conclusion, our study confirms the impact of HPV infection on commonly studied HNSCC markers *MT2A*, *MMP9*, *FLT1*, *VEGFA*, and

POU5F that were more highly expressed in HPV-negative HNSCC patients and also shows the relevance of studied markers in HPV-positive and HPV-negative HNSCC patients.

Keywords Head and neck cancer · Human papillomavirus · Tumor markers · Kaplan-Meier analysis

Introduction

Head and neck squamous cell carcinoma (HNSCC) is the sixth most common cancer worldwide. The 5-year survival rate is less than 50 % [1, 2]. Eighty to ninety percent of HNSCCs were traditionally attributed to tobacco and alcohol use. Nevertheless, the involvement of human papillomavirus (HPV) in oropharyngeal and oral carcinogenesis was suggested in the 1980s [3]. HPV was associated with 40 to 80 % of oropharyngeal tumors in the USA, whereas HPV cancer incidence in Europe changes from 90 % in Sweden to approximately 20 % in countries with the highest tobacco consumption [4]. About 100 subtypes of HPV have been described in humans [5]. In contrast to low-risk HPV types such as HPV6 and HPV11 which rarely cause cancer, the high-risk HPV types HPV16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, and 66 have all been implicated in oncogenesis [6], and except HPV16 and HPV18, other oncogenic HPVs were rarely detected in HNSCC [7]. The difference in the ability to induce tumor between high-risk and low-risk HPV could be caused by alternate splicing of E6 and E7 viral oncogenes in high-risk HPV [8]. The E7 protein of high-risk HPV targets many cell cycle regulatory proteins (including pRb, p107, p130, and p21) and thereby upregulates DNA synthesis and gene expression required for G1 to S-phase transition [9]. The physiological response of the host cell to this excessive proliferation would be to trigger senescence, growth arrest, and apoptosis.

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Another high-risk E6 protein is able to target many important proteins involved in regulation of cell damage, growth arrest, antiviral defense, and terminal differentiation of cells [10]. It is not surprising that HPV-positive (HPV+) HNSCCs may constitute a separate subclass with different clinical features and dissimilar biology. It could also represent a distinct cancer lineage formed through separate aetiological pathways of multistep tumorigenesis [9, 11–13]. Some studies suggest that HPV-positive HNSCC could be a sexually transmitted disease [14]. Accordingly, a strong association between sexual behavior and risk of oropharyngeal cancer has been shown [15, 16]. We assume that described variations in the biological and clinical characteristics of HPV+ and HPV– HNSCCs are likely reflected by shifts in gene expressions of commonly studied markers. Our study was mainly focused on possible HPV-specificity of molecular markers that could be key players in important steps of cancerogenesis such as (a) proliferative activity of tumor cells (*MKI67*), (b) acquisition of autonomous proliferative signaling (*EGF/EGFR*), (c) cell cycle and cell death modifications (*BAX*, *BCL-2*, *FOS*, *JUN*, *TP53*), (d) oxidative stress response (*MT1A*, *MT2A*), (e) angiogenesis (*VEGFA/FLT1*), (f) metastatic invasivity (*MMP2*, *MMP9*), and (g) pluripotency (*POU5F*).

Materials and methods

Tissue sample preparation

This study was approved by the ethical committee of St. Anne's Faculty Hospital, Brno, Czech Republic. All surgical tissue samples were obtained from HNSCC patients (Caucasian men; women were not included) after they signed the informed consent document. Histologically verified primary HNSCC tissue samples were collected. The tissue material harvested at surgery was placed into RNAlater Solution for RNA stabilization and storage (Ambion, Carlsbad, CA, USA). The material was maintained cold, and RNA was isolated within 24 h.

RNA isolation and reverse transcription

TriPure Isolation Reagent (Roche, Basel, Switzerland) was used for RNA isolation. The isolated RNA was used for cDNA synthesis. RNA (1000 ng) was transcribed using a transcriptor first strand cDNA synthesis kit (Roche, Switzerland), which was applied according to the manufacturer's instructions. The cDNA (20 μ l) prepared from the total RNA that was diluted with RNase-free water to 100 and 5 μ l was directly analyzed by using the LightCycler[®] 480 II System (Roche, Switzerland).

Quantitative real-time polymerase chain reaction

Quantitative real-time polymerase chain reaction (qRT-PCR) was performed using the TaqMan gene expression assays with the LightCycler[®] 480 II System (Roche, Switzerland), and the amplified DNA was analyzed by the comparative Ct method using β -actin as an endogenous control. The primer and probe sets for ACTB (assay ID Hs99999903_m1), MT2A (Hs02379661_g1), MT1A (Hs00831826_s1), TP53 (Hs01034249_m1), BAX (Hs00180269_m1), BCL-2 (Hs00608023_m1), VEGFA (Hs00900055_m1), FLT1 (Hs01052961_m1), MMP2 (Hs01548727_m1), MMP9 (Hs00234579_m1), FOS (Hs00170630_m1), JUN (Hs00277190_s1), MKI67 (Hs00606991_m1), EGF (Hs01099999_m1), EGFR (Hs01076078_m1), and POU5F1 (Hs04260367_gH) were selected from TaqMan gene expression assays (Life Technologies, USA). qRT-PCR was performed under the following amplification conditions: total volume of 20 μ l, initial incubation at 50 °C/2 min followed by denaturation at 95 °C/10 min, then 45 cycles at 95 °C/15 s and at 60 °C/1 min.

HPV detection

The 142 base pair long sequence of conservative *L1* gene was amplified using GP5 and GP6 primers for non-specific identification of HPV-positive subjects. The PCR mixture from New England Biolabs (UK) contained PCR buffer (10 mM Tris-HCl, pH 8.3, 50 mM KCl with 2.5 mM MgCl₂ included) with 0.05 mM of each dNTP and 0.05 mM of GP5 and GP6 primers. The DNA amplification was carried out during 40 cycles that included the denaturation at 94 °C for 30 s, annealing at 45 °C for 30 s, and the primer extension at 72 °C for 30 s.

The HPV-positive specimens were further analyzed with the HPV16 and HPV18 primers. The PCR amplicons reached lengths of 202 bp for HPV16 and 272 bp for HPV18. The DNA amplification was carried out during 40 cycles that included the denaturation at 94 °C for 30 s, the annealing at 58 °C for 30 s, and the primer extension at 72 °C for 30 s.

As internal quality control of the isolated DNA, β -actin gene (600 bp) was amplified. Each PCR product was analyzed using electrophoresis on 1 % agarose gels stained with ethidium bromide. Sequences of exploited primers are shown in Online resource 1.

HPV sequencing

GenomeLab DTCS Quick Start kit (Beckman Coulter, USA) with 20 ng of purified amplified DNA from the PCRs was used for the sequencing reaction. The cycling conditions were as follows: 30 cycles of denaturation at 96 °C for 20 s; annealing at 50 °C for 20 s; and 60 °C for DNA synthesis for 4 min. DNA fragments from this reaction were purified using

magnetic particles CleanSEQ (Beckman Coulter, USA). DNA sequencing was performed on Genetic Analysis System CEQ 8000 (Beckman Coulter, USA). After denaturation at 90 °C for 2 min, the fluorescence-marked DNA fragments were separated in 33-cm capillary with 75 µm i.d. (Beckman Coulter, USA), which was filled with a linear polyacrylamide denaturing gel (Beckman Coulter, USA). The separation was performed at capillary temperature of 50 °C and voltage of 4.2 kV for 85 min.

Data analysis

Difference between the two groups was calculated on log-transformed gene expression data using *t* test. Survival analysis was performed using Cox proportional hazard regression with tumor tissue gene expression as covariates. Consequently, Kaplan-Meier analysis was used with continuous data being recoded to three groups as follows: low expression (<33 percentile), medium expression (<66 percentile), and high expression (≥66 percentile). Relations of continuous variables were analyzed using Pearson's correlations and the principal component analysis. Unless noted otherwise, *p* level <0.05 was considered significant. Software Statistica 12 (StatSoft, Tulsa, OK, USA) was used for analysis.

Results

Clinicopathological characterization of HNSCC patients

In this study, 74 biopsy samples of tumors from patients with histologically verified spinocellular carcinoma and comprehensive patient histories were used. The locations of these tumors were as follows: oral cavity (9 samples), oropharynx (25 samples), hypopharynx (18 samples), and larynx (22 samples). A brief description of the patients is shown in Table 1. Sixteen patients were HPV16 positive (21.6 %), 9 were HPV18 positive (12.2 %), 27 harbored other unspecified HPV subtype (36.5 %), and 22 were HPV negative (29.7 %).

Association between the human papillomavirus status and clinical factors

In the group we studied, no statistically significant association between HPV-positive HNSCC and tobacco consumption, age, tumor grade, or stage has been demonstrated (evaluated by chi-square test). Smoking had no significant effect on expression of studied genes, $F(15, 67)=0.58, p=0.88$.

Gene expression analysis

Expression analysis of mRNA was performed in all tumor samples to characterize the expression profile of selected

Table 1 Characterization of the tested cohort

Factor	Valid <i>N</i>	Age mean (min-max)
HPV status		
Negative	22	66.22 (53–77)
Positive	52	62.96 (44–89)
HPV 16 status		
Negative	58	64.74 (47–89)
Positive	16	60.97 (44–73)
HPV 18 status		
Negative	65	64.57 (44–89)
Positive	9	59.24 (48–72)
Smoking status		
Not specified	4	58.51 (53–73)
Smoker	47	62.57 (44–79)
Non-smoker	23	67.63 (49–89)
TNM T staging		
1	10	62.75 (44–77)
2	24	65.16 (51–89)
3	16	65.25 (49–79)
3b	1	63.40 (63–63)
4	10	63.65 (54–76)
4a	10	62.41 (47–72)
4b	3	57.04 (53–60)
TNM N staging		
0	34	65.86 (44–89)
1	8	64.34 (51–72)
1b	1	63.50 (64–64)
2	6	63.78 (54–76)
2b	11	64.37 (47–77)
2c	9	59.38 (53–68)
3	5	57.57 (51–65)
TNM M staging		
0	73	64.00 (44–89)
1	1	58.14 (58–58)
Tumor grading		
1	3	63.01 (57–68)
2	46	65.40 (44–89)
3	22	60.66 (47–73)
4	1	54.46 (54–54)
n.s.	2	72.01 (67–77)
Tumor location		
Oral cavity	9	56.80 (44–67)
Oropharynx	25	63.12 (48–77)
Hypopharynx	18	61.78 (50–76)
Larynx	22	67.75 (54–89)

Age displayed as mean (minimum-maximum)

genes in HPV-positive and HPV-negative samples (see Table 2 and Online resource 2). Significantly higher expression of *MT2A* ($p=0.02$), *MMP9* ($p=0.02$), *FLT1* ($p=0.03$),

Table 2 Effect of HPV status on tissue gene expression

Gene	Expression fold change (95 % CI)	<i>p</i> level
BCL-2	0.41 (0.14 to 1.15)	0.089
BAX	0.53 (0.23 to 1.22)	0.134
TP53	0.59 (0.26 to 1.34)	0.207
MT1A	0.45 (0.11 to 1.89)	0.269
MT2A	0.32 (0.12 to 0.83)	0.020 ^a
MKI67	0.35 (0.11 to 1.11)	0.075
EGFR	0.48 (0.19 to 1.22)	0.120
EGF	0.39 (0.11 to 1.40)	0.146
MMP2	0.59 (0.24 to 1.47)	0.253
MMP9	0.25 (0.08 to 0.79)	0.020 ^a
FLT1	0.37 (0.16 to 0.89)	0.026 ^a
VEGFA	0.25 (0.09 to 0.76)	0.015 ^a
FOS	0.84 (0.33 to 2.14)	0.709
JUN	0.46 (0.20 to 1.03)	0.059
POU5F1	0.20 (0.05 to 0.79)	0.023 ^a

Results of statistical analysis. Displayed as a gene expression fold change (95 % confidence interval) compared to HPV-negative samples

^aStatistically significant changes

VEGFA ($p=0.02$), and *POU5F* ($p=0.02$) was found in HPV-negative tumor tissues (see Fig. 1a). HPV18 status had significant effect on *MT2A* tumor-tissue gene expression even if assessed independently ($p=0.001$; see Online resource 2).

To determine typical coexpression patterns, principal component analysis was done. To illustrate the model of HPV influence, two-factor analysis was chosen, which describes 50.68 and 73.54 % of the total variance of gene expression data for HPV+ tumors and HPV- tumors, respectively (see Fig. 1b, c). Based on this analysis, the majority of genes are clustered together. In contrast, gene vectors pointing in another direction are characteristic for infected or non-infected tumor tissue. The most significant shift of expression within the expression patterns was found for *MT1A* and *MT2A*. Consequently, we focused on *MT1A* and *MT2A* relationships with other studied genes within the expression profiles of HPV+ and HPV- tumor samples (see Online resource 3). Strong positive correlation with $r>0.50$ at $p<0.05$ specific only for HPV-negative tumor tissues were identified between *MT1A* and the following genes: *MKI67*, *MMP2*, and *FLT1*. *MT2A* correlated positively at an equally strong and significant level with *BAX*, *MMP2*, *MMP9*, *FLT1*, *VEGFA*, *FOS*, and *JUN*.

Survival analysis

Survival analysis showed no statistically significant shift in overall and disease-specific survival between HPV+ and HPV- HNSCC patients in our studied cohort of patients.

However, HPV+ HNSCC patients showed better survival during the first year of the disease (70 % in HPV-positive vs. 50 % in HPV-negative HNSCC group of patients; see Fig. 2a). Furthermore, overall survival and also disease-specific survival was better in HPV+ group of HNSCC patients with higher *TP53* ($p=0.03$) and *BAX* ($p=0.02$) expression and lower *BCL-2* expression (borderline statistical significance $p=0.055$). Hazard ratio (HR) was 0.001 for *TP53*, 0.03 for *BAX* and 10.5 for *BCL-2*, respectively (see Tables 3 and 4, and Fig. 2b, c). In contrast, high expression of *MMP9* was an unfavorable feature for disease-specific survival in the whole group of HNSCC patients (HR=5.81, $p=0.038$; see Table 4 and Fig. 2c). The Kaplan-Meier analysis showed that the disease-specific survival of patients with *MMP9* low-expressing tumors was 70 % after 2 years of follow-up, whereas it was only 30 % with *MMP9* high for moderate-expressing tumors.

Discussion

The incidence of HPV-positive tumors increased between the years 1970 and 2007 while the incidence of HPV-negative tumors simultaneously decreased [17]. Inasmuch as gene expression signatures of HPV-positive HNSCC have been repeatedly shown to be different from those of HPV-negative [5, 18, 19], it is very important to study the influence of HPV infection on the expression of biomarkers commonly examined in the context of HNSCC pathogenesis. It is very likely that some HPV-positive HNSCC-specific genes may not be relevant in HPV-negative head and neck cancer patients. For these reasons, our study was focused on expression analysis of molecular markers that present key functions related to HNSCC neoplastic transformation and have been intensively studied in recent years. Significantly higher expression of *MT2A* ($p=0.02$), *MMP9* ($p=0.02$), *FLT1* ($p=0.03$), *VEGFA* ($p=0.02$), and *POU5F* ($p=0.02$) was found in HPV-negative tumor tissues.

Many studies have confirmed that HPV-positive tumors in the head and neck region have a better prognosis compared with those that are HPV-negative [20–23]. In our study, survival analysis showed no statistically significant shift in overall and disease-specific survival between HPV+ and HPV- HNSCC patients. However, HPV+ HNSCC patients showed better survival during the first year of the disease. Several other authors were also unable to confirm the better long-term prognosis of HPV-positive HNSCC patients versus similar HPV-negative cancer patients [24–27]. This discrepancy in results could be caused by the different localization of tumors studied. Most of the studies that revealed a positive relationship between HPV-positivity and better survival were focused on oropharyngeal carcinoma. Studies focused on other HNSCC localizations often failed to confirm this

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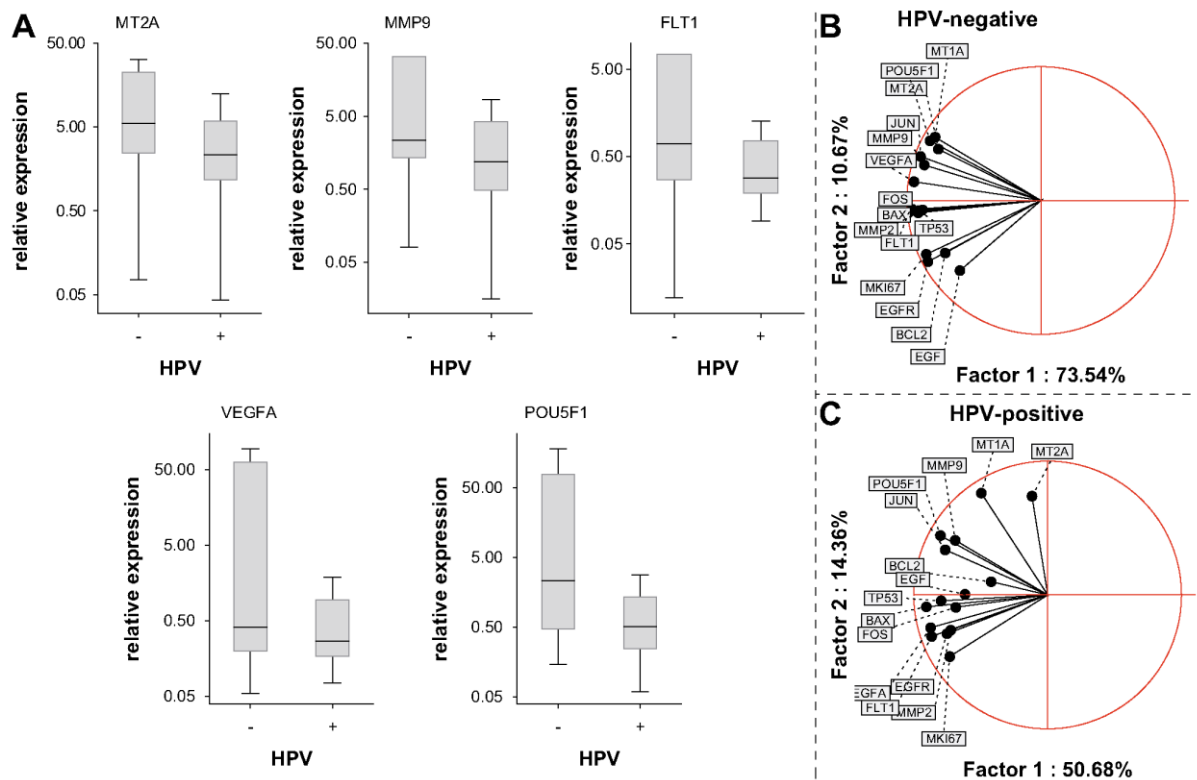


Fig. 1 Gene expression in HPV-positive and HPV-negative squamous cancer tissues. **a** Expression of genes differentially expressed ($p < 0.05$) in HPV-positive and HPV-negative tissues. Displayed as median, interquartile, and non-outlier range. **b** Principal component analysis

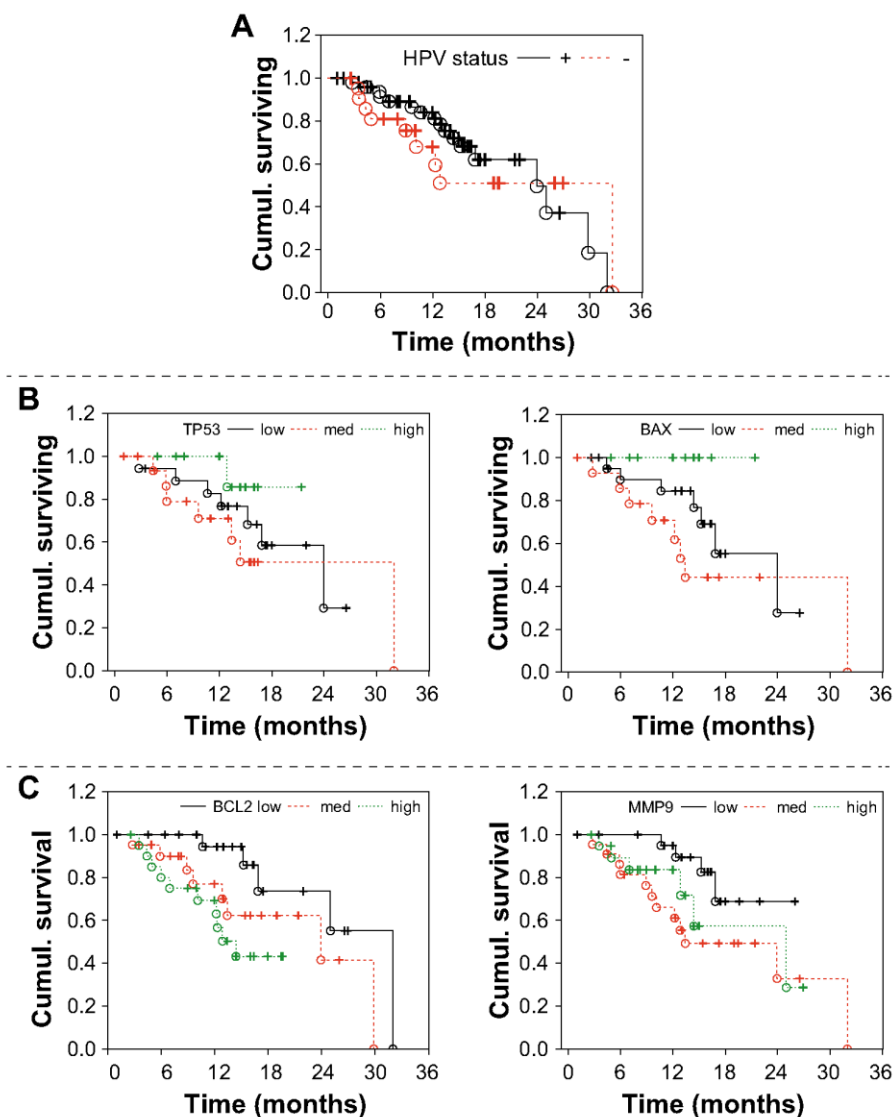
showing projection of variables (genes) on the factor plane of the first two factors in HPV-negative and **c** HPV-positive cases. Note that genes pointing to a different location than the “major gene cluster” are specific for a particular HPV condition. For details, see the “Results” section

observation. Furthermore, some evidence leads to the suggestion that loss of p53 function may be highly involved in the differences in prognosis between HPV-negative and HPV-positive HNSCC patients [28, 29]. Although HPV-positive tumors have reduced levels of p53 due to E6-mediated degradation, the p53 protein is not mutated and could be functional under stress conditions. Radiation-provoked increases in p53 levels may then be sufficient to trigger senescence or programmed cell death in the HPV-positive tumors [28]. Accordingly, overall survival and also disease-specific survival was better in our HPV+ group of HNSCC patients with higher *TP53* and *BAX* expression and lower *BCL-2* expression (borderline statistical significance $p = 0.055$), which confirm the importance of apoptosis disruption in HPV+HNSCC cancerogenesis. On the other hand, a high percentage of HPV-negative tumors have non-functional, mutated p53. The accumulation of such mutant p53 protein could lead to the production of anti-p53 antibodies, which was significantly associated with increased risk of relapse and death [30]. Given that mutation of *TP53* is an early event in the carcinogenesis of HNSCC [31, 32], HPV-positivity or negativity could play a more essential role in the early stages of HNSCC rather than in

advanced tumors. Differing study results can also result from the different structure of studied cohorts. Better survival rates of HPV+ HNSCC patients during the first year of the disease may be related to lower expression of genes playing a significant role in cancerogenesis, such as *MMP9*, *MT2A*, *FLT1*, *VEGFA*, and *POU5F* in comparison with HPV-negative patients.

The matrix metalloproteinases (MMPs) are secreted endopeptidases that require zinc for their catalytic activity [33]. These MMPs are capable of degrading all kinds of extracellular matrix protein components such as basement membrane collagens, proteoglycans, fibronectin, and laminin, which seems to be important in tumor growth and generation of metastases [34]. MMP2 and MMP9 overexpression in HNSCC tumor samples has been correlated with unfavorable prognosis in head and neck carcinoma [35–38]. In accordance with our results, Kim et al. found that HPV-negative specific gene set also included the MMP family [18]. Metallothioneins (MTs) are a group of low molecular weight, cysteine-rich, metal ion-binding proteins that have been linked with enhanced cell proliferation in squamous cell carcinoma of the esophagus [39]. They are involved in a variety of human

Fig. 2 Kaplan-Meier analysis of overall survival. **a** The effect of HPV status on overall survival rate. No significant difference was observed. **b** HPV-positive cases. Genes most distinctly characterizing overall survival in this cohort—TP53 and BAX. **c** HPV-positive and HPV-negative cases together. Tissue MMP9 expression correlates with patient outcome. The high/med/low scale is based on the 33th and 66th percentile of qRT-PCR expression of the particular gene



diseases and may be involved in the regulation of carcinogenesis and other important physiological processes [40]. A significantly positive association between MT staining and tumors (vs. healthy tissues) was observed in head and neck cancer [41]. Results by Cui et al. suggest that the physical interaction of tumor suppressor serine protease inhibitor Kazal-type 7 (also known as esophagus cancer-related gene 2 protein, ECRG-2) and MT2A could play an important role in the pathogenesis of esophageal cancer [42]. Furthermore, metallothioneins could play an important role in differences in HPV+ and HPV- cancerogenesis, inasmuch MT2A is generally highly expressed in HNSCC tumor tissue [43] and even more expressed in HPV-negative HNSCC tumor tissue. Ostrakhovitch et al. has shown that the semimetal-free apo-

form of MT interacts with p53 which may prevent binding of p53 to DNA. This could result in an inability of p53 to act as a transcriptional factor [44]. It is possible that metallothionein participates in p53 inactivation in HPV-positive tumors and is released for a different role in p53-defective HPV-negative tumors. Accordingly, the strong positive correlation specific only for HPV-negative tumor tissues were identified between *MT2A* and *MMP2*, *MMP9*, *FLT1*, *VEGFA*, *FOS*, and *JUN*, which prompt a possible MT2A role in angiogenesis enhancement and extracellular matrix remodeling in HPV-negative tumor tissue, which is in accordance with other studies [45–47]. In accordance, study by Nagel et al. proposed that the better prognosis of patients with HPV+ HNSCC is not related to intrinsic attributes of HPV+HNSCC tumor cells

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Table 3 Overall survival analysis using Cox proportional hazard regression with all genes included

Gene	All cases		HPV-positive	
	Hazard ratio (95 % CI)	<i>p</i> value	Hazard ratio (95 % CI)	<i>p</i> value
BCL-2	3.08 (0.75 to 12.58)	0.118	10.50 (0.95 to 116.15)	0.055 ^b
BAX	0.24 (0.02 to 3.44)	0.292	0.001 (0.001 to 0.34)	0.020 ^a
TP53	0.32 (0.06 to 1.64)	0.172	0.03 (0.00 to 0.44)	0.010 ^a
MT1A	1.11 (0.45 to 2.77)	0.815	0.20 (0.03 to 1.54)	0.122
MT2A	2.35 (0.63 to 8.73)	0.201	1.88 (0.26 to 13.40)	0.530
MKI67	0.88 (0.16 to 4.90)	0.880	21.12 (0.59 to 761.83)	0.095
EGFR	3.10 (0.55 to 17.34)	0.198	3.10 (0.14 to 67.44)	0.471
EGF	0.46 (0.21 to 1.02)	0.056 ^b	0.51 (0.09 to 2.96)	0.453
MMP2	0.70 (0.17 to 2.91)	0.622	0.03 (0.00 to 1.21)	0.063 ^b
MMP9	3.21 (0.90 to 11.39)	0.071 ^b	5.80 (0.63 to 53.54)	0.121
FLT1	3.73 (0.09 to 149.94)	0.485	0.54 (0.00 to 1110.43)	0.876
VEGFA	0.42 (0.04 to 4.38)	0.465	0.82 (0.00 to 194.61)	0.943
FOS	0.88 (0.16 to 4.87)	0.881	2.44 (0.14 to 43.02)	0.542
JUN	0.69 (0.06 to 7.51)	0.764	5.96 (0.04 to 984.91)	0.493
POU5F1	1.23 (0.15 to 9.96)	0.849	18.77 (0.89 to 394.56)	0.059 ^b

^a Statistically significant changes

^b Borderline statistical significance

cultured in cell line [48]. POU5F (POU domain, class 5, transcription factor 1) better known as octamer-binding transcription factor 4 (Oct4) plays a critical role in the development and self-renewal of embryonic stem cells and has been linked to oncogenic processes [49]. Overexpression of Oct4 was shown to enhance tumorigenic activities of oral squamous cell

carcinomas in vitro and also in vivo. These findings were coupled with epithelial-mesenchymal transition (EMT) [50]. In our study, significantly higher expression of *POU5F* ($p=0.02$) was found in HPV-negative tumor tissues. Conversely, Liu et al. found a higher expression level of Oct4 in HPV+ cervical cancer cell lines (HeLa and Caski)

Table 4 Disease-specific survival analysis using Cox proportional hazard regression with all genes included

Gene	All cases		HPV-positive	
	Hazard ratio (95 % CI)	<i>p</i> value	Hazard ratio (95 % CI)	<i>p</i> value
BCL-2	4.21 (0.79 to 22.28)	0.091	10.5 (0.95 to 116.15)	0.055 ^b
BAX	0.15 (0.01 to 3.03)	0.218	0.001 (0.001 to 0.34)	0.020 ^a
TP53	0.16 (0.03 to 1.07)	0.058 ^b	0.03 (0.00 to 0.44)	0.010 ^a
MT1A	0.93 (0.35 to 2.49)	0.886	0.20 (0.03 to 1.54)	0.122
MT2A	2.65 (0.71 to 9.81)	0.145	1.88 (0.26 to 13.40)	0.530
MKI67	1.39 (0.21 to 9.15)	0.734	21.12 (0.59 to 761.83)	0.095
EGFR	2.56 (0.32 to 20.30)	0.374	3.10 (0.14 to 67.44)	0.471
EGF	0.64 (0.25 to 1.64)	0.355	0.51 (0.09 to 2.96)	0.453
MMP2	0.69 (0.14 to 3.39)	0.647	0.03 (0.00 to 1.21)	0.063 ^b
MMP9	5.81 (1.10 to 30.59)	0.038 ^a	5.80 (0.63 to 53.54)	0.121
FLT1	0.55 (0.01 to 47.31)	0.793	0.54 (0.00 to 1110.43)	0.876
VEGFA	0.92 (0.08 to 10.92)	0.950	0.82 (0.00 to 194.61)	0.943
FOS	1.47 (0.19 to 11.35)	0.714	2.44 (0.14 to 43.02)	0.542
JUN	0.28 (0.02 to 4.47)	0.365	5.96 (0.04 to 984.91)	0.493
POU5F1	2.11 (0.27 to 16.55)	0.477	18.77 (0.89 to 394.56)	0.059 ^a

^a Statistically significant changes

^b Borderline statistical significance

than that in HPV-negative cervical cancer cell line C-33A [51]. This discrepancy could arise due to the influence of the surrounding tissues that are not present in cell line experiments.

In conclusion, our study confirms the impact of HPV infection on commonly studied HNSCC markers *MT2A*, *MMP9*, *FLT1*, *VEGFA*, and *POU5F* and also the different relevance of these markers in HPV-positive and HPV-negative HNSCC patients. In our previous studies [41, 43], we confirmed the importance of metallothioneins in HNSCC pathogenesis. In this study, we found a higher relevance of this marker in HPV-negative HNSCC patients. We assumed that treatment based on oxidative stress generating may be less effective in patients with high metallothionein expression. Nevertheless, further experiments are needed.

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Compliance with ethical standards

Conflicts of interest None

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4.7 Charakterizace buněk mikroprostředí

Nádorová tkáň je tvořena heterogenní populací nádorových buněk, cévami, buňkami imunitního systému, mezenchymálními buňkami a extracelulární matrix. Z hlediska vlivu na patogenezi nádorů a jejich progresi byly studovány ty populace nesoucí povrchové antigeny CD44 a CD90⁷⁷⁻⁷⁹. CD44-pozitivní buňky jsou typicky ty schopné iniciovat tumorigenezi⁸³⁻⁸⁶, migraci⁸⁰, a metastázování⁸⁷. Exprese CD90 je charakteristická mimo svoji úlohu v diferenciaci T-lymfocytů¹¹² také pro mezenchymální buňky – mj. fibroblasty^{91,92}. Obě tyto populace ovlivňují fenotyp nádoru; např. podíl CD90-pozitivních buněk ve spinocelulárních nádorech je v pozitivní korelaci s velikostí tohoto nádoru¹¹³.

S cílem popsat roli jednotlivých subpopulací CD44/CD90 v mikroprostředí byla vytvořena primokultura z dobře diferencovaného karcinomu dutiny ústní (muž, 57 let, nekuřák, HPV18+ T2N0M0 grade 1 tumor, dále označený jako primokultura 132P1, popis vzniku viz publikace Svobodova *et al.*¹⁰³ na str. 158). Mezi subpopulacemi byly prokázány následující rozdíly: CD44– nebyly schopny neomezeného růstu na misce (v souladu s očekáváním a literaturou), subpopulace CD44+/CD90– vykazovala nejvyšší míru buněčné migrace (str. 160), CD44+ buňky byly charakteristické vyšší mírou exprese MMP2 a EGFR (proteinová úroveň, str. 161), buňky CD90+ byly charakteristické vyšší mírou apoptózy/nekrózy a naopak CD44+ byly charakteristické vyšší mírou autofagie.

Z ko-kultivačních experimentů vyplývá, že „nádorové“ buňky CD44+/CD90– rostou rychleji, jsou-li přímo ko-kultivovány s „mezenchymálními“ buňkami CD44–/CD90+. Obdobně byla provedena také nepřímá ko-kultivace, při níž je možné dále provádět analýzy genové exprese. Z této analýzy bylo zjištěno, že jsou-li CD44+ „nádorové buňky“ ko-kultivovány s CD44+/CD90+ buňkami, dochází (u CD44+) zejména ke snížení markeru proliferace MKI67 a ke zvýšení poměru BAX/BCL2. Tedy – u nádorových buněk v důsledku působení buněk mezenchymálních dochází k ovlivnění růstu nádoru – populace CD44– mezenchymální buňky růst podporují proliferaci nádorových buněk a naopak CD44+ populace mezenchymálních buněk proliferaci nepodporuje a spouští apoptózu.

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Establishment of oral squamous cell carcinoma cell line and magnetic bead-based isolation and characterization of its CD90/CD44 subpopulations

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ABSTRACT

In this study, we describe the establishment of the human papillomavirus 18-positive, stage II, grade 1, T2N0M0 head and neck tumor primary cell line derived from oral squamous cell carcinoma of a non-smoking patient by using two different protocols. Furthermore, a preparation of subpopulations derived from this primary cell line according to the cluster of differentiation molecules CD44/CD90 status using magnetic bead-based separation and their characterization was performed. Impedance-based real-time cell analysis, enzyme-linked immunosorbant assay (ELISA), wound-healing assay, flow-cytometry, gene expression analysis, and MTT assay were used to characterize these four subpopulations (CD44⁺/CD90⁻, CD44⁻/CD90⁻, CD44⁺/CD90⁺, CD44⁻/CD90⁺). We optimised methods for establishment of primary cell lines derived from oral squamous cell carcinoma tissue samples and subsequent separation of mesenchymal (CD90⁺) and epithelial (CD90⁻) types of tumorous cells. Primary cell line prepared by using trypsin proteolysis was more viable than the one prepared by using collagenase. According to our results, CD90 separation is a necessary step in preparation of permanent tumor-tissue derived cell lines. Based on the wound-healing assay, CD44⁺ cells exhibited stronger migratory capacity than CD44⁻ subpopulations. CD44⁺ subpopulations had also significantly higher expression of *BIRC5* and *SOX2*, lower expression of *FLT1* and *IL6*, and higher levels of basal autophagy compared to CD44⁻ subpopulations. Furthermore, co-cultivation experiments revealed that CD44⁻/CD90⁺ cells supported growth of epithelial tumor cells (CD44⁺/CD90⁻). On the contrary, factors released by CD44⁺/CD90⁺ type of cells seem to have rather inhibiting effect. The most cisplatin-resistant subpopulation with the shortest doubling time was CD44⁻/CD90⁺, but this subpopulation had a low migratory capacity.

INTRODUCTION

Head and neck squamous cell carcinoma (HNSCC) is the sixth most frequent cancer worldwide. Regardless

of advances in diagnostic methods and therapy, survival of HNSCC remains almost unchanged with treatment resistance and metastases being the most important indicator of adverse outcome [1]. A recently disclosed

significant problem is the distinct cellular heterogeneity in the HNSCC tumor tissues that may contribute to formation of metastases or treatment resistance [2]. Recent data suggests that many potential cancer stem cell (CSC) markers are differentially expressed in different subpopulations of cells derived from a particular tumor [3]. One of the relevant characteristics of HNSCC subpopulations is the selective expression of surface receptors. In the context of HNSCC, CD44 and CD90 molecules are extensively discussed [4–6].

Surface glycoprotein CD44 is involved in cell-cell interactions, cell migration, and adhesiveness [7]. CD44 receptors are connected to the signalling cascade of EGFR and the PI3K/Akt and thus can significantly affect tumor progression [8]. The CD44⁺ phenotype is associated with head and neck, prostate, pancreatic, and breast cancer-initiator cells [9]. It has been also revealed that CD44 is important for metastasis as demonstrated on non-metastatic rat glioma cells. Those cells obtained metastatic ability when CD44 was over-expressed [10]. It was revealed that HNSCC cells positive for CD44 have the ability to produce tumors in immunocompromised mice. CD44⁺ cells are therefore often referred to as the CSCs [11, 12]. On the other hand, Lim et al. study puts the use of CD44 as a CSCs marker into question as they observed that both CD44⁺ and CD44⁻ cells extracted from squamospheres are able to regenerate these spheres [13]. Nevertheless, CD44⁺ cancer cell population in primary HNSCC is comprised of less than 10% of bulk tumor [14] and HNSCC-driven squamospheres

possessed enriched CD44⁺ cell population (53%) [13]. Furthermore, reduced CD44 expression resulted in a decreased proliferation and in an altered morphology of colonies suggesting a loss of stem cell character [15]. Moreover, CD44 expressed on cancer-associated fibroblasts (CAFs) seems to support the stemness and resistance of neighbouring malignant cells [16].

CD90 (also called THY1) was identified in the thymus as a T-cell differentiation and maturation marker [17], nevertheless human fibroblasts and cancer stromal cells also express abundant CD90 on their surface [18]. Immunohistochemical analysis showed overexpression of CD90 in cancer-associated stroma compared with non-cancer tissue stroma [17]. Furthermore, frequency of CD90-positive cells in HNSCC directly correlates with tumor volume [19]. Moreover, mesenchymal marker CD90 expressed on epithelial cells could be a marker of epithelial-mesenchymal transition (EMT) [4]. Lu et al. also suggested that CD90 could serve as an anchor used by carcinoma stem cells to attach tumor-associated monocytes and macrophages [20].

In this study, we describe the establishment of the head and neck tumor primary cell line derived from oral squamous cell carcinoma in a HPV18-positive, non-smoking patient (stage II, grade 1, T2N0M0) by using two different protocols. Furthermore, a preparation of subpopulations derived from this primary cell line according to the CD44/CD90 status using magnetic bead-based separation and their characterization was performed.

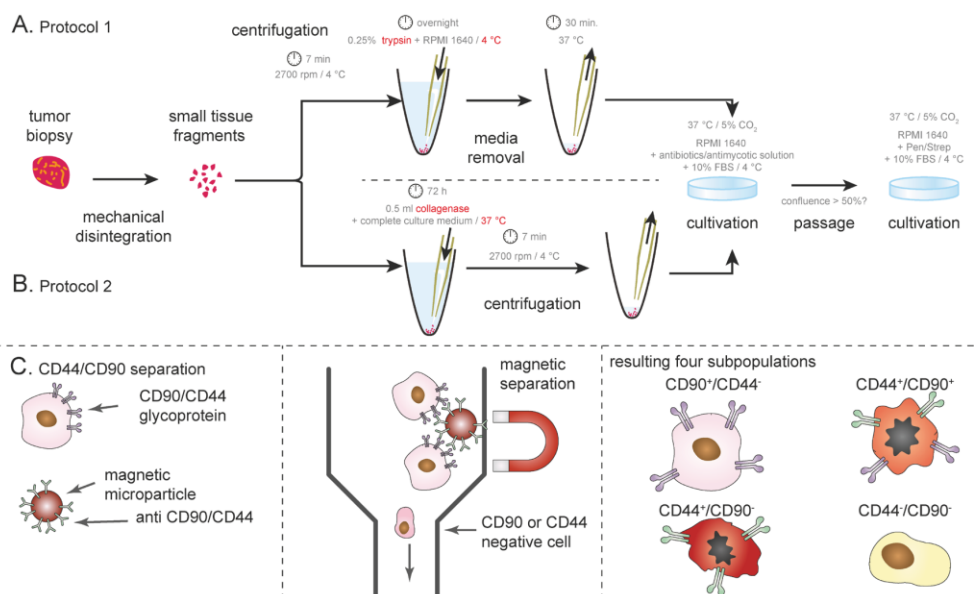


Figure 1: Schematic depiction of isolation protocol. (A) Protocol 1 using trypsin, **(B)** collagenase-based isolation protocol. **(C)** magnetic bead-based separation according CD44/CD90 surface antigens. Both positive- and negative-separation was utilized in order to obtain CD44/CD90 -positive and -negative cells. For details, see Material and Methods.

We also present an easy and cheap method for evaluation of the effects of factors released by distinct subpopulations in tumor tissue on cancer cell growth. In this study, we do not aspire to draw general principles of cancerogenesis and cellular interactions in tumor tissue. We would rather offer interesting methodics, procedures, and a new approach for cancer research.

RESULTS

Clinico-pathological characteristics of patient

The primary cell line was derived from tumor tissue taken from localized oral squamous cell carcinoma in a HPV⁺ (HPV16⁻; HPV18⁺), non-smoking male-patient (T2N0M0, stage II, grade I). The patient was at the age of 57 with BMI=22, had no previous history of tumors, no

diabetes mellitus, hypertension, heart ischemia, or chronic kidney disease. This patient has also not undergone stroke or myocardial infarction. This patient did not receive preoperative radiotherapy or chemotherapy. Patient achieved a complete remission after surgery.

Primary cell line establishment and characterization

The cell line was prepared by using two protocols; See the section Primary culture establishing and culture conditions. Primary cell line prepared according to the Protocol 2 was not as viable and the cell line designated 132P1 was successfully established according to the Protocol 1 (for details see “Primary culture establishing and culture conditions” section). The growth curve for the primary cell line 132P1 was as follows: lag phase

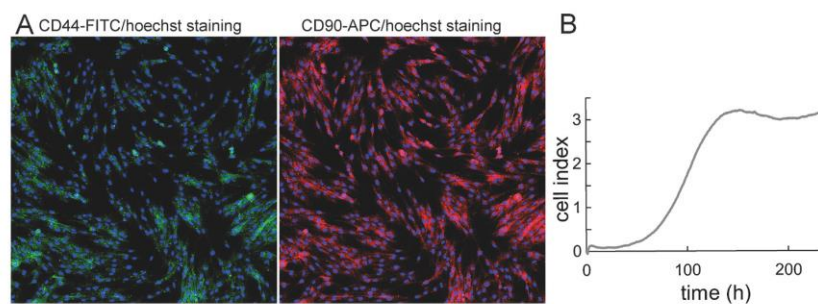


Figure 2: Fluorescence microscopy and growth curve of 132P1 unseparated primoculture. (A) CD44 and CD90 staining of the unseparated cell line 132P1 after passage 15. Fluorescent staining revealed prevailing representation of CD90⁺ cells; 10× magnification (B) Growth profile of unseparated cell line 132P1 using real-time cell analyser demonstrated as a dimensionless unit “cell index”.

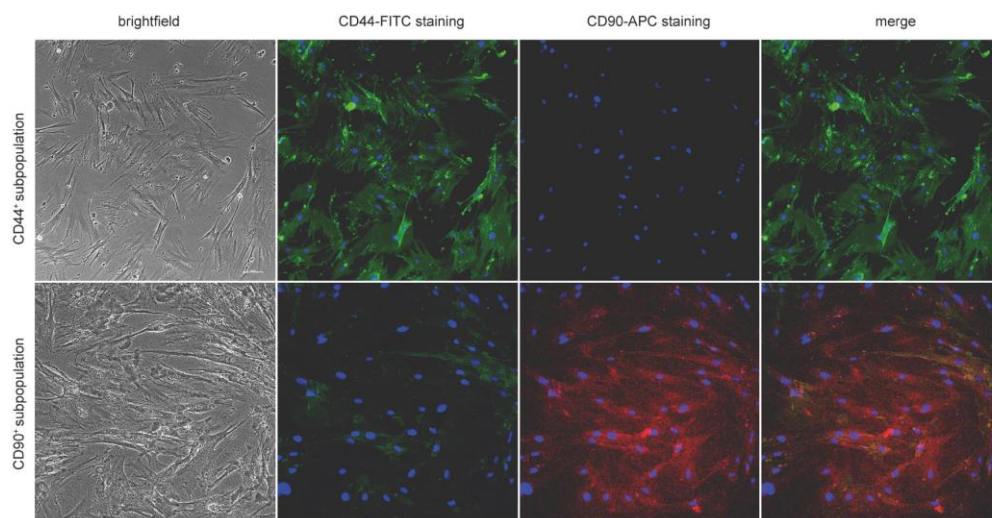


Figure 3: Fluorescent staining of CD44 and CD90 in separated subpopulations. Fluorescence confirms specificity of the sorting protocol; 20 × magnification.

87 h, exponential phase 25 h, and plateau phase after the 112th h. The doubling time was 22 h (Figure 2B). In our established primary cell line, other subpopulations were gradually overgrown by CD90⁺ cells (Figure 2A).

Morphology, growth and migratory characteristics of subpopulations

In the next step, the primary cell line 132P1 was separated according to the CD44 and CD90 status (CD44⁺/CD90⁻, CD44⁻/CD90⁻, CD44⁺/CD90⁺, CD44⁻/CD90⁺). Separation according to expression of CD44 and CD90 molecules was verified by CD44-FITC/CD90-APC fluorescent staining (see Figure 3). After separation of four subpopulations, all four types of subpopulations were able to grow long-term in cell culture (18 passages). Nevertheless, after 18th h and 20th h passage, respectively, CD44⁻/CD90⁻ and CD44⁺/CD90⁻ subpopulations have slowed down their growth extremely and nearly

stopped dividing, which was not observed in CD44⁺ subpopulations.

The growth curve for CD44⁻/CD90⁺ subpopulation was as follows: lag phase 80 h, exponential phase 37 h, and plateau phase after the 117th h. As soon as cells proceeded into the exponential growth during the exponential phase, the doubling time was measured, because the population is most uniform and cell viability is high in this phase. The doubling time was 26 h (Figure 4A). Morphology of this subpopulation is shown in the Figure 4B. The growth curve for CD44⁺/CD90⁺ subpopulation was as follows: lag phase 74 h, exponential phase 20 h, and plateau phase after the 94th hour. The doubling time was 30 h (Figure 4E). Morphology of this subpopulation is shown in the Figure 4F. The growth curve for CD44⁻/CD90⁻ subpopulation was as follows: lag phase 80 h, exponential phase 40 h, and plateau phase after the 120th hour. The doubling time was 34 h (Figure 4I). Morphology of this subpopulation is shown in the Figure 4J. The growth curve for CD44⁺/

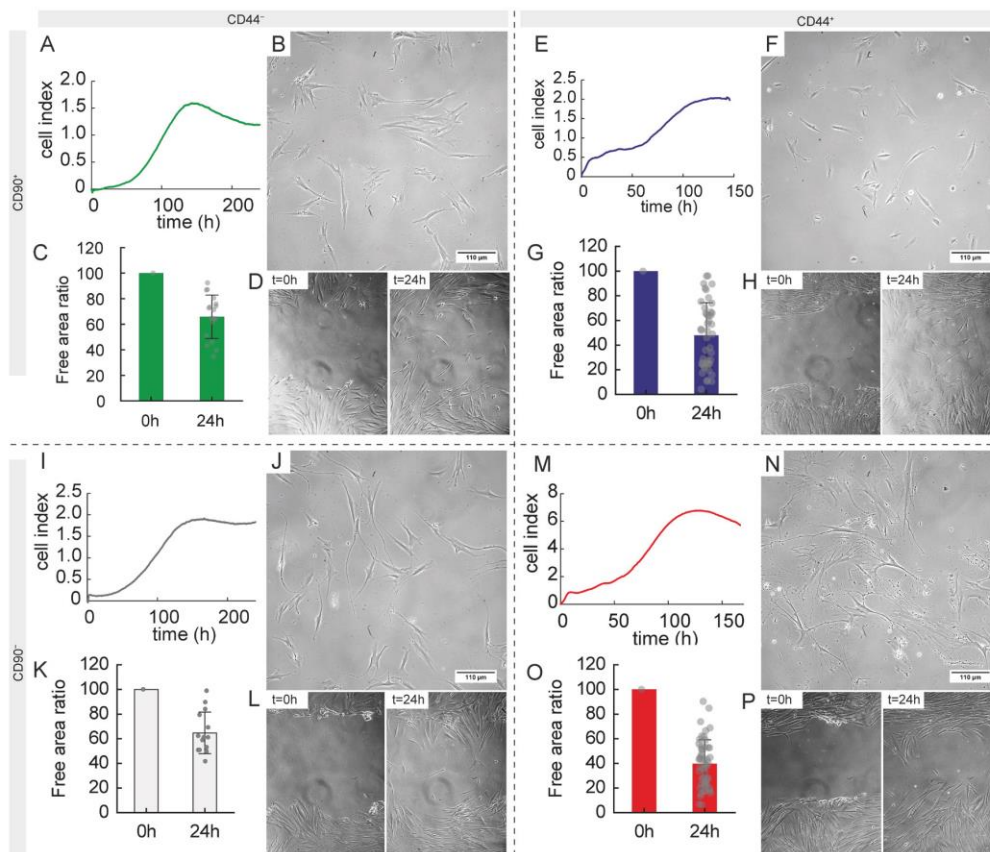


Figure 4: Morphological, growth and migratory characteristics of separated subpopulations according to CD44/CD90 features. (A, E, I, M) Growth profile using real-time cell analyser. (B, F, J, N) phase contrast microscopy of separated subpopulations, 20× magnification. (C, G, K, O) Wound healing (scratch) assay, percentage of free area (higher value mean slower migratory potential and thus lower invasiveness), (D, H, L, P) representative phase contrast images of wound healing assay used for analysis. 10× magnification A–D, CD44⁻/CD90⁺ subpopulation, E–H, CD44⁺/CD90⁺ subpopulation, I–L, CD44⁻/CD90⁻ subpopulation, M–P, CD44⁺/CD90⁻ subpopulation.

CD90⁻ subpopulation was as follows: lag phase 68 h, exponential phase 25 h, and plateau phase after the 92nd h. The doubling time was 30 h (Figure 4M). Morphology of this subpopulation is shown in the Figure 4N.

In the next step, migratory capacity of subpopulations was assessed using a wound healing assay after 24 h. Using univariate testing, CD44 status affected the migratory potential significantly, where cells

positive for this antigen demonstrated 1.5-fold higher migratory potential, $F(1, 123) = 23.46$; $p < 0.001$. CD90 status did not affected the migratory capacity significantly, $F(1, 123) = 1.10$, $p = 0.29$. Nevertheless, CD44⁺/CD90⁻ cells exhibited the strongest migratory capacity closely followed by CD44⁺/CD90⁺ subpopulation (1.2-fold lower, $p = 0.67$); see Figures 4C, 4D, 4G, 4H, 4K, 4L, 4O, 4P.

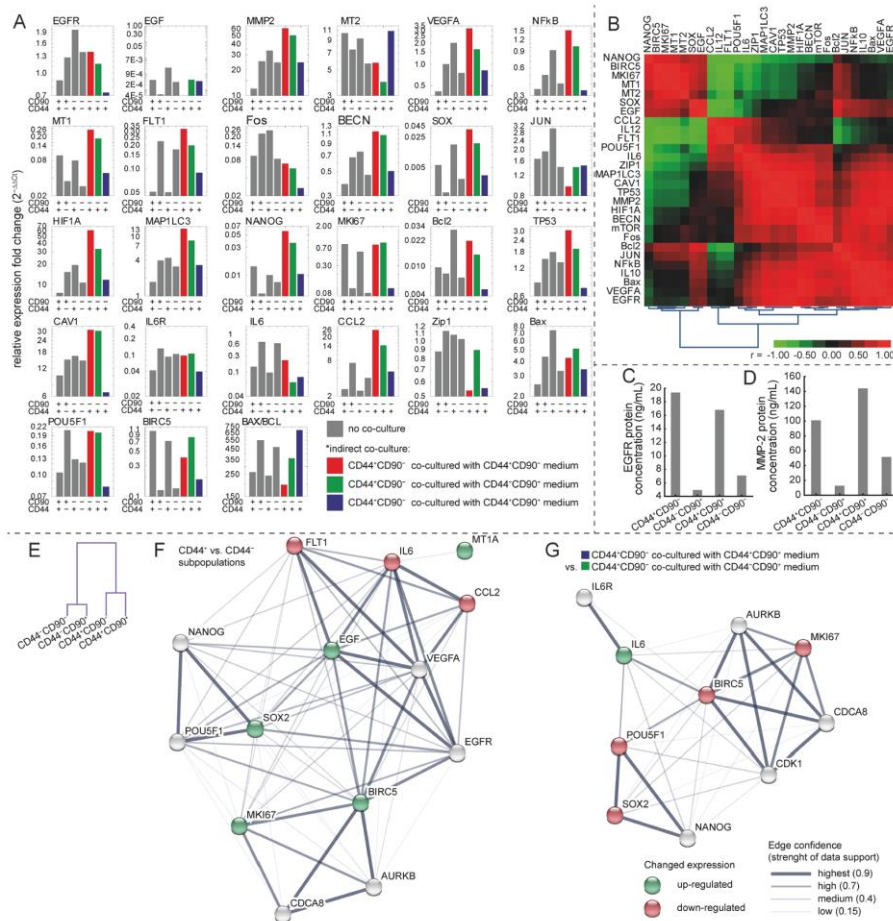


Figure 5: Gene expression in subpopulations and in co-culture experiment. (A) Gene expression using qRT-PCR. Gray bars indicate measurements without any type of co-culture, coloured bars indicate measurement of gene expression of CD44⁺/CD90⁻ subpopulation affected by medium from particular subpopulation (for details see Material and Methods section). (B) Clustered correlation heatmap based on a gene expression of subpopulations not exposed to co-culture experiment. (C) ELISA of EGFR in particular subpopulations. (D) ELISA of MMP-2 in particular subpopulations. (E) Hierarchical clustering of cases (subpopulations) based on the gene expression, no co-culture only. See the substantial effect of CD44 status on the gene expression. (F) Interactome network showing the genes, which expression differs significantly between CD44⁺ vs CD44⁻ subpopulations (green and red for up-, and down-regulation), analyzed using STRING software (version 10.0). Line thickness indicate strenght of data support. (G) Interactome network showing the genes, which expression differs significantly between CD44⁺CD90⁻ co-cultured with CD44⁺CD90⁺ medium and CD44⁺CD90⁻ co-cultured with CD44⁺CD90⁺ medium (groups coded blue and green at Figure 5A). For detailed statistical results, see Supplementary appendix 2, for Functional enrichments in the network of selected genes, see S3 appendix.

Characterization of basal cell death in subpopulations

Double-staining with fluorescein isothiocyanate (FITC)/propidium iodide (PI) was undertaken to determine basal levels of apoptosis and necrosis in particular subpopulations. First, non-stained cells (control) were analysed using flow-cytometry to set the annexin V /PI gating regions (Supplementary Appendix 1A). Consequently, non-treated cells from different subpopulations were analysed (Supplementary Appendix 1B). Four different phenotypes were distinguished: (a) annexin V-/PI- (lower left quadrant, Q3); (b) annexin V+/PI- (lower right quadrant, Q4, usually presumed as apoptotic); (c) annexin V-/PI+ (upper left quadrant, Q1); (d) annexin V+/PI+ (upper right quadrant, Q2, usually presumed as necrotic).

Average frequency of annexin V+/PI- cells was as follows: 14.9% in CD44⁻/CD90⁺ subpopulation; 23% in CD44⁺/CD90⁺ subpopulation; 12.3% in CD44⁺/CD90⁻ subpopulation; 12.6% in CD44⁻/CD90⁻ subpopulation. In sum, frequency of annexin V+/PI- cells in CD90⁺ subpopulations was lower than in CD90⁻ ones.

Furthermore, autophagosome formation in subpopulations was detected using the CYTO-ID Autophagy Detection Kit (Supplementary Appendix 1C). The levels of autophagy (CYTO-ID⁺ population) in subpopulations were as follows: 6.2% in CD44⁻/CD90⁺ subpopulation; 7.8% in CD44⁺/CD90⁺ subpopulation; 7.9% in CD44⁺/CD90⁻ subpopulation; 5.5% in CD44⁻/CD90⁻ subpopulation. In sum, the frequency of autophagy in CD44⁺ subpopulations was slightly higher than in CD44⁻ ones.

Expression patterns of HNSCC marker genes in CD44⁺/CD90⁺; CD44⁻/CD90⁺; CD44⁺/CD90⁻; CD44⁻/CD90⁻ subpopulations

This part of study is focused on the expression of genes potentially important for the development of cancer: 1) acquisition of autonomous proliferative signalling (*EGFR*, *EGF*); 2) proliferative activity of tumor cells (*MKI67*); 3) cell cycle and cell death modifications (*BCL2*, *BAX*, *FOS*, *JUN*, *TP53*, *BIRC5*, *MAP1LC3B*, *BECN1*, *NFKB1*, *MTOR*, *CAVI*); 4) angiogenesis (*VEGF/FLT1*); 5) metastatic potential (*MMP2*); 6) oxidative stress response

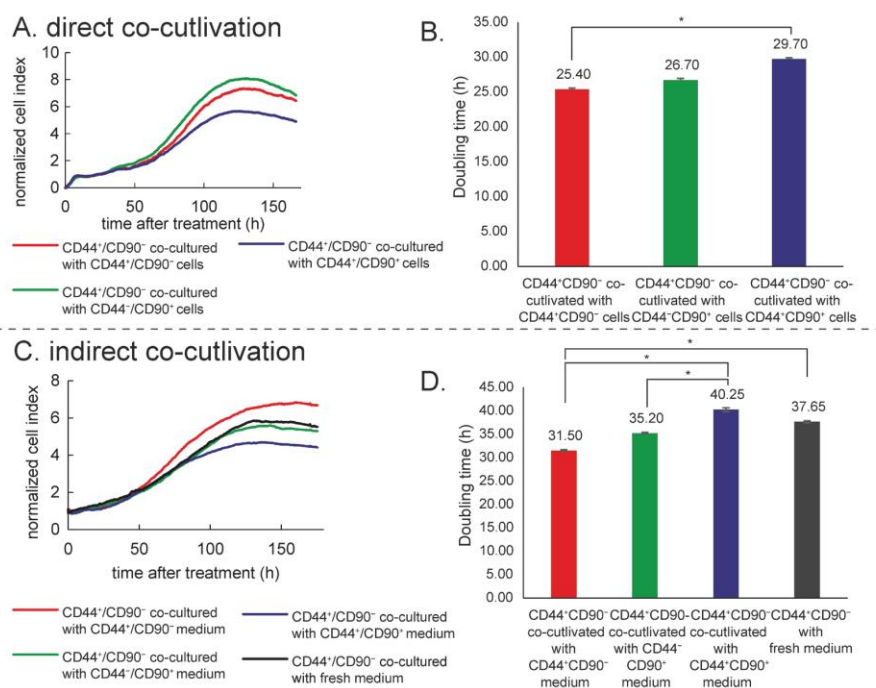


Figure 6: Co-culture experiment. (A) direct co-culture (measurement of the CD44⁺/CD90⁻ growth characteristics affected by cells in the insert without a direct contact). Real time cell analyser (RTCA). (B) doubling time of CD44⁺/CD90⁻ cells affected by particular subpopulation in a direct co-culture experiment. (C) indirect co-culture (measurement of the CD44⁺/CD90⁻ growth characteristics affected by a medium from particular subpopulations in a common RTCA setup). (D) doubling time of CD44⁺/CD90⁻ affected by indirect co-culture. Asterisk indicate significant difference at p < 0.05.

(*MT1A*, *MT2A*, *ZIP1*, *HIF1A*), pluripotency genes (*NANOG*, *SOX2*, *POU5F1*); and 7) immune response (*CCL2*, *IL6*, *IL6R*).

Abundantly expressed genes in all studied subpopulations were *MMP2*, *HIF1A*, *MT2A*, and *CAV1*. Subpopulation expressing CD44 had significantly higher expression of *BIRC5* and *SOX2* (CD44⁺/CD90⁺ vs. CD44⁻/CD90⁺ p=0.002 resp. p=0.017; CD44⁺/CD90⁻ vs. CD44⁻/CD90⁻ p=0.009 resp. p=0.006). No significant changes in expression between CD44⁺/CD90⁺ and CD44⁻/CD90⁻ or CD44⁺/CD90⁻ and CD44⁻/CD90⁺ were found. Thus, CD90 status did not affect the expression of studied genes significantly (see Supplementary Appendix 2). On the contrary, CD44⁺ subpopulations had lower expression of *FLT1* and *IL6* compared to CD44⁻ subpopulations (CD44⁺/CD90⁺ vs. CD44⁻/CD90⁺ p=0.03 resp. p=0.0001; CD44⁺/CD90⁻ vs. CD44⁻/CD90⁻ p=0.006 resp. 0.0001). No significant changes in expression between CD44⁺/CD90⁺ and CD44⁻/CD90⁻ were found (see the Figure 5A).

Based on the co-expression pattern of genes, hierarchical clustering revealed that there are two major clusters of subpopulations based on the CD44 status (Figure 5E). Nearness of CD44⁺ subpopulations in gene expression is clearly highlighted, while CD90 status did not affect the overall expression pattern substantially. Subsequently, interactome network showing the genes whose expression differs significantly between CD44⁺ vs CD44⁻ subpopulations was performed using STRING-DB software (Figure 5F). Based on this interactome network, it was revealed that biological processes relating to proliferation, migration, stemness, and angiogenesis were significantly affected by differentially expressed set of genes, (e.g GoMiner GO.0030335, GO.0050678, GO.0001525, GO.0022402, GO.0048646, GO.0016477). For the full list of significantly affected pathways and cellular components see S3 supplementary material.

According to the gene expression correlation analysis (see the Figure 5B), the proliferation marker *MKI67* was in no or even in a negative correlation with proliferative stimuli such as *IL6*, *VEGFA*, or *CCL2*. Additionally, the expression of receptors such as *EGFR*

and *FLT1* was not in a significant positive correlation with their ligands (*EGF*, *IL6*, and *VEGFA*). This implies that part of these factors could be exploited by the subpopulation in tumor mass which is not responsible for their production. For these reasons, experiments with co-cultivation were performed.

Furthermore, protein expression of MMP-2 and EGFR was assessed by ELISA (see figures 5C and 5D). According to ELISA, CD44⁺ subpopulations had higher levels of both tested proteins compared to CD44⁻. The lowest expression of both MMP-2 and EGFR was detected in CD44⁻CD90⁺ subpopulation of cells.

Direct co-culture

After 24 h of incubation, the inserts (containing CD44⁺/CD90⁻; CD44⁺/CD90⁺; or CD44⁻/CD90⁺ cells) were lowered into the E-plate (containing CD44⁺/CD90⁻ cells; anticipated as epithelial tumor cells). In this arrangement, two tested subpopulations were able to exchange factors released to medium and to influence each other's growth by paracrine signaling, but were not able to be in a direct contact. The grow response curve of the "lower" CD44⁺/CD90⁻ population was recorded. The rate of proliferation was monitored in real time using xCELLigence system (see Figure 6A). CD44⁻/CD90⁻ cells were not included in co-cultivation experiments, because they were not able to grow in inserts. Nevertheless, according to our expression and migration analysis, no significant differences between CD44⁺/CD90⁻ and CD44⁻/CD90⁺ subpopulations were observed.

Doubling time of CD44⁺/CD90⁻ cells co-cultivated with CD44⁺/CD90⁻ cells was significantly shorter than doubling time of CD44⁺/CD90⁻ cells co-cultivated with CD44⁺/CD90⁺ (25.40 ± 0.16 h vs. 29.70 ± 0.20 h, p = 0.028); see Figure 6B. Doubling time after co-cultivation with CD44⁻/CD90⁺ was shorter as well, but below the statistical significance (26.70 ± 0.22 h, p = 0.06). Doubling time of CD44⁺/CD90⁻ cells cultivated separately without any co-culture was 30 h; (see the section "Morphology, growth and migratory characteristics of subpopulations").

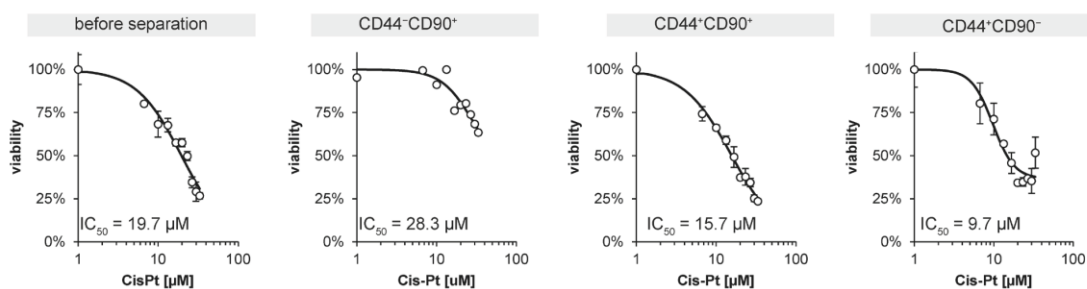


Figure 7: MTT assay of unsorted primoculture and subpopulations exposed to cisplatin treatment. IC_{50} means the half maximal inhibitory concentration.

Furthermore, the highest cell index was reached by co-cultivation of CD44⁺/CD90⁻ cells with CD44⁻/CD90⁺. On the contrary, when CD44⁺/CD90⁻ were cells co-cultivated with CD44⁻/CD90⁺, cell index (CI) value was the lowest. This dimensionless parameter is derived as a relative change in measured electrical impedance to represent cell status. When cells are not present or are not well-adhered to the electrodes, the CI is zero. Under the same physiological conditions, when more cells are attached to the electrodes, the CI values are larger. According to our results, factors released by mesenchymal type of cells, which do not express CD44 (CD44⁻/CD90⁺), are able to support a growth of CD44⁺/CD90⁻ type of cells (epithelial tumor cells). Moreover, CD44⁺/CD90⁻ cells also produce factors supporting their own growth.

Indirect co-culture with subpopulation-derived media

After 24 h-lasting cultivation of CD44⁺/CD90⁻ cells, medium was removed and replaced with a "foreign" media derived from another 24 h-lasting cultivation to display the effect of: a) CD44⁻/CD90⁺ derived medium, b) CD44⁺/CD90⁺ derived medium, c) CD44⁺/CD90⁻ derived medium from different Petri dish, and the effect of d) fresh control RPMI medium. In this arrangement, tested subpopulation was affected by factors released to the medium by other subpopulations (in this arrangement, factors released to medium were not a result of paracrine communication between different cell subtypes like in a direct co-culture experiment). An effect of exhausting of tested medium was also observed. The rate of proliferation was monitored in real time using xCELLigence system (see Figure 6C and 6D). Doubling times of CD44⁺/CD90⁻ cells were as follows: a) 35.20 ± 0.20 h with CD44⁻/CD90⁺-derived medium, b) 40.25 ± 0.41 h with CD44⁺/CD90⁺-derived medium, c) 31.5 ± 0.17 h with CD44⁺/CD90⁻-derived medium from different Petri dish, and d) 37.65 ± 0.22 h with fresh RPMI medium. Thus, cells supplemented with CD44⁺/CD90⁺ grew significantly slower compared to those supplemented with CD44⁺/CD90⁻ medium (p=0.004) and those supplemented with CD44⁻/CD90⁺-derived medium (p=0.030). Furthermore, the highest CI was reached by cultivation with CD44⁻/CD90⁺ derived medium. CI for CD44⁺/CD90⁺ derived medium, and fresh RPMI medium was almost identical. The lowest CI was observed by cultivation with CD44⁺/CD90⁺ derived medium. Inasmuch as tested medium was used for 24 h by other cell population, influence of released factors and nutrient exhausting could be presumed. Whereas CD44⁻/CD90⁺ derived medium from different Petri dish with CD44⁺/CD90⁻ population supported CD44⁺/CD90⁻ growth, CD44⁺/CD90⁺ derived medium rather inhibited CD44⁺/CD90⁻ cell growth in comparison with fresh medium.

Our qRT-PCR measurement has been performed after 72 h of CD44⁺/CD90⁻ cells' cultivation in "foreign" medium in medium derived from CD44⁺/CD90⁻ cells previously cultivated 24 h in a different Petri dish.

Exhausted medium derived from CD44⁺/CD90⁻ cells caused these particular changes in gene expression in comparison with CD44⁺/CD90⁻ cells in fresh medium (see Figure 5A): (a) Upregulation in *FLT1* (5.15 fold change, p=0.013), *NANOG* (4.58 fold change, p=0.034), *CCL2* (4.81 fold change, p = 0.039), and *IL6* (1.85 fold change, p=0.0001), (b) downregulation in *FOS* (0.25 fold change, p=0.024).

Medium derived from CD44⁺/CD90⁺ caused significant downregulation in expression of *SOX2* and *IL6* (p=0.01 resp. 0.0001) in CD44⁺/CD90⁻ cells (anticipated epithelial tumor cells) compared with medium derived from other CD44⁺/CD90⁻ cultivated separately (see Figure 5A).

Medium derived from CD44⁻/CD90⁺ caused significant downregulation in expression of *IL6* (p=0.0001) in CD44⁺/CD90⁻ cells (anticipated epithelial tumor cells) compared with medium derived from CD44⁺/CD90⁻ (see Figure 5A).

In conclusion, both tested media derived from mesenchymal subpopulations (CD44⁺/CD90⁺ and CD44⁻/CD90⁺) were able to decrease expression of *IL6* in CD44⁺/CD90⁻ cells in comparison with exhausted medium derived from CD44⁺/CD90⁻ cells. The effects on CD44⁺/CD90⁻ cells after a treatment with medium derived from CD44⁺/CD90⁺ cells differed significantly (was either higher or lower) when compared with other two types of partially exhausted subpopulation-derived media (compare Figure 5A). For instance, expression of proliferative marker *MKI67* triggered by tested medium was almost identical to cultivation with CD44⁺/CD90⁻ derived medium, CD44⁻/CD90⁺ derived medium, and fresh RPMI medium, which is in accordance with results obtained from xCELLigence system. CD44⁺/CD90⁺ derived medium rather inhibited *MKI67* expression in CD44⁺/CD90⁻ cells (medium derived from CD44⁺/CD90⁺ caused 0.23 fold change in *MKI67* expression in CD44⁺/CD90⁻ cells in comparison with medium derived from CD44⁻/CD90⁺; p=0.049). Furthermore, *SOX2* and *POU5F1* were also significantly down-regulated. Subsequently, interactome network showing the genes whose expression differs significantly between CD44⁺/CD90⁻ cells co-cultured with CD44⁺/CD90⁺ medium vs. CD44⁺/CD90⁻ co-cultured with CD44⁻/CD90⁺ medium was performed using STRING-DB software (Figure 5G). Based on this interactome network, it was revealed that biological processes relating to the cell division, cell cycle, and cellular response to interleukin-6 were significantly affected by differentially expressed set of genes, (e.g GoMiner GO.0051301, GO.0022402, GO.0071354). For the full list of significantly affected pathways and cellular components see S3 supplementary material.

Viability after cisplatin treatment

Viability of the primary cell line and the separate subpopulations after cisplatin treatment was tested by using MTT assay in order to assess the degree of resistance of individual subpopulation to this drug. The IC_{50} of primary cell line 132P1 for cisplatin was 19.7 μ M. The most resistant subpopulation was CD44⁺/CD90⁺ (IC_{50} = 28.3 μ M). Among CD44⁺ subpopulations, CD44⁺/CD90⁺ was more resistant to cisplatin than CD44⁺/CD90⁻ (IC_{50} value was 15.7 μ M and 9.7 μ M, respectively), see Figure 7.

DISCUSSION

In this study, we described the establishment of the HNSCC primary cell line designated 132P1 derived from well-differentiated, localized oral squamous cell carcinoma tissue obtained by the primary tumor biopsy and also establishment and characterization of CD44⁺/CD90⁻, CD44⁻/CD90⁻, CD44⁺/CD90⁺, and CD44⁻/CD90⁺ cell subpopulations derived from this primary cell line 132P1. Also of note, patient was a HPV18-positive, non-smoker, did not receive preoperative radiotherapy or chemotherapy and achieved a complete remission after surgery. Primary cell line prepared by using trypsin proteolysis was more viable than the one prepared by using collagenase (verified also in further primary cell lines preparations; unpublished results). In our established non-separated primary cell line, other subpopulations were gradually overgrown by mesenchymal (CD90⁺ cells) and therefore non-separated HNSCC tissue-derived cell lines are probably not a good model for testing of treatment response of epithelial cancer cells.

Magnetic bead-based separation was found to be suitable for separation of cells with distinct CD-features. All four types of subpopulations were able to grow long-term in cell culture. Nevertheless, after 20th passage, CD44⁻/CD90⁻ and CD44⁺/CD90⁺ subpopulations extremely slowed down their growth and nearly stopped dividing, which was not observed in CD44⁺ subpopulations. It implies that CD44⁺ cells could be more susceptible to immortalization and therefore more predisposed to establishment of permanent HNSCC cell lines. In accordance with our findings, Pries et al. found CD44 to be constitutively expressed on the surface of eight out of eight tested permanent HNSCC cell lines [21]. The doubling time of our primary cell line and derived subpopulations was rather fast (22–34 h) compared to other human tumor cell lines [22].

There is a growing number of studies that confirm a function of cancer-associated stroma in the carcinogenesis and tumor progression. Zhao et al. findings suggest that the phenotype of cells expressing high levels of CD90 is more tumor-promoting than the phenotype of cells expressing low CD90 [23]. According to our results, factors released by CD90⁺ type of cells, which do not express CD44, are

able to support growth of epithelial tumor cells (CD44⁺/CD90⁻). On the other hand, factors released by CD44⁺/CD90⁺ cells into medium seem to have rather inhibitory effect on epithelial tumor cell growth. Inasmuch the frequency of annexin V⁺/PI⁻ and also annexin V⁺/PI⁺ cells in CD44⁺/CD90⁺ subpopulation cultivated separately was higher than in other subpopulations, it is possible that CD44⁺/CD90⁺ cells are able to produce factors stimulating cell death in neighbouring cells. This observation is not in accordance with Spaeth et al. who showed that CD44 expression on tumor stromal precursors is necessary for their functionality within the tumor microenvironment as tumor supporting, angiogenesis inducing, activated fibroblasts [24]. Taking into account that our patient achieved a complete remission, we can speculate that the maintenance of tumor is a result of interactions in the cancer cell community and that tumor progression depends on the cooperation among all the members of tumor tissue, not just on the aggressiveness of its most abnormal subpopulation.

In our study, self-survival and self-renewal supporting factors such as *SOX2*, *NANOG*, and *BIRC5* [25–27] were highly expressed in CD44⁺/CD90⁺ subpopulation whereas CD44⁻/CD90⁺ subpopulation was an important producer of “public goods” such as chemokine (C-C motif) ligand 2 (*CCL2*) and interleukin-6 (*IL6*). *IL-6* has a possible function in the inducible generation of CSCs and their dynamic balance with non-stem cells [20, 28]. *CCL2* supports recruiting of inflammatory monocytes and facilitates metastasis [29]. Furthermore, *IL6* and *CCL2* are able to support tumor growth, EMT, stem cell migration, and finally treatment resistance [30–34]. Accordingly, we observed the highest cisplatin resistance in CD44⁺/CD90⁺ subpopulation. This subpopulation was more cisplatin-resistant than our primary unseparated cell line 132P1, which implies important role of this subpopulation in conferring of resistance in whole HNSCC tumor.

The most expressed gene in all subpopulations was matrix metalloproteinase 2 (*MMP2*). MMPs promote tumor progression and metastasis formation by degradation of the extracellular matrix. Dufour et al showed that transfection of COS-1 cells with *MMP-9*, *MMP-2*, or even with a proteolytically inactive mutant of *MMP-9* increases cell migration [35, 36]. ProMMP-2 was also shown to induce vascular endothelial growth factor (VEGF) expression via activation of PI3K/Akt/HIF-1 α , which may lead to an increased angiogenesis [37]. Other highly expressed genes in all types of subpopulations were *HIF1A*, *MT2A*, and *CAV1*. *HIF1A* is known to be involved in hypoxia-induced therapeutic resistance [38], *MT2A* that has been connected with oxidative stress [39], and *CAV1* that has been demonstrated to play an important role in EMT, glucose uptake, lactate accumulation, and ATP production [40–42].

CD44⁺ subpopulations were characteristic by higher gene expression of *BIRC5*, and *SOX2*, protein expression

of MMP2 and EGFR and by high basal autophagy. On the contrary, CD44⁺ subpopulations had lower expression of *FLT1* and *IL6*. BIRC alias survivin is involved *inter alia* in the ability to escape from accelerated senescence [43]. Of note, it is possible that senescence-escaped cells may transform into malignant cancer cells by the additional hits of several genes *in vivo*. Elevated levels of autophagy under basal conditions were observed in pancreatic cancer primary tumors and cell lines. Basal autophagy was shown to act as a cellular energy source and to prevent the accumulation of genotoxic levels of oxidative stress in pancreatic cancer cells. Conversely, inhibition of basal autophagy resulted in tumor regression [44]. Furthermore, the expression of *SOX2* gene connected with pluripotency [45] was abundant in both CD44⁺ subpopulations. It could mean that more than one subpopulation with stem cell abilities may exist in the same tumor (CD44⁺/CD90⁻ epithelial cancer stem cells [46] and CD44⁺/CD90⁺ tumor stem cells formed by EMT). Surprisingly, another studied pluripotency gene *POU5F* was abundantly expressed in the CD44⁺/CD90⁺ subpopulation. Mitchell et al. have recently showed that *POU5F*-induced plasticity in human fibroblasts results in a capability of responding to changes in the extracellular environment that could ultimately lead to the alteration of cell fate. This molecular state of human fibroblasts' plasticity was characterized by elevated levels of developmental genes, but not other genes involved in pluripotency [47]. We can speculate that *POU5F* expression in non-stem CD44⁺/CD90⁺ subpopulation demonstrates a "willingness" of this subpopulation to be manipulated by aggressive tumor cells.

Radiotherapy represents the standard treatment for head and neck squamous cell carcinoma (HNSCC) patients, often in combination with surgery. Nowadays, chemoradiotherapy has been incorporated in the treatment of advanced tumors. Cisplatin is the most common agent [11]. Based on the doubling time assessment, CD44⁺/CD90⁺ cells proliferated more rapidly than other cells and thus, they might be preferentially eliminated by cisplatin therapy. Nevertheless, the CD44⁺/CD90⁺ subpopulation showed the lowest sensitivity to cisplatin compared to other subpopulations and even compared to the primary cell line. This could be associated with a high expression of *FLT1* by this subpopulation (see Figure 5A) inasmuch as *FLT1* kinase was shown as a mediator of resistance in HNSCC [48]. Surprisingly, IC50 values for CD44⁺ subpopulations were lower than IC50 of primary cell line from which subpopulations were derived. This fact could point out on the important role of CD44-negative cells in the development of resistance; support of CD44⁺ by CD44⁻ may be one of the examples. In conclusion, we developed methodics for successful establishment of primary cell lines derived from oral squamous cell carcinoma tissue samples and methodics for removal of mesenchymal (CD90⁺) cells from this

cell line. Separation according to CD44 expression was also successful. CD90/44 status influenced growth rate, sensitivity to cisplatin, and migratory capacity of particular subpopulations derived from the same tumor. Furthermore, non-separated tumor-derived cell lines contained only mesenchymal types of cell after few passages and therefore they are not good model for testing of treatment response of epithelial cancer cells. According to our results, CD90 separation is a necessary step in preparation of permanent HNSCC-tissue derived cell lines. We also presented an indirect cocultivation as an easy and cheap method for evaluation of factors released by distinct subpopulations in tumor, inasmuch as results obtained from direct and indirect settings were comparable. Furthermore, some interesting characteristics of CD44-positive and CD44-negative subpopulations have arisen from our results. Nevertheless, these data are derived only from one HNSCC patient and need further confirmation.

MATERIALS AND METHODS

Primary culture establishment and culture conditions

This study was approved by St. Anne University Hospital Ethics Committee (Brno, Czech Republic) and informed consent was obtained from all subjects. All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards. Tissue for establishment of the primary cell line were obtained during surgery of patient with oral squamous cell carcinoma (T2N0M0, stage II, grade I) admitted to the Department of Otolaryngology-Head and Neck Surgery, St. Anne's University Hospital.

The first part of tumor tissue material obtained at surgery was placed into RNAlater (Ambion, USA), the second part into culture medium (RPMI 1640, Biochrom, USA) with an addition of 1% antibiotic-antimycotic solution (Santa Cruz Biotechnology, Texas), 10 µg/ml gentamycin sulphate (Santa Cruz Biotechnology, Texas) and 10 µg/ml ciprofloxacin (Santa Cruz Biotechnology, Texas) to prevent bacteria, fungi and yeast contamination. Within sterile environment and after rinsing the sample by 70% EtOH (Sigma-Aldrich, Germany), the most viable tissue was selected and any necrotic tissue was discarded. Leavings of EtOH were removed by PBS (Invitrogen, USA) washing. Tissue was mechanically dissociated into small pieces. For proteolysis were used: 1. Trypsin (PAA Laboratories GmbH, Austria) protocol 1 and 2. Collagenase (Sigma-Aldrich, Germany) (protocol 2).

Protocol 1

The small tissue fragments were added and stirred into sterile PBS (Invitrogen, USA) and centrifuged at 4°C, 2700 rpm for 7 min. The cell pellet was re-suspended into 0.25% trypsin in RPMI 1640 medium and left overnight at 4°C. Then medium was removed and tissue was incubated at 37°C for 30 minutes. The cell pellet was re-suspended in medium with an addition of antibiotic-antimycotic solution, gentamycin sulphate, ciprofloxacin and 10% FBS. Primary cell lines were cultivated at 37°C and 5% CO₂ in humidified atmosphere up to 50% confluence. As soon as cells were seen attaching to the flask surface, medium was changed. Tumor cells were no longer affected by the use of antibiotic-antimycotic solution, gentamycin sulphate, or ciprofloxacin that were added to the early culture. At this time, cells were grown only in Pen/Strep antibiotic solution (PAA Laboratories GmbH, Austria) in complete medium (penicillin 100 U ml⁻¹ and streptomycin 0.1 mg ml⁻¹; RPMI-1640 medium with 10% FBS (Biochrom, USA)). (Figure 1)

Protocol 2

The small tissue fragments were added and stirred into sterile PBS and centrifuged at 4°C, 2700 rpm for 7 min. The cell pellet was resuspended into 4.5 ml of complete culture medium with 0.5 ml (2000U/ml) of collagenase. The tissue was incubated at 37°C and 5% CO₂ for 72 hours and after that tissue was centrifuged at 4°C, 2700 rpm for 7 min. The cell pellet was resuspended in medium with an addition of antibiotic-antimycotic solution, gentamycin sulphate, ciprofloxacin and 10% FBS. Primary cell lines were cultivated at 37°C and 5% CO₂ in humidified atmosphere up to 50% confluence.

As soon as cells were seen attaching to the flask surface, medium was changed. Tumor cells were no longer affected by the use of antibiotic-antimycotic solution, gentamycin sulphate, or ciprofloxacin that were added to the early culture. At this time, cells were grown only in Pen/Strep antibiotic solution (PAA Laboratories GmbH, Austria) in complete medium (penicillin 100 U ml⁻¹ and streptomycin 0.1 mg ml⁻¹; RPMI-1640 medium with 10% FBS (Biochrom, USA)).(Figure 1)

Preparation of subpopulations according to the CD-molecules

Four cell subpopulations were derived from established HNSCC primary cell culture (CD44⁺/CD90⁺; CD44⁻/CD90⁺; CD44⁺/CD90⁻; CD44⁻/CD90⁻). For separation of subpopulation derived from primary cell line magnetic particles- MidiMACS™ Starting Kit (CD44 MicroBeads- human, FcR Blocking Reagent-human, LS Columns; Miltenyi Biotec, Germany) and MiniMACS™ Starting Kit (CD90 MicroBeads- human, MS Columns; Miltenyi Biotec, Germany) was used.

Dead cells were washed out with 0.5 M EDTA and viable cells were harvested by trypsin. Cell suspension was centrifuged at 300×g for 7 minutes at 4°C. Supernatant was aspirated completely. Cell pellet was resuspended in 1 ml of separating buffer (solution contained phosphate-buffered saline (PBS), pH 7.2, 0.5% bovine serum albumin (BSA), and 2 mM EDTA by diluting MACS® BSA Stock Solution 1:20 with autoMACS® Rinsing Solution). Buffer should be kept in cold (2–8°C). It is important to obtain a single-cell suspension before magnetic labeling. Therefore, cells were passed through 30 µm nylon mesh (Pre-Separation Filters (30 µm), Miltenyi Biotec, Germany) to remove cell clumps. Cell suspension was centrifuged at 300×g for 10 minutes at 4°C. Supernatant was aspirated. Cell pellet was resuspended in 60 µL of buffer and 20 µL of FcR Blocking Reagent per 10⁷ total cells. Then 20 µL of CD44 or CD90 MicroBeads was added. Solution was well mixed and incubated for 15 minutes in the dark in the refrigerator (2–8°C). Cells were washed by adding 1 mL of buffer per 10⁷ cells and centrifuged at 300×g for 10 minutes. Supernatant was aspirated completely. Cell pellet was resuspended in 500 µL of separating buffer. LS or MS column was placed in the magnetic field of a MACS Separator. Column was prepared by rinsing with 3 mL of buffer. Cell suspension was applied to the column. Flow-through containing unlabeled (CD44 or CD90 non-expressing) cells was collected. Column was washed with 3×3 mL of buffer. Collected unlabeled cells that pass through were combine with the flow-through from previous step and centrifuged at 300×g for 10 minutes. Supernatant was aspirated only 1 mL of buffer was left. These CD44 or CD90 negative cells were then cultivated in RPMI with 10% FBS and ATB. Then column was removed from the separator and was placed on a suitable collection tube. 5 mL of buffer were pipetted onto the column. the magnetically labeled cells were flush out by firmly pushing the plunger into the column. These CD44 or CD90 positive cells were then cultivated in RPMI with 10% FBS and ATB.

Cells that adhered to the flask were grown in complete medium (RPMI-1640 medium with 10% FBS, penicillin 100 U ml⁻¹ and streptomycin 0.1 mg ml⁻¹) until they reach 70% confluency, they were then passaged.

CD44 and CD90 fluorescent staining

At the logarithmic growth phase, cells were stained by CD90 and CD44 antibodies to detect their cell surface marker expression. The cells were washed three times with PBE (0.1 PBS containing 0.5% bovine serum albumin and 0.002 M EDTA (PAA Laboratories GmbH, Austria); pH 7.2) and stained with CD44-FITC/CD90-APC according to manufacturer's instructions (1:400, Miltenyi Biotec, Germany). Nuclei were co-stained with Hoechst 33258 (2 µM, Invitrogen, USA). Nikon Eclipse Ti-S equipped with appropriate set of filters (Japan) was used to visualize the cells. A bar represents 100 µm.

Cell number quantification

Total cell numbers were analysed using the Casy model TT system (Roche, Switzerland) and the following protocol: first, calibration was performed from viable and necrotic cells. For necrotic cells, 100 µl cell suspension and 800 µl Casy Blue solution was mixed and left for 10 min at room temperature. Subsequently, 9 ml Casy Tone was added. To prepare a viable cell standard, 100 µl of cell suspension was mixed with 10 ml of Casy Tone. All subsequent measurements were made in 100× diluted 100 µl cell suspension. Prior to each measurement, background was subtracted. All samples were measured in triplicates.

MTT viability assay

The MTT assay was used to determine cell viability. The suspension of cells in the growth medium was diluted to a density of 2000–10000 cells/column in 200 µl medium and transferred to wells 2–11 of standard microtiter plates. The medium (200 µl) was added to the first and to the last column (1 and 12). The plates were incubated for 2 days at 37°C to ensure the cell growth. The medium was removed from columns 2 through to 11. Columns 3–10 were filled with 200 µl of the medium containing different concentrations of cisplatin (0–34 µM). As a control, columns 2 and 11 were fed with the medium only. The plates were incubated for 48 h. After that, columns 1–11 were fed with 200 µl of the medium with 50 µl of MTT (5 mg/ml in PBS) and incubated for 4 h in a humidified atmosphere at 37°C, wrapped in the aluminium foil. After that, the medium was exchanged with 200 µl of 99.9% DMSO to dissolve MTT-formazan crystals. Then, 25 µl of glycine buffer was added to all wells with DMSO and the absorbance was recorded at 570 nm (VersaMax microplate reader, USA).

Real-time impedance based cell growth and proliferation

The impedance-based real-time cell analysis (RTCA) xCELLigence system was used according to the instructions of the supplier (Roche, Switzerland). Firstly, the optimal seeding concentration for proliferation and cytotoxic assay was determined. Optimal response was found for primary cell line (2,000 cells/well) and all subpopulations: CD44⁺/CD90⁻ (2,000 cells/well), CD44⁺/CD90⁺ (1,000 cells/well) CD44⁻/CD90⁻ (5,000 cells/well), CD44⁻/CD90⁺ (2,000 cells/well). After seeding a total number of cells in 200 µl of medium to each well in E-plate 16, the attachment and proliferation of the cells were monitored every 15 min. Duration of all experiments was 150 h. Results are expressed as relative impedance. In all of studied subpopulations doubling time was determined by using manufacturer's software.

Preparation of subpopulation-derived media

24 h prior to conducting experiments, CD44⁻/CD90⁺, CD44⁺/CD90⁺ and CD44⁺/CD90⁻ cells were trypsinized. 50,000 cells/ml of each cell line were cultured in 75 cm² flasks with medium. Media were removed from cultures after 24 h and used for the experiments.

Indirect co-culture

The rate of proliferation was monitored in real time using xCELLigence system (E-plate). 2000 CD44⁺/CD90⁻ cells per well were seeded in the E-plate 16. After 24 h of impedance reading, medium was removed from each well and replaced by media derived from foreign subpopulations (CD44⁻/CD90⁺, CD44⁺/CD90⁺) and by control medium derived from the same subpopulation (CD44⁺/CD90⁻) or by fresh RPMI medium. Impedance value was automatically monitored by the system for 200 h. This experiment was performed in duplicates.

Furthermore, determining of changes in gene expression after incubation with medium derived from foreign subpopulation was performed. The CD44⁺/CD90⁻ cells were cultivated at 37 °C and 5% CO₂ in humidified atmosphere up to 70% confluence. After 24 h medium was removed and replaced with foreign media: a) CD44⁻/CD90⁺, b) CD44⁺/CD90⁺ and c) by control medium (CD44⁺/CD90⁻). The experiment has been performed for 72 h than the relative expression of 15 genes related to HNSCC pathogenesis was determined by using qRT-PCR in all of these cases.

Direct co-culture

The rate of proliferation was monitored in real time using xCELLigence system (E-plate and E-insert). 2000 CD44⁺/CD90⁻ cells per well were seeded in the E-plate 16. CD44⁺/CD90⁻, CD44⁺/CD90⁺ and CD44⁻/CD90⁺ cells were seeded into insert at the density of 15000 cells per well. After 24 h of incubation at 37°C and 5% CO₂ the insert was lowered into the E-plate. Impedance value was automatically monitored by the system for 140 h. This experiment was performed in duplicates.

ELISA analysis

Levels of EGFR and MMP2 in homogenized cells were determined by commercial enzyme-linked immunosorbent assay (ELISA) kits (RayBiotech, USA) according to the manufacturer's instructions. The EGFR-ELISA is designed to detect human EGFR with a detection limit 4 pg/ml, a 10% intra-assay, and a 12% inter-assay variability. The EGFR antibodies were raised against the L25–S645 region of EGFR. The MMP2-ELISA is designed to detect human MMP2 with a detection limit 3500 pg/ml, a 10% intra-assay, and a 12% inter-assay variability.

RNA isolation and reverse transcription

TriPure Isolation Reagent (Roche, Basel, Switzerland) was used for RNA isolation. The isolated RNA was used for cDNA synthesis. RNA (1000 ng) was transcribed using transcriptor first strand cDNA synthesis kit (Roche, Switzerland), which was applied according to manufacturer's instructions. The cDNA (20 µl) prepared from the total RNA was diluted with RNase free water to 100 µl and the amount of 5 µl was directly analysed by using the LightCycler®480 II System (Roche, Basel, Switzerland).

Quantitative real-time polymerase chain reaction

qRT-PCR was performed using the TaqMan gene expression assays with the LightCycler®480 II System (Roche, Basel, Switzerland) and the amplified DNA was analysed by the comparative Ct method using β-actin as an endogenous control. The primer and probe sets for ACTB (assay ID: Hs99999903_m1), MT2A (Hs02379661_g1), MT1A (Hs00831826_s1), TP53 (Hs01034249_m1), BAX (Hs00180269_m1), BCL2 (Hs00608023_m1), VEGFA (Hs00900055_m1), FLT1 (Hs01052961_m1), MMP2 (Hs01548727_m1), FOS (Hs00170630_m1), JUN (Hs00277190_s1), MKI67 (Hs00606991_m1), EGF (Hs01099999_m1), EGFR (Hs01076078_m1), SOX2 (Hs01053049_s1), NFκB1 (Hs00765730_m1), BECN1 (Hs00186838_m1), HIF1A (Hs00153153_m1), MAP1LC3B (Hs00797944_s1), NANOG (Hs04260366_g1), CAV1 (Hs00971716_m1), MTOR (Hs00234508_m1), CCL2 (Hs00907239_m1), ZIP1 (also known as SLC39A1) (Hs00205358_m1), BIRC5 (Hs00153353_m1), IL6 (Hs00985639_m1), IL6R (Hs01075666_m1), and POU5F1 (Hs04260367_gH) were selected from TaqMan gene expression assays (Life Technologies, USA). qRT-PCR was performed under the following amplification conditions: total volume of 20 µl, initial incubation at 50°C/2 min followed by denaturation at 95°C/10 min, then 45 cycles at 95°C/15 sec and at 60°C/1 min. Gene expression experiments were performed in duplicates

Wound healing assay

After passage each cell line was resuspended and seeded into 24-well plate, the cell amount per well in 500 µl media was optimized to 60,000 for CD44⁺/CD90⁺, 50,000 for CD44⁺/CD90⁻, 40,000 for CD44⁻/CD90⁻ and 45,000 for CD44⁻/CD90⁺. After 48 h the cells were 100% confluent and the scratch into the cell monolayer was made. After gentle wash and change of media each well was photographed at time 0 and after 24 h at the very same spot. The photos were analysed according to instructions from the software creator [49]. The software computed the percent of open wound area. Each cell line was analysed in at least 24 repetitions.

Flow cytometric analysis of cell death

Double-staining with fluorescein isothiocyanate (FITC)/propidium iodide (PI) was undertaken using the Annexin V-FLUOS-staining kit (Roche Applied Science) according to the manufacturer's protocol in order to determine percentages of viable, apoptotic and necrotic cells. Briefly, the cells were harvested by repetitive pipetting and washed two times with PBS (centrifuged at 2000 rpm for 5 min), resuspended in 100 µl of Annexin-V-FLUOS labelling solution and incubated in the dark at 15–25°C (15 min.). Annexin V-FITC fluorescence was detected by flow cytometry (Partec GmbH, Münster, Germany); (FL1 filter for Annexin-V-FLUOS and FL3 filter for PI).

Flow cytometric detection of autophagosomes

Autophagosome formation in subpopulations were detected using the CYTO-ID Autophagy Detection Kit (Enzo, PA, USA) following the manufacturer's instruction. The CYTO-ID green fluorescent reagents specifically detect acid autophagic vacuoles formed during autophagy. Briefly, the cells were harvested by gentle repetitive pipetting, spun down and washed twice in RPMI 1640 with 5% fetal bovine serum (FBS), then were centrifuged at 2000 rpm for 5 min. The cells were resuspended in 500 µl of freshly diluted CYTO-ID staining reagent and incubated in the dark at 37°C for 30 min. CYTO-ID fluorescence of cells was immediately analysed by flow cytometry using the flow cytometry (Partec GmbH, Münster, Germany) (FL1 filter for CYTO-ID, SSC for cellular granularity). The percentage of cells with CYTO-ID staining was used to represent the formation of autophagosomes.

HPV detection

The 142 base-pair long sequence of conservative *L1* gene was amplified using GP5 and GP6 primers for non-specific identification of HPV-positive subjects. The PCR mixture from New England Biolabs (UK), contained PCR buffer (10 mM Tris HCl, pH 8.3, 50 mM KCl with 2.5 mM MgCl₂ included) 0.05 mM of each dNTP and 0.05 mM of GP5 (5'-TTTGTACTGTGGTAGATAC-3') and GP6 (5'-GAAAAATAAACTGTAAATCA-3') primers. The DNA amplification was carried out during 40 cycles that included the denaturation at 94°C for 30 s, annealing at 45°C for 30 s and the primer extension at 72°C for 30 s.

The HPV-positive specimens were further analysed with the HPV16 (FOR primer: 5'-CCCAGCTGTAATCATGCATGGAGA-3'; REV primer: 5'-GTGTGCCCATTAACAGGTCTTCCA-3') and HPV18 (FOR primer: 5'-CGACAGGAACGACTCCAACGA-3'; REV primer: 5'-GCTGGTAAATGTTGATGATTAAC-3') primers. The PCR amplicons reached length of 202 bp for HPV16

and 272 bp for HPV18. The DNA amplification was carried out during 40 cycles that included the denaturation at 94 °C for 30 s, the annealing at 58 °C for 30 s and the primer extension at 72 °C for 30 s. As internal quality control of the isolated DNA, β -actin gene (600 bp) was amplified (FOR primer: 5'CCTGAACCCTAAGGCCAACC3'; REV primer: 5'GCAATGCCTGGGTACATGGT3'). Each PCR product was analysed using electrophoresis on 1% agarose gels stained with ethidium bromide.

Statistical analysis

Pearson correlation and cluster analysis were performed to reveal associations between cases and variables. These analyses were performed on standardized data; the cluster analysis was performed using Ward's method. Data were analysed using factorial ANOVA following planned comparisons. All charts are depicted with means and standard deviations. P value < 0.05 was considered significant. Software Statistica (StatSoft, Tulsa, OK, USA) was used for analysis. The annotation analyses were performed using the GoMiner (<http://discover.nci.nih.gov/gominer/index.jsp>), interactome network was constructed using the STRING software (<http://string-db.org/>) using Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway database (<http://www.genome.jp/kegg/>), which provides gold standard sets of molecular pathways.

Author contributions

MS wrote manuscript, coordinated lab experiments, performed tissue culture experiments; MR wrote manuscript, performed gene expression analysis; JG analysed results, performed statistics; JB performed flow cytometry; MF performed MTT; MK performed real time cell growth experiments, cell migration analysis; HP performed ELISA.

ZHor. performed biopsies, work with patient; RK provided clinical feedback; PB performed microscopy; ZHeg. performed HPV detection; MM designed experiment.

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CONFLICTS OF INTEREST

The authors declare no conflicts of interest.

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4.8 Radiosenzitivita primokultur a buněčných linií HNSCC

Nádory hlavy a krku jsou v blízkosti k vitálním strukturám, užití radikálního chirurgického přístupu tak není vždy ideální. Nechirurgické postupy (chemo/radioterapie) jsou proto často preferovanými modalitami. Přibližně u 50 % nádorů ale dochází k rozvoji radiorezistence, kterou současnými diagnostickými prostředky není možné predikovat^{46,48} (blíže viz kapitola 1.1.3 Markery odpovědi na (radio)terapii).

Následující studie Falk *et al.* (str. 175) se zaměřuje na analýzu spojitostí mezi mírou reparace dvouřetězcových zlomů pomocí imunofluorescenční analýzy γ H2AX/53BP1 foků a rezistencí k radioterapii. Jaderné foky γ H2AX/53BP1 jsou obecně uznávaným markerem dvouřetězcových zlomů. Nejdříve byla provedena podrobná analýza indukce a oprav dvouřetězcových zlomů DNA u „referenčních“ buněčných linií: (1) nemaligních lidských kožních fibroblastů (NHDF) a u modelových HNSCC buněčných linií (2) Detroit 562 a (3) FaDu. FaDu je buněčná linie pocházející z hypofaryngálního karcinomu bez mutace PI3K, která by umocňovala radiorezistenci. Detroit 562 je buněčná linie odvozená z metastatického pleurálního výpotku karcinomu faryngu nesoucí aktivační mutaci PI3K, lze tedy očekávat radiorezistentnější fenotyp; radiosenzitivita těchto buněčných linií však dosud nebyla v literatuře popsána.

Na základě detekovaných foků a jejich rychlosti reparace jsme potvrdili, že linie Detroit může být využita jako model relativní radiorezistence a linie FaDu je oproti ní k radiaci senzitivnější (obr. na str. 179). Tato rezistence k ozáření byla potvrzena i vyšší schopností formovat kolonie, jak ukázáno u modelu metastatické buněčné linie Detroit 562 (colony-forming assay). Obě nádorové linie vykazovaly vyšší míru genetické nestability v porovnání s nenádorovou linií NHDF.

Stejným způsobem jsme analyzovali tři primární kultury odvozené z tkáně HNSCC pacientů. Na základě vzniku a oprav γ H2AX/53BP1 foků bylo potvrzeno že vyšší míra radiosenzitivity se vyskytuje u nádoru TNM T1 a T2, zatímco u T3 tumorů byla prokázána vyšší míra rezistence. Ta byla potvrzena také pomocí průtokové cytometrie (barvení annexinu V/7AAD). 91,5 % buněk T3 přežilo ozáření 2 Gy (γ -záření; 1Gy / min), str. 179.

Na základě těchto poznatků byl proveden screening u dalších 35 HNSCC pacientů. Foky byly zaznamenány ve třech nejdůležitějších časových intervalech po ozáření, ve kterých jsme rovněž měřili životaschopnost buněk čtyřmi různými přístupy (pozitivita buněk na trypanovou modř měřeno na CellCounteru, průtoková cytometrie s annexinem V/7-AAD, průtoková cytometrie s MUSE Cell count and viability kitem a pomocí colony-forming testu). Předběžné analýzy potvrzují shora popsané závěry, tj. vztah mezi účinnější opravou DSB definovanou jako nižší

počet buněk s vysokou hustotou γ H2AX/53BP1 foků (8 hodin po ozáření nebo později) a přežití buněk po ozáření. Vypozorovány byly také FaDu-podobné a Detroit-podobné skupiny HNSCC nádorů.

Obdobně jako v předchozí studii charakterizující CD44/CD90 fenotypy (str. 157) byla u těchto subpopulací popsána rozdílná citlivost, resp. rozdílná míra reparace po ozáření. Smíšené kultury CD90+ a CD90- kultivované dohromady vykazovaly nižší tvorbu a rychlejší opravy γ H2AX / 53BP1 foků, než bylo pozorováno u stejných dvou subpopulací kultivovaných odděleně (str. 179). Celkově tyto výsledky naznačují, že přinejmenším u některých nádorů by mohly být γ H2AX/53BP1 foky slibným markerem radiosenzitivity daného nádoru.

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γ H2AX/53BP1 foci as a potential pre-treatment marker of HNSCC tumors radiosensitivity – preliminary methodological study and discussion^{*}

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Abstract. In order to improve patients' post-treatment quality of life, a shift from surgery to non-surgical (chemo)radio-treatment is recognized in head and neck oncology. However, about half of HNSCC tumors are resistant to irradiation and an efficient marker of individual tumor radiosensitivity is still missing. We analyzed whether various parameters of DNA double strand break (DSB) repair determined *in vitro* can predict, prior to clinical treatment initiation, the radiosensitivity of tumors. We compared formation and decrease of γ H2AX/53BP1 foci in 48 h after irradiating tumor cell primocultures with 2 Gy of γ -rays. To better understand complex tumor behavior, three different cell type primocultures – CD90⁻, CD90⁺, and a mixed culture of these cells – were isolated from 1 clinically radioresistant, 2 radiosensitive, and 4 undetermined HPV-HNSCC tumors and followed separately. While DSB repair was delayed and the number of persisting DSBs increased in the radiosensitive tumors, the results for the radioresistant tumor were similar to cultured normal human skin fibroblasts. Hence, DSB repair kinetics/efficiency may correlate with clinical response to radiotherapy for a subset of HNSCC tumors but the size (and therefore practical relevance) of this subset remains to be determined. The same is true for contribution of different cell type primocultures to tumor radioresistance.

1 Introduction

Head and neck squamous cell cancer (HNSCC; shortened here as HN) are usually aggressive neoplasms with high recurrence rate and poor prognosis. Due to their proximity to vital structures, efficient radical surgery results in patients' mutilation with impaired quality of life. Non-surgical (chemo-radiotherapy) approaches are therefore preferred but bear the risk of radioresistance resulting in the tumor persistence or even progression after treatment, which cannot always be salvaged by surgery. Indeed, about 52% of HN tumors resist to irradiation and results of the salvage surgery are in principle incomparable to those of primary surgery, with protracted healing and risk of unrecognizable tumor growth in the irradiated terrain [1,2]. Oncologists thus permanently face to a serious dilemma of the optimal first-line therapy for a particular patient (reviewed in [3]).

Unfortunately, the radioresistance markers allowing tumor radiosensitivity estimation prior to therapy are still unknown. Their discovery is largely complicated by genetic and functional heterogeneity of tumors that seems to be particularly high in HN. Unlike some other cancer types, HN tumors can be considered neither radiosensitive nor radioresistant, since these tumors occupy both extremes of the radiosensitivity spectrum (reviewed e.g. in [4]). Though some genes have been repeatedly found to be mutated in HN, there are not common 'founder' mutations associated with these malignancies ([5] and citations therein) and their radiosensitivity.

The radiosensitivity/radioresistance markers might be logically associated with complex cell response to DNA damage. Most relevant in this sense is probably repair of DNA double strand breaks (DSBs) since DSBs represent the most serious lesions being extensively introduced into the DNA molecule by ionizing radiation and some kinds of chemotherapy [6]. However, also genetic or epigenetic defects affecting other processes [7–16] such as resistance to apoptosis [7], defects in cell cycle regulation [8], ability

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to divide with damaged genome [9] or competency to re-enter cell cycling from senescence [10] (reviewed in [11,12]) can significantly contribute to final cell radioresistance. Eventually, those mechanisms might even play a major role.

Hence, it would not be surprising to discover that the basis of radioresistance differs among individual tumors. This expectation then almost precludes usage of model systems, such as permanent cell lines or transgenic mice, to study HN tumor biology and behavior. Moreover, even single tumors are highly heterogeneous and dynamic systems. Still undetermined source of radioresistance heterogeneity thus also comes from characteristics and proportion of different cell types, their specific clones, and mutual interactions among all these cells [17–19].

In this study, by using immunofluorescence confocal microscopy for sensitively quantifying γ H2AX/53BP1 foci formation and decrease in post-irradiation (PI) time, we attempt to find out how individual HN tumors vary in DNA double-strand break (DSB) repair kinetics and efficiency, whether these characteristics correlate with tumor cells' radiosensitivity, and whether in vitro monitoring of DSB repair could be predictive of tumors' clinical response to radiotherapy. To address these questions and in a need to deeper explore biological determinants of HN tumors' radioresistance, we prepared from patients' tumors three different cell primocultures – the primoculture of epithelial tumor cells characterized by absence of CD90 surface antigen (CD90⁻ cells), the primoculture of remaining cells that were CD90 positive (CD90⁺ cells), and a mixed culture of both these cell types. CD90 cluster of definition is expressed in several cell types, including a fraction of fibroblasts; CD90⁺ cells used in our experiments thus contain a significant fraction of tumor-associated fibroblasts (TAFs) that are, in addition to CD90⁻ tumor cells, expected to influence tumors' biology and characteristics [17–19]. We describe here our first results comparing DSB repair between tumors for each specific cell primoculture and between the primocultures for each particular tumor.

2 Methods

2.1 HN tumor biopsy extraction

HN tumor biopsy extraction was performed in the Department of Otorhinolaryngology and Head and Neck Surgery, St. Anne's University Hospital and Faculty of Medicine, Masaryk University, Brno, Czech Republic. Patients were completely examined clinically and the tumor staging was determined using radiodiagnostic approaches (CT, MRI, PET). Only newly diagnosed patients with none previous therapeutic history and with HN squamous-cell carcinoma (HNSCC) confirmed histopathologically were included in the study, after signing the informed consent. Biopsy cell samples were obtained by endoscopy under local or total anesthesia.

2.2 Tumor cells primocultures

Tumor cells primocultures were prepared in the Department of Pathological Physiology, Faculty of Medicine, Masaryk University, Brno, Czech Republic. The tumor tissue material obtained at surgery (see Sect. 2.1) was placed into culture medium (RPMI 1640, Biochrom, USA) with an addition of 1% antibiotic-antimycotic solution (Santa Cruz Biotechnology, Texas), 10 $\mu\text{g ml}^{-1}$ gentamicin sulphate (Santa Cruz Biotechnology, Texas) and 10 $\mu\text{g ml}^{-1}$ ciprofloxacin (Santa Cruz Biotechnology, Texas) to prevent bacteria, fungi and yeast contamination. Within sterile environment and after rinsing the sample by 70% EtOH (Sigma-Aldrich, Germany), the most viable tissue was selected while any necrotic tissue was discarded. Leavings of EtOH were removed by PBS (Invitrogen, USA) washing. Tissue was mechanically dissociated into small pieces and Trypsin (PAA Laboratories GmbH, Austria for proteolysis were used) was used according to Protocol 1 (below) to separate the cells.

Protocol 1. The small tissue fragments were added and stirred into sterile PBS (Invitrogen, USA) and centrifuged at 4 °C, 2700 rpm for 7 min. The cell pellet was re-suspended into 0.25% trypsin in RPMI 1640 medium and left overnight at 4 °C. Then medium was removed and tissue was incubated at 37 °C for 30 min. The cell pellet was re-suspended into medium with an addition of antibiotic-antimycotic solution, gentamicin sulphate, ciprofloxacin and 10% FBS. Primary cell lines were cultivated at 37 °C and 5% CO₂ in humidified atmosphere up to 50% confluence. As soon as the cells were seen attaching to the flask surface, medium was changed. Tumor cells were no longer affected by the use of antibiotic-antimycotic solution, gentamicin sulphate, or ciprofloxacin that were added to the early culture. At this time, cells were grown only in Pen/Strep antibiotic solution (PAA Laboratories GmbH, Austria) in the complete medium (penicillin 100 U ml⁻¹ and streptomycin 0.1 mg ml⁻¹; RPMI-1640 medium with 10% FBS (Biochrom, USA)).

For separation of subpopulation derived from primary cell line magnetic particles-MiniMACSTM Starting Kit (CD90 MicroBeads-human, MS Columns; Miltenyi Biotec, Germany) was used. Cells that adhered to the flask were grown in complete medium (RPMI-1640 medium with 10% FBS, penicillin 100 U ml⁻¹ and streptomycin 0.1 mg ml⁻¹) until they reach 70% confluency; they were then passaged. For each tumor, we prepared separated primocultures for CD90⁻, CD90⁺, and their mixed co-culture serving to study possible interactions between the cell types. The whole procedure is described in Svobodova et al. (2017) (Oncotarget; DOI: 10.18632/oncotarget.19914).

2.3 Irradiation with γ -rays

The cells were irradiated at the Institute of Biophysics, Czech Academy of Sciences, Brno, Czech Republic. In our first experiments, presented here, we irradiated the cell lines with a single dose of 2 Gy ($D = 1 \text{ Gy/min}$) of γ -rays

Table 1. Tumors characteristics.

Patient	Sex	Age [y]	Tumor	Locality	Stage	Grade	Therapy	RT response	Current status, other characteristics
T1	m	60	SCC	OP	IV T4 N2b	G3	RT + BT	S	UT
T2	m	70	SCC	HL	T3 N1	G3	S + RT	S	BT: cetuximab REM (6 month) total laryngectomy + adjuv. RT
T3	m	66	SCC	L	II T2N0	G2	RT	R	UT tripl CA (mammary + renal + HN)
T4	m	77	SCC	OP	IV T4b N3	G2	None	?	†
T5	m	70	SCC	LHP	IV T3N1	G3	S + RT	?	REM (4 month) total laryngectomy + adjuv. RT
T6	f	90	SCC	OHP	IV T4N2	G3	None	?	†
T7	m							?	

Legend: m: male, f: female, OP: oropharynx, L: Larynx, HL: hypolarynx, LHP: laryngohypopharynx, OHP: orohypopharynx, RT: radiotherapy, S: surgery, BT: biological treatment, R: radioresistant (none/poor response), S: radiosensitive (good response), †: died, UT: under treatment, REM: remission.

(^{60}Co , Chisostat, Chirana, CR). Cells were irradiated in RPMI 1640 medium (37 °C, normal atmosphere) [20].

2.4 Evaluation of DNA double strand break (DSB) induction and repair in tumor cell primocultures

The evaluation of DSB induction and repair was performed at the Institute of Biophysics, Czech Academy of Sciences, Brno, Czech Republic. DSBs were quantified in different periods of time post-irradiation (5 min–48 h PI) by means of γH2AX and 53BP1 foci immunodetection combined with high-resolution 3D confocal microscopy. For more detailed description of visualization of γH2AX and 53BP1 in spatially (3D) fixed cells see [21].

2.5 3D high-resolution confocal microscopy

The microscopy of samples was performed at the Institute of Biophysics, Czech Academy of Sciences, Brno, Czech Republic. Leica DM RXA microscope [22] (equipped with DMSTC motorized stage, Piezzo z -movement, MicroMax CCD camera, CSU-10 confocal unit and 488, 562, and 714 nm laser diodes with AOTF) was used for acquiring detailed cell images (100 \times oil immersion Plan Fluotar lens, NA 1.3). The equipment was controlled by the Acqarium software developed in collaboration with Masaryk University [23]. Modern Leica SP5 microscopy system, equipped with white laser for multicolor microscopy, allowed “high-throughput” cell imaging [21]. Images were reconstructed and analysed in Acqarium (FI MU, Brno), LAS AF (Leica), Adobe Photoshop CS5 (Adobe), and ImageJ software. DSB repair foci were scored also manually by two experienced examiners. Though absolute numbers of foci were lower for software analyses, the trends for manual and software scoring were the same. SigmaPlot Scientific Software (SPSS, Systat Software, Inc.) was used for statistical evaluation of data.

3 Results

3.1 Patients/tumors characteristics

HNSCC tumor biopsies were taken from 5 patients' primary tumors after confirming SCC by conventional histopathology and signing the informed consent. Patients were completely examined clinically and basic tumor characteristics were determined (Tab. 1). Radiodiagnostic approaches (CT, MRI, PET) were employed to determine tumors' staging. Only patients with newly diagnosed HPV–HNSCC with none therapeutic history and recommended for non surgical treatment were included into the study; the purpose for this decision was to minimize unwanted biological/experimental variability and allow later comparison of results obtained in vitro to histopathological characteristics of tumors and their response to radiotherapy in vivo. From 7 tumors currently included into the study, 1 tumor (T3) was radioresistant, 2 tumors (T1 and T2) were radiosensitive, and the status of remaining tumors was unsure (patients died without treatment, etc.).

Regarding oncologic prognosis and quality of life, our collected therapeutic results from last 15 years show that the optimal treatment strategy for an individual patient can be still determined only with difficulty and low fidelity if it is only based on clinical data and/or tumors' response to chemotherapy [1,2]. Identification of marker(s) allowing radiosensitivity estimation prior to the therapy initiation therefore still remains of utmost importance.

3.2 Methodological strategy and results

3.2.1 Preparation and characterization of CD90[−] and CD90⁺ cell primocultures

In order to deeper comprehend the phenomenon of tumor radiosensitivity, we decided to compare DSB repair

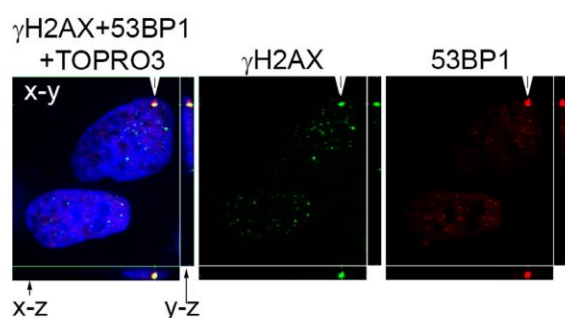


Fig. 1. Sensitive detection of DSBs by means of immunofluorescence confocal microscopy. Two DSB markers – γ H2AX (green) and 53BP1 (red) – are detected simultaneously in spatially (3D) fixed cells. A single DSB detected in one of displayed non-irradiated human normal skin fibroblasts (top one) is indicated by colocalizing green and red signals (white arrow). This approach currently brings the maximum sensitivity and precision in DSB quantification. A single confocal slice ($0.3 \mu\text{m}$ thick) through the cell nuclei in the plane of detected DSB is shown. Chromatin counterstaining by TOPRO3 (artificially blue); magnification 100 \times .

for two important cell types inhabiting the tumors – CD90^- and CD90^+ cells – and for their mixed culture ($\text{CD90}^- + \text{CD90}^+$). For this purpose, we developed and optimized [24] a protocol for immunoseparation of CD90^- and CD90^+ from tumors according to their CD90 cluster of definition (surface CD antigens [25]). Using the procedure described in Section 2, the two cell types were successfully separated and their primocultures prepared and basically characterized in terms of gene expression. Interestingly, expression of some important genes, such as EGFR, MMP2 and MT2 in CD90^+ cells isolated from tumors resembled more tumor CD90^- cells than normal CD90^+ fibroblasts (not shown).

3.2.2 Introduction of immunofluorescence confocal microscopy for DSB repair monitoring in CD90^- and CD90^+ cell primocultures

Immunofluorescence confocal microscopy of γ H2AX foci currently represents the most sensitive method to quantify DSBs [26]. This is demonstrated also by present results (Fig. 1) successfully revealing even occasional DSBs occurring in non-irradiated nonmalignant human skin fibroblasts (NHDF cells). Therefore, in this work, we tested applicability of γ H2AX foci immunodetection as a tool to predict tumors' radiosensitivity/radioresistance in vitro and to study complex response of tumor cells to irradiation. To further maximize sensitivity and fidelity of the method, we decided to analyze two independent DSB markers – γ H2AX and 53BP1 foci – in spatially (3D) fixed cells simultaneously (Fig. 1) [20,27]. Successful application of γ H2AX/53BP1 foci immunofluorescence confocal microscopy to monitor DSB repair kinetics and efficiency in tumor cell primocultures is illustrated in Figure 2.

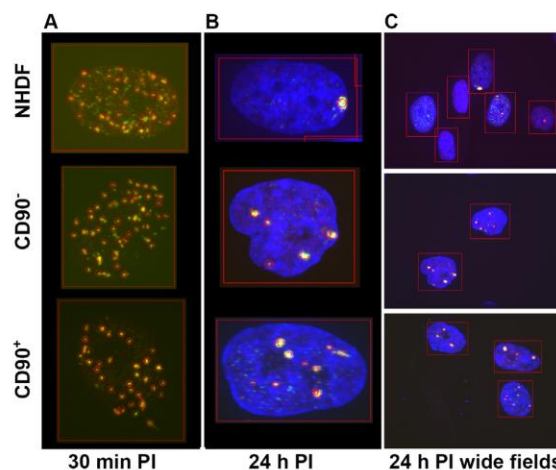


Fig. 2. γ H2AX (green) and 53BP1 (red) repair foci co-detected by immunofluorescence confocal microscopy in irradiated (2 Gy of γ -rays; $D = 1 \text{ Gy/min}$) normal human skin fibroblasts (NHDF) and CD90^- and CD90^+ cell primocultures obtained from the radioresistant tumor T3 (see Tab. 1 for the tumor characteristics). The cells were spatially (3D) fixed and immunostained at 30 min (A) and 24 h (B, C) post-irradiation, respectively. Panel C shows wide-field images with more cells. Maximum images composed of 30 confocal slices $0.3 \mu\text{m}$ wide are shown. In B and C, chromatin is counterstained with TOPRO3 (artificially blue) while this staining is absent in A in order to make γ H2AX (green) + 53BP1 (red) foci better visible. Foci detected by automatic software analyses are indicated by red circles (A, B, C). Magnification 100 \times .

3.3 DSB repair in CD90^- and CD90^+ cell primocultures

Figure 2 shows illustrative microscopy images for normal human skin fibroblasts (NHDF) and CD90^- and CD90^+ cells isolated from the radioresistant tumor T3 (see Tab. 1 for characteristics); mutually colocalizing γ H2AX and 53BP1 foci were immunodetected at 30 min and 24 h after irradiation of the cells with 2 Gy (1 Gy/min) of γ -rays. While formation of γ H2AX/53BP1 foci at 30 min PI (maximum DSB induction) was similar for all three cell types, an increased presence of foci at 24 h PI (persistence of unrepaired DSBs), relative to normal NHDF, could be seen in CD90^- and CD90^+ radioresistant tumor primocultures. Figure 3 then provides detailed quantitative comparisons on DSB repair kinetics and efficiency for normal cultured fibroblasts and the mixed $\text{CD90}^- + \text{CD90}^+$ primocultures isolated from radiosensitive (T1 and T2) and radioresistant (T3) tumors, respectively. Data for tumors T4–T7 are not displayed for their unknown clinical radiosensitivity and to allow better readability of the graphs. For all tumors, irrespective of their radiosensitivity status, the maximum DSB induction appeared at 30 min PI; however, the kinetics of γ H2AX/53BP1 foci disappearance varied with samples: While DSB repair kinetics for the radioresistant tumor T3 closely resembled that of normal cultured fibroblasts, a significant delay of this process

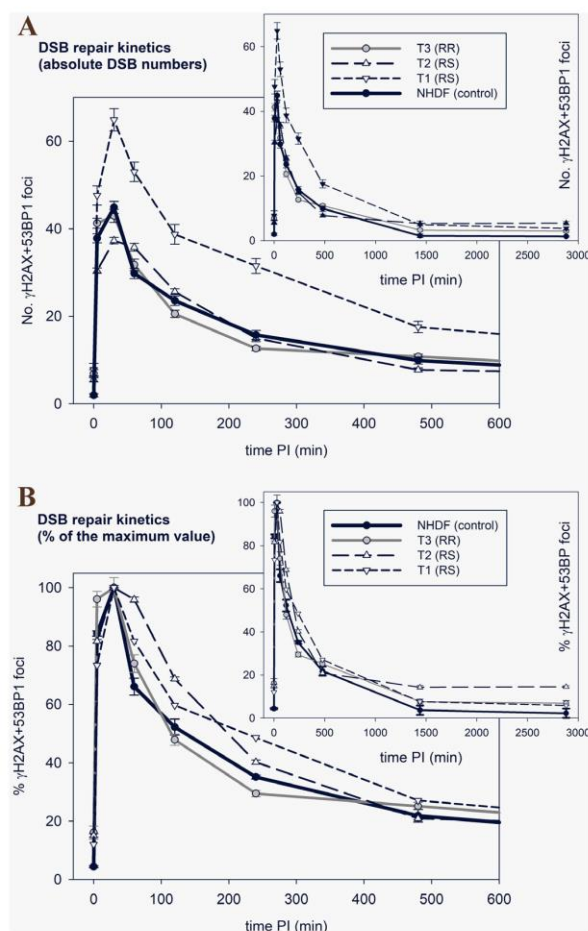


Fig. 3. γ H2AX/53BP1 foci formation, disappearance and persistence (DSB repair kinetics and efficiency) compared for normal human skin fibroblasts (NHDF) and CD90⁺ tumor cells primocultures derived from clinically radiosensitive (T1 and T2) and radioresistant (T3) tumors, respectively. See Table 1 for the tumors' characteristics. A: The mean numbers of γ H2AX/53BP1 foci per nucleus during the time post-irradiation with 2 Gy of γ -rays. The values obtained by immunofluorescence confocal microscopy in spatially (3D) fixed cells are shown. Error bars represent standard deviations (T1, T2 and T3) or standard errors of means (NHDF) calculated for two independent experiments. B: As A but the percentage of γ H2AX/53BP1 foci per nucleus is shown (100% correspond to the maximum value detected for all samples at 30 min PI).

appeared in the case of both radiosensitive tumors, T1 and T2 (Fig. 3). Nevertheless, the reason for this repair delay differed: In T1, the average number of DSBs per nucleus induced by 2 Gy of γ -rays dramatically exceeded that in NHDF fibroblasts and also all other tumors. This situation followed from an enormous size of T1 cells and extremely slowed the removal of DSBs (Fig. 3A), though the repair efficiency seemed to be unaffected (Fig. 3B). In contrast, both the average maximum number of γ H2AX/53BP1 foci

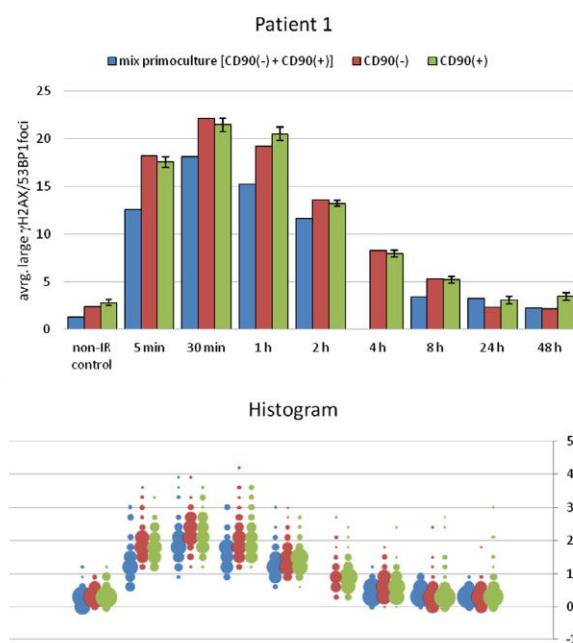


Fig. 4. DSB induction and repair compared for CD90⁻ and CD90⁺ cells and for their mixed culture (CD90⁻ + CD90⁺); all primocultures were derived from the radiosensitive tumor T1. Mean values of large γ H2AX/53BP1 foci per nucleus are shown with standard errors.

per nucleus and DSB repair efficiency were low in tumor T2 (Figs. 3A and 3B).

Moreover, the amount of γ H2AX/53BP1 foci detected in non-irradiated cells (genomic instability) and the amount of foci persisting in cells long periods of time post-irradiation (48 h PI; DSB repair inefficiency/DSB tolerance) were increased (as compared to NHDF) in all tumors but especially in both radiosensitive tumors (Fig. 3). For the radiosensitive tumors T1 and T2 the numbers of persisting foci exceeded the average value measured for NHDF significantly (Fig. 3).

Experiments with separated CD90⁻ and CD90⁺ cell primocultures provided the results that were mutually comparable and roughly resembled those described above for the mixed CD90⁻ + CD90⁺ cultures; however, in several cases, the mixed CD90⁻ + CD90⁺ cultures showed lower formation and faster disappearance of γ H2AX/53BP1 foci than we observed for both CD90⁻ and CD90⁺ cells. The results for tumor T1 are provided as an example in Figure 4.

4 Discussion

While only about 50% of HN tumors respond to irradiation [1,2], radiotherapy is being applied more or less "randomly" since any effective and reliable method to identify radiosensitive tumors has not been implemented yet. HN tumors to be treated by radiotherapy are therefore

only selected on the basis of their clinical parameters and/or response to neoadjuvant chemotherapy. However, our clinical experience from past 15 years (180–220 newly diagnosed patients/year) shows that the chemosensitivity of HN tumors (with the highest share of laryngeal, oropharyngeal and hypofaryngeal locality, mostly in advanced stage) does not sufficiently correlate with the radiosensitivity. Searching for a more direct and reliable HN tumor radiosensitivity/radioresistance marker thus still represents an important task of radiobiological research.

Though many other processes may also contribute, the repair of DSBs could be suspected of substantially determining the tumors' radiosensitivity/resistance. This is because DSBs represent the most lethal DNA damage being introduced into DNA of affected cells by radiotherapy and some kinds of chemotherapy. In this work, therefore, we tested this hypothesis for HN tumors and analyzed the possibility whether evaluation of DSB repair in tumor cell primocultures irradiated *in vitro* might open new way to predict an individual-specific response to radiotherapy [28].

We succeeded with introducing methods for preparing separate primocultures of different cell types from HN tumors and employed currently the most sensitive method – immunofluorescence confocal microscopy of γ H2AX/53BP1 repair foci [27] – to monitor DSB induction and repair in these primocultures prior to and upon irradiation. We have demonstrated already earlier that results of γ H2AX/53BP1 immunofluorescence microscopy well correlate with comet assay, the gold standard method in radiobiology to directly quantify DSBs [27]. Taking advantage of the described approach, we compared various parameters of DSB repair for CD90⁻, CD90⁺ and CD90⁻ + CD90⁺ tumor cell primocultures derived from 7 HN tumors, where 1 tumor was clinically radioresistant, 2 tumors were radiosensitive and remaining tumors were of unknown status. The reason for separating cells according to the CD90 surface antigen positivity is as follows: though there are some uncertainties in the literature about interpretation of CD90 expression, we can reasonably suppose that CD90⁻ cells in our study represent epithelial tumor cells while CD90⁺ cells contain a predominant fraction of tumor-associated fibroblasts (TAFs). Important roles of TAFs in influencing malignant potential and treatment response of tumors have repeatedly been described (e.g. [29] and citations therein). The mixed CD90⁻+CD90⁺ primoculture allowed us to reveal potential influence of CD90⁻ and CD90⁺ cell interactions on DSB repair.

Cultured human skin fibroblast (NHDF) provided us DSB repair characteristics for normal, non-malignant cells and served thus as the patient-independent DSB repair standard. Comparisons of results to normal mucosa cells extracted from histologically normal HN tissues (e.g. tonsils) of corresponding HN cancer patients were impossible for present tumors; however, we hope to obtain such data at least for some tumors in future. This information will allow for determining the patient-specific DSB repair efficiency-ratio between normal and tumor cells, while comparison with NHDF cell line may reveal potential functional (DSB repair) or even pre-malignant alter-

ations in histologically normal patients' tissues far distant from the tumor [30,31]. The results may contribute to our better understanding of tumor development as well as to better therapy planning in future.

We first analyzed presence of DSBs in non-irradiated NHDF cells and tumor primocultures (see Fig. 3). The results revealed that even non-irradiated CD90⁻, CD90⁺, and CD90⁻ + CD90⁺ primocultures derived from the radioresistant tumor T3 show markedly higher average numbers of γ H2AX/53BP1 foci per nucleus than NHDF cells. Since increased numbers of γ H2AX/53BP1 repair foci appeared in the majority of cells, we suppose this observation reveals increased genomic instability in all three tumor T3 primocultures, rather than their higher mitotic activity. Though, both these possibilities might be not mutually exclusive and further experiments are necessary to shed more light on this phenomenon. Interestingly, non-irradiated primocultures isolated from radiosensitive tumors T1 and T2 also obtained increased foci numbers, higher than NHDF cells. Hence, more tumors must be analyzed to find out whether the genomic instability may point more generally to a higher tumor radioresistance. One explanation could consist in the fact that tumors with a higher level of genetic heterogeneity contain increased frequencies of cell clones, where some of them might exhibit radioresistant features. However, the genomic instability may also point to the cell radiosensitivity arising due to a dysfunction of DSB repair.

Consequently, we followed DSB repair kinetics and efficiency in NHDF fibroblasts and tumor cell primocultures after irradiation with a single dose of 2 Gy (1 Gy/min) of γ -rays. While the maximum average numbers of DSBs per nucleus induced by irradiation in CD90⁻, CD90⁺, and CD90⁻ + CD90⁺ primocultures varied with tumors, DSB repair kinetics was quite similar to (or even faster than in) NHDF fibroblasts for the radioresistant tumor analyzed (see Fig. 3).

On the other hand, the primocultures derived from the radiosensitive tumors (T1 and T3, Tab. 1) showed, relative to NHDF, significantly delayed DSB repair with a substantial fraction of DSBs persisting in cells for a long period of time (48 h) after irradiation. Hence, though general validity of described results and their connection to tumors' radioresistance at molecular level remain to be determined, it seems that radiosensitive tumors may exhibit defects or deregulation of DSB repair and at the same time do not tolerate persistent DSBs. On the other hand, it seems that radioresistant tumors can tolerate unrepaired DSBs and benefit from them. Unrepaired DSBs may increase genetic "dynamics" of radioresistant tumors and their adaptability (not only) to radiation-induced stress.

The defects in repair processes might be of epigenetic origin since otherwise the same genetic mutations would appear both in CD90⁻ and CD90⁺ cells of the tumor. However, even the "mutation" alternative does not seem to be unprecedented. For instance, in colon cancer, we revealed genetic changes even in cells of histologically normal tissue taken 10 cm far from the tumor [30].

Finally, CD90⁻ and CD90⁺ primocultures did not show striking differences in DSB repair characteristics.

However, for several tumors, lower numbers of γ H2AX/53BP1 repair foci appeared upon irradiation in mixed CD90⁻ + CD90⁺ co-cultures than in CD90⁻ or CD90⁺ cells cultured separately (see Fig. 4); and existing foci also disappeared sooner from the former cells. In accordance with these results, we revealed that expression of some important genes in CD90⁺ primocultures resembles more the situation in CD90⁻ cells than in CD90⁺ cells taken from histologically normal HN tissue. More efficient repair in CD90⁻ + CD90⁺ co-cultures may point to interactions between CD90⁻ and CD90⁺ cells that stimulate DSB repair.

5 Conclusions

In this work, we described our first results on DSB repair kinetics and efficiency in CD90⁻ and CD90⁺ cell primocultures isolated from radiosensitive and radioresistant HNSCC tumors, respectively. We demonstrated our ability to prepare CD90⁻ and CD90⁺ primocultures and follow DSB repair in these cells in vitro with highest possible sensitivity and precision. While the only radioresistant tumor in our study showed characteristics of DSB repair similar to normal human skin fibroblasts, both radiosensitive tumors exerted genetic instability and markedly delayed repair kinetics and increased persistence of unrepaired DSBs. Nevertheless, whether these results are more generally valid and monitoring of DSB repair can be used to predict the response of individual tumors to radiotherapy must be further studied.

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Author contribution statement

Martin Falk and Michal Masarik designed the project, supervised the experiments and analyzed/interpreted the data. M.F. prepared the manuscript; Zuzana Horakova and Hana Binkova were responsible for clinical part of all works (patients diagnostics and follow up, tumors biopsies); Z.H. prepared clinical part of the manuscript; Jaromir Gumulec, Marketa Svobodova and Martina Raudenska isolated cells from patients tumors

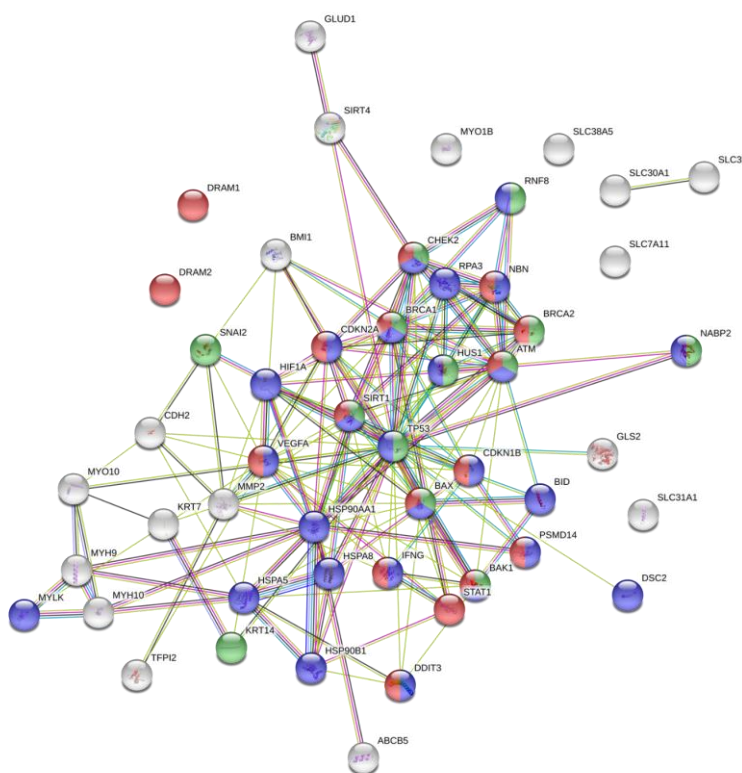
and prepared CD90⁻ and CD90⁺ cell primocultures; they also basically characterized gene expression of the primocultures; Olga Kopecna, Iva Falkova and Alena Bacikova participated in cell culturing, irradiated cells, and performed all immunofluorescence experiments on detection of γ H2AX/53BP1 repair foci; O.K. also contributed to image analyses; Daniel Depes acquired microscopy images of γ H2AX/53BP1 repair foci and analyzed the image data.

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4.9 Změny v transkriptomu po ozáření

S cílem popsat komplexní molekulárně-biologické změny v nádorových buňkách po ozáření byla provedena microarray umožňující studovat rozdíly v komplexní genové expresi 372 genů spojených s opravou DNA, buněčnou smrtí, metabolismem nádorových buněk, buněčnou motilitou, kmenovostí, epiteliálně-mezenchymální tranzicí a angiogenezí. Jako model HNSCC byly použity buněčné linie FaDu a Detroit 562. Jako model netransformovaných buněk byly použity nemaligní lidské kožní fibroblasty (NHDF). Linie FaDu je odvozena z primárního dlaždicobuněčného karcinomu faryngu a v této studii je použita jako model „primárního nádoru“, linie Detroit 562 je naproti tomu odvozena ze sekundárního ložiska faryngálního karcinomu v pleuře, je použita jako model „sekundárního ložiska“. Fibroblastová linie NHDF zde figuruje jako „nenádorová reference“; fibroblasty jsou po epiteliálních buňkách nejčastější komponentou mikroprostředí. Zkoumali jsme, zda buněčné linie FaDu a Detroit vykazují po ozáření odlišnosti v genové expresi ve srovnání s nenádorovými NHDF a jaké jsou rozdíly mezi buňkami FaDu a Detroit a jak tyto odlišnosti korelují s účinností oprav DSB.



Obrázek 1 Interaktom genů signifikantně up-regulovaných u buněčné linie FaDu barevně kódovaných dle biologického procesu, jehož se účastní. červená, apoptotické proces (17 genů), modrá, buněčná odpověď na stres (26 genů), zelená, odezva na ionizující záření (13 genů). Vytvořeno pomocí databáze StringDB.

Díky tomuto přístupu byly nalezeny expresní vzorce charakteristické pro jednotlivé linie (viz **Obrázek 1**). Tyto expresní vzorce budou později využity k charakterizaci tkání pacientů. Pro opravu DSB u nádorových buněk zřejmě hraje velkou roli ATM (ataxia-telangiectasia mutated) a BRCA2, jejichž role je menší u nenádorových NHDF. Radiosenzitivní buňky FaDu také udržují déle vysokou expresi genů souvisejících s opravami DNA. Na základě souboru up-regulovaných genů bylo zjištěno, že signální dráha GO0044767 (single-organism developmental process) je charakteristicky zapojena do reakce na záření v nádorových buňkách a nezapojuje se u NHDF. Pokud jde o charakteristické geny pro jednotlivé buněčné linie po ozáření, bylo prokázáno, že u NHDF jsou nejvíce up-regulované geny pro: Fibronectin 1; Proteasome (Prosome, Macropain) 26S Subunit, Non-ATPase, 14; Myosin IB; Interferon Gamma; Replication Protein A3; Intercellular Adhesion Molecule 1; Programmed Cell Death 1 Ligand 1; Growth Arrest And DNA Damage-Inducible Alpha; ER Membrane Protein Complex Subunit 7; UV Radiation Resistance Associated; Zinc Finger E-Box Binding Homeobox 2; Nejvíce utlumená byla pak exprese genů pro: Nuclear Factor, Erythroid 2 Like 2; BRCA2; KRAS, viz Tabulka 1.

U linie Detroit 562 byly nejvíce up-regulované geny pro: Platelet Derived Growth Factor Receptor Alpha; BCL2 Antagonist/Killer 1; Connective Tissue Growth Factor; Myosin IB. Nejvíce utlumená byla pak exprese genů: Cathepsin S/ indicative of poor prognosis; Cell Division Cycle 42; autophagy related 10.

U linie FaDu byly nejvíce up-regulované geny pro: Cadherin 2; Proteasome (Prosome, Macro-pain) 26S Subunit, Non-ATPase, 14; Checkpoint Kinase 2; Myosin IB; Tumor Protein P53; Heat Shock Protein Family A (Hsp70) Member 5; Ring Finger Protein 8; Fibronectin 1; Heat Shock Protein 90 Alpha Family Class A Member 1; Vascular Endothelial Growth Factor A. Nejvíce utlumená byla pak exprese genů: Cathepsin S; Split Hand/Foot Malformation; Cell Division Cycle 42; Nuclear Factor, Erythroid 2 Like 2; Fibroblast Growth Factor 2.

Tabulka 1: Odezva genové exprese na ionizující záření. Geny současně up/down-regulované u sledovaných buněk po ozáření.

<i>Geny</i>	<i>Odpověď na iradiaci (buněčná linie)</i>		
	<i>NHDF</i>	<i>FaDu</i>	<i>Detroit 562</i>
ATM		↑	↑
BAK1	↑	↑	↑
BAX	↑	↑	↑
BRCA1		↑	
BRCA2	↓	↑	↑
CHEK2		↑	
HUS1		↑	
KRT14		↑	
NABP2		↑	
RNF8		↑	
SIRT1		↑	↑
SNAI2	↑	↑	
TP53		↑	
NBN	↑	↑	↑

5 Diskuse výsledků

Výzkum nádorové biologie posledních dekad byl výrazně fokusován na popis vlastnosti nádorových buněk samotných; velkou měrou bylo toto umožněno vytvořením modelů buněčných linií. Po vytvoření linie karcinomu děložního čípku HeLa v padesátých letech¹¹⁴ se postupně objevily další buněčné modely, díky nimž byly popsány zásadní buněčné procesy a pochopená molekulární úroveň patologických stavů¹¹⁵. Přes významný přínos naráží tento přístup na významné limitace: Buněčné linie se dělí tisíce generací v buněčné kultuře, v prostředí radikálně odlišném oproti přirozenému. V průběhu času se buňky adaptovaly na „život“ v Petriho misce a ztratily mnohé vlastnosti svého původního fenotypu¹¹⁶. Nepřítomnost přirozeného extracelulárního stromatu s jeho fyzikálně-chemickými vlastnostmi není jediným důvodem. Zatímco buněčné linie jsou charakteristicky jednoho buněčného typu, nádory *in situ* obsahují také fibroblasty, myelofibroblasty, neuroendokrinní buňky, adipocyty, buňky imunitního systému, krevní a lymfatické cévy a tím tvoří mikroprostředí nádoru. To významně participuje na všech krocích tumorigeneze – iniciace, progresu i metastázování¹¹⁷ a pro jejich komplexní chápání není možná redukce na jeden buněčný typ.

Analogicky, častým přístupem při hledání „tkáňového nádorového markeru“ je prosté porovnání nádorové tkáně pacienta s tkání nádoru přilehlou, histologem označenou jako „normální“. Nicméně, v průběhu progresu nádoru ovlivňují nádorové buňky přilehlé stroma s cílem vytvořit tumor-podporující prostředí¹¹⁸. Typicky jsou ovlivněny fibroblasty – bylo prokázáno, že až 80 % morfologicky normálních fibroblastů karcinomu prsu vykazuje odlišný fenotyp „cancer-associated fibroblastů“ (CAF)¹¹⁹. Role CAF je obdobná myelofibroblastům při hojení ran – secernují prozánětlivé cytokiny, růstové faktory s cílem vytvořit permisivní prostředí¹¹⁸. Není proto možné s jistotou považovat nádoru přilehlou tkáň jako referenční, protože právě zde tvoří fibroblasty buněčnou majoritu, byť jsou morfologicky normální⁷².

V naší práci Raudenska *et al*⁹⁹ (dostupné na str. 103) byly proto použity dvě referenční tkáně – tkáň nádoru přilehlá a tkáň pacientů po tonzilektomii. Vzorky získané při tonzilektomii představují tkáň vystavenou chronickému zánětu, která však dosud neprošla procesem nádorové transformace. V práci bylo zjištěno, že transkriptom tkáně přiléhající k tumoru byl v mnoha ohledech „podobnější“ nádorové tkáni – expresí (tumor-podporujících) růstových faktorů EGF,

jejich receptorů, markerů proliferace MKI67, antioxidačně působících MT2A a apoptózu regulujících BAX genů. Lze teoreticky předpokládat, že tkáně přilehlé k nádoru mohou produkci EGF podporovat růst nádoru, což je v souladu s dalšími studii^{21,120-122}. Vyjma EGF a jiných genů dochází také k významně vyšším hladinám miRNA – miR-29c-3p a miR-375 v přilehlé tkáni oproti tkáni nádorové, jak bylo ukázáno ve studii Hudcova *et al.*¹⁰⁰ (dostupné na str. 123). Prostřednictvím epigenetické regulace tak mohou být ovlivněny buněčné pochody imunitní odpovědi (GO:0006955), buněčné adheze (GO:0007155), buněčné komunikace (GO:0007154) aj., jak bylo zjištěno za pomoci kombinace bioinformatických nástrojů TargetScan 7.2 (www.targetscan.org) a Gene ontology Enrichment analysis (Panter 13.1, geneontology.org).

Fakt, že transkriptom nádorové tkáně se liší oproti histologicky stejné tkáni pacienta bez nádoru má také diagnostický potenciál – charakteristická exprese výše uvedených genů jednak predikuje malignitu, jednak může být využita k ověření okrajů při chirurgickém odstranění nádoru. Nádory jsou dynamickým onemocněním a proto pouhé porovnání bioptických vzorků nádorový okraj/střed nádorů zdaleka nepopisuje heterogenitu spektra buněk. V průběhu tumorigeneze dochází k vývoji a selekci geneticky odlišných subpopulací nádorových buněk uvnitř nádorového ložiska, propůjčující nádorům schopnost rezistence¹²³. Pochopení a charakterizace této heterogenity je tak nezbytným krokem pro vývoj cílených léčebných strategií budoucí personalizované léčby. Pro heterogenitu spinocelulárních nádorů hlavy a krku hrají zásadní roli povrchové antigeny CD90 a CD44⁷⁷⁻⁷⁹. CD44-pozitivní buňky jsou typicky ty schopné iniciovat tumorigenezi⁸³⁻⁸⁶, migraci⁸⁰ a metastázování⁸⁷. Exprese CD90 je charakteristická pro mezenchymální buňky – tedy – v případě nádorové tkáně zejména CAFs^{91,92}. S cílem popsat roli jednotlivých subpopulací CD44/CD90 v mikroprostředí byla vytvořena primokultura z dobře diferencovaného karcinomu dutiny ústní (viz publikace Svobodova *et al.*¹⁰³ na str. 158). Tyto subpopulace (CD44+/CD90–, CD44+/CD90+, CD44–/CD90+ a CD44–/CD90–) se mezi sebou lišily v mnoha ohledech (viz kapitola 4.7); z perspektivy vlivu mikroprostředí je nejzásadnějším zjištěním, že subpopulace pozitivní na CD90 a negativní na CD44, tj. „s nádorem asociované fibroblasty“ podporovaly růst „nádorové“ populace CD44+/CD90–, zatímco populace negativní na CD44 (a pozitivní na CD90) nádorovou populaci v agresivním fenotypu spíše tlumily a způsobovaly prudký nárůst poměru BAX/BCL2. U těchto nádorových buněk ovlivněných buňkami CD90+/CD44+ (CAFs) bylo zjištěno, že na základě změn v genové expresi dochází k aktivaci genů souvisejících s embryonálními buněčnými procesy (GO:0001711 pathway analýza), což je ve shodě s literaturou. Chen *et al* souhlasně prokázali, že u buněk plicního nádoru dochází po ovlivnění fibroblasty asociovanými s nádorem ke zvýraznění „cancer stem cell“ fenotypu provázeného zvýšením exprese markeru NANOG¹²⁴. Vyjma uvedeného

bylo ve studii potvrzeno, že na základě genové exprese dochází právě u subpopulace CD44 k aktivaci buněčných kaskád souvisejících s regulací proliferace epiteliálních buněk (GO:0050678), buněčného cyklu (GO:0051726), regulací apoptózy (GO:0042981), migrací monocytů (GO:0071674), aj., zatímco u buněk CD90+ oproti buňkám negativním na tento marker nedochází ke změně v transkriptomu sledovaných genů, ani ke změnám v migračním potenciálu.

Závěry vycházející pouze z analýzy transkriptomu indikují, že právě CD44+ jsou populací zodpovědnou za agresivní fenotyp nádorů. Populace CD90, ač nevykazující zásadní rozdíly v aktivaci genů „hallmarks of cancer“ nicméně podporují CD44 populaci v růstu, jak demonstrováno migračními analýzami. Navíc, z perspektivy reakce subpopulací na ozáření bylo patrné, že kultury CD90+ a CD90– kultivované dohromady vykazovaly nižší míru tvorby dvouřetězcových zlomů DNA a rychlejší dynamiku jejich oprav (str. 179). Nabízí se spekulace, že míru agresivity tumoru není možné stanovit na základě (jednoduché analýzy) transkriptomu jedné subpopulace, ale takovými metodami, integrujícími poznatky o vzájemné interakci jednotlivých buněčných populací v mikroprostředí. Tyto metody jsou nicméně metodicky a časově náročné a v současné chvíli nejsou jednoduše redukovatelné do podoby přímočarého klinicky využitelného testu.

Mikroprostředí je typicky definováno svou složkou buněčnou a extracelulární – tedy složky organismu vlastní, byť nádorově změněné. Fenotyp mikroprostředí je nicméně definován také exogenními biologickými vlivy – pro HNSCC má nejzásadnější význam přítomnost HPV. I když jsou HPV⁺, tak HPV⁻ charakteristické ovlivněním zejména supresoru TP53, u obou typů nádorů dochází k tomuto jevu zcela odlišnými cestami; zatímco u HPV⁻ se toto děje typicky v důsledku genové nestability a z toho vyplývajících mutací TP53, HPV⁺ tumory toto činní prostřednictvím virových proteinů E6 a E7⁷¹. Ty inhibují (jinak intaktní) tumor supresor⁶⁷⁻⁷⁰. TP53 „dysfunkce“ je proto do jisté míry reverzibilní. I když je podstatná část TP53 inhibována virovými proteiny, při nárůstu buněčného stresu (např. v důsledku chemo/radioterapie) dochází ke zvýšení exprese TP53 a tato malá, dosud neinhibovaná frakce pravděpodobně umožní zástavu buněčného cyklu a naměřování buněk do buněčné smrti, jak popsáno ve studii Westra *et al.*⁷¹. Toto bylo nepřímě potvrzeno i v naší studii – celkové přežití HPV⁺ pacientů se liší v závislosti na míře tkáňové exprese pro-apoptotického BAX a TP53. Přežívání s vyšší expresí obou těchto genů je lepší, viz str. 152.

Popsání prognostických biomarkerů pro karcinomy hlavy a krku, resp. absence biomarkerů pro schopných predikovat efekt nechirurgické léčby je významnou limitací současné diagnostiky a terapie tohoto onemocnění. Protože existují tyto zásadní rozdíly v závislosti na HPV statusu,

je nutné očekávat, že potenciální panel biomarkerů bude odlišný pro HPV⁺ a HPV⁻ tumory, jak demonstrováno v naší studii. Na základě změn v tkáňové expresi (nižší exprese MMP9, MT2A, FLT1, VEGFA a FOU5F1 u HPV⁺ tumorů) bylo prokázáno zapojení do kaskád souvisejících se signalizací VEGF (GO:0038084) a „embryonální vývoj“, (GO:0009790). V tomto ohledu je zajímavá zejména asociace s MT2A. Tento protein, ač primárně zapojený do antioxidačních funkcí, do patogeneze choroby zasahuje pravděpodobně významnou měrou; v meta-analýze Gumulec *et al.* byla ověřena spojitost vysoké exprese s nepříznivou prognózou,⁴². Ostrakhovitch *et al* popsali, že MT je schopen zabránit vazbě TP53 na DNA a tím ovlivnit jeho tumor-supresorové působení¹²⁵.

Určitou strategií pro stanovení efektu léčby, např. právě diskutované radioterapie, může být analýza genové nestability a míry reparace dvouřetězcových zlomů DNA buněk získaných z biopsie a ozářených *in vitro*, jak popsáno v práci Falk *et al* dostupné na str. 175. Touto metodou bylo prokázáno, že míru radiorezistence nádorové tkáně je možné predikovat. Nádory o vyšším stádiu vykazovaly vyšší míru radiorezistence a přinejmenším u některých nádorů je touto metodou možné predikovat radiosenzitivitu a tím výhledově objektivněji volit léčbu konkrétnímu pacientovi. Současně je touto metodou možné odlišit zejména extrémně radiorezistentní, resp. extrémně radiosenzitivní tkáně; jeden ze tří pacientů, u nichž je na základě kliniky očekávána radiosenzitivita, vykazuje enormní míru tvorby dvouřetězcových zlomů. Naopak vysoká míra radiorezistence byla patrná u jednoho pacienta ze čtyř, projevující se velmi rychlou reparací vzniklých zlomů, rychlejší či stejně rychlou jako u buněčné linie fibroblastů.

Mikroprostředí, resp. jeho heterogenita, významně ovlivňuje progresi nádorů. Výše uvedené výsledky – heterogenní odpověď radioterapie, rozdílná schopnost subpopulací migrovat – jasně dokládají, že charakterizace mikroprostředí je nezbytná pro volbu optimálního terapeutického postupu konkrétního pacienta.

6 Závěr

Hlavním faktorem podílejícím se na letalitě nádorových onemocnění a selhání nechirurgické léčby je vysoká míra heterogenity buněčné nádorové populace¹²⁶. Tato heterogenita je u různých nádorů variabilní, nádory hlavy a krku jsou charakterizovány její poměrně vysokou měrou. Přes množství studií popisujících tuto variabilitu mikroprostředí a její klinickou relevanci stagnuje vývoj nových léčebných postupů a způsobů, kterak problematiku „dynamické evoluce“ uchopit klinickými studiemi. Téma heterogenity nádorového mikroprostředí je v práci studováno víceúrovňově – na úrovni analýzy séra, tkáňových vzorků, ale také na úrovni jednotlivých subpopulací buněk získaných z nádorových vzorků pacientů se spinocelulárními karcinomy. Zásadním problémem léčby spinocelulárních karcinomů je existence rezistence k nechirurgickým terapeutickým postupům, kterou není možné predikovat před zahájením léčby. Klinicky využitelné prognostické biomarkery podobné PSA pro nádor prostaty, či estrogenovému receptoru či HER2 pro nádor prsu^{9,10} pro nádory hlavy a krku neexistují. „Biomarkery“ jsou tradičně vnímány jako produkt nádorových buněk. Díky komplexnosti mikroprostředí dochází v důsledku přítomnosti nádoru k ovlivnění jinak histologicky normální „k nádoru přilehlé“ tkáně a tato tkáň poté mění svůj transkriptom. Ty nejenže jsou schopny podporovat růst vlastní nádorové tkáně, jsou také schopny na základě spektra transkriptomu nádorovou tkáň predikovat. Zásadní poznatky byly získány studiem interakce subpopulací jednotlivých buněk charakteristických přítomností povrchových antigenů CD44 a CD90. Nejenže bylo zjištěno, že populace CD44 je významně zapojena do patogeneze prostřednictvím své účasti v molekulárně-biologických procesech de-diferenciace, proliferace, apoptózy, bylo také pozorováno, že populace „nenádorová“ CD90, jinak nevykazující „fenotyp agresivního nádoru“, zásadně podporuje růst vlastní nádorové populace a propůjčuje rezistenci k radioterapii jiným subpopulacím. Populace buněk s povrchovým antigenem CD90, tedy populace s nádorem asociovaných fibroblastů je významným podpůrným článkem nádorového mikroprostředí. Spinocelulární nádory hlavy a krku, podobně jako většina solidních tumorů, nejsou monoklonální neoplázií. Přístup k vývoji nových diagnostických a (zejména pak) léčebných strategií musí tuto heterogenitu mikroprostředí reflektovat, protože právě toto je jedna z cest – možná cesta zásadní – pro personalizovanou medicínu schopnou radikálního zlepšení prognózy pacientů s pokročilými nádory.

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