

Universitätsmedizin Rostock

Institut für Pathologie

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**Neue diagnostische, prognostische und prädiktive
Untersuchungen im nicht - kutanen
Plattenepithelkarzinom des Kopf - Hals - Bereichs**



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1 Einleitung

Zur besseren Lesbarkeit wird in dieser Schrift das generische Maskulinum verwendet. Die in dieser Arbeit verwendeten Personalbezeichnungen beziehen sich – sofern nicht anders kenntlich gemacht – auf alle Geschlechter.

Das nicht - kutane Plattenepithelkarzinom des Kopf - Hals - Bereichs (*Head and Neck Squamous Cell Carcinoma, HNSCC*) ist die sechsthäufigste Krebserkrankung weltweit und betrifft die Schleimhäute der Mundhöhle, des Rachens und Kehlkopfes¹. Die weltweit höchste Inzidenz des oralen Plattenepithelkarzinoms (OSCC) findet sich in Südasien, wo das Kauen von Betelnüssen die Tumorgenese begünstigt (Abb. 1). Klassische Risikofaktoren für die Entwicklung eines HNSCC in Deutschland sind Nikotin - und Alkoholabusus sowie eine persistierende Infektion der Mund - und Rachenschleimhaut mit Hochrisiko - Typen (HR) humaner Papillomviren (HPV)^{2,3}. Der Anteil HPV - assoziierter HNSCC hat in den vergangenen Jahren weltweit zugenommen (Abb. 2)^{4,5}.

Die Bestimmung des HPV - Status hat große Relevanz, da die Prognose HPV - assoziierter HNSCC des Mundrachens als günstiger gilt^{2,3,6}. Dies beruht auf einer andersartigen Tumorbilogie mit verbessertem Ansprechen der Tumorzellen auf Radio - beziehungsweise Radiochemotherapie⁷.

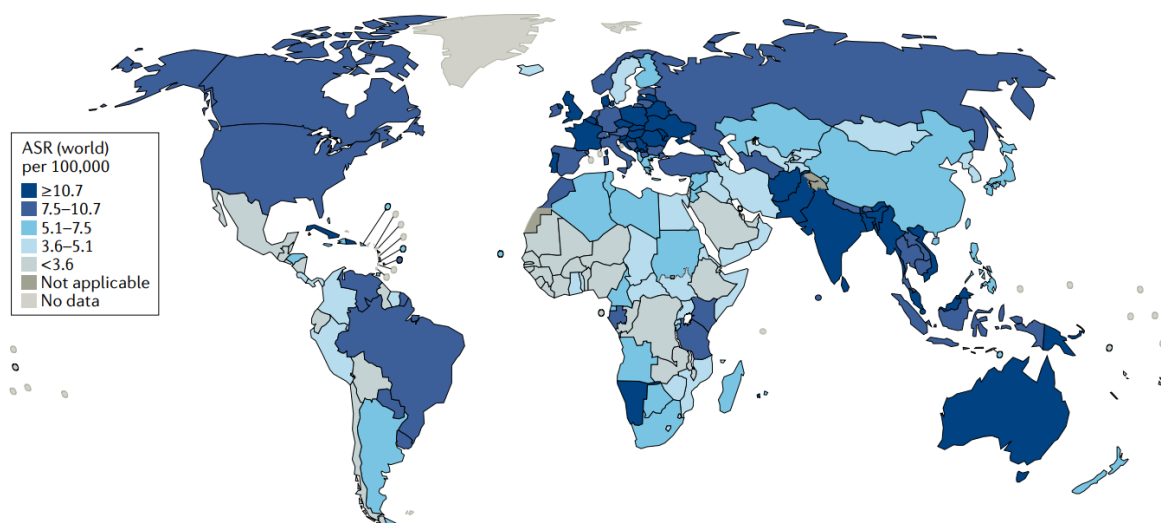


Abb. 1 | Globale Inzidenz nicht - kutaner Plattenepithelkarzinom des Kopf - Hals - Bereichs (HNSCC). Die geschätzten altersstandardisierten Raten (ASRs) der Inzidenz von Plattenepithelkarzinomen aller HNSCC weltweit sind für Männer und Frauen zusammen dargestellt. Quelle: ¹

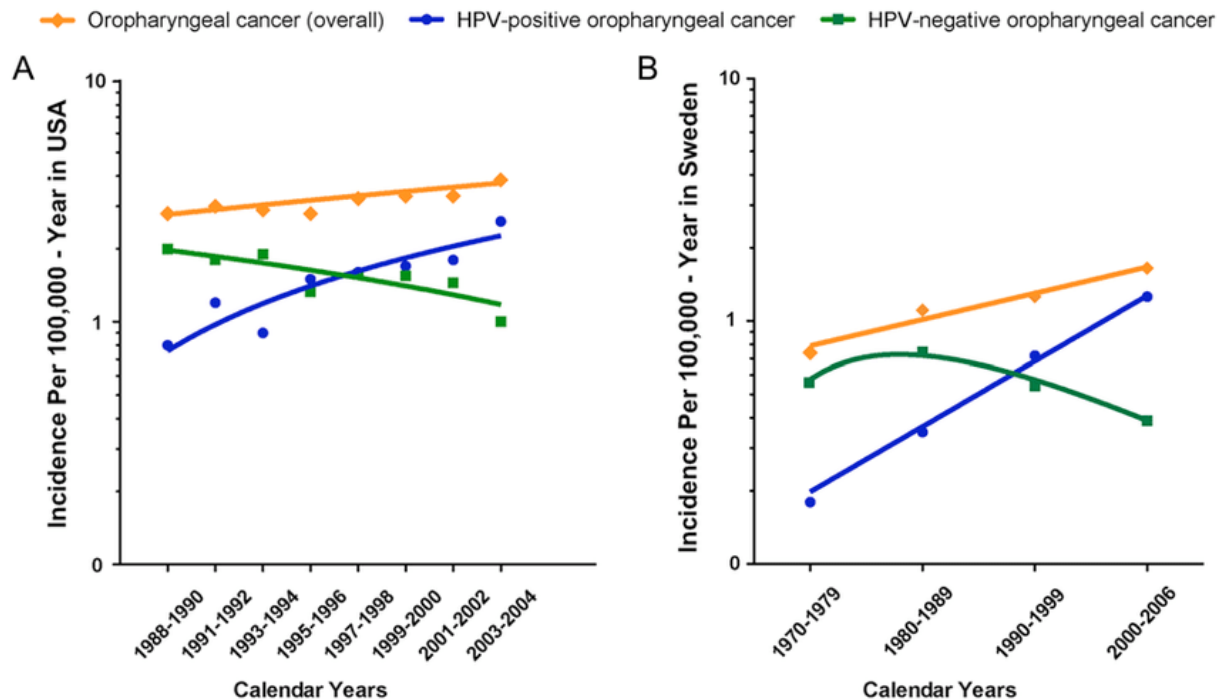


Abb. 2 | Inzidenzraten von HPV - assoziierten Oropharynxkarzinomen und HPV - negativen Oropharynxkarzinomen.

(A) in den Vereinigten Staaten von Amerika zwischen 1988 und 2004 und (B) in Schweden, zwischen 1970 und 2006. Quelle: ⁵

Während die Entstehung von Malignomen durch HR - HPV - Typen in extra - oropharyngealen Lokalisationen des oberen Aerodigestivtrakts gesichert ist, wird der Einfluss der HPV - Assoziation auf das Krankheits - spezifische Überleben in oralen (OSCC) und laryngealen Plattenepithelkarzinomen (LSCC) diskutiert ⁹⁻¹⁵.

Kontroverse Ergebnisse entsprechender Studien können einerseits durch global unterschiedliche karzinogene Risikokonstellationen einschließlich Sexualverhalten und HPV - Prävalenz, andererseits durch eine nicht - standardisierte Bestimmung von HPV - assoziierten extra - oropharyngealen HNSCC erklärt werden ^{16,17}. Eine Fragestellung des Habilitationsprojekts war daher, den Stellenwert der p16 - Immunhistochemie (alleine oder in Kombination zu weiteren Zellzyklus - assoziierten Proteinen) als Surrogatmarker einer onkogen transformierenden HPV - Infektion im OSCC und LSCC zu prüfen ¹⁸⁻²⁴. Abbildung 3 veranschaulicht schematisch die virale Infektion des Epithels ¹.

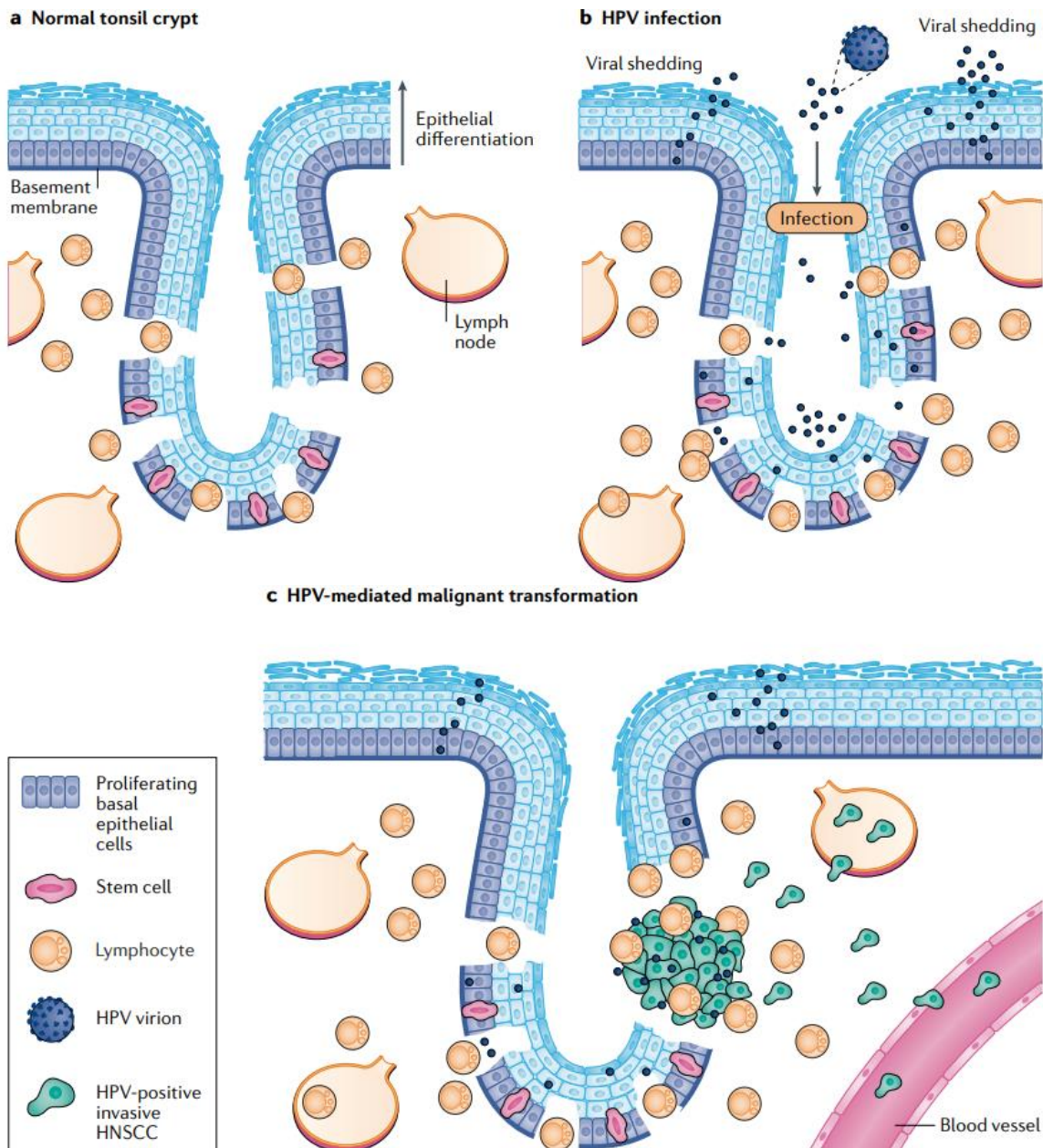


Abb. 3 | HPV - Infektion der Tonsillenkrypten und Entwicklung eines HPV - positiven Plattenepithelkarzinome des Kopfes und Halses (HNSCC).

a | Im gesamten Tonsillenepithel bilden proliferierende Basalepithelzellen die an die Basalmembran angrenzende Zellschicht. Die Differenzierung der Basalepithelzellen resultiert in ihrer Ablösung von der Basalmembran und ihrer Wanderung nach oben. Diese fortschreitende Differenzierung führt zu terminal differenzierten, nicht mehr proliferierenden Zellen. Die Gaumen - und Zungenmandeln sind durch zahlreiche Gewebeeinwüchse gekennzeichnet (Krypten), die an der Basis besonders reich an Stammzellen sind. Die Kriptenstruktur ist von einem einzigartigen, retikulierten Plattenepithel ausgekleidet. In der Basalmembran und der Basalschicht treten zudem Spalten / Fissuren auf. Diese Spalten ermöglichen es Lymphozyten in die Krypten einzudringen und direkt mit fremden, externen Antigenen zu interagieren.

b | Bei einer Infektion mit dem humanen Papillomavirus (HPV) ermöglicht die netzartig zerrissene Beschaffenheit des Plattenepithels der Krypten den Zugang des Virus zu Stammzellen bzw. proliferierenden Basalzellen. Infiltrierende Immunzellen kommen ebenfalls mit den Viruspartikeln in Kontakt. Bei einer produktiven Infektion werden verschiedene virale Gene und Proteine während der verschiedenen Stadien der Epithelzelldifferenzierung induziert und / oder aktiviert, was in der Produktion und Ausscheidung neuer Viruspartikel resultiert.

c | Stammzellen oder proliferierende Basalzellen stellen wahrscheinliche Ursprungszellen für HPV - positive HNSCC dar. Die stabile Integration des viralen Genoms in das Wirtsgenom und die konzertierte Wirkung der HPV - Proteine E6 und E7 auf zellulären p53 - bzw. Retinoblastom - Spiegeln fördert die Zelltransformation. Die Anhäufung zusätzlicher genetischer Veränderungen ist erforderlich, um eine vollständige Transformation zu bewirken, einschließlich des Erwerbs von invasiven und metastatischen Phänotypen. Quelle: ¹

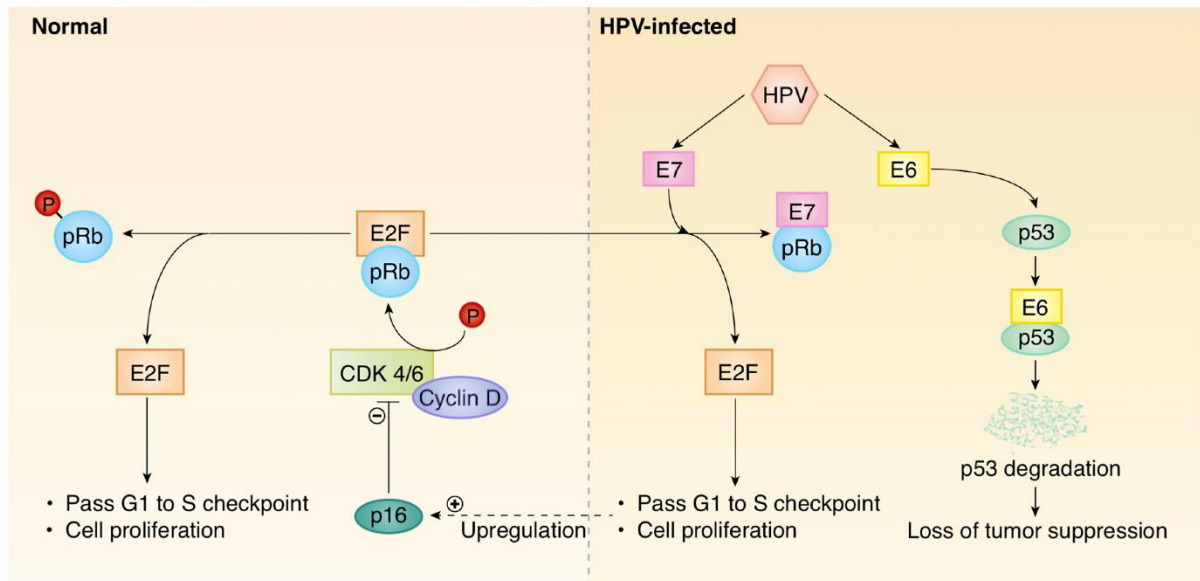


Abb. 4 | Schaubild zum molekularen Mechanismus der p16 - Akkumulation in Humanen Papillomavirus (HPV) assoziierten Karzinomen.

Links: Normale, nicht infizierte Zelle: Der Cyclin - abhängige Kinase (CDK) 4/6 - Komplex leitet die Phosphorylierung des Tumorsuppressorproteins Retinoblastom (pRb) ein. Die Hyperphosphorylierung von pRb führt zur Freisetzung des Transkriptionsfaktors E2F in seinen aktiven Zustand, der die Expression von nachgeschalteten Genprodukten antreibt und der Zelle den Übergang von der G1 - zur S - Phase ermöglicht. Als Cyclin - Kinase - Inhibitor ist p16 ein Tumorsuppressor und negativer Regulator des CDK 4/6 - Komplexes.

Rechts: HPV - infizierte Zelle: Wenn der Transkriptionsfaktor E2F an pRb gebunden ist, bleibt er inaktiv. Die Überexpression des E7 - Onkoproteins durch Hochrisiko - HPV - Subtypen unterbricht den E2F - pRb - Komplex, indem E2F verdrängt und an pRb gebunden wird. Die anschließende Freisetzung von E2F in seinen aktiven Zustand treibt die Expression nachgeschalteter Genprodukte an und ermöglicht der Zelle den Übergang von der G1 - zur S - Phase. In einem regulatorischen Rückkopplungsversuch zur Hemmung der weiteren Zellproliferation wird p16 hochreguliert und kann somit als Surrogatmarker für HPV - assoziierte Tumoren dienen. Das überexprimierte E6 - Onkoprotein wirkt über einen anderen Mechanismus. E6 bindet an das Tumorsuppressorprotein p53 und führt letztlich zum Abbau von p53. Der Verlust der regulatorischen Funktion von p53 führt zu einem ungehemmten Zellzyklus mit verminderter Apoptose. Modifiziert nach Quelle: ²⁵

Abbildung 4 zeigt den Mechanismus der Anreicherung des p16 - Proteins, das vom Tumorsuppressor Cyclin - abhängige Kinase Inhibitor 2A kodiert wird, im zytoplasmatischen und nukleären Kompartiment einer HPV- infizierten Zelle ²⁵⁻²⁸.

Der Anteil der HPV - assoziierten Mundhöhlenkarzinome liegt derzeit unter 10 % aller OSCC²⁹. Hauptrisikofaktoren für die Tumorentstehung sind Rauchen und Alkoholkonsum ³⁰. Orale Plattenepithelkarzinome können zudem auf dem Boden chronischer Mundschleimhautveränderungen entstehen ³¹. Unabhängig der Ätiologie präsentieren sich präkanzeröse Vorläuferläsionen der Mundschleimhaut klinisch als auffällige, häufig weißliche (= leukoplakische) Veränderungen ³².

Der stärkste Prädiktor für die maligne Entartung potenziell maligner Veränderungen der Mundschleimhaut (aus dem Englischen *oral potentially malignant disorders*, OPMD) ist der nach WHO - definierte Grad einer oralen epithelialen Dysplasie (OED) (Abb. 5).

Die Dysplasie samt Schweregrad wird am Biopsiematerial eines klinisch auffälligen Schleimhautareals diagnostiziert ³². In Abhängigkeit definierter Kriterien, welche zytologische Auffälligkeiten und Störungen in der Epithelarchitektur graduieren, liegt eine gering - , mäßig - oder schwergradige Epitheldysplasie vor (Tabelle 1) (Abb. 5) ³³.

Tabelle 1. Morphologische Kriterien der Epitheldysplasie gemäß der aktuellen WHO - Klassifikation

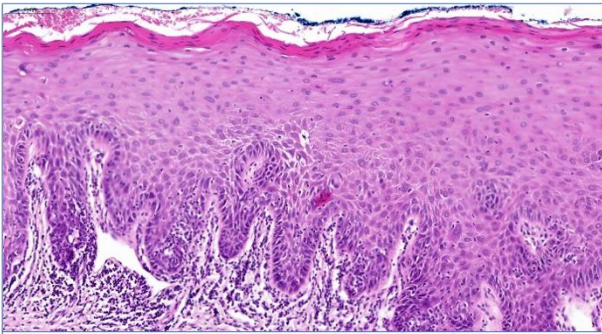
Architekturelle Veränderungen	Zytologische Veränderungen
Irreguläre Schichtung	Abnorme Variation der Kerngröße
Polaritätsverlust basaler Zellen	Abnorme Variation der Kernform
Tropfenartige Reteleisten	Abnorme Variation der Zellgröße
Cluster - /Nesterbildung der basalen Zellen	Abnorme Variation der Zellform
Verbreitertes proliferatives Kompartiment	Erhöhte mitotische Aktivität
Mitosen in oberflächlichen Schichten	Vergrößerte Kerne
Mitosen in ausreifenden Zellen	Erhöhtes Kern - Zytoplasma - Verhältnis
Generalisierte „premature“ Verhornung	Atypische Mitosefiguren
Keratinperlen in den Reteleisten	Erhöhte Zahl und Größe von Nukleoli
Verminderte Kohäsion der Keratinozyten	Einzelzellverhornungen
Verändertes Verhornungsmuster nach anatomischer Region	Nukleäre Hyperchromasie
Verruköse/papilläre Architektur	Apoptotische Mitosen
Veränderungen bis in Ausführungsgänge von Speicheldrüsen	
Scharfe Grenzen zum normalen Epithel	
Verschiedene Dysplasie - Muster	

Mit zunehmenden zytologischen Veränderungen, wie beispielsweise einer zugunsten des Kerns verschobenen Kernplasmarelation, steigt das Risiko der malignen Entartung. Parallel zu Einzelzell - basierten Charakteristika ist eine Architektur - bzw. Reifungsstörung des oralen Plattenepithels ein Prädiktor für die Krebsentstehung ³².

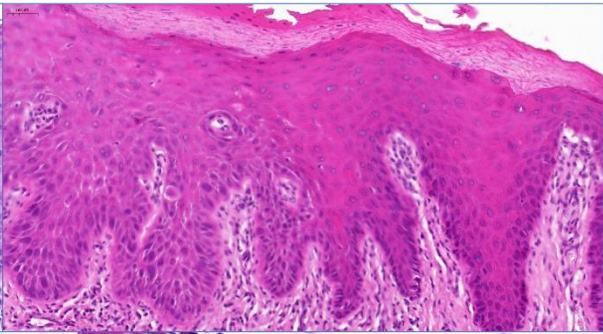
Die OED - Variante, welche sich vorrangig durch eine Reifungsstörung bei weitgehend unauffälliger Zytomorphologie definiert, wird aktuell differenzierte Dysplasie genannt ³⁴⁻³⁶.

Im Rahmen einer zahnärztlichen Promotion wurde diese Variante der OED untersucht ³⁷. Als Teilprojekte ergaben sich die Fragen nach der Häufigkeit der differenzierten Dysplasie, ihrer Diagnostik am zytologischen und bioptischen Material sowie der assoziierten Transformationsrate in ein orales Plattenepithelkarzinom.

A: geringgradige orale Epitheldysplasie



B: mäßiggradige orale Epitheldysplasie



C: schwergradige orale Epitheldysplasie

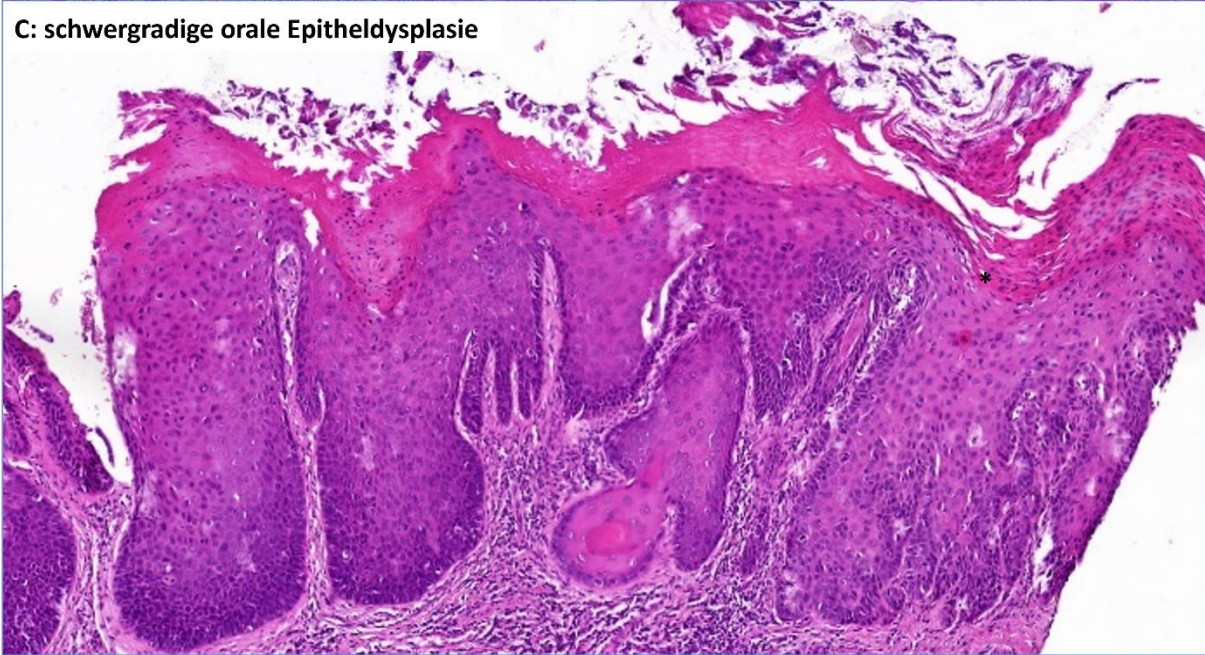


Abb. 5 | Orale Epitheldysplasie.

a) geringgradige Dysplasie mit vorrangig architekturellen Veränderungen in Form verlängerter Reteleisten, vorzeitiger Verhornung und basaler Hyperchromasie. b) mäßiggradige Dysplasie mit verlängerten, tropfenförmig ausgezogenen Reteleisten, vermehrten Mitosen, reduzierter Zellkohäsion und variierender Zellkerngröße bis in das Stratum spinosum mit scharfem Übergang zum angrenzenden Epithel (rechts). c) schwergradige Dysplasie mit deutlichen architekturellen und zytologischen Veränderungen (alle Bilder 200x).

Mundhöhlenkarzinome stellen in Abhängigkeit von der dominierenden Risikofaktoren typischerweise die größte anatomische Gruppe aller HNSCC dar ¹.

In fortgeschrittenen Stadien, insbesondere im rezidierten oder metastasierten (r/m) Setting, werden diese Tumore ebenso wie Plattenepithelkarzinome übriger anatomischer Lokalisationen des oberen Aerodigestivtrakts mit einem multimodalen Therapiekonzept behandelt ^{1,38}.

Neben einer lokalen Bestrahlung beinhaltet diese im deutschsprachigen Raum typischerweise Platinhaltige Chemotherapeutika. Dieses Zytostatikum hemmt in Zellen die DNA - Replikation durch Querverknüpfungen zweier benachbarter Guanin - Basen eines DNA - Strangs ³⁹. Dies führt zu Veränderungen in der DNA - Struktur. Der Zellstoffwechsel kommt zum Erliegen und die Zelle leitet die Apoptose ein.

Resistenzen gegen Cisplatin werden insbesondere in rezidierten Tumoren beobachtet und verantworten Therapieversagen und eine schlechte Prognose ⁴⁰.

In der Regulation des programmierten Zelltods nimmt das Anti - Apoptoseprotein BCL2 (B - cell lymphoma 2) eine tragende Rolle ein (Abb. 6) ⁴¹⁻⁴⁵. Wenngleich der Apoptoseregulator auf Proteinebene in allenfalls 20 % aller HNSCC im kaukasischen Patientenkollektiv nachweisbar ist, zeigen Patienten mit BCL2 - positiven Tumoren nach gängigen Auswerteschemata ein kürzeres Überleben und eine höhere Rezidivrate als BCL2 - negative Vergleichsgruppen ^{43,46-51}.

Selektive BCL2 - Inhibitoren sind Standard in der Behandlung hämatologischer Neoplasien ⁵². Auch im HNSCC scheinen entsprechende Analysen, die BCL2 in Kombination zu anderen Therapieschemata wie Radiatio *in vitro* untersuchten, vielversprechend ^{53,54}.

Ein Teilprojekt der vorliegenden Arbeit untersuchte den Einfluss des selektiven BCL2 - Inhibitors Venetoclax auf unterschiedliche Zellfunktionen einschließlich Apoptoserate allein oder in Kombination mit einer Cisplatin - Monotherapie. Hierbei prüften wir auch den Einfluss auf die Methuoserate, einer alternativen Form des Zelltods.

Die Wortschöpfung stammt vom griechischen *methuo* (*bis zum Rausch trinken*), denn charakteristisch für diese Form des Zelltods ist die Verdrängung des Zytoplasmas durch große, flüssigkeitsgefüllte Vakuolen ⁵⁵. Der Zelltod ähnelt vielen Formen der Nekrose, da es zu einem Verlust der Stoffwechselkapazität und der Integrität der Plasmamembran kommt, ohne dass die Zelle schrumpft und der Zellkern fragmentiert, wie es bei der Apoptose der Fall ist.

In enger Kooperation mit der Medizinischen Klinik III für Hämatologie, Onkologie und Palliativmedizin unserer Universitätsmedizin führten wir Zellkulturmodelle an Patienten - abgeleiteten Zelllinien durch, die im Vorfeld von der Arbeitsgruppe um PD Dr. rer. nat. Claudia Maletzki etabliert wurden ^{56,57}. Im Hinblick auf die fragliche klinische Einsatzfähigkeit von Venetoclax in HNSCC - Patienten nutzen wir Gewebeproben eines retrospektiven Kollektivs, um das Potential von BCL2 als Biomarker im Sinne translationaler Forschung zu analysieren.

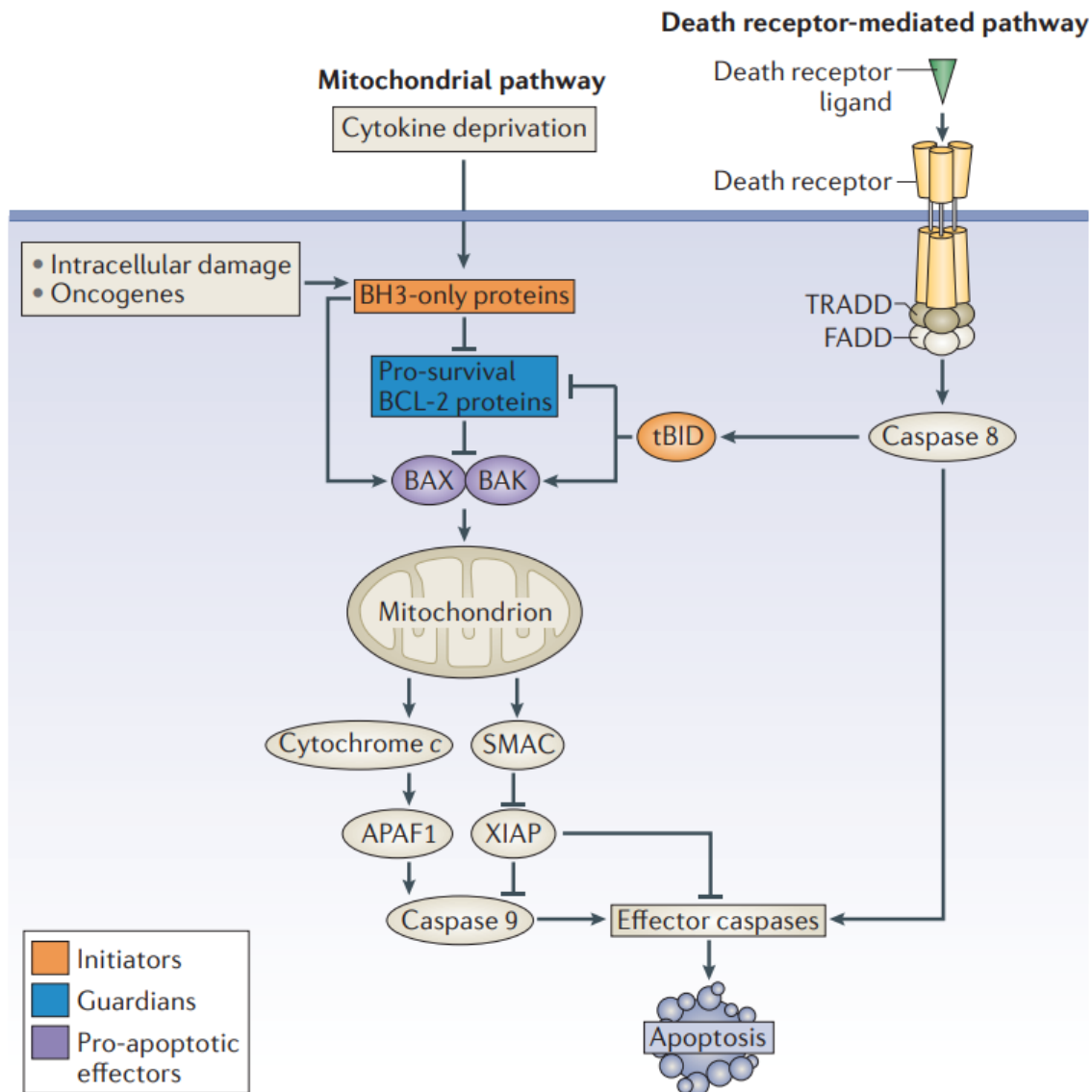


Abb. 6 | Schaubild der mitochondrialen und durch Todesrezeptoren vermittelten Wege zur Apoptose unter Berücksichtigung der B - cell lymphoma 2 (BCL2) - Proteine.

Verschiedene zytotoxische Stimuli, darunter onkogener Stress und Chemotherapeutika, sowie entwicklungsbedingte Reize setzen den mitochondrialen Weg in Gang, der durch Mitglieder der **BCL2 - Familie** reguliert wird. Diese Stimuli aktivieren **BH3 - Familienmitglieder (Initiatoren)**, die die überlebenswichtigen **BCL2 - ähnlichen Proteine (Guardians)** hemmen. So werden die proapoptotischen Effektoren **BAX und BAK** aktiviert, die die äußere Membran der Mitochondrien zerstören. Das aus den Mitochondrien freigesetzte Cytochrom c fördert die Aktivierung von Caspase 9 am Gerüstprotein APAF1 (apoptotische Protease activating factor 1), während das freigesetzte Protein SMAC (second mitochondria derived activator of caspases) den Caspase - Inhibitor XIAP (Xlinked inhibitor Apoptose - Protein) blockiert.

Der Todesrezeptor - vermittelte (oder extrinsische) Weg der Apoptose wird aktiviert, wenn bestimmte Todesrezeptor - Liganden der Tumor - Nekrose - Faktor (TNF) Familie (wie der FAS - Ligand und TNF) ihre entsprechenden Todesrezeptoren (FAS bzw. TNFR1) an der Plasmamembran zeigen, was zur Aktivierung von Caspase 8 über das FAS - assoziiertes Todesdomänenprotein (FADD) und TNFR - assoziiertes Todesdomänen Protein (TRADD) führt.

Die beiden Pfade konvergieren bei Aktivierung der Effektor - Caspasen. Darüber hinaus kann die verkürzte Form von **BID (tBID)**, die durch Caspase 8 - vermittelte Proteolyse von BID im Todesrezeptor - vermittelten Weg entsteht, den mitochondrialen Weg aktivieren, um die apoptotische Reaktion zu verstärken. Modifiziert nach Quelle: ⁴²

Das Management des rezidierten / metastasierten (r / m) HNSCC wird seit Jahrzehnten von der multimodalen Radiochemotherapie dominiert. Diese wird entweder allein oder in Ergänzung zu einer chirurgischen oder medikamentösen, gegen den epidermalen Wachstumsfaktorrezeptor (EGFR) gerichteten Therapie (Cetuximab) eingesetzt (Abb. 7) ^{1,58}.

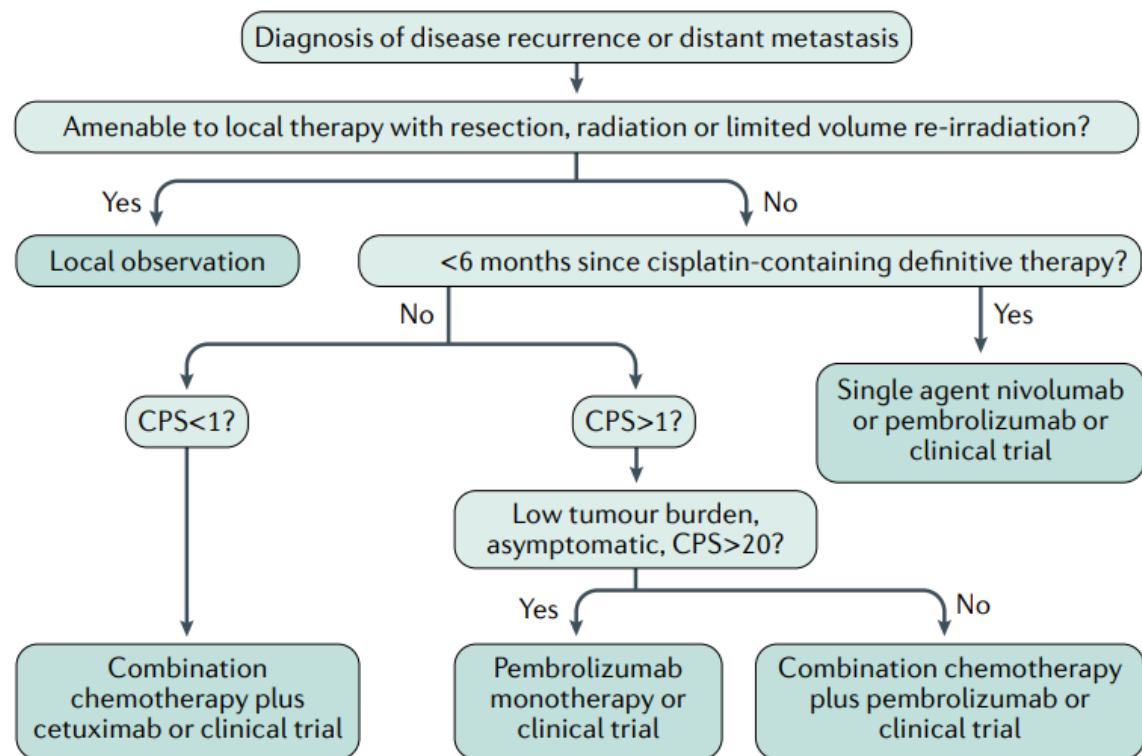


Abb. 7 | Algorithmus zur Entscheidungsfindung bei der Behandlung von rezidiertem und / oder metastasiertem HNSCC.

Nach der Diagnose eines Krankheitsrezidivs oder einer Fernmetastasierung erhalten Patienten mit einer Erkrankung, die einer lokalen Therapie zugänglich ist, eine Resektion oder Bestrahlung und werden anschließend beobachtet. Bei Patienten, die auf diese Therapien nicht ansprechen, ist eine systemische Therapie angezeigt. Pembrolizumab ist bei Patienten mit PD - L1 Expression (Combined Positive Score (CPS) > 1; CPS ist die Anzahl der PD - L1 - färbenden Tumor - und Immunzellen dividiert durch die Gesamtzahl der gezählten lebensfähigen Tumorzellen) × 100) als alleinige Therapie bei hoher Expression (CPS ≥ 20) und / oder asymptomatischer Erkrankung oder in Kombination mit einer platinbasierten Chemotherapie bei Patienten mit geringerer Expression (CPS 1 - 19) oder höherer Symptomlast zugelassen. HNSCC, nicht kutanes Plattenepithelkarzinom des Kopf - / Halsbereichs. Quelle: ¹

Die Ansprechraten bleiben jedoch moderat.

Ähnliches gilt für eine neuere Klasse monoklonaler Antikörper, den sogenannten Immuncheckpoint - Inhibitoren (u.a. anti - PD1, anti - PD - L1), welche spezifisch gegen die Tumorzell - induzierte Immunevasion in verschiedenen soliden Neoplasien eingesetzt werden. Obgleich Immuncheckpoint - Inhibitoren in verschiedenen Malignomen einen klaren Überlebensvorteil für entsprechend behandelte Patienten erzielten, blieb ein vergleichbarer Erfolg im HNSCC aus ⁵⁹.

Ein Grund ist das heterogene, nicht prognostizierbare Ansprechen auf Inhibitoren gegen das Molekül Programmed Cell Death Protein - 1 (PD - 1), welche für Patienten mit r / m oder primär inoperablen HNSCC zugelassen sind ¹. Obwohl ein Combined Positive Score (CPS) von mindestens eins für den PD1 - Rezeptor Programmed Cell Death Ligand - 1 (PD - L1) als Mindestwert für die PD - L1 - Positivität auf Tumorzellen und assoziierten Immunzellen für die Therapie gefordert wird, kann der onkologische Nutzen nicht sicher durch die PD - L1 - Expression vorhergesagt werden.

Das auf Tumor - und Immunzellen exprimierte Molekül PD - L1 wird durch CMTM6 (CKLF like MARVEL transmembrane domain containing 6) auf posttranslationaler Ebene stabilisiert ^{60,61}. Ein hoher CMTM6 Proteinstatus korreliert in anderen epithelialen Neoplasien mit dem Ansprechen auf eine Immuncheckpoint - Blockade, z.B. durch den monoklonalen Antikörper Pembrolizumab gegen den PD - 1 ⁶². Im HNSCC gilt CMTM6 in Abhängigkeit angewandter Erhebungs - Assays insbesondere im asiatischen Raum als schlechter prädiktiver Marker für eine Cisplatinhaltige Chemotherapie ^{63,64}. In der Immunhistochemie zeigt CMTM6 typischerweise eine membranöse Reaktivität in Tumorzellen und eine membranöse und zytoplasmatische Reaktivität auf assoziierten Immunzellen.

Aufgrund der widersprüchlichen Datenlage zu CMTM6, seiner biologischen Wertigkeit für PD - L1 und der nach wie vor schlechten Ansprechraten von HNSCC - Patienten auf konventionelle Therapien, haben wir CMTM6 mit etablierten Scores in einem retrospektiven HNSCC - Kollektiv untersucht. Unsere Ergebnisse haben wir mit dem Überleben in Anhängigkeit adjuvanter Behandlungen unter Berücksichtigung des Tumormikromileus und anderer Immuncheckpoint - Moleküle abgeglichen, um die prognostische und prädiktive Wertigkeit von CMTM6 zu eruieren.

2 Zusammenfassung der Fragestellungen

Im Rahmen des Habilitationsprojekts sollen neue Aspekte der histopathologischen Diagnostik sowie der Prädiktion auf multimodale Therapiekonzepte unter Berücksichtigung des Tumormikromilieus betrachtet werden und sind folgenden Teilprojekten mit entsprechenden Originalien zugeordnet:

- 1) **Differenzierte Dysplasie der Mundhöhle als potentiell maligne Vorläuferläsion der Mundschleimhaut:** Wie häufig ist die, nach heutigem Verständnis definierte, differenzierte Dysplasie bei leukoplakischen Läsionen? Wie hoch ist ihre maligne Transformationsrate? Welche Zusatzuntersuchungen können helfen, die differenzierte Dysplasie zu diagnostizieren?
- 2) **HPV - Diagnostik in Mundhöhle und Larynx:** Ist die p16 - Immunhistochemie ein ausreichend guter Surrogatmarker in der Detektion HPV - assoziierter HNSCC extra - oropharyngealer Lokalisationen kaukasischer Patienten? Profitiert die diagnostische Performance des p16 - Tests extra - oropharyngealer Tumoren durch Hinzunahme der Färbungen weiterer Zellzyklus - assoziierter Proteine? Beeinflusst eine HPV - Assoziation das Überleben der Patienten?
- 3) **Stellenwert von BCL2 und dessen medikamentöse Inhibition additiv zur Chemotherapie im HNSCC:** Bestätigt sich BCL2 als schlechter Biomarker? Welchen Einfluss hat die alleinige oder mit Cisplatin - Monotherapie kombinierte BCL2 Inhibition in HNSCC - Zelllinien auf Zelltod, Proliferation und epitheliale - mesenchymale Transition?
- 4) **Prognostische und prädiktive Wertigkeit ausgewählter Immuncheckpoint - Moleküle einschließlich CMTM6 im HNSCC:** Korreliert CMTM6 mit dem Immuncheckpointmolekül PD - L1 oder spezifischen Immunzellpopulationen? Lassen sich anhand entsprechender Marker deskriptive Zusammenhänge zum Patientenüberleben oder Ansprechen auf eine Immuncheckpoint - Blockade herstellen?

3 Betrachtung der Teilprojekte

3.1 Potentiell maligne Veränderungen der Mundschleimhaut mit Fokus auf dem Konzept der differenzierten Dysplasie (Originalarbeit I)

Um die strikt auf architekturellen Abnormalitäten beruhende differenzierte Dysplasie als Variante der oralen epithelialen Dysplasie hinsichtlich diagnostischer Kriterien, Prävalenz und Entartungsrisiko zu klassifizieren, wurde ein retrospektives Kollektiv (Einschlusszeitraum Januar 2014 bis März 2023) von Gewebeproben von Patienten mit der klinischen Diagnose „Leukoplakie“ erstellt. Um die histologische Variabilität einschließlich physiologischer Keratinexpression des Plattenepithels der Mundhöhle zu berücksichtigen, nahmen wir nur Proben der auskleidenden Mundschleimhaut auf. Dieses unverhornte Plattenepithel kleidet Mundboden, Zungenunterseite, Mundvorhof, Weichgaumen und den Bezirk um die Alveolarfortsätze aus. Es exprimiert Keratin 13, nicht aber Keratin 17⁶⁵⁻⁷¹. Ein reziprokes Expressionsmuster dieser beiden Intermediärfilamente findet sich u.a. bei entzündlichen oder präneoplastischen Veränderungen⁷¹.

Anhand der Morphologie wurden definierte zytoarchitektonische Kriterien in differenzierten Dysplasien sowie Kontrollfällen häufiger OPMDs untersucht. Das Expressionsmuster von Keratin 13 / 17 und die proliferative Aktivität anhand des Markers Ki67 wurden analysiert. Verfügbare Ergebnisse der oralen Bürstenzytologie wurden aus den klinischen Unterlagen entnommen. Ebenso wurden klinische und pathomorphologische Daten mit dem malignen Transformationsrisiko abgeglichen.

Wir schlossen 159 Biopsien von 112 Patienten ein (33 % differenzierte Dysplasie; 27 % Keratose ohne Dysplasie sowie 9 % leichte, 8 % mittelschwere und 7 % schwere orale Epitheldysplasie bzw. invasive Karzinome). Auf histomorphologischer Ebene zeigte die differenzierte Dysplasie die höchste Hypergranulose - Rate. Der Verlust von Keratin 13 und der Zugewinn von Keratin 17 war in den Fällen mit differenzierter Dysplasie höher als in der Diagnosegruppe der Keratose ohne Dysplasie ($p < 0,0001$).

Im gesamten Kollektiv war die Expression von Keratin 17 mit einer höheren malignen Transformationsrate verbunden ($p = 0,0028$). Transformationsrate und -zeit waren zwischen der Gruppe von Dysplasien mit zytologischen Veränderungen und der differenzierten Dysplasie - Gruppe vergleichbar. Diese beiden Dysplasieformen wiesen jeweils höhere Progressionsraten und kürzere Progressionszeiten als die Kontrollfälle ohne Dysplasie auf ($p = 0,08$). Die zytologischen Untersuchungen der Fälle mit der histologischen Diagnose einer differenzierten Dysplasie waren zu 100 % negativ, d.h. sie zeigten unauffälliges Epithel.

Zusammenfassend stellt die differenzierte Dysplasie innerhalb des WHO - Einstufungssystems das eine Ende des Spektrums der oralen Dysplasie dar und definiert sich durch rein architektonische Anomalien. Ein Verlust von Keratin 13 bei abnormer Expression von Keratin 17 erleichtert die Diagnosestellung. Unsere Daten bestätigen die Annahme, dass die orale Bürstenzytologie die differenzierte Dysplasie nicht detektiert. In unserer Kohorte hatten Patienten mit differenzierter Dysplasie ein vergleichbares Risiko für eine Malignisierung als dysplastische Läsionen mit zytonukleären oder gemischt zytonukleären / architektonischen Merkmalen.

3.2 HPV - Diagnostik in Mundhöhle, Oropharynx und Larynx (Originalarbeit II)

Das humane Papillomavirus ist ein zunehmender Risikofaktor für Plattenepithelkarzinome des Kopfes und Halses ⁷²⁻⁷⁵. Die Gruppe der durch HPV - Hochrisikotypen induzierten oropharyngealen Plattenepithelkarzinome (OPSCC) sind mit einem günstigen Outcome assoziiert, was eine therapeutische De - Eskalation bedingt ⁷⁶.

Im diagnostischen Alltag dient die p16 - Färbung als Surrogatmarker für eine onkogene HPV - Infektion bei OPSCC ^{27,77}. Tumoren entsprechender Patienten werden mittels Immunhistochemie durch das *Blockstaining* - Muster für p16 identifiziert. In dieser anatomischen Lokalisation verbessern die wildtypische Anfärbung von p53 in der Immunhistochemie sowie der Verlust des Tumorsuppressors Retinoblastom (pRb) und des mit pRB interagierenden Onkogens Cyclin D1 die Spezifität von p16 ^{78,79}.

Bei OSCC und LSCC ist die p16 - Immunhistochemie, allein oder in Kombination mit anderen zellzyklusbezogenen Proteinen, für die Identifikation HPV - bedingter Karzinome weniger untersucht⁸⁰⁻⁸². Auch ist die aktuelle Datenlage bzgl. der onkologischen Relevanz der HPV - Assoziation für HNSCC mit Lokalisation in Mundhöhle, Larynx oder Hypopharynx in Hinblick auf Überleben und Therapieansprechen unklar.

Mittels Immunhistochemie wurde die Expression von p16, pRb, Cyclin D1 und p53 in 327 HNSCC - Proben aller Lokalisationen untersucht⁷⁹. Der HPV - Status der 310 OPSCC wurde mittels HPV - DNA - PCR bestimmt^{83,81}. In den 119 nicht - OPSCC wurde zusätzlich eine RNA *in situ* Hybridisierung durchgeführt. Der HPV - Status wurde mit Färbemustern, p53 - Status und klinischen Daten korreliert.

Unter Verwendung der Cut - off - Kriterien von > 70% zytoplasmatischer und nukleärer Färbung zeigten die OPSCC in 36 % der Fälle eine Blockfärbung für p16. Weitere 8 % aller OPSCC zeigten in der p16 - Färbung kein eindeutiges positives oder negatives Ergebnis und wurden als zweifelhaft klassifiziert. Bei 57 % der OPSCC wurde ein molekularer HPV - Test durchgeführt, 53% waren positiv für HPV - DNA. Die HPV - Assoziation korrelierte mit dem Verlust von pRb und Cyclin D1 und einem günstigen Outcome. Bei den Nicht - OPSCC wiesen 18 % eine p16 - Blockfärbung und 13 % eine E6 / E7 - RNA Positivität auf. Sechs von sieben HPV - positiven OSCC und acht von acht LSCC verloren pRb und Cyclin D1. Im Vergleich zu HPV - negativen Fällen berichteten Patienten HPV - positiver Karzinome einen geringeren Alkoholkonsum, ferner war die nicht - keratinisierende Morphologie unter den HPV - positiven Fällen häufiger. Die HPV - positiven OSCC hatten ein längeres Gesamtüberleben ($p < 0,05$).

Schlussfolgernd gehen HPV - assoziierte OSCC und LSCC analog zum OPSCC mit einer Überexpression von p16 und einem Verlust von pRb - und Cyclin D1 einher. Die Färbungen dieser beiden Marker scheinen die diagnostische Leistung von p16 nicht zu verbessern. Bei fraglichen p16 - Färbergebnissen, für die keine molekularen HPV - Tests zur Verfügung stehen, kann ein Wildtyp - p53 - Färbemuster die HPV - bedingte Karzinogenese bekräftigen.

3.3 Perspektiven der Inhibition des Anti - Apoptoseproteins BCL2 in Ergänzung zur klassischen Chemotherapie in der Behandlung fortgeschrittener HNSCC (Originalarbeit III)

Eine therapeutische Limitation im HNSCC sind hohe Rezidivraten, welche u. a. auf Resistenzen gegen platinbasierte Chemotherapeutika zurückzuführen sind ⁴⁰. Das anti - apoptotische Protein BCL2 ist an der Apoptose - bzw. Chemoresistenz sowie der Invasion / Migration von Tumorzellen beteiligt ^{44,84-86}.

Daher untersuchten wir, ob die immunhistochemische Überexpression von BCL2 das Ansprechen von HNSCC auf eine Cisplatin - basierte Radiochemotherapie vorhersagt und eine selektive BCL2 - Inhibition *in vitro* additiv zu Cisplatin - induzierten Zellveränderungen wirkt.

Hierzu korrelierten wir den BCL2 - Immunstatus mit klinischen Parametern, einschließlich Überlebensdaten nach Therapie, in einer hinsichtlich adjuvanter Therapieregimen weitgehend einheitlichen HNSCC - Kohorte. In von ausgewählten Tumoren dieses Kollektivs etablierten Zelllinien wurde die Kombinationstherapie von ABT - 199 (Venetoclax), einem BCL2 - Inhibitor, und Cisplatin untersucht, wobei die Fähigkeit zur Koloniebildung und die Art des Zelltods analysiert wurden ^{56,57}.

Patienten mit prä - therapeutisch BCL2 - positiven Tumoren (44 / 254; Behandlung entweder Bestrahlung, Cisplatin - Monotherapie oder beides) hatten sowohl ein kürzeres Gesamt - als auch progressionsfreies Überleben (log - rank; $p = 0,048$) sowie eine höhere Rate an Rezidiven (Fisher's Exact Test; $p = 0,0032$). Die *in vitro* Experimente zeigten, dass die alleinige BCL2 - Hemmung in keiner der drei untersuchten Zelllinien, unabhängig vom BCL2 - Status, einen wesentlichen Einfluss auf die Zellviabilität hatte, die Cisplatin - induzierten Effekte jedoch verstärkte: Unter der Kombinationstherapie verdoppelten sich die Raten für Autophagie und Zelltod einschließlich Methuose. Ferner zeigten unsere Experimente, dass die Kombinationstherapie aus BCL2 - Inhibition und Platingabe in den Zellen die epithelial - mesenchymale Transformation (EMT) hemmt.

Die EMT - Inhibition könnte der Resistenzentwicklung gegen Platinhaltige Therapien entgegenwirken. Daher stellt die zusätzliche BCL2 - Hemmung parallel zu Cisplatinhaltigen Therapieschemata einen vielversprechenden Ansatz dar, die Chemoresistenz einschließlich EMT in Patienten mit fortgeschrittenen HNSCC zu behandeln.

Die Immunhistochemie für BCL2 könnte perspektivisch als prädiktiver Biomarker eingesetzt werden, um Subpopulationen mit BCL2 - positiven Tumoren zu identifizieren, die eine höhere Wahrscheinlichkeit haben, auf die Therapie anzusprechen.

3.4 CMTM6 als prognostischer und prädiktiver Biomarker im HNSCC (Originalarbeit IV und V)

Die nach wie vor unbefriedigende Prognose von HNSCC - Patienten mit einer 5 - Jahres - Überlebensrate von etwa 65 % macht es notwendig, nach geeigneteren Therapien zu forschen¹. Die Immuntherapie ist ein solcher Behandlungsansatz, wobei das Ansprechen auf Immuncheckpoint - Inhibitoren im klinisch fortgeschrittenen Stadium nicht zuverlässig prognostizierbar ist^{8,87}. Um die Eigenschaften von HNSCCs, die sich dem Immunsystem entziehen (Evasion), besser zu verstehen und perspektivisch der individuell besten Behandlungsoption zuzuführen, ist ein tieferes Verständnis des Immunkontextes in den Karzinomen notwendig⁵⁹. Ein entscheidender Mechanismus der Immunevasion ist die Expression sogenannter Immuncheckpoints wie PD - L1⁸⁸. Die Interaktion von PD - L1 auf Tumorzellen mit PD1 auf T - Zellen führt zur T - Zellerschöpfung und ausbleibender antitumoralen T - Zellantwort. Das Medikament Pembrolizumab blockiert diesen inhibitorischen Mechanismus, wobei ein positiver PD - L1 Status auf Tumor - und assoziierten Immunzellen prädiktiv für ein Therapieansprechen ist. Auf posttranslationaler Ebene wird PD - L1 durch CMTM6 stabilisiert, welches im asiatischen HNSCC - Kollektiv als schlechter prognostischer und prädiktiver Marker beschrieben ist^{64,89–91,64,92}.

Da die Assoziation von PD - L1, CMTM6 und Tumor - infiltrierenden Lymphozyten (TILs) einschließlich ausgewählter lymphozytärer Subpopulationen für kaukasische Patienten in der Literatur bisher nicht beschrieben wurde, untersuchten wir die gewebständige Expression dieser Proteine (Originalarbeit IV).

Die Ergebnisse korrelierten wir mit dem Gesamtüberleben (OS) und dem progressionsfreien Überleben (PFS) in einer retrospektiven Kohorte der Unimedizin Rostock. Im ersten Teilprojekt zur Ermittlung der prognostischen Wertigkeit untersuchten wir retrospektiv 129 behandlungs - naive HNSCC - Proben hinsichtlich der Expression von PD - L1 und CMTM6, TILs und tumorassoziierter Makrophagen (TAMs).

Dabei analysierten wir an Ganzflächenschnitten die wechselseitigen Beziehungen zwischen diesen Markern bzw. Zellpopulationen.

In ~70 % der HNSCC - Proben wurden PD - L1 ($\text{CPS} \geq 1$) und CMTM6 ($\text{CPS} \geq 10$) nachgewiesen. Der Großteil der PD - L1 - positiven Fälle wies eine gleichzeitig hohe CMTM6 - Expression in vergleichbaren Färbemustern auf. Die Gruppe PD - L1 - positiver ($p < 0,0001$) und / oder CMTM6 - hoch exprimierender Tumore ($p < 0,05$) zeigten das beste OS.

Eine hohe Dichte an TILs ($p < 0,01$), CD8^+ T - Zellen ($p < 0,001$) und ein $\text{CD68} / \text{CD163}$ - Verhältnis > 1 waren prognostisch günstig. Neben PD - L1 und CD8^+ T - Zellen wurde CMTM6 in der multivariaten Cox - Regressionsanalyse als unabhängiger prognostischer Faktor identifiziert. PD - L1 und CMTM6 korrelierten mit TILs und CD8^+ Zellen, nicht aber mit dem HPV - Status.

Unsere Ergebnisse zeigen, dass CMTM6 ein wichtiges Molekül in der Interaktion zwischen TILs, CD8^+ T - Zellen und PD - L1 ist, der auch für die Wirksamkeit onkologischer Behandlungen und Prognose von Bedeutung zu sein scheint.

Das zweite Teilprojekt (Originalarbeit V) sollte das prädiktive Potential der Marker herausarbeiten. Hierfür vergrößerten wir das oben beschriebene Kollektiv durch weitere Patienten der Universitätsmedizin Rostock; die Rostocker Kohorte bildete die Testkohorte. Parallel kooperierten wir mit Wissenschaftlern des Universitätsklinikums Schleswig - Holstein Campus Lübeck, welche uns ein Vergleichskollektiv an HNSCC - Tumoren für Validierungszwecke zur Verfügung stellten (= Validierungskohorte)⁹³⁻⁹⁷.

In der Testkohorte untersuchten wir das Expressionsprofil von CMTM6, PD - L1, CTLA - 4 und FOXP3 anhand von Tumorproben 177 kaukasischer HNSCC - Patienten aller Tumorstadien und verschiedener Therapiemodalitäten. Die Abundanz einzelner Marker in Tumor - und Immunzellen korrelierten wir mit den Behandlungsergebnissen.

Patienten mit Tumoren, die CMTM6 hoch ($\text{CPS} \geq 10$) exprimierten, hatten unabhängig von der Behandlung ein längeres Gesamtüberleben. Dieser prognostische Vorteil von CMTM6 wurde in der Validierungskohorte bestätigt.

In der Testkohorte war eine hohe CMTM6 - Expression in Tumoren ein guter prädiktiver Marker für Patienten, die eine alleinige Strahlentherapie erhielten ($p = 0,07$), was sich für andere Therapiemodalitäten nicht zeigen ließ. CMTM6 korrelierte mit PD - L1 - , CTLA - 4 - und FOXP3 - Positivität, wobei Patienten mit hoher Abundanz von CMTM6 und FOXP3 unabhängig von der Behandlung das längste Überleben aufwiesen. Bei den mit Chemotherapie behandelten Patienten war die PD - L1 - Positivität mit einem längeren progressionsfreien Überleben assoziiert ($p < 0,05$).

Bei den 27 Patienten, die eine Immuntherapie erhielten, zeigte die Genexpressionsanalyse in der Respondergruppe (definiert als partielles oder vollständiges Therapieansprechen) niedrigere CTLA - 4 - und FOXP3 - Werte, während für CMTM6 oder PD - L1 kein Einfluss nachgewiesen wurde.

Zusammenfassend zeigten Tumore mit hohem CMTM6 - Status kaukasischer HNSCC - Patienten in unabhängigen Kohorten ein längeres Überleben. Ein hoher CMTM6 - Status korrelierte mit PD - L1 - , CTLA - 4 - und FOXP3 - positiven regulatorischen T - Zellen und war mit einem besseren Ansprechen auf Strahlentherapie vergesellschaftet^{98,99}. Während weder der PD - L1 - noch der CMTM6 - Status das Ansprechen auf Immuncheckpoint - Inhibitoren vorhersagte, war eine niedrige CTLA - 4 - Expression mit einem guten Ansprechen verbunden. Die Kombination dieser immunmodulatorischen Marker scheint eine interessante Signatur für HNSCC - Patienten zu sein, mit der individualisierte Therapien optimiert werden könnten^{98,100,101,98}.

4 Zusammenfassung und Ausblick

Das Habilitationsprojekt hatte das Ziel, Ansätze aufzuzeigen, die Diagnostik einschließlich Früherkennung und Prognostik von HNSCC zu verbessern und mögliche zukünftige Behandlungsoptionen unter Einsatz verschiedener methodischer Ansätze zu untersuchen. Die Identifizierung geeigneter, im Alltag anwendbarer diagnostischer Verfahren könnte langfristig neue Perspektiven für eine standardisierte Diagnostik, Harmonisierung internationaler Forschungsergebnisse und eine optimierte personalisierte Therapie für die Patienten eröffnen.

Die Diagnose einer differenzierten Dysplasie als potentiell maligne Veränderung der Mundschleimhaut und Vorläuferläsion eines OSCC stützt sich neben morphologischen Charakteristika auf immunhistochemische Färbungen, nicht aber auf orale Bürstenabstriche. Wenngleich die differenzierte Dysplasie in unserem Kollektiv ein der klassischen oralen epithelialen Dysplasie geringen Grades vergleichbares Malignitätspotential aufweist, werden weitere Untersuchungen den Stellenwert dieser Dysplasieform prüfen müssen, bevor sie abschließend in den Kontext der präneoplastischen Veränderungen eingeordnet werden kann.

Die Diagnostik HPV - assoziierter, insbesondere durch den Subtyp 16 ausgelöster HNSCC durch die p16 - Färbung gelingt auch in extra - oropharyngealen Tumoren zuverlässig. Viral induzierte OSCC scheinen in unserem Kollektiv mit einem besseren Überleben vergesellschaftet. Andere Arbeitsgruppen finden demgegenüber keine Überlebensunterschiede für HPV - assoziierte HNSCC extra - oropharyngealer Lokalisationen^{16,102,81,103-106,29,21,107}. Weitere Untersuchungen sind notwendig, um die pathogenetische Relevanz der Tumor - treibenden HPV - Infektion in Mundhöhle und Larynx abschließend einordnen zu können. Anzumerken ist, dass die Rate HPV - positiver OPSCC in unserer Kohorte mit etwa einem Drittel im Bundesvergleich eher niedrig ist¹⁰⁸. Dies bedingt sich durch den Einschlusszeitraum mit Start im Jahr 2000 und damals noch geringer Prävalenz HPV - positiver OPSCC und die vergleichsweise hohe Rate Noxen - assoziierter Karzinome in Mecklenburg - Vorpommern.

Auch unsere *in vitro* Ergebnisse bzgl. des BCL2 Inhibitors ABT - 199 bedürfen weiterer (klinischer) Studien, ehe sie in den Alltag übersetzt werden können. Die Hemmung dieses anti - apoptotischen Moleküls in Kombination zur Chemotherapie könnte potenziell helfen, die Cytotoxizität zu erhöhen und die EMT als Cisplatin - Resistenzmechanismus zu umgehen.

Der am HNSCC - Tumormaterial prüfbare BCL2 Status könnte bessere Vorhersagen ermöglichen, um perspektivisch individuellere Therapieentscheidungen zu finden und das Konzept der personalisierten Medizin zu fördern ^{48,53}.

Immuncheckpointmoleküle wie CMTM6 oder CTLA - 4 könnten interessante Marker für zielgerichtete Therapieentscheidungen darstellen und weitere Anstrengungen zur Forschung im Bereich der Immunevasion erscheinen sinnvoll. Bisherige Studien zeigten keinen Benefit einer adjuvanten CTLA - 4 - Hemmung im HNSCC ^{100,101,109,109}. Zudem kann die Tumorzell - ständige Expression dieses Moleküls anders als PD - L1 nicht mittels Immunhistochemie innerhalb eines Arbeitstages ermittelt werden. Dies schränkt das Potential von CTLA - 4 als Biomarker in der Prädiktion für anti - CTLA - 4 - gerichtete Therapien ein.

Eine Phase IIa Studie zeigte 2021, dass die neoadjuvante Immuncheckpoint - Blockade durch Nivolumab und Ipilimumab in etwa einem Drittel der evaluierbaren Fälle (n = 23) das Tumolvolumen um > 90% reduzierte ¹¹⁰. In der Gruppe mit diesem guten Ansprechen war der PD - L1 CPS höher als in der Gruppe ohne Therapieansprechen (p = 0,1). Dies stärkt den Stellenwert der PD - L1 Immunhistochemie in ihrer Funktion als Biomarker. Trotz der vielversprechenden Daten sind neben den Kosten bei der Gabe immunmodulatorischer Therapien Nebenwirkungen (Enteritis, Hepatitis u.a.) zu bedenken ¹¹¹. Aktuelle Untersuchungen prüfen, ob neben Pembrolizumab weitere der in Abbildung 8 gezeigten, für unterschiedliche Tumorentitäten aktuell zugelassenen Immuncheckpoint - Inhibitoren nach Nutzen - / Risikoabwägung eine Standardmäßige Anwendung im HNSCC finden werden ¹¹².

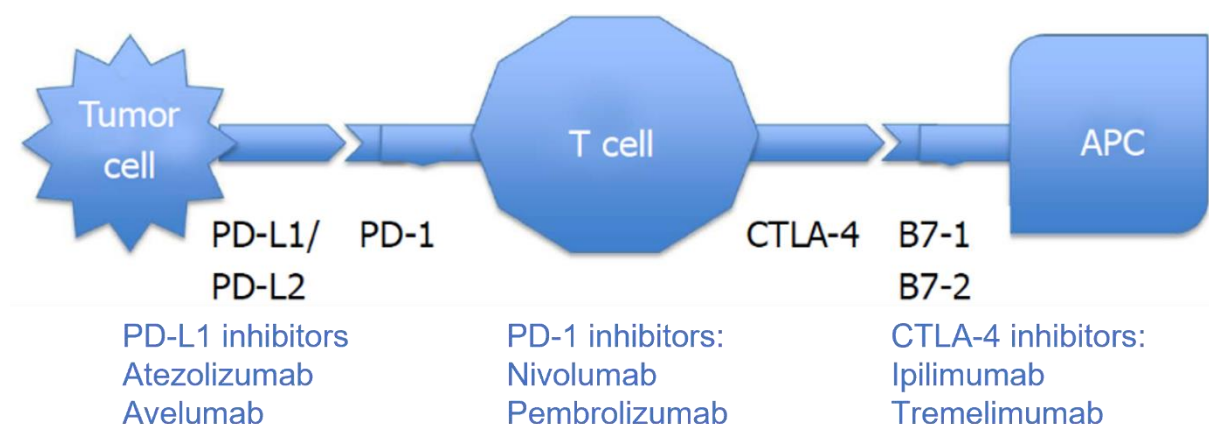


Abb. 8 | Schematische Darstellung von Checkpoint - Inhibitoren.

APC, Antigen präsentierende Zelle; PD - L1, Programmed cell death ligand 1; PD - 1, Programmed cell death protein 1; CTLA - 4, Cytotoxic T - Lymphocyte - Associated Protein 4; B7, Oberflächenligand auf Immunzellen mit kostimulatorischer Funktion für die Immunzellregulation. Modifiziert nach Quelle: ¹¹²

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6. Abkürzungsverzeichnis

- BCL2 - B - cell lymphoma 2
- CMTM6 - CKLF Like MARVEL Transmembrane Domain Containing 6
- CPS – Combined Positive Score
- CTLA4 - Cytotoxic T - Lymphocyte - Associated Protein 4
- EMT – epitheliale mesenchymale Transition
- EGFR – Epidermal Growth Factor Receptor
- HNSCC - Head and Neck Squamous Cell Carcinoma
- FOXP3 - forkhead box P3
- HPV - Humanes Papillomavirus
- IFN – Interferon
- LSCC – Laryngeal Squamous Cell Carcinoma
- NGS - Next Generation Sequencing
- OED – Oral Epithelial Dysplasia
- OPMD – Oral potentially malignant Disorders
- OS – Overall Survival
- OPSCC – Oropharyngeal Squamous Cell Carcinoma
- OSCC – Oral Squamous Cell Carcinoma
- PD - 1 – Programmed Cell Death Protein 1
- PD - L1 - Programmed Cell Death Ligand 1
- pRb – Retinoblastom (Protein)
- TILs – Tumor Infiltrating Lymphocytes
- TAMs – Tumor Associated Macrophages
- UICC - Union for International Cancer Control
- WHO - World Health Organization

7 Curriculum vitae und Publikationsverzeichnis

7.1 Curriculum vitae

Aus datenschutzrechtlichen Gründen entfernt.

7.2 Publikationsverzeichnis

Originalarbeiten:

- Inhibition of Platin - induced BCL2 Increase Overcomes Chemoresistance In Squamous Cell Carcinoma of the Head and Neck Through Resensitization to Cell Death. **Becker AS**, Klauk F, Freitag T, Strüder DF, Schneider B, Zimpfer AH, Maletzki C. Transl Oncol. 2025 Feb 18;53:102308. doi: 10.1016/j.tranon.2025.102308. Impact Factor 4,5
- CMTM6 status predicts outcome in head and neck squamous cell carcinoma. **Becker AS**, Zonnur S, Zimpfer AH, Krause M, Schneider B, Strüder DF, Burmeister A, Erbersdobler A, Junghanss C. Maletzki C. Discov Oncol. 2024 Dec 4;15(1):745.doi: 10.1007/s12672 - 024 - 01554 - 4. Impact Factor 2,8
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- p16 overexpression identifies oncogenic high - risk HPV infection in non - oropharyngeal squamous cell carcinoma of the head and neck. **Becker AS**, Merkl J, Strüder DF, Maletzki C, Zimpfer AH. Head Neck. 2024 Oct;46(10):2569 - 2581.doi: 10.1002/hed.27764. Impact Factor 2,4
- Diagnosis of differentiated dysplasia as a variant of oral epithelial dysplasia. **Becker AS**, Holm M, Liese J, Engel N, Zimpfer AH. Oral Dis. 2024 Oct;30(7):4185 - 4194.doi: 10.1111/odi.14846. Impact Factor 2,9
- Differential Expression of CKLF - like MARVEL Transmembrane Domain - Containing Protein 6 and Programmed Cell Death Ligand 1 as Prognostic Biomarkers in Upper Tract Urothelial Carcinoma. Kdimati S, Christoph C, Glass Ä, Engel N, Dräger DL, Maletzki C, **Becker AS**, Zimpfer A. Int J Mol Sci. 2024 Mar 20;25(6):3492. doi: 10.3390/ijms25063492. PMID: 38542462. Impact Factor 4,9
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- Evaluation of the Cellular Dissociation Grading, Based on Tumor Budding and Cell Nest Size, in Squamous Cell Carcinoma of the Penis. Derani H, **Becker AS**, Hakenberg O, Erbersdobler A. *Cancers*. 2022 Oct 9;14(19):4949. doi: 10.3390/cancers14194949. Impact Factor 6,6
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- Neutrophil Granulocytes in Ovarian Cancer - Induction of Epithelial - To - Mesenchymal - Transition and Tumor Cell Migration. Mayer C, Darb - Esfahani S, **Meyer AS**, Hübner K, Rom J, Sohn C, Braicu I, Sehoul J, Hänsch GM, Gaida MM. *J Cancer*. 2016 Mar 10;7(5):546 - 54. doi: 10.7150/jca.14169. Impact Factor 3,6
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Sonstige Originalarbeiten:

- Clinical characteristics, treatment outcomes and potential novel therapeutic options for patients with neuroendocrine carcinoma of the prostate. Apostolidis L, Nientiedt C, Winkler EC, Berger AK, Kratochwil C, Kaiser A, **Becker AS**, Jäger D, Hohenfellner M, Hüttenbrink C, Pahernik S, Distler FA, Grüllich C. *Oncotarget*. 2019 Jan 1;10(1):17 - 29. doi: 10.18632/oncotarget.26523

Peer Review:

- mit geteilter Erstautorenschaft*:
Becker AS*, Oehmcke - Hecht S*, Dargel E, Kaps P, Freitag T, Kreikemeyer B, Junghanss C, Maletzki C. Preclinical *in vitro* models of HNSCC and their role in drug discovery - an emphasis on the cancer microenvironment and microbiota. Expert Opin Drug Discov. 2025 Jan;20(1):81 - 101. doi: 10.1080/17460441.2024.2439456. Impact Factor 6,0.
- Im März 2025 *under Review*:
CME - Artikel zum Thema „Mundhöhlenkarzinom und orale epitheliale Dysplasie: prä - und postoperative Diagnostik“. Liese J, **Becker AS**. Pathologie. Impact Factor 0,9.

Vorträge:

- **AS. Becker**: Stellenwert von HPV - Hochrisikotypen in der Karzinogenese und Prognose oraler und laryngealer Plattenepithelkarzinome unter Berücksichtigung der neuen WHO - Klassifikation. Eingeladener Vortrag im Rahmen des Journal Clubs am 12.01.2024 der Klinik und Poliklinik für Hals - , Nasen - , Ohrenkrankheiten, Kopf - und Halschirurgie der Uniklinik Greifswald, 12.01.2024, Uniklinikum Greifswald.
- **AS. Becker**: Immunsystem, PDL1 und CMTM6 im fortgeschrittenen HNSCC. Regionaltagung der Pathologen/innen aus Mecklenburg - Vorpommern 2023, 24.06.2023, Burg Stagaad.

Kongressbeiträge / zitierfähige Abstracts:

- **Becker AS**, Holm M, Liese J, Zimpfer A: The concept of “differentiated” dysplasia in oral potential malignant disorders and its translation to daily practice. 106. Jahrestagung der Deutschen Gesellschaft für Pathologie, 01.06. - 03.06.2023, Leipzig
- Kluge C, Zimpfer A, Strüder D, Erbersdobler A, Maletzki C, **Becker AS**: CMTM6 - Status in Head & Neck Squamous Cell Cancer Predicts Survival and Response to Radiochemotherapy. 106. Jahrestagung der Deutschen Gesellschaft für Pathologie, 01.06. - 03.06.2023, Leipzig
- **Becker AS**, Zimpfer A, Schofeld C, Schneider B, Strueder D, Erbersdobler A, Maletzki C: CMTM6 - Status in Head & Neck Squamous Cell Carcinomas predicts Survival and Response to Radiochemotherapy and PD - 1 Inhibitor Treatment. USCAP Annual Meeting 2023, 11.03. - 16.03.2023 New Orleans, Louisiana, USA

- **Meyer AS**, Kriegsmann M, Waldburger N, Sinn P: Frequency and significance of basal phenotype in ER - positive breast cancer. 101. Jahrestagung der Deutschen Gesellschaft für Pathologie, 22.06. - 24.06.2017, Erlangen
- **Meyer AS**, Wan S, Roessler S, Pinna F, Marquardt J, Schirmacher P, Breuhahn K: Identification of potential driver genes involved in chromosomal instability and tumor cell migration in hepatocarcinogenesis. 31. Jahrestagung der Deutschen Arbeitsgemeinschaft zum Studium der Leber 2015, München
- **Meyer AS**, Schumacher C, Möller P, Lennerz JK: MET expression status predicts TKI - response in EGFR - mutant lung adenocarcinoma. USCAP Annual Meeting 2014, 01.03.2014 - 07.03.2014, San Diego, California, USA
- **Meyer AS**, Möller P, Lennerz JK: Colocalization algorithms for the conversion of immunohistochemistry into virtual multicolor stains. USCAP Annual Meeting 2013, 02.03. - 03.03.2013 01.03.2014 - 07.03.2014, Baltimore, Maryland, USA

Für die 108. Jahrestagung der Deutschen Gesellschaft für Pathologie 2025 eingereichte Abstracts (im Frühjahr 2025 *under Review*):

- Folkers K, Obermeit L, Hühns M, **Becker AS**: Frequency Of Human Papillomavirus - associated Oral Epithelial Dysplasia And Its Potential For Malignant Transformation.
- Vetter A, Hühns M, **Becker AS**: HPV Status and *TP53* mutations in nasal squamous cell carcinomas.

Drittmittel, Stipendien, Preise und Engagement in beruflichen Gesellschaften:

- Forschungsförderung der Medizinischen Fakultät der Rostocker Universität (FORUN - Programm 2025) zum Thema „Identifikation onkogener Treiber in der adulten laryngealen Papillomatose mit maligner Transformation“
- Forschungsförderung der Medizinischen Fakultät der Rostocker Universität (FORUN - Programm 2022) zum Thema „Identification of Predictive Biomarkers for Immuncheckpoint - Inhibitors in Head and Neck Squamous Cell Cancer“

- Fördermittel aus „Stiftungen und Preise 2014“ der Medizinischen Fakultät Heidelberg zum Thema „Motogene Faktoren im humanen HCC“
- Promotionsstipendium “Experimentelle Medizin” der Universität Ulm, Förderungsphase 01.03.2012 - 31.12.2012: „c - Met als Biomarker im EGFR - mutierten nichtkleinzelligen Lungenkarzinom“
- Promotionspreis der Deutschen Gesellschaft für Pathologie 2012, 96. Jahrestagung der Deutschen Gesellschaft für Pathologie 20.05.2012 - 23.05.2012, Berlin: “Colocalization algorithms for the conversion of immunohistochemistry into virtual multicolor stains”
- Seit 2024 Vorsitz der Arbeitsgruppe Kopf - Hals - Pathologie der Deutschen Gesellschaft für Pathologie

Pandemie - bedingte Einschränkungen zu wissenschaftliche Themen:

Akzeptierter Beitrag (Vortrag) auf der 104. Jahrestagung der Gesellschaft für Pathologie 2020 (Pandemie - bedingt bei virtueller Tagung zurückgezogen) zum Thema:

Immunohistochemical analysis of potential biomarkers predicting response to Immunotherapy in Advanced Melanoma

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Background

Metastatic cutaneous Melanoma (MM) is an aggressive malignancy. Immune checkpoint inhibitors (ICI) are now standard therapy, but are only effective in a subset of patients and reliable biomarkers (BM) predicting clinical benefit are needed. To identify simple assessable BM, we have retrospectively analyzed eight potential BM (PD - L1 / - L2, CD3/4/8 = TILs, β 2 - Microglobulin (β 2M), HLA - ABC / - DR) in stage IV MM patients before receiving ICI.

Methods

Whole slide sections of archived metastatic tumor tissue from. stage IV MM patients starting ICI between 2015 and 2017 were stained for the named markers above. Results were evaluated using common scores (PD - L1/2: TPS/MEL - Score; CD3/4/8: semiquantitative TIL - grade counting density and distribution of TILs at the tumor periphery and within the tumor by a 4 - tiered system; β 2M, HLA - ABC / - DR: IRS on tumour cells and TIL - grade on immune cells) and correlated with ICI - response (partly after exclusion of non - interpretable TIL - staining results in biopsy specimen).

Results

Of the 50 patients, 70% were male, 30% female, median age at start ICI was 63 years, the tissue available for examination was most commonly obtained from lymph node metastasis (42%). ICI was first line therapy in 32% of the patients and 8% showed CR, 6% PR, 18% SD, 14% mixed response and 54% PD. Tumour samples (76% resections, 24% biopsies) were in 24% positive for PD - L1 (TPS>5%), which correlated significantly with expression of β 2M ($r=0,35$; $p=0,04$), HLA - ABC ($r=0,3$; $p=0,04$), HLA - DR ($r=0,39$; $p=0,006$) on tumour cells and the amount of CD8+ intratumoural TILs ($r=0,36$; $p=0,01$). Intratumoural CD3+ as well as CD8+ TILs were lower in patients with PD compared to the others (CD3: $p=0,0008$; CD8: $p=0,07$ and $p=0,02$ in the subgroup of all examined non - LK - Metastases). Patients with expression of β 2M or HLA - ABC (IRS >4) tended to have a longer PFS ($p=0,07$; correlation of the both $r=0,84$; $p<0,0001$). Tumour cells of a long - time responder (PFS 31 months) showed loss of β 2M in a re - biopsy at timepoint of progression. PD - L1 / - L2 status and CD4+ - TILs did not correlate with response to ICI.

Conclusion

In the diverse setting of metastatic melanoma, we found that the presence of CD3+ - and CD8+ - TILs, β 2M, HLA - ABC and HLA - DR significantly correlated with response to ICI. In one patient, loss of β 2M was associated with secondary resistance to ICI. However, PD - L1/2 expression showed no significant association with response to ICI.

8 Selbstständigkeitserklärung

Aus datenschutzrechtlichen Gründen entfernt.

9 Danksagung

Aus datenschutzrechtlichen Gründen entfernt.

10 Schriftenverzeichnis der zugrundeliegenden Originalarbeiten

Die kumulative Habilitation basiert auf den folgenden Originalarbeiten:

- I. Becker, Anne - Sophie; Holm, Maximiliane; Liese, Jan; Engel, Nadja; Zimpfer, Annette Helene (2024): Diagnosis of differentiated dysplasia as a variant of oral epithelial dysplasia. In: *Oral diseases* 30 (7), S. 4185–4194. DOI: 10.1111/odi.14846. Impact Factor: 2,9
- II. Becker, Anne - Sophie; Merkel, Jenny; Bozkurt, Inci; Strüder, Daniel Fabian; Maletzki, Claudia; Hühns, Maja; Zimpfer, Annette Helene (2024): p16 overexpression identifies oncogenic high - risk HPV infection in non - oropharyngeal squamous cell carcinoma of the head and neck. In: *Head & Neck* 46 (10), S. 2569–2581. DOI: 10.1002/hed.27764. Impact Factor: 2,4
- III. Becker, Anne - Sophie; Klauk, Friederike; Freitag, Thomas; Strüder, Daniel Fabian; Schneider, Björn; Zimpfer, Annette; Maletzki, Claudia (2025): Inhibition of platin - induced BCL2 increase overcomes chemoresistance in squamous cell carcinoma of the head and neck through resensitization to cell death. In: *Translational oncology* 53, S. 102308. DOI: 10.1016/j.tranon.2025.102308. Impact Factor: 4,5
- IV. Becker, Anne - Sophie; Wieder, Nicolas; Zonnur, Sarah; Zimpfer, Annette; Krause, Mareike; Schneider, Björn; Strüder, Daniel Fabian; Burmeister, Ann - Sophie; Erbersdobler, Andreas; Junghanß, Christian; Maletzki, Claudia (2024): CMTM6 status predicts survival in head and neck squamous cell carcinoma and correlates with PD - L1 expression. In: *Discover oncology* 15 (1), S. 745. DOI: 10.1007/s12672 - 024 - 01554 - 4. Impact Factor: 2,8
- V. Becker, Anne - Sophie; Kluge, Cornelius; Schofeld, Carsten; Zimpfer, Annette Helene; Schneider, Björn; Strüder, Daniel Fabian; Zunke, Emily; Redwanz, Catarina; Ribbat - Idel, Julika; Idel, Christian; Maletzki, Claudia (2023): Identifying Predictive Biomarkers for Head and Neck Squamous Cell Carcinoma Response. *Cancers* 2023, 15, 5597. In: *Cancers* 16 (20). DOI: 10.3390/cancers16203554. Impact Factor: 5,2

11 Anhänge (zugrundeliegende Originalarbeiten)

ORIGINAL ARTICLE

Diagnosis of differentiated dysplasia as a variant of oral epithelial dysplasia

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Abstract

Objectives: The World Health Organization's definition of oral epithelial dysplasia includes differentiated dysplasia, which is defined by purely architectural abnormalities of oral mucosa without cytological changes. We analysed differentiated dysplasia's frequency, progression risk and correlation with oral brush cytology.

Materials and Methods: Cytoarchitectural criteria and expression patterns of keratin 13/17 and ki67 were studied in oral biopsies clinically diagnosed with leukoplakia. Biopsies were assessed for dysplasia and its grade. Available brush cytology findings were obtained from clinical records.

Results: We included 159 biopsies from 112 patients (33% differentiated dysplasia; 27% keratosis without dysplasia; oral epithelial dysplasia with atypia of mild, moderate and severe degree including invasive cancers in 9%, 8% and 7%, respectively). Keratin 13 loss and keratin 17 gain were higher in differentiated-dysplasia cases ($p < 0.0001$), which had the highest hypergranulosis frequency. Keratin 17 expression was associated with higher malignant-transformation rates ($p = 0.0028$). The transformation rate and time were comparable between dysplasia with atypia and differentiated-dysplasia cases, which had higher progression rates and shorter time periods than keratosis cases without dysplasia ($p = 0.08$). Cytology prior to differentiated dysplasia all indicated normal oral mucosa.

Conclusions: Keratin 17 but not oral brush cytology can help identify patients with differentiated dysplasia with higher risk for malignant transformation.

KEYWORDS

differentiated dysplasia of the mouth, keratin 13, keratin 17, oral brush biopsy cytology, oral potentially malignant disorders, oral squamous cell carcinoma

1 | INTRODUCTION

Oral potentially malignant disorders (OPMDs) include diseases where the oral mucosa shows increased risk of development of oral squamous cell carcinoma (OSCC) (Warnakulasuriya et al., 2021).

As defined in the 5th edition of the World Health Organization's (WHO's) Classification on Head and Neck Tumours the spectrum of OPMD covers different entities with diverse disease-causing mechanisms (Muller & Tilakaratne, 2022; Vigneswaran et al., 2022). The term 'potentially malignant' indicates a variable transformation rate

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with dependency on the underlying condition (Odell et al., 2021). Patients remain under surveillance at specialized oral cancer centres according to the exact form of OPMD, patient history and prior treatments.

Lesions with a high risk of progression need to be identified to optimize surveillance, prevent OSCC and minimize the workload of regular monitoring. In addition to clinical risk factors such as smoking or anatomic location, tissue-based risk stratification of OPMD biopsies is used to classify patients into groups of high and low risk. Lesions presenting clinically as oral leukoplakia are the most common manifestation of OPMD, with an estimated global prevalence of 2%–3% (Reibel et al., 2017). In histopathological assessment, oral leukoplakia mostly shows hyperkeratotic oral mucosa. If such keratotic lesions show cytological or architectural changes, they are dysplastic (Reibel et al., 2017).

Among all OPMDs, oral epithelial dysplasia (OED) has the highest risk of developing OSCC (Woo, 2019). Dysplasia with atypia is graded as mild, moderate and severe, which have increasing risks for cancer development over time (Reibel et al., 2017). Morphological changes in dysplastic lesions may be cytological, architectural or mixed. Most dysplastic lesions are considered mixed. Because dysplasia can have any combination of features, purely architectural changes in the absence of cytologic changes warrant labelling of lesions as 'dysplastic' (Mahmood et al., 2022; Odell et al., 2021). This includes samples that have overlapping features with keratosis without dysplasia but have increased risk of malignant progression.

In daily practice, such dysplastic lesions might be missed due to bland cytonuclear morphology and could be diagnosed as keratosis without dysplasia (Odell et al., 2021). The OED variant based purely on architectural features showing an altered differentiation of keratinocytes is called differentiated or architectural dysplasia (Brouns et al., 2022; Wils et al., 2020). Differentiated dysplasia is distinguished in this work from the other manifestations of OED with cytomorphological or mixed characteristics, which is referred to simply as OED hereafter. Although there are diagnostic criteria for purely architectural dysplasia, there is a lack of precise data on the exact risk of malignant potential for these lesions (Brouns et al., 2022).

Oral epithelial dysplasia commonly loses expression of keratin 13 (K13) and gains expression of keratin 17 (K17) (Wils et al., 2020). K13 is expressed in the healthy lining mucosa of the oral cavity but is lost in OED and well-differentiated OSCC (Boricic et al., 2022; Matsuhira et al., 2015). K17 is normally not expressed in the non-keratinized stratified epithelium but increases if cancerous transformation occurs (Dasgupta et al., 2018; Farrukh et al., 2015; Ikeda et al., 2020; Matsuhira et al., 2015; Nobusawa et al., 2014; Podoll et al., 2017; Vaidya et al., 2022; Wils et al., 2020; Yamashina et al., 2014). Wils et al. (2020) recently showed that a decreased expression of K13 and an increased expression of K17 are indicative markers for differentiated dysplasia of the mouth. In their cohort, K13 loss in oral leukoplakia samples without overt cytomorphological abnormalities identified patients at increased risk for progression.

Besides the invasive method of taking a biopsy to diagnose an OPMD as the gold standard, brush biopsy cytology can be easily performed with less cost and discomfort to the patient (Alsarraf et al., 2018; H Alsarraf et al., 2018; Idrees, Farah, et al., 2022; Idrees, Shearston, et al., 2022; Kujan et al., 2021; Wang et al., 2023). This technique can be used to diagnose OPMDs (Alsarraf et al., 2018; Neumann et al., 2022). Diagnostic accuracy is best for high-grade OED (moderate to severe dysplasia) and invasive OSCC, but other OPMDs such as oral lichen planus (OLP) or proliferative verrucous hyperplasia (PVL) can also be diagnosed, especially with the addition of immunohistochemistry (Böcking et al., 2011; Idrees, Shearston, et al., 2022; Kujan et al., 2021; Maraki et al., 2006; Yamashina et al., 2014).

The frequency and risk of malignant progression in differentiated dysplasia of the mouth have been shown to be comparable to those of dysplastic lesions showing cytological or mixed features (OED) (Brouns et al., 2022). However, to our knowledge, the value of oral brush biopsy cytology in identifying differentiated dysplasia is unclear. The aims of this study were to (a) examine the frequency and risk of progression of differentiated dysplasia, (b) check whether K13 and K17 immunohistochemistry helps in recognizing differentiated dysplasia and (c) check whether brush biopsy cytology contributes to the identification of differentiated dysplasia.

2 | METHODS

2.1 | Study cohort

This hospital-based retrospective study used biopsies obtained from the lining oral mucosa (vestibular, buccal, tongue, floor of mouth) in which a definitive clinical diagnosis of oral leukoplakia was recognized according to strict criteria (Brouns et al., 2014). Samples from patients with lesions that were clinically suspicious for OLP were evaluated in line with clinical data using direct immunofluorescence to exclude autoimmune blistering diseases of the oral mucosa (Cheng et al., 2016; Müller, 2017; Odell et al., 2021). The inclusion period was January 1, 2014, through March 31, 2023.

The definitive histopathologic diagnosis of specimens was made with respect to clinical data using criteria according to the 4th WHO classification (Reibel et al., 2017) and included the following conditions: (1) differentiated dysplasia, (2) keratosis without dysplasia, (3) OLP, (4) hyperplastic candidiasis, (5) PVL, (6) mild OED, (7) moderate OED, (8) severe OED with purely cytological or mixed cytological-architectural features and (9) OSCC. All data were obtained from clinical and pathological records. If oral brush biopsy cytology was performed up to 6 months prior to the biopsy, results were included in this study.

The four-tiered German schema for reporting cytology results was translated into the Modified Bethesda Cytology system with the aid of DNA-cytometry results (Alsarraf et al., 2018; Andratschke et al., 2015; Bechstedt et al., 2022). For patients with multiple biopsies, the earliest available biopsy was included. Follow-up was



calculated from the time of the first available biopsy and was updated until July 15, 2023, using clinical records and anonymized data from the local cancer registries. Patients were defined as non-progressors when malignant transformation did not occur during follow-up. Patients were defined as progressors when severe dysplasia or OSCC developed during follow-up.

Patients who developed a malignancy within 3 months of biopsy were excluded. Patients with shorter follow-up may not have had a representative biopsy. Informed consent was obtained from all patients. The study was performed in accordance with the Declaration of Helsinki and approved by the local ethics committee of Rostock University Hospital (A2018-0003; A2022-0120).

2.2 | Histology and immunohistochemistry

Sections were re-evaluated using haematoxylin–eosin (HE) stains for the (1) presence and grade of oral dysplasia with atypia, (2) the presence of differentiated dysplasia or (3) any other form of OPMDs. Furthermore, distinct histomorphological patterns (acanthosis, hyperkeratosis, parakeratosis, hypergranulosis, architecture of rete ridges, lichenoid reaction within the superficial submucosa) were graded from 0 (absent) to 3 (severe) by two experienced oral pathologists (AHZ, ASB). Due to the somewhat small sample size and high interobserver variability, verrucous architecture was not included as a parameter in our scheme. Verrucous and papillomatous lesions were excluded.

Periodic acid shift (PAS) stained sections were analysed to identify *Candida albicans* and glycogen distribution patterns. Cases presenting with differentiated dysplasia combined with candidiasis were categorized as differentiated dysplasia. Biopsies originally coded as keratosis without dysplasia but showing architectural anomalies were re-classified as differentiated dysplasia.

Immunohistochemical staining was performed on thin 2-µm sections of formalin-fixed paraffin-embedded tissue after deparaffinization and antigen retrieval (cell conditioning, Ventana Medical Systems Inc., Tucson, AZ, USA, for 24 min). Incubation was then performed with primary antibodies (K13: mouse monoclonal antibody against human K13, clone KS-1A3, 1:50, Thermo Fisher Scientific, Waltham, MA, USA; K17: mouse monoclonal antibody against human K17, SP95, 1:50, Spring Bioscience, Cambridge, UK; and Ki67: 1:500, Mib-1 Dako, Glostrup, Denmark). Next, 3,3'-diaminobenzidine (DAB) was detected using the Dako-kit K8000 according to the manufacturer's instructions.

Keratin expression in the epithelium was classified as 'normal' or 'aberrant' in the most affected area of each sample. K13 loss was defined as aberrant (meaning negative) when K13 was absent in that part of the mucosa. K17 expression was considered aberrant (meaning positive) when the mucosa showed detectable levels, even if only partially. Nuclear staining for Ki67 was evaluated with emphasis on an expanded basal proliferation unit and scored as (1) normal, (2) reactively increased with Ki67-positive cells within but not expanding the suprabasal compartment and

(3) increased (meaning Ki67 positive cells reaching above the suprabasal layer).

2.3 | Statistical analysis

Morphological differences of differentiated dysplasia and other WHO-defined OPMDs within groups were assessed with Fisher's exact or a chi-squared test. A Kruskal-Wallis test compared times and morphological parameters between differentiated dysplastic lesions and the remaining OPMD samples. Survival curves were analysed by a log-rank test and plotted using the Kaplan–Meier method. All statistical analyses were performed using GraphPad Prism (Silicon Valley, CA, USA). Statistical significance was indicated by p -values < 0.05 .

3 | RESULTS

3.1 | Diagnostic groups

Of the 112 patients included in our study, 76% were male and 54% were documented smokers. The median age at diagnosis was 58 years, and follow-up times ranged from 4 to 290 months (median 52 months). Including re-biopsies, 159 samples were included and time-matched brush biopsy cytology was available for 44% of the samples. With the aid of keratin stains, the diagnosis was (1) differentiated or architectural dysplasia in 33%, (2) keratosis without dysplasia in 27%, dysplasia with atypia of (3) mild degree in 9%, (4) moderate degree in 8%, (5) severe degree in 7%, (6) early invasive OSCC in 7%, (7) hyperplastic candidiasis in 3%, (8) lichen planus in 4% and PVL in 2.5% of the samples (Table 1).

The 14 samples fitting in more than one diagnostic group were coded for the one with the highest assumed risk for progression. Differentiated dysplasia was combined with candidiasis (two cases) and oral lichen planus (five cases), but not with carcinoma. Figure 1 shows examples of purely differentiated dysplasia in the oral mucosa. No significant clinicopathological risk factors for differentiated dysplasia were detected.

3.2 | Hypergranulosis and aberrant keratin expression help in detecting differentiated dysplasia

Morphological profiles were compared among the included categories of OPMDs (Table 2). All cases with differentiated dysplasia showed a small-calibre basal cell layer, an expanded stratum intermedium with enlarged eosinophilic cells presenting at least slightly hyperchromatic nuclei with detectable nucleoli, and prominent intercellular bridges. Lichenoid reactions of varying degrees were more frequent than in other OPMDs ($p = 0.03$, Kruskal-Wallis test). In our cohort, one unique characteristic of differentiated dysplasia was hypergranulosis (Figure 1). Although the thickening or de-novo presence of the stratum

TABLE 1 Diagnostic groups and clinicopathological parameters^a.

Diagnostic group	Sample size ^a	Male	Female	Smoking yes/no	Alcohol yes/no	Localization	Trans-formation ^b
Differentiated dysplasia	33	58	42	54/28 18 na	40/39 21 na	Tongue: 32 FOM: 23 Buccal: 28 Vestibule: 17	16
Keratosis without dysplasia	27	73	27	37/38 25 na	25/55 20 na	Tongue: 14 FOM: 23 Buccal: 50 Vestibule: 13	0
Mild dysplasia	9	71	29	86/7 1 na	43/50 1 na	Tongue: 14 FOM: 50 Buccal: 29 Vestibule: 7	0
Moderate dysplasia	8	79	21	69/0 4 na	62/8 4 na	Tongue: 23 FOM: 69 Buccal: 8 Vestibule: 0	15
Severe dysplasia	7	58	42	58/25 17 na	50/33 17 na	Tongue: 17 FOM: 33 Buccal: 33 Vestibule: 17	0
OSCC	7	56	54	82/0	54/0	Tongue: 18 FOM: 45 Buccal: 18 Vestibule: 18	-
Lichen planus	4	33	67	17/50 33 na	0/66 33 na	Tongue: 16,5 FOM: 16,5 Buccal: 67 Vestibule: 0	33
Hyperplastic candidosis	3	100	0	60/20 20 na	60/20 20 na	Tongue: 20 FOM: 0 Buccal: 60 Vestibule: 20	0
PVL	2.5	100	0	50/50	50/50	Tongue: 25 FOM: 25 Buccal: 25 Vestibule: 25	75

Note: Values in %, rounding differences total 100.5%.

Abbreviations: FOM, floor of mouth; na, not assessed; OSCC, oral squamous cell carcinoma; PVL, proliferative verrucous hyperplasia.

^aReferring to all samples ($n = 159$).

^bMalignant transformation referring to all patients ($n = 113$) using the diagnostic code of the first biopsy for patients with rebiopsies.

granulosum was a rather rare finding, it was frequently found in differentiated dysplasia ($p < 0.001$). The confluence of rete ridges was pronounced in these lesions but was also seen in other OPMDs.

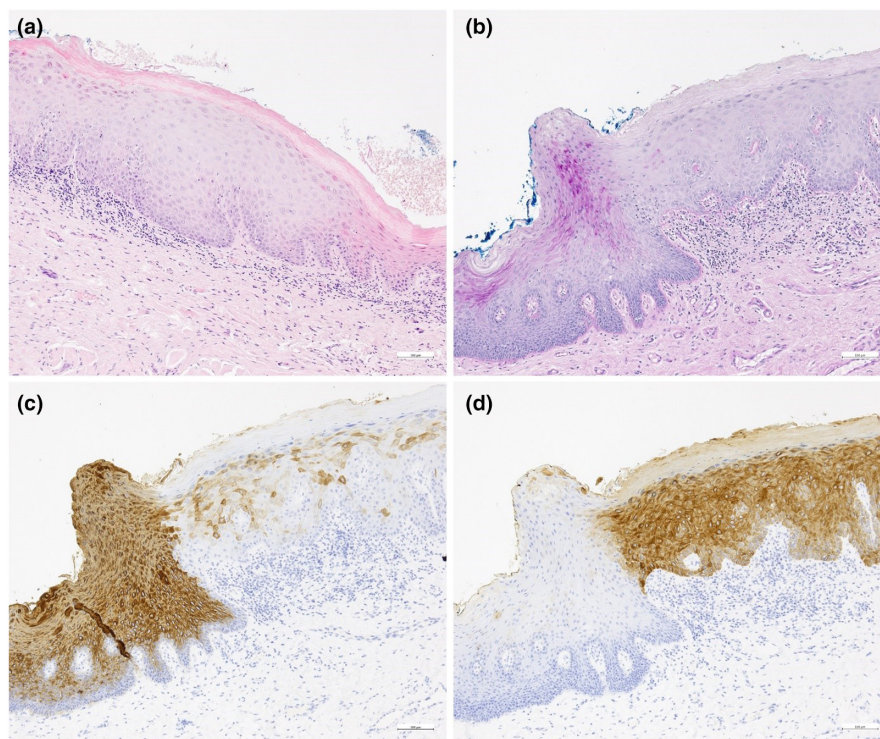
The specimens classified as differentiated dysplasia according to morphological criteria demonstrated a combination of K13 negativity and reciprocal K17 positivity in 88% of cases (Figure 1). This abnormal pattern was detected in 50% of mild dysplasia, 62% of moderate dysplasia, 77% of severe OED/OSCC and 17% of lichen planus cases. The four cases of PVL showed a reciprocal aberrant staining pattern for both keratins. Biopsies originally classified as keratosis without dysplasia were re-classified as differentiated dysplasia if they exhibited at least one morphological criterion of differentiated dysplasia and aberrant keratin expression (K13 or K17 or both), which affected 61 samples (38%). The proliferating compartment (nuclear

Ki67 positivity in squamous cells) was expanded to the suprabasal compartment in 89% of differentiated dysplasia cases. This rate was significantly higher compared to that of proliferation indices for keratosis without dysplasia but lower than that of OED ($p < 0.0001$ for each).

3.3 | K17 expression identifies patients with a higher risk of malignancy

In the 112 patients without invasive cancers at the first presentation, the transformation rate was 15%, while the rate was 7% for patients diagnosed with differentiated dysplasia, 15% for moderate OED, 2.5% for PVL and 2% for lichen planus. None of

FIGURE 1 Example of differentiated dysplasia with PAS, CK13, CK17 staining. The HE-stained section shows a basal layer of small cells with unsuspicious nucleoli but enlarged cells in the suprabasal compartment with eosinophilic cytoplasm, spongiosis and noticeable desmosomes as expression for reduced epithelial cohesions. A lichenoid reaction of moderate degree and hypergranulosis are also present (a). The periodic acid shift-stained section emphasizes reduced glycogen storage and proves the absence of *Candida albicans* (b). Loss of K13 (c) and gain in K17 (d) are depicted, which are not pathognomonic for detecting differentiated dysplasia but are helpful. K13, cytokeratin 13; K17, cytokeratin 17 (100 $\times$).



the included patients diagnosed with mild or severe OED developed (invasive) cancer during the follow-up period. The median transformation time overall was 50.5 months (Figure 2). Patients diagnosed with dysplastic lesions of any degree showing purely cytological and/or mixed features developed carcinoma in 17% of cases. Purely differentiated dysplasia turned malignant in 16% of cases (Table 1).

The time and risk of malignant transformation were comparable between differentiated and OED of any degree, but their difference, either alone or in combination, was not significant when compared to keratosis without dysplasia ($p=0.08$, log-rank-test; Figure 2c). The median transformation time for patients diagnosed with differentiated dysplasia was 110 months, while it was 112 months for patients with OED and undefined for cases of keratosis without dysplasia. None of the patients with low ($n=14$) or severe ($n=2$) OED progressed during follow-up. One case with moderate OED at initial diagnosis progressed within 4 months. One patient with PVL also progressed to OSCC within 4 months. The time to malignant transformation was longer than 6 months in the remaining progressors.

With three of four patients developing an OSCC, PVL had the highest transformation rate and the shortest transformation period for (median 25.5 months). In the entire cohort, patients whose biopsies showed reciprocal abnormal staining for K13/K17 had a higher transformation rate ($p=0.0249$, log-rank test; Figure 2e). Independently of K13 expression, samples showing abnormal K17 expression turned malignant significantly more frequently with a median transformation time of 110 months. In contrast, the median time was 226 months in the K17-negative group ($p=0.0028$; log-rank test; Figure 2d).

In samples of keratosis without dysplasia, differentiated dysplasia and OED of low, moderate and severe degree together ($n=100$), the aberrant keratin pattern of K13/17 identified patients with a significantly shorter transformation time ($p=0.04$; log-rank test; Figure 2f). In the subgroup of differentiated dysplasia, where all samples showed K13 loss and/or K17 gain, there was no statistically significant difference in the transformation time between lesions with single aberrant (either K13 loss or K17 gain) or double aberrant keratin expression. The morphological parameters assessed here (lacking cytological features) failed in detecting patients with a higher transformation rate.

3.4 | Brush biopsy cytology results are unsuspicious in differentiated dysplasia

In the total cohort, brush biopsy cytology results were available in 78 cases, which were all performed within 6 months prior to the biopsy. Of these, 74% were graded as normal oral mucosa, 12% were low-grade squamous intraepithelial lesions, 1% were high-grade squamous intraepithelial lesions and 13% were oral squamous cell carcinoma. Matched biopsies showed a sensitivity of 82% and specificity of 90%.

Of the OED samples showing purely cytological or mixed cytological-architectural features of any degree, 68% were screened by brush biopsy cytology. Of these, 50% showed abnormal results. From the biopsies categorized as differentiated dysplasia, 50% were screened by brush biopsy cytology and all were classified as normal oral mucosa. Therefore, DNA cytometry was not performed for these patients.

TABLE 2 Selected morphological parameters in differentiated dysplasia and other OPMDs^a.

	Differentiated dysplasia	Other OPMDs	
	N = 50	N = 109	
Histological features	Number (%)	Number (%)	p-Value
Acanthosis			
Absent	0	2 (2)	0.47
Minimal	16 (31)	26 (24)	
Moderate	26 (15)	54 (50)	
Brisk	9 (17)	25 (23)	
Hyperkeratosis			
Absent	0	4 (4)	0.95
Minimal	29 (56)	35 (33)	
Moderate	11 (21)	48 (45)	
Brisk	11 (21)	20 (19)	
Parakeratosis			
Absent	8 (15)	14 (13)	0.31
Minimal	34 (65)	48 (45)	
Moderate	5 (10)	44 (41)	
Brisk	4 (8)	1 (1)	
Confluence of rete ridges			
Absent	3 (6)	35 (33)	0.1
Minimal	14 (27)	48(45)	
Moderate	13(25)	14 (13)	
Brisk	19 (37)	10 (9)	
Hypergranulosis			
Absent	15 (29)	81 (76)	<0.001
Minimal	29(56)	19 (18)	
Moderate	7 (13)	7 (7)	
Brisk	0	0	
Lichenoid reaction			
Absent	3 (6)	37 (35)	0.3
Minimal	22 (42)	27 (25)	
Moderate	17 (33)	26 (24)	
Brisk	7 (13)	16 (15)	
Proliferative unit			
Normal	15 (29)	22 (21)	0.047
Suprabasal expanded	30(58)	60 (56)	
Increased	6 (12)	26 (24)	
Keratin 13			
Retained	2 (4)	52 (49)	<0.001
Absent (aberrant)	49 (94)	56 (52)	
Keratin 17			
Absent	6 (12)	56 (52)	<0.001
Present (aberrant)	45 (88)	52 (49)	

Note: Percentages are <100% in cases where not all samples were available for evaluation; >100% in other cases due to rounding (including keratosis without dysplasia, oral epithelial dysplasia with atypia, hyperplastic candidiasis, oral lichen planus, proliferative verrucous hyperplasia).

Abbreviations: OPMDs, oral potentially malignant disorders; K13, cytoK13; K17, cytoK17.

^aValues referring to all samples (n = 159).

4 | DISCUSSION

We investigated the frequency and malignant transformation rate of differentiated or architectural dysplasia of the mouth using biopsies with a clinical diagnosis of oral leukoplakia. We compared the morphology, expression of K13 and K17 and proliferation activity to those of other OPMDs with the aim of optimizing the diagnostic criteria for differentiated dysplasia to harmonize future studies. To determine whether differentiated dysplasia might be missed by brush biopsy cytology, we compared oral brush cytology results of patients who subsequently obtained biopsies yielding a diagnosis of differentiated dysplasia.

In our well-documented but pre-selected cohort of White patients, the general clinicopathologic parameters were representative with rather high rates of having a history of tobacco smoking and alcohol drinking. Differentiated dysplasia is slightly more frequent than keratosis without dysplasia and OED. Differentiated dysplasia can be missed by morphology alone and may be underdiagnosed as keratosis without dysplasia, but we have been using K13 and K17 immunostaining for its detection. This approach might increase the frequency of differentiated dysplasia because K13 can increase or decrease while K17 does the opposite in lining oral mucosa with inflammatory damage but no premalignant transformation (Clausen et al., 1986; Jacques et al., 2009; Ouhayoun et al., 1985).

To minimize the rates of false-positive differentiated dysplasia, we used strict histomorphological criteria to correctly identify samples. Matching to our diagnostic work-up, the event of and time to malignant transformation in our sample was similar for differentiated and OED showing either purely cytologic or mixed features. When compared to keratosis without dysplasia, both the dysplastic groups (differentiated and OED of any degree) showed a trend of shorter progression periods. However, this was not statistically significant (for either group alone and for the combination of groups).

In general, our findings are in line with the conclusions from Wils et al. (2020) who showed a comparable risk of malignancy for differentiated oral dysplasia to that of OED showing cytologic or mixed features, but they observed a longer transformation time in the latter. In their sample, oral leukoplakia specimens with lost expression of K13 had the highest risk of progression. They concluded that patients with no dysplasia who retained K13 positivity in their oral leukoplakia biopsies have a very low risk of malignant transformation and could thus have less intensive surveillance.

In our cohort, the K13-positive subgroup included mainly patients with keratosis without dysplasia. Within the samples of differentiated dysplasia, only 2 of 51 samples stained positive for K13. This underpins the concept of an oral preneoplastic lesion missing overt cytonuclear changes even with disturbed maturation, which can be highlighted by abnormal keratin expression. Partly due to the diagnostic criteria applied, all our samples classified as differentiated dysplasia exhibited aberrant K13 and/or K17 expression. We observed this pattern in more than half of all our OED tissue samples, as previously described (Boricic et al., 2022; Nobusawa et al., 2014), but not in keratosis without dysplasia. In contrast to the findings by Wils et al. (2020) K13 negativity was associated with

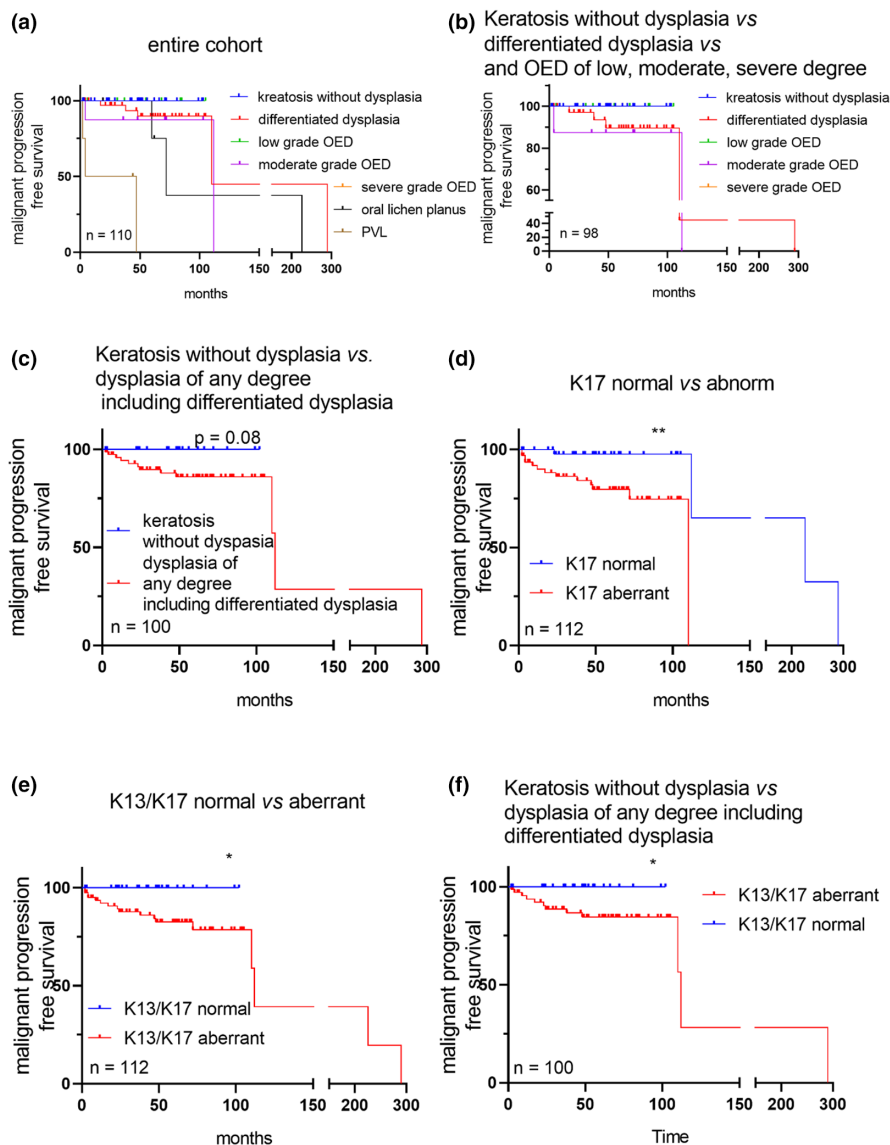


FIGURE 2 Malignant progression-free survival of the cohort stratified by (a) WHO-defined OPMDs with the incorporation of differentiated dysplasia. The Kaplan-Meier survival curve demonstrates that the malignant progression-free survival of patients presenting with differentiated dysplasia (red) equals that of patients with OED with purely cytomorphological or mixed features (green: low degree, purple: moderate degree, orange: severe degree). (b) A Kaplan-Meier survival curve demonstrating the malignant progression-free survival of patients clinically presenting with oral leukoplakia, which were coded as keratosis without dysplasia (blue), differentiated dysplasia (red) or OED of any degree (green) by histomorphology. None of the patients showing keratosis without dysplasia with regular K13/K17 expression at first presentation progressed during follow-up. (c) A Kaplan-Meier survival curve showing the trend of a shorter malignant progression-free survival of oral leukoplakia patients that presented without (blue) or with (red) any type of dysplasia, either differentiated or with either purely cytomorphological or mixed features. (d,f) Kaplan-Meier survival curves showing a shorter malignant progression-free survival of oral leukoplakia patients that presented regularly without (blue) or abnormally with (red) K17 expression in their biopsies in the entire cohort (d: $p=0.0028$) and the subgroup presenting with keratosis without dysplasia or any type of dysplasia (f: $p=0.0442$). (e) A Kaplan-Meier survival curve shows a shorter malignant progression-free survival of patients with K13 loss/K17 gain ($p=0.0249$). None of the patients showing regular K13/K17 expression at first presentation progressed during follow-up. Diagnoses used samples at the time of first sampling and refer to the entire collective. OED, oral epithelial dysplasia with atypia; OPMDs, oral potentially malignant disorders; PVL, proliferative verrucous hyperplasia; K13, cytoK13; K17, cytoK17.

a significantly shorter transformation time in both the entire cohort and in any subgroup. On the contrary, increased K17 helped to identify patients with shorter progression periods compared to their K17-negative counterparts. This was observed for all patients, different subgroups and in combination with K13 loss.

The immunoprofile of K13 negativity and reciprocal K17 positivity was seen in 17% of cases with lichen planus. Relying on this ancillary technique therefore carries the risk of overdiagnosing lichen planus as dysplasia. Although this frequency of altered keratin expression leaves the question concerning the malignant potential in lichen planus

unresolved, the diagnosis of this entity requires a clinicopathologic correlation and one should not rely on K13/K17 staining. Besides lichen planus, another differential diagnosis of differentiated dysplasia includes early lesions of PVL. These lesions are usually flat without atypia, but do show a host immune response. In such cases, the diagnosis must be changed to PVL at a later stage when the diagnosis becomes apparent to respect the higher transformation rate and different management (Afkhami et al., 2023; Gillenwater et al., 2014).

Independent of keratin staining, we focused on morphology and identified hypergranulosis within certain parameters as a reliable finding in differentiated dysplasia. A prominent granular layer can occur in any type of OPMD but is frequently observed in PVL (Gillenwater et al., 2014). With regard to the concept of differentiated dysplasia, we emphasize the diagnosis of PVL. OPMDs include a spectrum of lesions primarily defined by differentiated changes like verrucous hyperplasia with mostly bland cytomorphology, especially at early stages. Although its transformation rate is one of the highest among all OPMDs, mutational status in PVL differs from that of typical OSCC with a lower copy number variation but higher frequencies of telomerase reverse transcriptase (TERT) promoter mutations (Afkhami et al., 2023).

In cases of differentiated dysplasia with performance of brush biopsy cytology up to 6 months earlier than the diagnosis of differentiated dysplasia, all results showed normal oral mucosa according to the modified Bethesda Cytology system. This was to be expected because the technique itself relies on mainly cytonuclear changes. As the technique performed well in the other OPMD samples, additional staining of smears for K13/K17 might help to identify patients with differentiated dysplasia, which might be too intensive in terms of time and costs. However, to our knowledge, this method has not been analysed for its capacity in screening differentiated dysplasia of the oral cavity, and leukoplakia samples with bland brush biopsy cytology fit best in the category of normal oral mucosa, independently of altered epithelial architecture.

According to morphology, an oral biopsy is considered as architecturally dysplastic if there are architectural changes, especially within the lower and middle third of the lining oral mucosa, in combination with aberrant expression of K13 and/or K17. Concerning the associated risk for malignant transformation in differentiated dysplasia, further studies are needed to elucidate its molecular background. If potential genetic alterations, possibly other than p53, are found, they might prove the concept of differentiated dysplasia. Before characterizing corresponding samples at the molecular level, strict diagnostic criteria are needed.

K13/K17 stains help to diagnose differentiated dysplasia in addition to the proposed diagnostic criteria by morphology using HE and PAS-stained sections. Compared to keratosis without dysplasia, the proliferation rate is higher in differentiated dysplastic lesions. In doubtful cases, Ki67 assessment serves as an additional diagnostic aid that supports the diagnosis of differentiated dysplasia if Ki67-positive nuclei are located in the basal and suprabasal layer according to brush biopsy cytology. However, it has no additional diagnostic value if no obvious cytonuclear atypia suggesting OED are visible.

This study has several limitations. Our small single-centre cohort had limited follow-up. Although the follow-up times were comparable to other studies in this field, the time spans to malignant progression, which sometimes happens years after the initial diagnosis of oral leukoplakia, warrant longer observation periods. The wash-out definition of differentiated dysplasia represents a grey zone for oral epithelial dysplasia with mild atypia.

K13 and K17 expressions help to define the entity but occur in inflammatory conditions or dysplasia with atypia as well. K13 and K17 were only measured by IHC, not at the mRNA level. Two included cases showed differentiated dysplasia with *C. albicans* and were coded as differentiated dysplasia without knowing the patients' outcomes after antimycotic treatment. Finally, there was a limited number of brush biopsy cytology results matched to corresponding biopsies.

5 | CONCLUSIONS

Within the WHO grading system for oral dysplasia, differentiated dysplasia represents the end of the spectrum and is characterized by purely architectural abnormalities. In our cohort, cases of differentiated dysplasia had a comparable risk for malignant transformation to that of dysplastic lesions showing cytonuclear or mixed cytonuclear/architectural features. Oral brush cytology seems to be of no value in detecting differentiated dysplasia. Using histology, differentiated dysplasia can be identified by abnormal K13/K17 staining in keratosis without dysplasia showing altered keratinization patterns without brisk cytologic atypia. Patients without oral epithelial dysplasia and normal K13/K17 staining have a very low risk of malignant progression.

AUTHOR CONTRIBUTIONS

Anne-Sophie Becker: Methodology; investigation; data curation; validation; writing – original draft; writing – review and editing; software; formal analysis; visualization. **Maximiliane Holm:** Investigation; writing – review and editing. **Jan Liese:** Resources; writing – review and editing. **Nadja Engel:** Resources; writing – review and editing. **Annette Helene Zimpfer:** Project administration; investigation; conceptualization; methodology; writing – review and editing; data curation; validation; supervision; software; visualization.

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interest.

DATA AVAILABILITY STATEMENT

The datasets generated during and/or analysed during the current study are available from the corresponding author on reasonable request.

CONSENT TO PARTICIPATE

Informed consent was obtained from all individual participants included in the study.

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ORIGINAL ARTICLE

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p16 overexpression identifies oncogenic high-risk HPV infection in non-oro-pharyngeal squamous cell carcinoma of the head and neck

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Abstract

Background: Human papillomavirus (HPV) is an increasing risk factor for cancer. HPV-associated oropharyngeal squamous cell carcinoma (OPSCC) is associated with a favorable outcome. Blockstaining for p16 is a surrogate marker for HPV+ OPSCC. In oral and laryngeal squamous cell carcinoma (OSCC/LSCC), the relevance of p16 immunohistochemistry, alone or in combination with other cell cycle-related proteins, to identify HPV-driven non-OPSCC is less well understood.

Methods: We stained for p16, pRb, cyclin D1, and p53 in 327 HNSCC. In 310 OPSCC, HPV-status was assessed by HPV DNA PCR. In 119 non-OPSCC, RNA in situ hybridization was additionally performed. HPV-status was correlated with staining patterns, p53 and clinical data.

Results: The OPSCC showed blockstaining for p16 in 36%, 8% were equivocal. Of these, HPV-testing was performed in 57%, and 53% were positive for HPV DNA. HPV-association correlated with absence of pRb and cyclin D1 and favorable outcome. In non-OPSCC, 18% showed p16-blockstaining, and 13% showed E6/E7 RNA. Six of seven HPV+ OSCC and 8/8 LSCC lost pRb and cyclin D1. Compared to HPV-negative counterparts, patients with HPV+ cancers had lower rates of alcohol consumption and keratinizing morphology. HPV-positive OSCC had a longer overall survival ($p < 0.05$). HPV subtype 16 was the most common.

Conclusions: We conclude that HPV-positive non-OPSCC are associated with p16 overexpression and low levels of pRb and cyclin D1. High expression of pRb and cyclin D1 indicates HPV-negativity.

KEYWORDS

cyclin D1, head and neck squamous cell carcinoma, human papillomavirus, immunohistochemistry, p16, p53, retinoblastoma protein

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1 | INTRODUCTION

An increasing incidence of human papillomavirus (HPV) has been identified in head and neck squamous cell carcinoma (HNSCC).¹ The presence of oncogenic HPV infection varies in different anatomical sites of HNSCC.² High-risk HPV types (HR-HPV), mainly type 16, are associated with a subset of oropharyngeal squamous cell carcinoma (OPSCC) with increasing incidence in Western countries.^{3,4} HPV-related OPSCC, located in the base of the tongue, tonsils, and oropharynx, have a better prognosis and response to chemoradiation than the HPV-negative counterpart.^{5,6} For non-oropharyngeal HNSCCs (non-OPSCC), the influence of HPV-driven carcinogenesis on outcome is less understood, with a conflicting contribution of HPV in oral squamous cell carcinoma (OSCC) and laryngeal squamous cell carcinoma (LSCC).⁷

Transcriptionally active HR-HPV infection is typically associated with p16 protein overexpression.⁸ Strong nuclear and cytoplasmic staining for p16 in $\geq 70\%$ of tumor cells is a surrogate marker for HR-HPV infection and part of the 8th edition of the Union for International Cancer Control (UICC) tumor node metastasis (TNM) classification.^{9,10} Although the sensitivity of p16-immunohistochemistry (IHC) for HR-HPV infection is high, its specificity depends on the prevalence of HPV infection.¹¹ While the concordance rate between p16 protein overexpression and molecularly confirmed HR-HPV infection is approximately 92%–100% in OPSCC of the Western world,^{12,13} the concordance rates are lower (83%–93%) in areas with lower prevalence of HPV-driven cancers, such as India.^{2,13} Therefore, p16 is not a perfect stand-alone surrogate marker for HR-HPV infection, especially in lower prevalence areas and non-OPSCC.¹⁴ p16-overexpressing but HPV-negative tumors may be misclassified as HPV-positive HNSCC, leading to inconsistent observations of patient outcomes.¹⁵ Molecular testing complements HPV analysis but is not widely available due to technical and financial constraints.¹¹ In OPSCC, not only high p16 but also low retinoblastoma protein (pRb), low cyclin D1, and wild-type p53 protein levels assessed by IHC surrogate biologically active HPV.^{10,16–18} The combination of these cell cycle-associated proteins has been demonstrated in independent study cohorts with slightly different cut-offs and serves as an optimized marker for HR-HPV without additional molecular testing.¹⁸

Since the addition of pRb, cyclin D1, and p53 improves the specificity of p16-IHC to identify OPSCC with active HPV infection, we investigated the capacity of these markers in identifying HPV-driven non-HNSCC, and their prognostic potential HR-HPV for infected versus HPV-negative patients. Upfront, we validated this diagnostic set in a large series of OPSCC.

2 | MATERIALS AND METHODS

2.1 | Patient and tissue selection

Specimens of formalin-fixed paraffin-embedded (FFPE) tissue of histologically proven primary OPSCC diagnosed from 2000 to 2022 were selected from the Department of Pathology as training set. Then, tumor tissue of primary HNSCC located in any non-oropharyngeal compartment, but with routinely upfront performed p16-IHC diagnosed from 2015 to 2022 were included. Age- and T-stage matched squamous cell carcinomas of the same anatomic site served as controls. Clinicopathological data were obtained from patients' archives. Tongue carcinomas located near the base of tongue were verified for their exact anatomic location by reviewing clinical examination forms. Follow-up data were obtained from the regional Cancer Registries. Cancers diagnosed before 2017 were re-classified using the 8th edition of the UICC TNM. Written informed consent was obtained from patients. Studies on the archived tumor material were conducted in accordance with the Declaration of Helsinki and approved by internal board review (Ethics Committee of Rostock University; A2018-0003 and A2022-0120).

2.2 | Immunohistochemistry

Antigen retrieval was performed on 4 μ m thick tissue slides with a high pH buffer (20 min at 97°C). Five minutes of incubation in peroxidase-blocking buffer followed by incubation with primary antibody (p16: 1:100, G175-405, BD Bioscience, San Jose, CA; pRb: 1:200, SAB4502590, Sigma-Aldrich, St. Louis, MO; cyclin D1: ready to use, NCL-L-CYCLIN-D1-GM, Leica Biosystems, Wetzlar, Germany; p53: 1:100, Do-7, Dako, Glostrup, Denmark) and 3,3'-diaminobenzidine (DAB) detection using the Dako-kit K8000 according to the manufacturer's instructions were performed in an Autostainer link 48 instrument (Leica Biosystems, Wetzlar, Germany) before counterstaining with hematoxylin.

2.3 | Microscopic evaluation

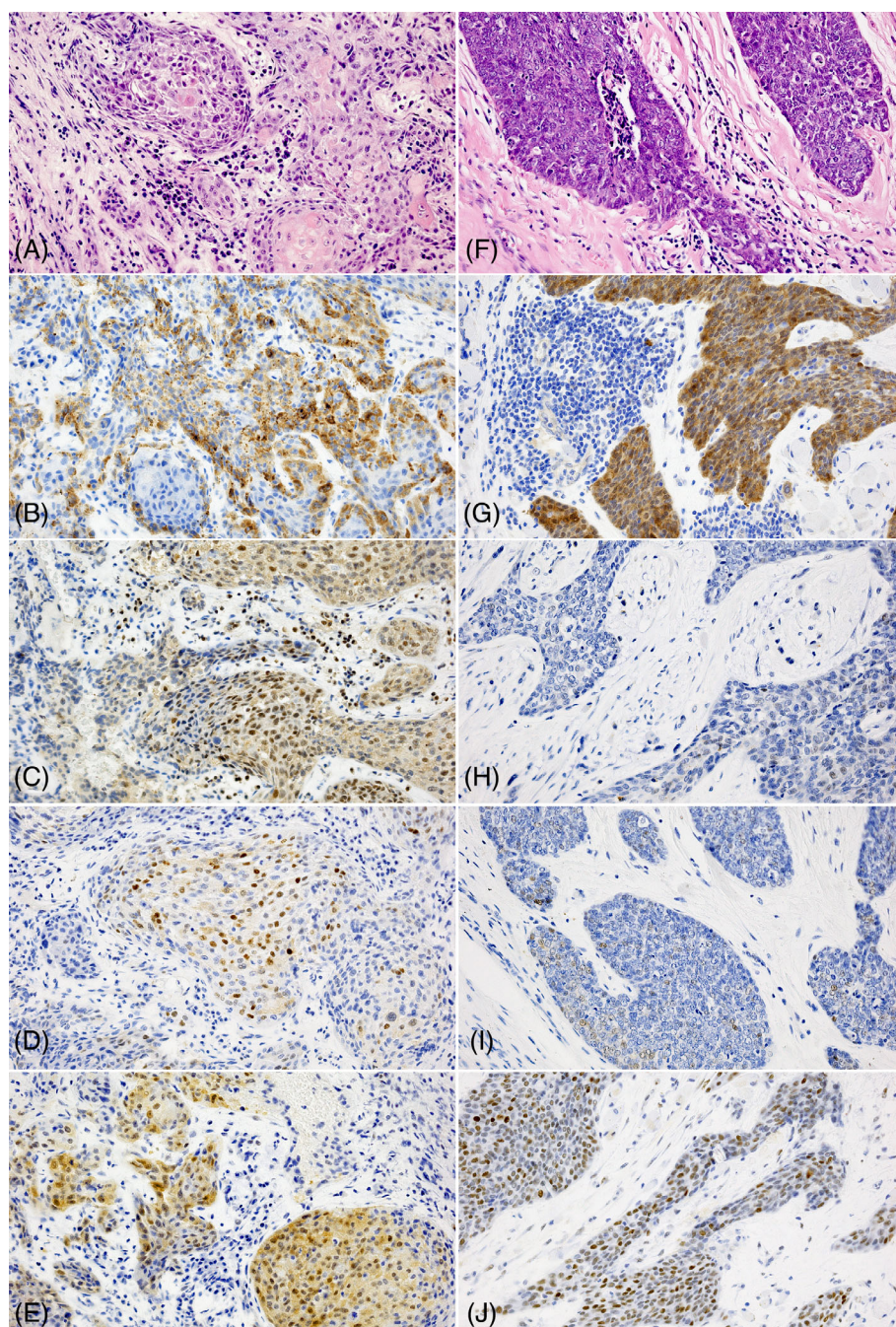
Hematoxylin & Eosin-stained slides were checked for invasive HNSCC including grading and morphology. The p16 expression was considered positive when $\geq 70\%$ of tumor cells showed strong and diffuse nuclear and cytoplasmic staining.¹⁹ Unequivocal staining patterns were discussed by ASKB and AHZ until a final score was built.

Samples without blockstaining but with moderate intensity in $\geq 70\%$ of cells or strong staining reactivity in $\geq 50 < 70\%$ of cells were categorized as *equivocal*. Expression of pRb and cyclin D1 was scored as *high* (preserved expression) or *low* (loss) based on the proportion of positive cancerous nuclei with a cut-off set by $\geq 70\%$. Nuclear/cytoplasmic p53 reactivity in tumor cells defined p53-status (weak scattered nuclear expression in $< 50\%$ = *wild-type*, strong nuclear expression $\geq 50\%$ or any cytoplasmic expression = *mutant*, absent expression ("null pattern") = *deleted*).¹⁰ Exemplary staining patterns are shown in Figure 1.

2.4 | HPV status by molecular pathology

OPSCC with *equivocal* p16-IHC and non-OPSCC with positive or *equivocal* p16-IHC were prepared for molecular testing. Tumor cells were selectively dissected from 4- μ m-thick serial tissue sections of the same paraffin block, genomic DNA was isolated using a QIAamp DNA FFPE tissue kit (Qiagen, Hilden, Germany) according to manufacturer's instruction. Isolated DNA was checked for amplifiability. A multiplex PCR was used, consisting of 10 different primers that generated PCR products between 100 and 400 bp. Primer sequences and PCR

FIGURE 1 Illustrative histology, expression of p16, pRb, CyclinD1, and p53 in high-risk human papillomavirus (HR-HPV) negative (A–E) and positive (F–J) oral squamous cell carcinomas: (A) Keratinizing squamous cell carcinoma (hematoxylin and eosin). (B) Weak to moderate nuclear and cytoplasmic expression of p16 by IHC. (C) Cancer cells show retained expression of pRb and (D) CyclinD1 expression by IHC cancer cells show a loss of nuclear pRb and (D) CyclinD1 expression by IHC. (E) Cytoplasmic staining for p53 indicating a *TP53* mutation. (F) Non-keratinizing squamous cell carcinoma (hematoxylin and eosin). (G) Diffuse nuclear and cytoplasmic expression of p16 by IHC. (H) Cancer cells show a loss of nuclear pRb and (I) CyclinD1 expression by IHC, while non-neoplastic squamous epithelium and internal lymphocytes are positive. (J) Markedly increased ($> 50\%$) nuclear expression of p53, caused by underlying *TP53* mutation. All $20\times$. [Color figure can be viewed at wileyonlinelibrary.com]



conditions are given in Data S1, Supporting Information. PCR products were analyzed by agarose gel electrophoresis. The VisionArray HPV Chip 1.0 (Zytovision, Bremerhaven, Germany) was used for HPV type identification according to manufacturer's protocol.

In situ hybridization (ISH) was performed using digoxigenin-labeled oligonucleotides (~2.2 ng/μl) specific for HPV types 16/18/31/33/35/39/45/51/52/56/58/59/66/68/82, targeting DNA sequences encoding the HPV 16/18/31/33/35/39/45/51/52/56/58/59/66/68/82 proteins E6, E7, and/or L1 (ZytoFast® HPV High-Risk [HR] Types Probe [ZytoVision GmbH, Bremerhaven, Germany]). The probe also targets the corresponding messenger RNA sequences of the E6, E7, and/or L1 proteins that are expressed during some stages of infection. The chromogenic in situ hybridization (CISH) analysis was performed due to manufacturer's instruction. A positive and negative control were included in each series. Integrated E6/E7 RNA in a single dot-like signal within tumor cell nuclei, either alone or in combination to an episomal (=diffuse nuclear signal) pattern was set RNA+. RNA+ was defined as evidence of oncogenic HPV infection, and RNA+ positive specimens were considered HPV-associated.

2.5 | Next generation sequencing

Somatic variants of *TP53* were sequenced with the AmpliSeq Cancer HotSpot Panel (Illumina) due to manufacturer's protocol. Sequencing was performed on an Illumina iSeq100 with an input of 50 ng of genomic DNA. The variants were annotated using the Local Run manager (Illumina) and the Integrative Genomics viewer (IGV version 2.8.9; Broad Institute).

2.6 | Statistics

Statistical evaluation was performed using GraphPad PRISM software, version 8.0.2 (GraphPad, San Diego, CA). Clinical and pathologic data were tested univariately (Fisher's exact test). Kaplan-Meier survival curves were analyzed using the log rank (Mantel Cox) test. Receiver operator characteristics (ROC) curve including area under the curve (AUC) calculation was used to test the behavior of IHC results in diagnosing HPV RNA+ non-OPSCC. The software SPSS 28.0.0.0 (IBM, Ehingen, Germany) was used for the survival analyses. Cox regression was performed to select the factors influencing OS and to quantify their influence by calculating their hazard ratios (HRs). The variable selection step was performed using a univariable Cox regression approach.

Subsequently, a multiple (final) Cox model was applied with all selected variables to calculate the adjusted HRs and their respective *p*-values. Backward selection was carried out for the multivariable analysis. *p*-values <0.05 were considered to indicate statistical significance.

3 | RESULTS

3.1 | HPV infection and impact on outcome in OPSCC

The clinicopathologic characteristics of the 310 OPSCC are summarized in Table 1. The median overall survival time was 33 months, ranging from 1 to 249 months, respectively (Figure 2). Tumor relapse occurred in 9% until the end of the follow-up, and median progression-free survival (PFS) was 29 months.

Molecular testing for HPV DNA by PCR was performed in 37% of all OPSCC and in 57% (77/134) cases with positive (=blockstaining) or equivocal p16-IHC. Among these, HR-HPV was detected in 92% (71/77) of the tested cases, with type 16 being most prevalent in 59 samples, twice in combination with type 33. Among the molecularly tested specimens with p16 blockstaining (*n* = 68), four were HR-HPV negative, including two with low-risk HPV type 6 infection and concurrent oncogenic *TP53* variants as demonstrated by IHC and next-generation sequencing. Regarding the other markers, the four HPV DNA negative specimens within the p16-positive subgroup all had a *TP53* driver mutation. Two of them had lost and two of them had preserved staining for pRB and cyclin D1, respectively. The 12 p16-negative specimens for which HPV testing was performed were all HPV-negative. Of the 21 cases with equivocal p16 staining, 9 were tested by HPV DNA PCR, revealing type 16 infection in 7 and HPV-negativity in two. Calculating only on samples that were either negative or positive for p16-IHC with molecular HPV data available for the latter group (total *n* = 245), the sensitivity, specificity, positive predictive value (PPV), and negative predictive value (NPV) for p16-IHC alone were 100%, 98%, 94%, and 100%, respectively. The combination of p16 with either pRB, cyclin D1, or p53 changed the specificity and PPV to 96% each. Nine of the 176 p16-negative cases showed loss of cyclin D1, with synchronous loss of pRB in three cases and preserved pRB expression in the remaining ones. By p16-IHC, 36% of all OPSCC were strongly positive suggesting HR-HPV infection, 7% showed equivocal staining and 57% were negative. Using p16-status as surrogate marker for HPV-positivity, stratification by p16 showed well-established clinicopathologic associations, as previously shown (Table 1).³

TABLE 1 Clinicopathological characteristics of the cohort.

Characteristic <i>n</i>	OPSCC <i>n</i> = 310			OSCC <i>n</i> = 69			LSCC <i>n</i> = 50		
	p16 negative [#]	p16 positive	<i>p</i> - value	HR-HPV RNA negative	HR-HPV RNA positive	<i>p</i> -value	HR-HPV RNA negative	HR-HPV RNA positive	<i>p</i> -value
Median age (range)	60 (46–86)	60 (36–83)	n.s.	66 (29–95)	65 (60–79)	n.s.	69 (49–87)	68 (57–85)	n.s.
Sex			**			n.s.			n.s.
Male	52	26		77	7		78	16	
Female	8	14		13	3		6	0	
Smoking			****			0.08			n.s.
>10 py	49	13		52	9		60	12	
<10 py	11	27		36	1		24	4	
Alcohol			****			*			**
>3 U/d	49	3		33	0		46	2	
<3 U/d	11	37		57	10		38	14	
Tumor stage			*			*			**
T1/2	29	32		29	7		38	14	
T3/4	31	8		60	3		46	2	
Nodal stage			**			*			n.s.
N0	28	7		71	4		48	6	
N+	32	33		19	6		36	10	
Treatment			**			n.s.			n.s.
Surgery/ radiation	25	28		32	6		30	9	
+adjuvant [#]	35	12		58	4		54	7	
2-year overall survival rate	65	79	***	89	100	****	86	100	****
HR-HPV DNA			****			****			****
Negative	67 ⁺ (14/21)	6 ⁺⁺ (4/68)		58/62	0/7		39/42	0/8	
Positive	33 ⁺ (7/21)	94 ⁺⁺ (64/68)		4/62	7/7		3/42	8/8	

Note: [#]Including 21 equivocal cases with weak to moderate nuclear and cytoplasmic staining in $\geq 50 < 70\%$ of tumor cells; [#]including radiochemotherapy, chemotherapy, radiation. ^{+/++}Numbers referring to ⁺*n* = 21 (including 9 cases with doubtful p16 positivity and HPV HR DNA+ in 7 of those), and ⁺⁺*n* = 68 cases with molecular HPV DNA PCR-testing.

Abbreviations: HPV, high-risk human papillomavirus; n.s., not significant; py, pack years.

p* < 0.05; *p* < 0.01; ****p* < 0.001; *****p* < 0.0001. Values are given in %.

3.2 | Prevalence of HPV infection in non-oro-pharyngeal HNSCC and clinicopathological findings

One hundred and nineteen HNSCC with a tumor epicenter outside the oropharynx were included (Table 1). Tumors were located in the oral cavity in 69 patients, in the larynx in 48 patients, and in the hypopharynx twice (included in the laryngeal cohort).

OSCC were p16-positive in 10 cases and p16-negative in 59 cases (Table 2). Three tumors showed equivocal p16 staining. Molecular testing revealed 7 RNA+ specimens (all p16-positive). Further PCR-based analysis revealed HR-HPV type 16 5× and types 26 and 33 each. The three samples with equivocal p16 reactivity, while negative for HPV RNA, contained HR-HPV (type 16) infection as shown by PCR. Tumor tissue from a p16-negative OSCC tested positive for low-risk HPV type

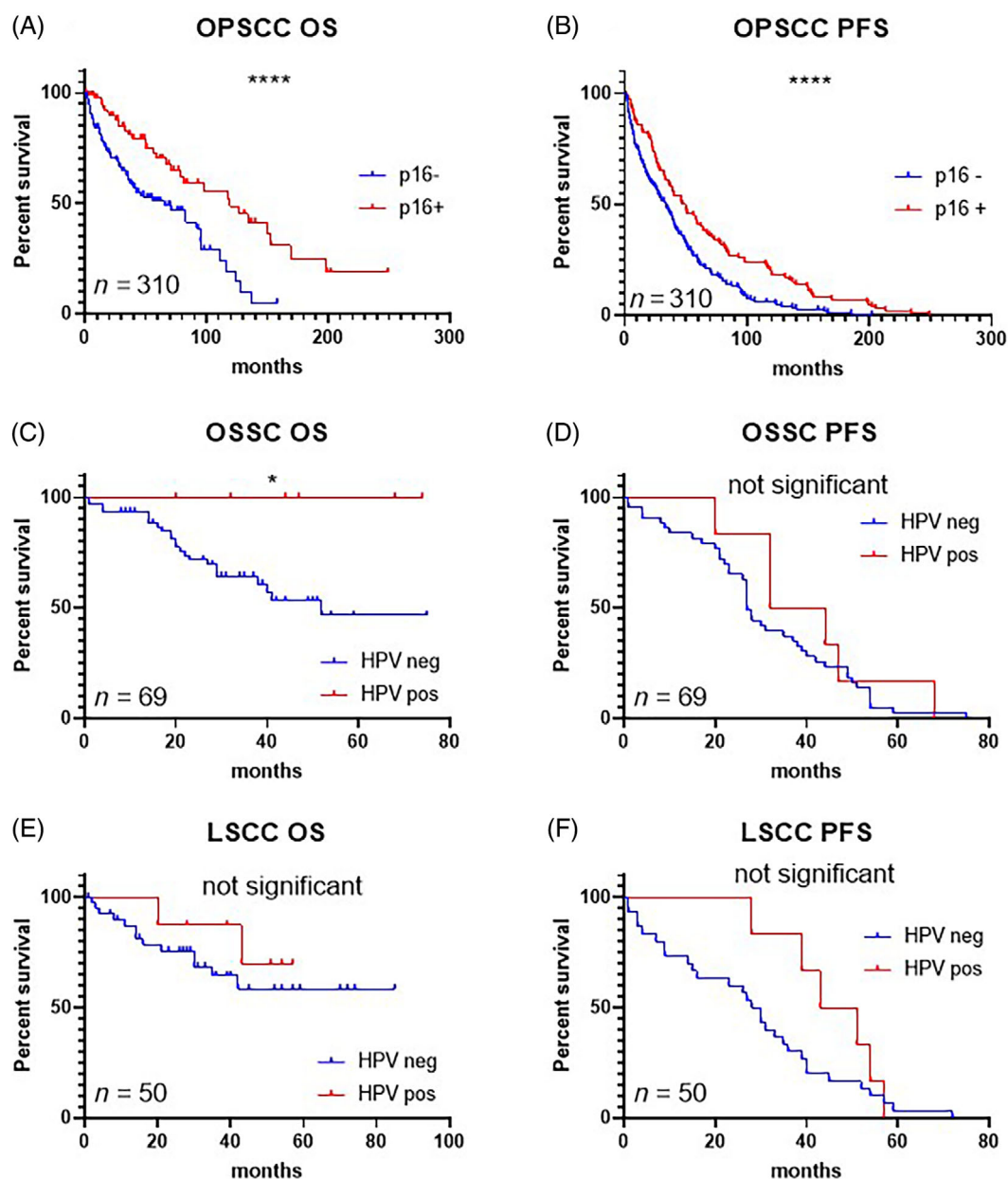


FIGURE 2 Overall and progression free survival in dependency of HPV-status and anatomical site: (A, B) p16 negative versus p16 positive oropharyngeal, (C, D) HPV DNA positive versus negative oral, and (E, F) HPV DNA positive versus negative laryngeal tumors. LSCC, laryngeal squamous cell carcinoma; OPSCC, oropharyngeal squamous cell carcinoma; OSCC, oral squamous cell carcinoma. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$. [Color figure can be viewed at wileyonlinelibrary.com]

6. Overall, 10% of the oral carcinomas showed oncogenic HPV infection.

Of the 50 LSCC, 11 were p16-positive. The remaining 39 were p16-negative (Table 2). Molecular testing revealed 8 RNA+ specimens (all p16-positive), all of which showed HR-HPV type 16 by PCR. Overall, 16% of laryngeal cancers showed oncogenic HPV infection. Alcohol, but not tobacco, consumption was significantly lower in HPV-driven OSCC and LSCC (Table 1). Non-keratinizing morphology was more common in HPV-driven LSCC. This trend was also observed in OSCC, but was not statistically significant (Table 2).

3.3 | Prognostic impact of HPV in non-oropharyngeal HNSCC

With a median follow-up of 2.9 years, two of the 13 HR-HPV-positive patients (both LSCC) and 37 of the 106 HPV-negative patients died. Stratification of the 119N-HNSCC (13% HR-HPV positive) by HPV RNA status showed a longer OS for patients with HPV RNA+ oral cancer using univariate Kaplan-Meier analysis ($p = 0.049$, log-rank test; Figure 2C). There were no significant differences for LSCC (Figure 2E,F). Multivariate Cox proportional hazard models adjusted for age, sex, cell

TABLE 2 Association between cell-cycle protein expression and HR-HPV RNA E6/E7 status.

<i>n</i>	OSCC cohort	HR-HPV RNA negative	HPV 16	Non-HPV 16	<i>p</i> -value	LSCC cohort	HR-HPV RNA negative	HPV 16	Non-HPV 16	<i>p</i> -value
		62	5	2			42	8	0	
p16-IHC					****					****
Negative		56/62	0/5	0/2			39/42	0/8		
Positive		3/62	5/5	2/2			3/42	8/8		
Equivocal		3/62	0/5	0/2			0	0		
pRB					****					****
Loss		2/62	4/5	2/2			3/42	8/8		
Regular		60/62	1/5	0/2			39/42	0		
Cyclin D1					****					****
Loss		2/62	4/5	2/2			2/42	8/8		
Regular		60/62	1/5	0/2			40/42	0		
P53					****					**
Wild-type		8/62	4/5	2/2			8/42	6/8		
Mutant		54/62	1/5	0/2			34/42	2/8		
p16+/pRb					****					****
High/loss		1/62	4/5	2/2			1/42	8/8		
Other		61/62	1/5	0/2			41/42	0		
p16+/cyclin D1					****					****
High/loss		1/62	4/5	2/2			1/42	8/8		
Other		61/62	1/5	0/2			41/42	0		
p16+/p53					****					****
High/wt		2/62	4/5	2/2			2/42	6/8		
Other		60/62	1/5	0/2			40/42	2/8		
p16/pRB/p53					****					****
High/loss/wt		1/62	4/5	2/2			0/42	6/8		
Other		61/62	1/5	0/2			42/42	2/8		
NKSCC					0.07					*
Yes vs. absent		26/62	3/5	2/2			18/42	7/8		

Note: +Including three cases with weak to moderate nuclear and cytoplasmic staining in $\geq 50 < 70\%$ of tumor cells.

Abbreviations: HR-HPV, high-risk human papillomavirus; HPV 16, type 16; LSCC, hypopharyngeal ($n = 2$) and laryngeal ($n = 48$) squamous cell carcinoma; NKSCC, non-keratinizing squamous cell carcinoma morphology; n.s., not significant; OSCC, oral squamous cell carcinoma; pRB, retinoblastoma protein; wt, wild-type.

* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$.

cycle markers, oncogenic HPV infection (=HPV RNA+), clinical stage, T stage, N stage, and recurrence status showed a statistically significant association with better OS only for patients without recurrent disease (Table 3). On univariate analysis, stratification by pRB and cyclin

D1 showed that patients in the respective groups had comparable survival, and patients with an oncogenic *TP53* mutation (OSCC $n = 55$; LSCC $n = 36$) had a shorter OS than their wild-type counterparts ($p = 0.016$, log-rank test).

TABLE 3 Univariable and multivariable Cox regression for overall survival non-opharyngeal squamous cell carcinoma.

Parameter	Univariable regression		Multivariable regression			
	p-value	95% CI	HR	p-value	95% CI	adj. HR
OSCC						
Age: * <66 years vs. ≥66 years	0.575	0.549–2.945	1.271			
Sex: *Male vs. female	0.979	0.460–1.315	0.777			
p16: *Absent vs. present	0.110	0.026–1.447	0.194			
HPV RNA+: *Absent vs. present	0.215	0.000–6.573	0.039			
p53: *Wt vs. mutant	0.309	0.593–5.206	1.758			
pRB: *Normal vs. aberrant	0.536	0.148–2.702	0.632			
Cyclin D1: *Normal vs. aberrant	0.616	0.161–2.949	0.689			
UICC: *Low (I/II) vs. high (III/IV)	0.345	0.630–3.743	1.536			
pT: *T1 vs. T2 vs. T3 vs. T4	0.471	0.786–1.684	1.150			
c/pN: *N0 vs. N1 vs. N2	0.439	0.744–1.976	1.213			
Recurrence: *No vs. yes	<0.001	1.983–10.71	4.609	<0.001	1.898–10.297	4.421
LSCC						
Age: * <66 years vs. ≥66 years	0.760	0.434–3.139	1.167			
Sex: *Male vs. female	0.667	0.082–4.949	0.638			
p16: *Absent vs. present	0.227	0.566–11.0	2.494			
HPV RNA+: *Absent vs. present	0.377	0.442–8.664	1.956			
p53: *Wt vs. mutant	0.303	0.550–6.817	1.936			
pRB: *Normal vs. aberrant	0.170	0.641–12.43	2.823			
Cyclin D1: *Normal vs. aberrant	0.127	0.721–14.03	3.180			
UICC: *Low (I/II) vs. high (III/IV)	0.271	0.577–7.109	2.024			
pT: *T1 vs. T2 vs. T3 vs. T4	0.105	0.934–2.050	1.384			
c/pN: *N0 vs. N1 vs. N2	0.062	0.974–2.785	1.647			
Recurrence: *No vs. yes	0.012	1.319–9.550	3.549	0.014	1.293–9.763	1.462

Note: Cox proportional hazards analysis was used adjusted for age, sex, cell cycle markers, oncogenic HPV infection (=HPV RNA+), clinical stage, T-stage and N-stage, and recurrence status; significant HR are in bold. *Reference category. For selecting OS influencing factors for the multivariable regression approach, the cut-off was set to $\alpha = 0.250$.

Abbreviations: CI, confidence interval; HR, hazard ratio; LSCC, hypopharyngeal ($n = 2$) and laryngeal ($n = 48$) squamous cell carcinoma; OSCC, oral squamous cell carcinoma; pRB, retinoblastoma protein; wt, wild-type.

3.4 | Reliability of p16 in detecting HPV infection in non-opharyngeal HNSCC

For both OSCC and LSCC, the sensitivity and NPV of p16 positivity for predicting HR-HPV infection were 100% (Table 4). In OSCC, three p16-positive but RNA-negative cases tested positive for HPV DNA types 16, 26, and 33, respectively, resulting in a specificity of 95%. Three cases with p16 staining classified as equivocal included one transient type 16 infection (HPV RNA-negative), one low-risk type 6 infection, and one HR-HPV negative specimen. The two HPV DNA-positive cases showed an episomal pattern in the ISH. In OSCC, Youden's index to assess the accuracy of the different markers in detecting HPV-associated cancers was best for p16 alone (0.95).

In LSCC, three p16-positive but RNA-negative cases tested positive for HPV DNA type 16 twice and 35 once, resulting in a specificity of 93%. The two HPV DNA type 16 cases showed an episomal pattern on ISH, while the tumor with type 35 was negative. In LSCC, Youden's index was best for p16 in combination to CyclinD1 (0.95).

3.5 | Cell cycle protein marker combinations and their associations with HPV infection in non-opharyngeal HNSCC

In OSCC, p16 blockstaining had 100% sensitivity and 95% specificity for detecting HPV RNA+ cancers. Focusing on

TABLE 4 Sensitivity, specificity, PPV, and NPV of p16 and pRb/Cyclin D1 to predict oncogenic HR-HPV infection in non-oro-pharyngeal squamous cell carcinoma.

	Sensitivity	Specificity	PPV	NPV	AUC (95% CI)	Youden's index
OSCC: HPV prevalence 10%						
p16+ alone ^a	100	95	70	100	0.52 (0.43–0.62)	0.95
p16+/pRb loss	86	98	86	98	0.5 (0.4–0.6)	0.84
p16+/cyclin D1 loss	86	98	86	98	0.5 (0.4–0.6)	0.84
p16+/p53wt	86	97	75	98	0.54 (0.44–0.63)	0.83
p16+/pRb loss/p53wt	86	98	86	98	0.51 (0.41–0.6)	0.84
LSCC: HPV prevalence 16%						
p16+ alone	100	93	73	100	0.53 (0.42–0.64)	0.93
p16+/pRb loss	100	93	73	100	0.51 (0.4–0.62)	0.93
p16+/cyclin D1 loss	100	95	80	100	0.51 (0.4–0.62)	0.95
p16+/p53wt	75	95	75	95	0.5 (0.39–0.61)	0.7
p16+/pRb loss/p53wt	75	100	100	95	0.52 (0.41–0.63)	0.75

^aThe three OSCC cases with *equivocal* p16 staining results were included in the p16 *negative* group.

Abbreviations: AUC, area under the curve; CI, confidence interval; NPV, negative predictive value; LSCC, hypopharyngeal ($n = 2$) and laryngeal ($n = 48$) squamous cell carcinoma; OSCC, oral squamous cell carcinoma; PPV, positive predictive value; pRb, retinoblastoma protein; wt, wild-type.

pRb and cyclin D1, 6/7 HPV RNA+ oral cancers showed loss of pRb and cyclin D1 with one carcinoma having normal expression (with a type 16 infection by PCR). One of the type 16 RNA+ OSCC had an oncogenic *TP53* mutation. On the contrary, *TP53* mutation was observed in 87% of RNA-negative OSCC, with loss of pRb and cyclin D1 expression in one p16-negative case. Interestingly, the p16 equivocal RNA-negative case with HPV type 16 DNA lost pRb and cyclin D1 and showed a wild-type pattern for p53. The two p16 equivocal but HR-HPV negative cancers had preserved pRb and cyclin D1 but mutant p53. The diagnostic accuracy of p16 alone in detecting HPV RNA+ OSCC is moderate, as the AUC is 52% using ROC analysis. It increases slightly to 54% when p16-IHC is combined with p53, but decreases when combined with the other two cell cycle markers (see Tables 2 and 4 for test results).

In LSCC, p16 blockstaining had 100% sensitivity and 93% specificity for detecting HPV RNA+ cancers. Focusing on pRb and cyclin D1, 8/8 HPV RNA+ laryngeal cancers (all positive for type 16 by PCR) showed loss of pRb and cyclin D1. Within the HPV RNA negative group, three lost pRb expression, two of which also lost cyclin D1. Two of the RNA+ LSCC had an oncogenic *TP53* mutation, but *TP53* mutation was observed in 81% of the RNA-negative OSCC. Notably, the three p16-positive but RNA-negative cases all lost pRb and cyclin D1 and twice showed a wild-type pattern for p53. The AUC of p16 alone in the detection of HPV RNA+ OSCC is 53% and decreases when combined with the other markers.

4 | DISCUSSION

Identification of HPV association typically relies on blockstaining for the surrogate marker p16 by IHC combined with detection of HPV nucleic acids by molecular methods. In scenarios where adequate tumor material or molecular testing is not available, loss of pRb combined with p16-IHC optimizes specificity for the identification of HR-HPV OPSCC. Here, we validate this diagnostic algorithm for the detection of HR-HPV-positive OPSCC and examine its ability to detect HPV-associated HNSCC located outside the oropharynx.

In our OPSCC training set, molecular testing for HPV used the PCR-based approach. Due to its high specificity, this technique is subject to false positive contamination and may indicate latent, non-oncogenic HPV infection. Validation data demonstrating oncogenic infection mediated by the viral oncogenes E6/E7 were not available. Next, in approximately 50% of our OPSCC samples, the DNA quality was too poor for molecular analysis. As a result, half of the p16-positive tumors could not be tested for HPV DNA. Working only with samples for which molecular HPV data were available confirmed the well-known fact that HPV-positive carcinomas lose pRb and CyclinD1 while being p53 wild-type. Therefore, the OPSCC cohort served well as our training set to become confident enough with the different stains before evaluating them in the extra-oro-pharyngeal tumors. As the ability of p16 to detect HPV-associated OPSCC has been established, we chose the approach of classifying all p16-positive OPSCC as HPV-associated.¹⁹

Using p16 as a surrogate marker for HPV infection, p16 positivity was detected in 37% of the OPSCCs correlating with a favorable prognosis. This is consistent with previous studies.^{16–18} The rather low prevalence of HPV in our OPSCC cohort can be explained by an enrollment starting in 2000, when the incidence of HPV-related tumors was lower than today, and high frequency of smoking-related OPSCC in Northern Germany.²⁰ As shown previously, p16-positive cases had a loss or a rather mosaic-like staining pattern for pRb and cyclin D1. The rate of oncogenic *TP53* mutations was higher in the HPV-negative group, consistent with their induction by tobacco and smoking. In line with current analyses, eight patients with p16-positive/HPV-negative tumors had an intermediate survival (median OS in months: 82 for p16^{pos}/HPV^{neg}, 152 for p16^{pos}/HPV^{pos}, 36 for p16^{neg}/HPV^{neg}).¹³

In our extra-oralopharyngeal test set, molecular testing for HPV used first the PCR-based approach and second CISH for E6/E7 RNA of different HR types to validate oncogenic infection. RNA+ OSCC and LSCC were considered HPV-driven. We tested p16-IHC and its combination with pRb, cyclin D1, and p53 to detect HPV-driven non-OPSCC in preselected oral and laryngeal tumors. The search for HPV-associated tumors in our database explains the 13% incidence of HPV-positive non-OPSCC in our cohort, which is usually <10% for this anatomical site in Germany.^{21–24}

Unlike in the oropharynx, the mucosa of the oral cavity and larynx does not consist of physiologically patchy lymphoepithelial tissue on the surface, but of intact squamous epithelium. The intact surface reduces the risk of a potentially cancer-causing infection by HPV types of the high-risk group of the deeper, still proliferatively active epithelial layers.^{25,26} Similar to the transitional epithelium of the female cervix, microlesions of the epithelium can be assumed to be the cause of the rare persistent HPV infections in stem cells.²⁷ If viral oncoproteins are integrated into the cell genome, the tumor suppressors pRb, p53, and p16 are severely restricted in their function according to mechanisms analogous to the oropharynx. Although p16 may accumulate in tumor cells by molecular mechanisms other than its interaction with viral oncoproteins, and pRb, CyclinD1, and p53 may show altered expression patterns by IHC independent of E7/E6, their expression changes in HPV-driven carcinomas.^{28,29} Therefore, they may be useful in the identification of HPV-driven non-OPSCC.

In this LSCC cohort with a 16% prevalence of HPV RNA+ tumors, p16 alone detected all HPV RNA+ samples (all HPV type 16). Adding the information of the CyclinD1 stains to p16, diagnostic test accuracy as measured by Youden's index increased slightly (0.93–0.95),

while p16 alone was superior to other marker combinations. Therefore, the other three markers do not appear to be of additional value in identifying HPV-positive laryngeal cancers in our cohort. This may differ in other regions where HPV prevalence and HPV strains vary.^{1,30,31} Loss of pRb and cyclin D1 and wild-type staining for p53 were higher in HPV-positive cancers, confirming previous studies.^{10,16} All three p16-positive but HPV RNA- negative specimens turned out to be positive for HPV HR DNA by PCR (including once the rare type 35) with reduced/lost expression of pRb and CyclinD1. Although this suggests a latent, non-oncogenic infection, the altered cell cycle markers favor the incorporation of viral-associated abnormalities into the host cell, including genomic, epigenetic or post-translational modifications. One explanation would be that E6/E7 RNA was very little, and that the ISH technique was not sensitive enough for its detection. Unfortunately, the more precise reverse transcriptase-PCR is not available in our lab. Another reason could be that HPV acts in a slightly different manner to cause cancer in non-OPSCC sites, and that E6/E7 may not be the only key mediators inducing cancer.^{32,33}

In OSCC, all seven HPV RNA+ cases were p16 positive, and the diagnostic accuracy of p16 for detecting HPV-driven oral cancer is excellent (Youden's index 0.95). Youden's index, one of the oldest measures of diagnostic accuracy, serves as a global measure of test performance and is used to assess the overall discriminatory power of a diagnostic test.³⁴ Nevertheless, identification of HPV RNA+ cancers in the oral cavity by p16-IHC was more difficult than in the LSCC cohort (Table 4). This may be partly explained by the lower prevalence of HPV RNA+ tumors (10% in the OSCC subgroup). Next, HPV strains other than type 16 accounted for 28% of oncogenic infections. The non-type 16 HPV RNA+ cases were p16-positive, *TP53* wild-type, and showed loss of expression of pRb and cyclin D1. In contrast, the two non-type 16 HPV RNA- negative cases were p16-positive, *TP53* wild-type, but showed restored expression for pRb and cyclin D1, highlighting the importance of different HPV strains as recently reported.¹⁴ Finally, three cases were classified as equivocal, which turned out to be HPV RNA negative but HPV DNA positive (type 16) once and HPV DNA negative twice, but all with loss of pRb (twice with concomitant loss of CyclinD1). This suggests more complex mechanisms in connection to viral oncoproteins in mediating OSCC, which should to be tested in more detailed in vitro models. Of all the markers, p16 positivity alone had the strongest association with HR-RNA positivity.

Although there are established cut-off criteria for p16 positivity in HNSCC, routine diagnosis includes samples

that are difficult to label: samples without blockstaining but with moderate intensity in $\geq 70\%$ of cells or strong staining reactivity in $\geq 50 < 70\%$ of cells (=equivocal). As seen in our HPV RNA+ OSCC, this weakens the specificity of p16-IHC, where all three equivocal cases were HPV RNA negative. In two cases, staining for p53 indicated mutation with preserved pRB and CyclinD1 expression, and no HPV DNA was detected by PCR. In one case, pRB and cyclin D1 were lost and a *TP53* mutation was found. In this case, HPV type 16 DNA was detected by PCR, while ISH did not demonstrate oncogenic infection. As dysregulations in oncogenic pathways occur prior to cancer development, one cannot argue that these staining results argue for a transient infection becoming oncogenic. Another view would be that certain oncogenic pathways terminate in a common terminal pathway to affect an elementary signaling axis via different mechanisms for oncogenesis.²⁹ In conclusion, the use of pRB and CyclinD1 in combination with p16 for HPV detection is questionable for routine diagnostics. First, staining procedures need to be established, and second, evaluation can be challenging. Since cyclin D1 rather than pRB is part of the antibody repertoire in many laboratories, cyclin D1 alone can be added to p16-IHC to address this first point. Although we observed a high concordance rate for pRB and cyclin D1 independent of anatomic compartment or HPV status, two p16-positive OPSCCs showed divergent staining results for pRB and cyclin D1. The second issue, the interpretation of pRB and cyclin D1 in tumor cells, was addressed by applying a binary cutoff scheme. The mechanisms of regulating cell cycle are far more complex and a more detailed scoring, for example, using partial or complete loss as suggested by Jirumaru et al., would be advantageous.¹⁶ Nevertheless, our results are comparable to theirs.

A key finding of our work is simply the presence of HPV-driven non-OPSCC in Caucasian patients. While tobacco use was not lower, reported alcohol consumption was lower in HPV RNA+ patients. This is consistent with findings in OPSCC, where the non-keratinizing tumor morphology is more common in HPV-driven carcinomas. Here, basaloid morphology was also more common in the HPV RNA+ non-OPSCC subgroup. Regarding survival, HPV RNA+ OSCC had a longer OS, which was not evident in multivariate analyses in our cohort. The short follow-up period (median 43 months HPV-positive) may explain the comparable outcome for HPV-positive and negative laryngeal tumors. No significant survival benefit was observed for the subgroup of HPV DNA+, but RNA-negative tumors. This fits to observations of other groups, and strengthens the importance of identifying those patients with really HPV-driven carcinomas, and not with HPV-infection of uncertain, maybe latent relevance.^{21,35,36}

Previous studies investigating whether the presence of HPV affects survival in non-OPSCC have shown mixed results.⁷ This may be explained by divergent genetic and environmental risk factors^{37,38}; a potential beneficial effect of HPV-positivity in OSCC and LSCC may be abolished by carcinogen-induced nucleic acid damage, resulting in additional driver mutations.³⁹ Tumors with combined carcinogenesis may be more aggressive than either HPV- or toxin-mediated counterparts.^{40,41} The high mutational load of OSCC and LSCC reflects the prolonged exposure to carcinogens such as tobacco and alcohol in these compartments.^{42,43} Improved outcomes for HPV-driven tumors, particularly for laryngeal cancers treated with chemoradiation, have been reported in a few larger studies.^{37,44,45} While adjuvant therapy regimens were similar for patients in our groups, PFS for HPV-positive non-OPSCC were comparable to their HPV-negative counterpart. Future studies are needed to confirm this observation. Ideally, these studies should stratify patient according to systemic therapy.

A weakness of our study is the detection of HPV by the use of DNA PCR in the OPSCC cohort. Although DNA PCR is very sensitive, it may detect transient or irrelevant HPV infections of adjacent tissues and may miss infections with unusual HPV types. While acceptable for our training set, we optimized the method for HR-HPV testing in the OSCC and LSCC group: to prove biologically relevant HPV infection we performed ISH additionally because it identifies transcriptionally active HPV infection and indicates translation of viral E6 and E7 oncoproteins.⁴⁶ An additional confounding factor may be large multi-level, and originally *oropharyngeal* tumors, that were misdiagnosed as *non-OPSCC*. We tried to address this by critically reviewing clinical examination forms to correctly classify primary cancers.

5 | CONCLUSION

HPV-positive non-OPSCC is associated with p16 overexpression. Using cut-off criteria of $>70\%$ cytoplasmic and nuclear staining, our results demonstrate that p16 blockstaining is a reliable diagnostic tool for HPV infection in OSCC and LSCC. Low levels of pRb and cyclin D1 are more common in HPV-positive than HPV-negative tumors. Their protein signatures as well as p53 IHC do not seem to improve the diagnostic performance of p16 alone. In scenarios of cases with equivocal p16 staining where molecular testing is not available, an additional p53 wild-type staining pattern may favor HPV-driven carcinogenesis. The prognostic and predictive value of HPV infection in non-OPSCC needs to be further investigated in studies.

AUTHOR CONTRIBUTIONS

ASB: Performed development of methodology; data analysis and acquisition and interpretation of data; writing and revision of the paper. **JM and IB:** Performed TMA assembly and the majority of its evaluation (data analysis). **DFS:** Assisted in data acquisition and critically reviewed the paper. **CM:** Assisted in interpretation of data; created the graphical abstract; critically reviewed the paper. **MH:** Did the molecular HPV testing including data analysis and interpretation; critically reviewed the paper. **AZ:** Performed study concept and design; data analysis; acquisition and interpretation; reviewed the manuscript critically. All authors read and approved the final paper.

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflicts of interest.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

ETHICS STATEMENT

The institutional ethic committee at the University Hospital Rostock approved the study (A2018-0003 and A2022-0120). The study was performed in accordance with the Declaration of Helsinki.

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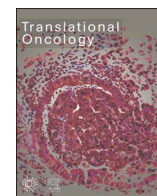
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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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Original Research

Inhibition of platin-induced BCL2 increase overcomes chemoresistance in squamous cell carcinoma of the head and neck through resensitization to cell death

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ABSTRACT

The clinical outcome of head and neck squamous cell carcinoma (HNSCC) remains poor with high recurrence rates, in part due to resistance to concurrent platinum-based chemotherapy. The anti-apoptotic BCL2 protein is involved in apoptosis resistance and tumor cell invasion/migration. Here, we test whether BCL2 overexpression predicts poor therapeutic response of HNSCC to cisplatin-based chemoradiotherapy and the effects of selective BCL2 inhibition on cisplatin-induced cell changes *in vitro*.

BCL2 immunostatus was correlated with survival after chemoradiotherapy in a uniformly treated HNSCC cohort. The combination therapy of ABT-199, a BCL2 inhibitor, and cisplatin was evaluated *in vitro* using corresponding patient-derived cell lines. Colony formation and the mode of cell death were analyzed in-depth.

Patients with BCL2-positive tumors (44/254) prior to treatment (either radiation, cisplatin monotherapy, or both) had shorter overall and progression-free survival (log-rank; $p = 0.048$) and a higher rate of tumor relapse (Fisher's exact test; $p = 0.0032$). BCL2 inhibition alone had no effect on cell functions in our triple panel of cisplatin-sensitive cell lines but enhanced cisplatin-induced effects. Rates of autophagy and cell death, including methuosis, were doubled, while epithelial-mesenchymal transformation was inhibited.

As selective inhibition of BCL2 is available and standard of care in other malignancies, its immunohistochemical assessment could help personalize therapy by identifying a subpopulation to overcome chemoresistance, particularly in locally advanced HNSCC.

Introduction

Head and neck cancers are a heterogeneous group of malignancies with increasing incidence worldwide. The majority of cancers are squamous cell carcinomas arising from the epithelium of the oral and nasal cavities, pharynx, and larynx [1]. Existing treatments can improve survival rates, but serious consequences can occur, including disruption of vital anatomy, aesthetic impairment, and associated psychosocial problems [2].

Chemotherapy is an essential component of multimodal treatment strategies for HNSCC. Approximately 60 % of patients with advanced disease receive either curative or palliative chemotherapy during treatment [3]. Depending on clinical presentation, radiotherapy may be

combined with chemotherapy, which is mainly cisplatin [1]. Cisplatin crosslinks DNA in several ways, interfering with cell division through mitosis. The damaged DNA triggers DNA repair mechanisms, which in turn activate apoptosis when repair proves impossible [4]. This tumor cell death is counteracted by resistance mechanisms, which is a limitation to treatment options [5]. The avoidance of therapy-induced apoptosis is a hallmark of acquired resistance. Inhibition of apoptosis is considered an essential step in the transition of normal to malignant cells. Among the key regulators of apoptosis, the B-cell lymphoma 2 (BCL2) protein family plays an important role.

BCL2 is an anti-apoptotic protein and is normally not upregulated in HNSCC. Aberrant overexpression occurs in approximately 15–25 % of HNSCC tumors, resulting in resistance to cisplatin-induced apoptosis [6,

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7]. A substantial body of research shows that targeting BCL2 family members has synergistic activity with standard therapies that use cytotoxic agents such as cisplatin to kill HNSCC cells [8,9]. While pre-clinical studies were promising, translation into clinical practice was not feasible due to poor oral absorption or toxicity related side effects [10]. ABT-199 (= venetoclax), which exclusively targets the BCL2 protein, is EMA/FDA-approved for the treatment of several types of leukemia [11, 12]. Venetoclax re-sensitized leukemic tumors towards chemotherapy [13]. In combination to other drugs, the selective inhibition of BCL2 by venetoclax mediated chemotherapeutic effects in breast, cervical and colon cancer as well as in osteosarcoma ([14–16]. While the pan-BCL-inhibitor AT-101 is a competent enhancer of radiation-induced apoptosis in HNSCC *in vitro* [17], venetoclax has not been studied in HNSCC patients receiving cisplatin. Venetoclax in combination with radiation and the anti-EGFR antibody cetuximab enhanced antitumoral effects in HNSCC cells, including inhibition of proliferation, invasion/migration, and overcoming apoptosis resistance [7].

The aim of this study is to evaluate the potential of venetoclax as a chemotherapeutic sensitizer, as (radio)chemotherapy is a fundamental part of treatment strategies in HNSCC. Therefore, we investigated the effects of combined venetoclax cisplatin treatment on cellular expression patterns and cytotoxic effects in patient-derived HNSCC cell lines. We also analyzed BCL2 protein expression in HNSCC samples as a potential therapeutic option.

Material and methods

Patient cohort

Pretreatment samples from locally advanced or recurrent/metastatic HNSCC patients, diagnosed between 2000 and 2021, receiving either cisplatin, radiation or radiochemotherapy as first line adjuvant treatment were collected from Rostock Pathology archives. Patients' characteristics including HPV status and genomic alterations were obtained from clinical records and pathology reports. Status of PD-L1, CMTM6 and density of tumor infiltrating lymphocytes (TIL) were extracted from prior studies [18]. Using pseudonyms, follow-up data were obtained from the regional Cancer Registries. Tumor tissue was obtained from formalin-fixed, paraffin-embedded (FFPE) specimens. Tissue microarrays (TMAs) of three representative areas were constructed using 1 mm cores with a manual tissue microarrayer (Beecher Instruments, Silver Spring, MD, USA). Tissues were collected with patient consent. The institutional ethic committee at the University Hospital Rostock approved to the study (A2018–0003; A2022–0120) which was conducted in accordance with the Declaration of Helsinki of 1975.

Immunohistochemistry

Slides of 4 µm were used. Heat-induced antigen retrieval was performed with a high pH buffer (20 min at 97 °C). The following steps were performed in an Autostainer link 48 instrument (Dako, Hamburg, Germany): 5 min of incubation in peroxidase-blocking buffer followed by 20 min of incubation with primary antibody (BCL2: 1:100, SC06–86, GeneTex, Alton Pkwy Irvine, CA, USA) and 3,3'-diaminobenzidine (DAB) detection using the Dako-kit K8000 according to the instructions of the manufacturer. Slides were counterstained with hematoxylin. Lymphocytes served as positive controls.

Tumor cells with membranous and/ or cytoplasmic BCL2 staining of any intensity were counted manually in the 400x- magnification, and scoring was based on the percent of positive cells as negative (<29 %) or positive (≥30 %).

Cell culture

The HNSCC 16 (HPV^{neg}/ p16^{neg}), 46 (HPV^{neg}/ p16^{pos}) and 48 (HPV^{neg}/ p16^{neg}) cell lines were derived from patient xenograft models

and established by our group [19]. Cells' characteristics including detailed molecular profiling and chemosensitivity have been described previously [19]. Cells were cultured in DMEM supplemented with 10 % fetal bovine serum (FBS), 100 U/mL penicillin, 0.1 g/L streptomycin, and 6 mM l-Glutamine.

Colony formation assay

Cells were seeded in 24-well plates at 500 cells/well. After 24 h, cells were treated for 2 × 72 h with cisplatin (University Hospital Rostock Pharmacy), venetoclax (MedChemExpress, NJ, USA), or a combination of both at doses determined before: abemaciclib [HNSCC16 P1 M1/ HNSCC46 P0 M2: 1 µM, HNSCC48 P0 M1: 0.1 µM], cisplatin [HNSCC16 P1 M1/ HNSCC46 P0 M2: 1 µg/mL, HNSCC48 P0 M1: 0.05 µg/mL]. Readout was performed using crystal violet staining. Colonies were counted with ImageJ (Hersteller). The Bliss Independence model was used to analyze potential synergistic and additive effects between the compounds.

RNA isolation, cDNA synthesis, and quantitative real-time PCR

Total RNA was isolated with RNeasy Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. RNA was reverse-transcribed into cDNA from 1 µg RNA using 1 µl dNTP mix (10 mM), oligo (dT)15 primer (50 ng/µl), 1 µl reverse transcriptase (100 U) and 4 µl 5x RT buffer complete (all purchased from Bioron GmbH, Ludwigshafen, Germany). cDNA synthesis conditions were as follows: 70 °C for 10 min, 45 °C for 120 min, 70 °C for 10 min. Target cDNA levels of HNSCC cell lines were analyzed by quantitative real-time PCR using TaqMan Universal PCR Master Mix (Thermo Fisher Scientific, Darmstadt, Germany) and predesigned BCL2 TaqMan gene expression assay labeled with 6-FAM-3' BHQ-1: Hs00608023_m1 (Thermo Fisher Scientific). GAPDH was used as housekeeping gene. Primers were self-designed (Hersteller) 5' HEX BHQ-TGCCATCAATGACCCCTTCATTG-3'; for: 5' -TCACCAGGGCTGCTTTTAAC-3'; rev: 5' -GGGTGGAATCATATTG-GAACA-3'. Reaction was performed 12.5 ng cDNA with the following PCR conditions: 95 °C for 10 min, 40 cycles of 15 s at 95 °C, and 1 min at 60 °C. All reactions were run in triplicates. The mRNA levels of target genes were normalized to GAPDH. The general expression level of each sample was considered by calculating 2-ΔΔCT resulting from the difference between ΔCT_{target}-ΔCT_{Calibrator}. Untreated cells were used as calibrator.

Flow cytometry

Apoptotic/necrotic cells were detected by Yo-Pro-1 and propidium iodide (PI) staining as described before [20]. Briefly, early and late apoptotic cells were detected by either Yo-Pro-1 or Yo-Pro-1/propidium iodide (PI) positivity. Necrotic cells were defined as Yo-Pro-1 negative/PI positive.

Extended flow cytometry included assessment of BCL2 (APC REA anti-human BCL, please see below for details), as well as several cell death and DNA-damage associated markers. Therefore, two in-house panels were applied. Panel 1 and 2 were used to study apoptosis, DNA damage, autophagy, cell stress, and methuosis. Panel 3 was used to identify epithelial-mesenchymal transition (EMT) and glucose transporters. For this purpose, 0.5 × 10⁶ cells per panel were taken and processed. All procedures were performed using staining buffer (PBS, 2 mM EDTA, 2 % BSA).

Panel 1: Extracellular staining was done using Apotracker green (Stock: 80 µM, 1:400 final, Biolegend, San Diego, USA), CYTO-ID (0.5 µl/sample, CYTO-ID® Autophagy detection kit 2.0, Enzo Life Sciences GmbH, Lörrach, Germany). Membrane permeabilization was done as first step (True Nuclear Transcription Factor Buffer Set, Biolegend, True-Nuclear™ 1X Fix concentrate, 45 min, RT). Then, the True-Nuclear™ 1X Perm Buffer (Biolegend) was added, cells were washed (350 x g, 5 min)

and stained with antibodies for intracellular staining (in 100 µl True-Nuclear™ 1X Perm Buffer): Alexa Fluor 700 mouse anti-human cleaved PARP (Clone F21–852, BD Biosciences, Heidelberg, Germany), PE/Cyanine7 mouse anti-H2A.X phospho (clone: 2F3, 1:40, Biolegend), V450 rat anti-histone H3 (1:40, BD Biosciences), and Alexa Fluor 647 rabbit anti-human LC3B (clone: 1251A, R&D Systems, Minneapolis, USA). Staining was done for 30 min at RT, reaction was stopped with True-Nuclear™ 1X Perm Buffer, followed by two washing steps (350 x g, 5 min). Cells were resuspended in 0.35 ml staining buffer.

Panel 2: Extracellular staining was done for 20 min at RT in staining buffer (in 100 µl): FITC mouse anti-human CD107a (clone: H4A3, Biolegend) and Alexa Fluor 594 rat anti-human Rab7a (clone: W16034A Biolegend). Intracellular staining was done after permeabilization as described for Panel 1 using the following antibodies: APC REA anti-human BCL2 (clone: REA872, Miltenyi Biotec, Bergisch-Gladbach, Germany) and Alexa Fluor 405 mouse anti-human Hif1α (clone: 241,812, R&D). All subsequent steps were done as described above. Cells were resuspended in 0.35 ml staining buffer.

Panel 3: Extracellular staining was done for 20 min at RT in staining buffer (in 100 µl): PE/Cyanine7 mouse anti-human CD325 (1:25, N–Cadherin, clone: 8C11, Biolegend), and APC-Vio 770 mouse anti-human CD324 (1:15, E-Cadherin, clone 67A4, Biolegend). Afterwards, cells were washed two times followed by membrane permeabilization (BD Transcription Factor Buffer Set, BD, 1x Fix/Perm Working solution, 45 min, 4 °C). Then, the 1x Perm/Wash Buffer (BD) was added, cells were washed (350 x g, 5 min) and were stained with antibodies for intracellular staining (in 100 µl 1x Perm/Wash Buffer): Alexa Fluor 647 mouse anti-human Glut1 (1:500, BD) and Alexa Fluor 700 mouse anti-human Glut4 (1:100, clone: # 925,932, R&D). Staining was done for 30 min at room temperature, reaction was stopped with 1x Perm/Wash Buffer, followed by two washing steps (350 x g, 5 min). Cells were finally suspended in 0.35 ml staining buffer.

All measurements were done on a spectral flow cytometer (Cytek Aurora, Cytek Biosciences, Fremont, California, United States) in the Core Facility for Cell Sorting and Cell Analysis, University Medical Center Rostock, Rostock, Germany.

Immunofluorescence

Cells were fixed with 2 % paraformaldehyde (PFA) w/o methanol (15 min, Thermo Fisher Scientific), permeabilized, and blocked with 0.5 % Triton X-100 (Thermo Fisher Scientific,) in 2 % BSA (PAN-Biotech, Aidenbach, Germany) for 60 min. Slides were incubated with alexa Fluor® 594 anti-Vimentin antibody (clone: O91D3, Biolegend) over night at 4 °C, washed three times with PBS, followed by phalloidin green staining (1:50 in PBS) for 20 min. Nuclei were counterstained with DAPI (1:1.000, Biomol, Hamburg, Germany) and cells were analyzed using a Zeiss microscope Axiovert A1 (Zeiss, Oberkochen, Germany).

Statistics

All values are given as mean ± SD (*in vitro* analysis) or mean ± SEM (*in vivo* approach). Statistical evaluation was performed using GraphPad PRISM software, version 8.0.2 (GraphPad Software, San Diego, CA, USA). The criterion for significance was set to $p < 0.05$. *In vitro* experiments were executed in biological triplicates and summarized as mean values with standard deviation. After proving the assumption of normality (Shapiro–Wilk test), one-way ANOVA (Dunnett's multiple comparison), two-way ANOVA (Tukey's multiple comparison) or *t*-test was performed. If the normality test failed, the Kruskal–Wallis or U-Test was performed. Survival distributions were compared using the log-rank test.

Results

Patients with HNSCC tumors expressing BCL2 had a higher risk of recurrence

Overall, 17 % of the 254 included HNSCC samples were BCL2 positive (Fig. 1A and B). Details of the representative cohort are shown in suppl. Table 1. 28 % of patients suffered from p16-positive tumors. In BCL2-positive tumors, the staining intensity was homogeneous. Intratumoral heterogeneity, defined as a percentage of labeled tumor cell fractions (35 to 70 %), was observed in 50 % of positive cases. No correlation with PD-L1 expression as assessed by the combined positive score nor its post-translational stabilizer CMTM6 not tumor infiltrating lymphocytes (TILs) was found in cases where data on these markers were available ($n = 151$). The recurrence rate was significantly higher for BCL2 positive tumors compared to the BCL2 negative counterpart ($p = 0.0032$, Fisher's exact test). Patients whose tumors were BCL2 positive had a significantly shorter OS regardless of treatment ($p = 0.048$; log rank; Fig. 1C). Further stratification revealed that this effect was significant only in patients receiving radiochemotherapy ($n = 128$; $p = 0.01$), but not in the cisplatin- or radiation-treated subgroups (Fig. 1D). Progression-free survival (PFS) was significantly shorter in patients with BCL2 positive tumors ($p = 0.003$) than in the BCL2 negative group. This effect was significant in patients receiving radiation ($n = 68$) or radiochemotherapy ($p < 0.0001$ and $p = 0.003$, respectively), but not in the cisplatin-treated subgroup (Fig. 1E and F).

We examined whether BCL2 expression correlated with other clinical characteristics to test the hypothesis that BCL2 expression is a marker of poor therapeutic outcome. However, none of the characteristics, including anatomic site and p16 status, differed significantly after stratification according to BCL2 status (Suppl. Table 1).

BCL2 in cell lines and its treatment-related changes

Three patient-derived cell lines from our HNSCC biobank were included [21,22]. BCL2 expression was confirmed in all three cell lines with, however, with cell line dependent differences (Fig. 2A). Cisplatin treatment increased BCL2 expression levels in 2/3 cases. Like illustrated in Fig. 2B, BCL2 levels rise after cisplatin treatment with the highest increase in cell line HNSCC16, where this anti-apoptotic molecule normally shows low mRNA levels. Venetoclax alone slightly inhibited BCL2 expression in all three cell lines, but this effect is lost in the combination. In BCL2^{low} HNSCC16, treatment with either cisplatin or venetoclax increased the rate of BCL2 positive cells, and the fraction was higher when both drugs were combined (Fig. 2C).

Then, the effect of either treatment on colony formation was studied (Fig. 2D). While venetoclax alone was not able to reduce colony formation, its combination with cisplatin reinforced this effect in all three cell lines, irrespectively of the pre-existing BCL2 mRNA expression status. Notably, this growth inhibitory effect was synergistic in HNSCC16 and HNSCC46 (Fig. 2E). Subsequent flow cytometric apoptosis/necrosis measurement confirmed antitumoral effects, which were mainly attributable to necrotic cell death (Fig. 3A). While this was highly significant in HNSCC16 and HNSCC46, no such therapeutic effects were detectable in the BCL2^{high} HNSCC48 cells, which were classified as resistant.

Taken together, venetoclax reinforces the cytotoxic effects of cisplatin monotherapy.

Combination of venetoclax and cisplatin and its effect on the mode of cell death

To gain a deeper understanding of the mechanisms of response and eventually resistance, a full spectrum flow cytometric approach was applied to HNSCC16 cells (Fig. 3B). With this analysis, complex mechanisms of cell death were identified and the effects of cisplatin

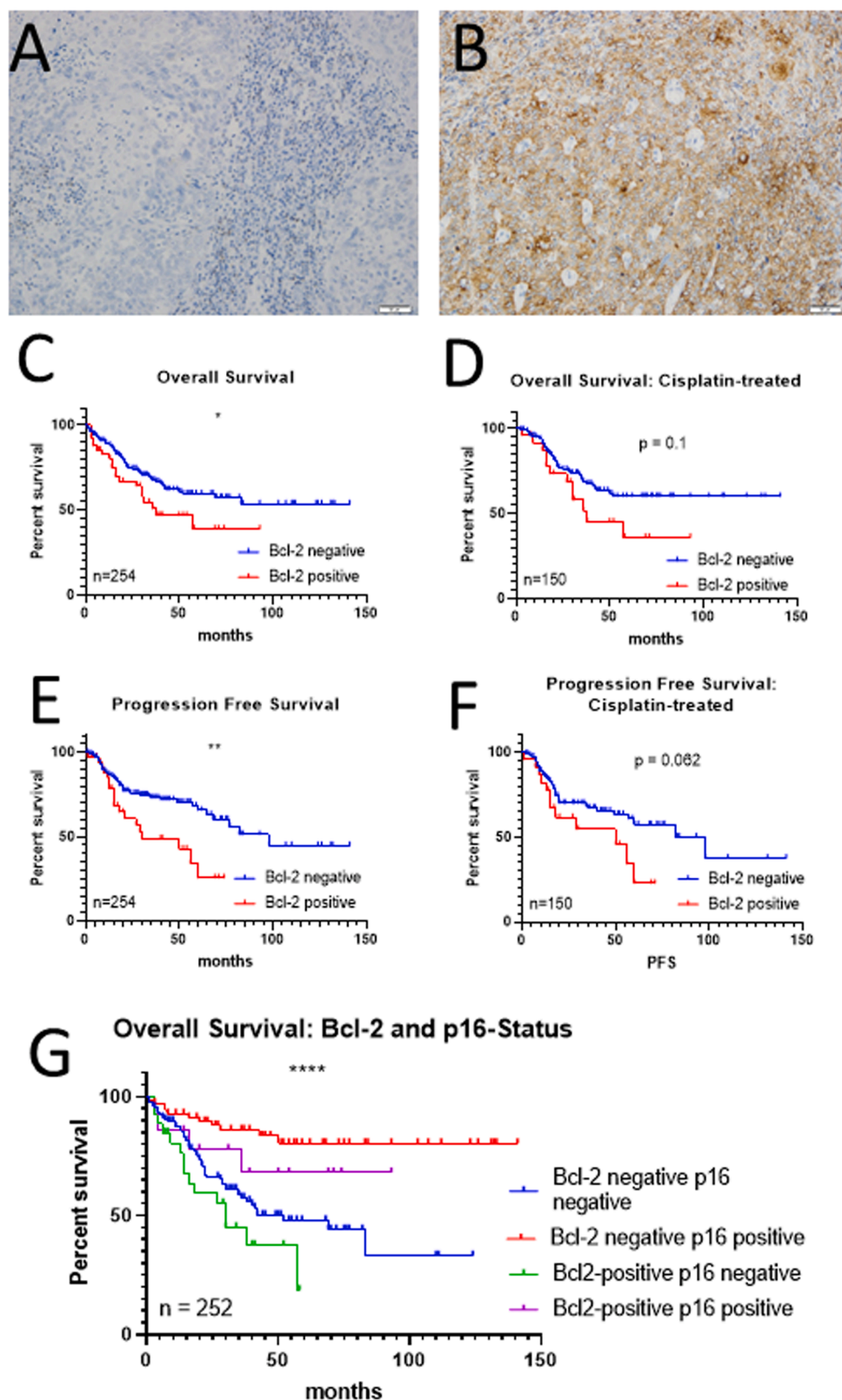


Fig. 1. Immunohistochemical staining for BCL2 and its impact on survival:

A, B) representative images of a BCL2 negative (A) and positive HNSCC specimen (B), respectively (200x). C-F) Kaplan-Meier plots of overall (C, E) and progression-free (D, F) survival for the whole cohort (receiving cisplatin, radiation, or radiochemotherapy) (C, E) and the Cisplatin-treated subgroup (D, F). BCL2 expression was related to a shortened survival with patients of BCL2-positive, p16^{negative} tumors having the shortest survival (G). * $p < 0.05$; ** $p < 0.01$; **** $p < 0.0001$.

Table 1
Clinicopathological characteristics of the cohort (n = 254).

Characteristic	Bcl-2 No Cisplatin	negative + Cisplatin	Bcl-2 No Cisplatin	positive + Cisplatin	p- value*
n	88	122	16	28	
Median age (range)	59,5 (36–70)	60 (40–83)	53 (45–67)	54 (46–67)	n.s
Gender			n.s		n.s
male	59	100	16	26	
female	29	22	3	2	
Localization			n.s		n.s
Oral	15	34	5	9	
Oropharyngeal	43	41	8	12	
Hypopharyngeal	8	22	1	2	
Laryngeal	22	24	2	5	
UICC stage					n.s
I/II	48	27	5	8	
III/IV	40	95	11	20	
Treatment					n.s
surgery/radiation only	32	–	6	–	
+ radiation	56	–	10	–	
+ chemotherapy	–	60	–	15	
+ chemoradiation	–	62	–	13	
2-year OS	71 %	57 %	50 %	54 %	n.s
Recurrence					
Yes	20 %	34 %	44 %	54 %	**
P16 [#]					n.s
negative	58	85	12	20	
positive	28	37	4	8	

* Significance level: * p < 0.05; ** p < 0.01; # n = 252; n.s, not significant.

monotherapy were mostly enhanced by the addition of venetoclax. In detail, we identified significantly more cleaved PARP⁺ apoptotic cells, higher levels of DNA damage (γH2A.X⁺), and reduced amounts of cells in G2-phase (pH3⁺). Another rather unexpected finding was the massive induction of autophagy (Cyto-ID⁺LC3b⁺), accompanied by high levels of HIF1α and signs of methuosis. The latter was detected by LAMP-1/Rab7a positivity and the number of cells undergoing this type of cell death almost doubled when both drugs were combined. Thus, the addition of venetoclax to cisplatin enhances the inhibitory and pro-apoptotic effects of both monotherapies *via* the induction of autophagy and methuosis.

Combination of venetoclax and cisplatin and its effect on epithelial mesenchymal transition

Epithelial-mesenchymal transition (EMT), *i.e.* the conversion of immobile epithelial cells into motile mesenchymal cells and the alteration of cell–cell adhesion and extracellular matrix is an unwanted but common resistance mechanism after cisplatin-based chemotherapy. To examine if this mechanism may also play a role here, we analyzed the status of different EMT markers. As shown in Fig. 4A, the fraction of E-cadherin⁺/N–Cadherin⁺ cells increased in HNSCC16 cells as a result of cisplatin monotherapy or its combination with venetoclax indicating the so-called “cadherin switch”. The ratio of SNAIL⁺/Vimentin⁺ cells as another indicator of EMT increased tenfold after cisplatin treatment of BCL2^{low} HNSCC16 cells. Notably, the addition of venetoclax to cisplatin partially reversed this effect by approximately 50 %. This reduction implies a significant inhibition of cisplatin-induced EMT by venetoclax. In support of this, the levels of Glut1⁺/Glut4⁺ cells were lower in the combination, compared to cisplatin monotherapy. Using phalloidin green as an epithelial and vimentin as a mesenchymal marker, this effect is illustrated in Fig. 4B, where vimentin positive cells are markedly reduced in the combination (Fig. 4B). Although this did not reach statistical significance, we would like to mention this positive trend of reversed EMT in both HNSCC16 and HNSCC46 cells. Besides, cells in the combination group showed morphological signs of cell stress (as evidenced by the presence of stress fibers, please see orange box for

visualization). Since this was not seen after monotherapy, we conclude that enhanced cell stress is a contributing factor to the increased cell death in the combination.

Discussion

Our study confirms that HNSCC patients with BCL2-positive tumors have a shorter PFS and OS. We demonstrate a significant inhibition of cell proliferation and an increase in cell death when HNSCC cells are treated with the combination of cisplatin and venetoclax. Inhibition of BCL2 by this drug together with cisplatin reduced EMT capacities in cells, independent of pre-existing BCL2 status. Combined therapy targeting anti-apoptotic pathways, which normally promote chemotherapy resistance, may overcome cisplatin resistance-related tumor escape by re-inducing apoptosis and inhibiting EMT.

BCL2 has been studied in HNSCC for decades [23] with conflicting predictive value. As Michaud et al. conclude in their 2010 study, this can be partly explained by the heterogeneity of collectives and different scoring systems. Using a simple binary scoring system, BCL2 is associated with shorter PFS in patients receiving radio-chemotherapy. This is consistent with the findings of Michaud, who induced BCL2 expression in cell lines using a retroviral expression vector and observed an increased cisplatin resistance. Here, we use venetoclax to inhibit BCL2 activity and see congruent effects. More recently, a French group reported increased tumor cell death *in vitro* and delayed tumor growth with prolonged lifespan in tumor-bearing mice after treatment with venetoclax, cetuximab (an EGFR inhibitor) and radiation. The addition of another potent but less selective BCL2 inhibitor (ABT-263, or navitoclax) also increased tumor cell apoptosis *in vitro* independent of p53 status and significantly delayed tumor growth in mice [9]. The authors concluded a senolytic-mediated clearance of senescent HNSCC cells. In addition to inhibiting BCL2, navitoclax also inhibits its family members Bcl-xL and Bcl-w, which may add to the toxicity for HNSCC patients, reducing its clinical utility [8]. Despite efforts to treat patients with advanced HNSCC, median survival remains poor [1]. Venetoclax in combination with radio-chemotherapy may represent a new therapeutic option, particularly for patients with cisplatin-resistant tumors, and further clinical trials are needed to demonstrate its efficacy [24].

While the poor outcome of BCL2-positive HNSCC patients and the ability of venetoclax to inhibit cell proliferation, invasion/migration, and resistance to apoptosis have been demonstrated, a novel and unique finding of our study is the enhanced cisplatin-mediated cytotoxicity in combination with venetoclax [6,7,25,26]. Cell death was identified by autophagy and methuosis. The latter was originally defined in glioblastoma cells after ectopic expression of activated RAS [27]. In this context, RAS signaling is of interest because ectopic expression of an activated form of the GTPase HRAS oncoprotein (G12 V) induces massive cytoplasmic vacuolization and caspase-independent cell death in cultured glioblastoma and gastric cancer cells [28]. Methuosis is responsible for the increased rate of non-apoptotic cell death observed in our cell lines, when cisplatin and venetoclax are combined. While other mechanisms may induce methuosis in parallel with oncogenic RAS, our data suggest a more complex mechanism of action for venetoclax.

Consistent with previous findings, BCL2 expression in HNSCC specimens was associated with shorter OS and PFS [26]. This, in contrast to the questionable BCL2 specificity of venetoclax, argues in favor of this protein as a potential predictive biomarker, as immunohistochemical staining is simple, *i.e.*, either scoring as negative or positive (membranous/cytoplasmic staining in ≥30 % of tumor cells).

Some studies describe methuosis-like features upon exposure to small molecules, particularly in cells resistant to pro-apoptotic drugs [27]. The Cancer Genome Atlas profiling revealed mutations in *HRAS* in approximately 4 % of HNSCCs tested. These mutations were all detected in HPV^{neg} cases, often with inactivating *CASP8* mutations, and wild-type *TP53* [29]. Furthermore, cells with an oncogenic RAS were extremely sensitive to the inhibition of protein kinase C and BCL2 could antagonize

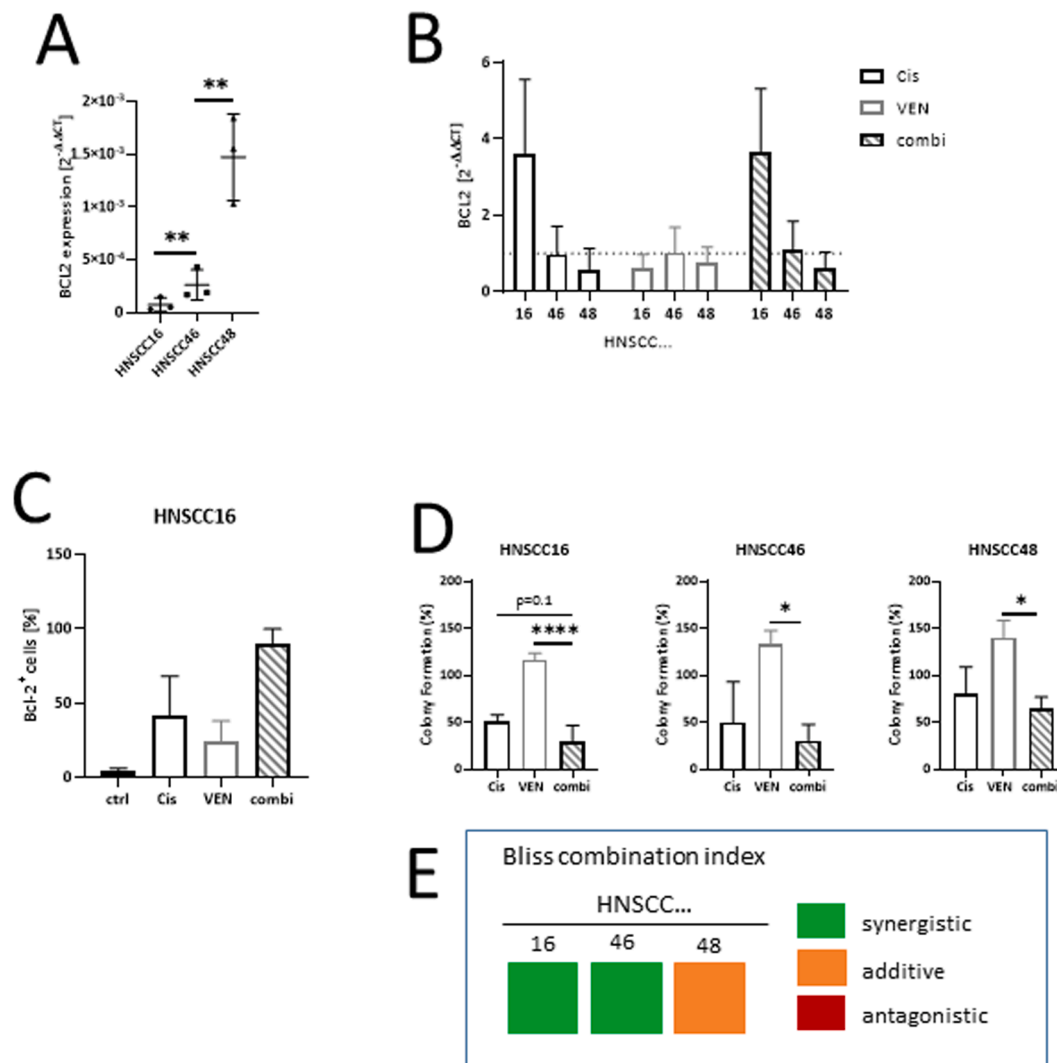


Fig. 2. BCL2 status in patient-derived HNSCC cells & impact of treatment on cell growth:

(A) Pre-treatment mRNA expression levels with lowest levels in HNSCC16 and highest level in HNSCC48, which was derived from a patient with recurrent disease. ** $p < 0.01$; one-way ANOVA (Tukey's multiple comparison test). (B) Treatment-induced changes in mRNA expression levels. (A, B) mean $\pm$ SD, $n = 3$ independent experiments. Values are given as $2^{-\Delta\Delta CT}$. Untreated cells were used as calibrator. (C) Treatment-induced changes in protein levels using HNSCC16. Notably, residual cells in the venetoclax/cisplatin combination group showed highest BCL2 levels (y-axes related to non-treated controls). mean $\pm$ SD, $n = 3$ independent experiments. (D) Colony formation assay. While venetoclax monotherapy had no effect on colony formation, the cisplatin induced inhibition doubles in all cell lines when venetoclax and cisplatin are combined (y-axes related to non-treated controls). mean $\pm$ SD, $n = 3$ independent experiments. * $p < 0.05$; **** $p < 0.0001$; one-way ANOVA (Tukey's multiple comparison test) (E) Bliss combination index for cisplatin + venetoclax therapy in the three cell lines to calculate synergistic effects of the combination in comparison to either monotherapy.

this apoptotic process [7,30]. Additionally, mechanisms of resistance to venetoclax-based therapy in acute myeloid leukemia include *TP53* gene mutations or inactivation of the p53 protein, and activating kinase mutations such as RAS. Given that *TP53* mutations (a) occur in $\sim 72\%$ of HNSCC cases with enrichment in the HPV^{neg} group, (b) confer cisplatin resistance, (c) are currently undruggable [31,32], and (d) can co-occur with *HRAS* mutations, venetoclax represents a realistic treatment option for a subset of HNSCC patients.

The patient of cell line HNSCC48 was suffering from recurrent oral cancer and was treated with chemoradiation after the initial diagnosis. While the initial tumor did not stain for BCL2 by immunohistochemistry, the recurrent tumor tissue taken to establish the cell line stained positive for BCL2 in 50 % of tumor cells (Supplementary Figure 1). This suggests that the BCL2 may be associated with a more aggressive phenotype. In addition, EMT is a major contributor to drug resistance and, conversely, may be driven by cisplatin through the up-regulation of Snail and N-cadherin in HNSCC cells [5,33]. Evasion of the immune response by

altering the expression of molecules involved in immunosuppression or immune evasion is another important mechanism of EMT-driven resistance. Upregulation of BCL2 is associated with cisplatin resistance via inhibition of Bax translocation [34]. In cervical cancer, a lower dose of cisplatin is feasible when combined with BCL2 silencing as an adjuvant treatment to reduce resistance [35]. The addition of venetoclax to cisplatin reduced cisplatin-induced EMT in our cell lines. While the beneficial cytotoxic effect of BCL2 inhibition in parallel with standard therapy has been shown in HNSCC cell lines [7,25], the inhibitory potential of EMT is a novel finding. Since cisplatin increases BCL2 levels, the inhibitory function of venetoclax targets EMT independent of treatment-naïve BCL2 status. It is known that BCL2 family members regulate EMT through calcium signaling [36] and that BCL2 induces EMT in mammalian cell lines [37]. The exact interplay is complex and not fully understood. In the context of a possible broader pharmacological axis for venetoclax beyond BCL2, this agent may be integrated into cisplatin-based treatment regimens for selected patients.

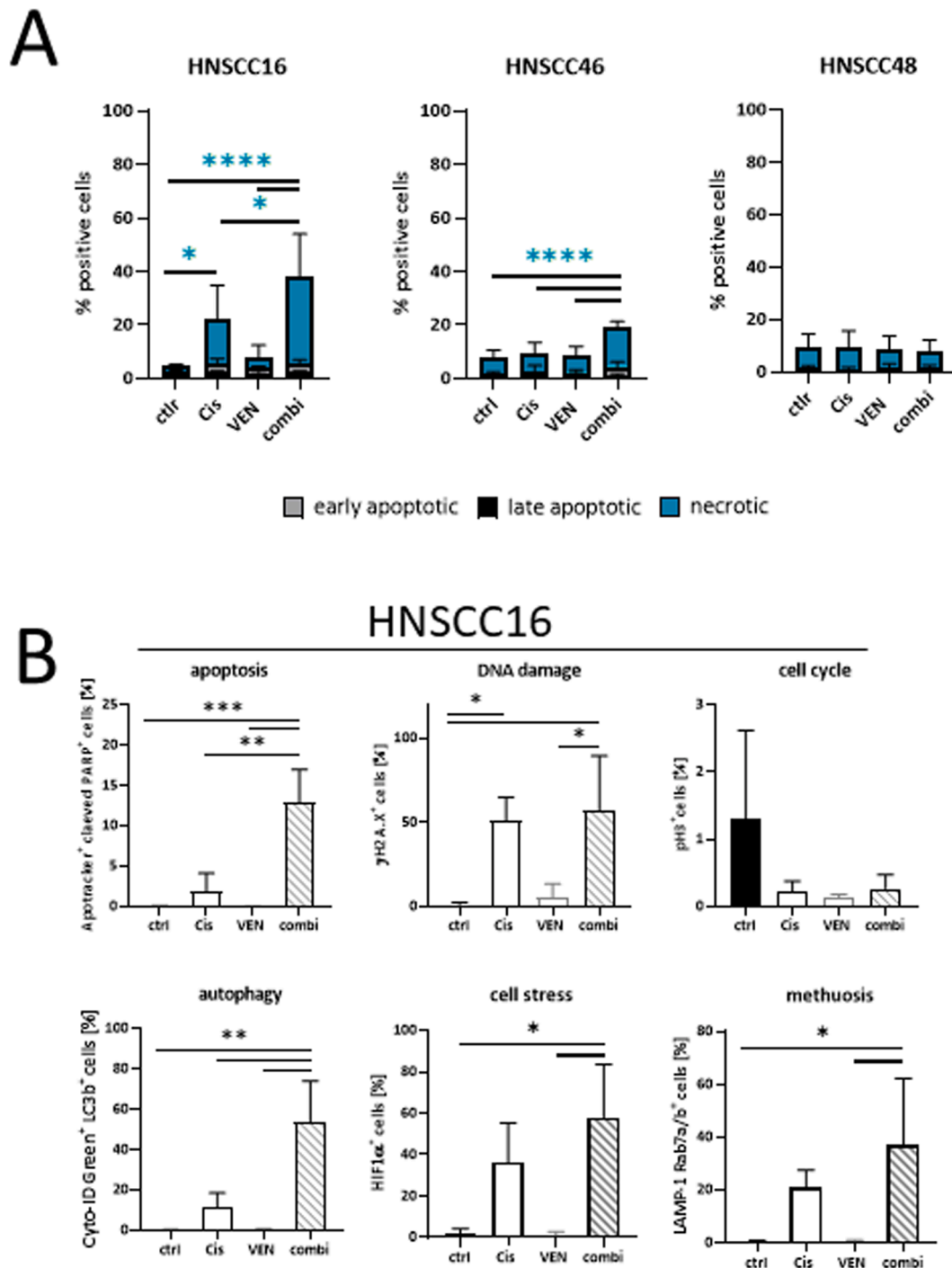


Fig. 3. Treatment-related changes in patient-derived HNSCC cells (flow cytometry).

(A) Apoptosis/necrosis assay. Cells were stained with Yo-Pro 1 iodide and PI. Cells that were positive for Yo-Pro 1 iodide were defined as early apoptotic, cells that were positive for PI were defined as necrotic, and double positive cells were defined as late apoptotic. Apoptosis/necrosis assay was done after 2×72 h. mean $\pm$ SD, $n = 3$ independent experiments. * $p < 0.05$; **** $p < 0.0001$; one-way ANOVA (Tukey's multiple comparison test) (B) Changes in apoptosis, DNA damage, cell cycle, autophagy, cell stress, and methuosis are shown for HNSCC16. While cisplatin alone induced apoptosis, DNA damage, autophagy, cell stress, and methuosis by reducing cell cycle at the same time, effects are stronger using double therapy with a significantly increase for cells undergoing apoptosis, autophagy, and methuosis. Venetoclax had no impact on cell death. mean $\pm$ SD, $n = 3$ independent experiments. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; one-way ANOVA (Tukey's multiple comparison test).

This study has some limitations. First, experiments could have been performed with more cell lines. We attempted to address this by using three patient-derived cell lines from different anatomical sites, including one cell line from a recurrent cancer, each with a different molecular profile. Second, the *in vitro* part did not consider HPV-related effects, although HPV-driven HNSCC respond better to chemoradiation than

their HPV-negative counterpart. As we did not have HPV-positive cell lines established in our lab, we focused on the HPV-negative counterpart, and observed no differences in the treatment effect when therapies were combined.

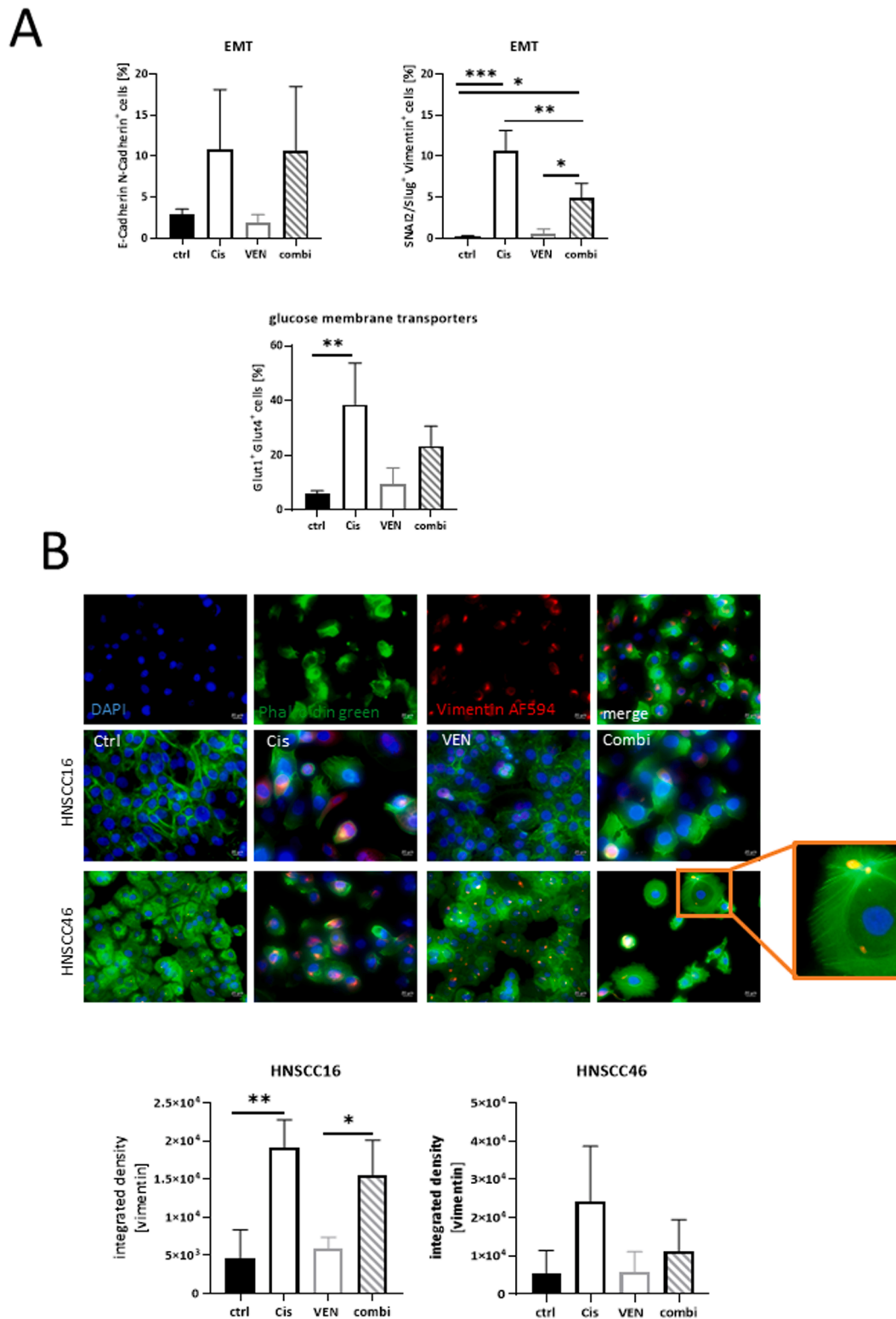


Fig. 4. Impact of BCL2 blockade on epithelial-mesenchymal transition (EMT).

(A) Flow cytometric staining for EMT markers in HNSCC16. Adding venetoclax to cisplatin has no effect of cisplatin induced changes in the ratio of cells expressing E-Cadherin/N—Cadherin but reduced the ratio of cisplatin monotherapy-induced increased numbers SNAI⁺/Vimentin⁺ cells. mean $\pm$ SD, $n = 3$ independent experiments. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; one-way ANOVA (Tukey's multiple comparison test). (B) Immunofluorescence to confirm flow cytometry results. Semi-quantitative analysis of EMT marker vimentin was done for HNSCC16 and HNSCC46 cells after treatment. With this analysis, the potent antitumoral effect of the venetoclax/cisplatin combination was confirmed, but had minor influence of vimentin intensity in residual cells. Original magnification 400x. mean $\pm$ SD, $n = 3$ independent experiments. * $p < 0.05$; ** $p < 0.01$; one-way ANOVA (Tukey's multiple comparison test).

Conclusion

Cisplatin is widely used to treat advanced HNSCC, but therapy resistance often occurs. Adding the BCL2 inhibitor venetoclax to chemotherapy may reinforce cisplatin-based cell death and help to overcome therapy resistance by weakening EMT and re-inducing cell death. Before this drug, which is standard of care for some hematological malignancies, can be established in HNSCC patients, biomarkers are needed to identify patients who will benefit most from a combined therapy.

Ethics approval and consent to participate

The institutional ethic committee at the University Hospital Rostock approved the study (A2018–0003; A2022–0120). The study was performed in accordance with the Declaration of Helsinki.

CRediT authorship contribution statement

Anne-Sophie Becker: Writing – review & editing, Writing – original draft, Project administration, Methodology, Formal analysis, Data curation, Conceptualization. **Friederike Klauk:** Methodology, Data curation. **Thomas Freitag:** Methodology, Data curation. **Daniel Fabian Strüder:** Writing – review & editing, Supervision, Project administration. **Björn Schneider:** Validation, Resources, Methodology, Investigation. **Annette Zimpfer:** Validation, Supervision, Resources. **Claudia Maletzki:** Writing – review & editing, Writing – original draft, Supervision, Resources, Project administration, Investigation, Formal analysis, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at [doi:10.1016/j.tranon.2025.102308](https://doi.org/10.1016/j.tranon.2025.102308).

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Research

CMTM6 status predicts survival in head and neck squamous cell carcinoma and correlates with PD-L1 expression

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Abstract

We retrospectively analyzed 129 treatment-naïve head and neck squamous cell carcinomas (HNSCCs) for the expression of programmed death ligand 1 (PD-L1), CKLF-like MARVEL transmembrane domain-containing 6 (CMTM6), tumor-infiltrating leukocytes (TILs), and tumor-associated macrophages (TAMs). We evaluated the relationships among these markers, human papilloma virus (HPV) status, and overall survival (OS). PD-L1 and CMTM6 (combined positive score (CPS) ≥ 1 and ≥ 5) were detected in ~70% of HNSCCs. HPV status had insignificant effects on marker expression. Most PD-L1-positive cases showed concomitant CMTM6 expression with comparable staining patterns. While *PD-L1* and *CMTM6* mRNA expression levels correlated with PD-L1 and CMTM6 protein status, no significant correlation was observed for PD-L1 and CMTM6 mRNA expression. Tumors expressing PD-L1 ($p < 0.0001$) and/or CMTM6 ($p < 0.05$) were associated with the best OS. A high density of TILs ($p < 0.01$), CD8⁺ T cells ($p < 0.001$), and CD68/CD163 ratio > 1 were prognostically relevant. In addition to HPV status, PD-L1 and CD8⁺ T cells, CMTM6 was identified as an independent prognostic factor using a multivariate Cox regression analysis. PD-L1 and CMTM6 correlated with TILs and CD8⁺ cells but not with HPV. Our results identified CMTM6 as an important interaction partner in the crosstalk between TILs, CD8⁺ T cells, and PD-L1, which mediates anticancer efficacy. Assessments of CMTM6 may be helpful for prognostic prediction, and it may serve as a reliable biomarker for immunotherapy selection.

Keywords HNSCC · TIL · CMTM6 · Programmed death ligand 1 · Overall survival

1 Introduction

Head and neck squamous cell carcinoma (HNSCC) is the eighth most common type of malignancy worldwide. HNSCC often develops because of chronic alcohol and tobacco use or oncogenic human papillomavirus (HPV) infection [1, 2]. Accordingly, these tumors are categorized as HPV^{pos} or HPV^{neg}, with each subtype exhibiting distinct anatomical locations, molecular signatures, and clinical presentations [3, 4]. This heterogeneity, coupled with the fact that

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patients are often diagnosed at a locally advanced or metastatic disease stage, poses a challenge to conventional treatment approaches such as surgery or chemoradiotherapy. Although immune checkpoint inhibitors (ICIs) are a potential cutting-edge treatment option for HNSCC, early studies have shown mixed results, with only 20% of patients benefiting from ICIs [5–7].

Stromal composition and tumor microenvironment (TME), including the number of CD8⁺ T cells, interferon (IFN- γ) signature, high tumor mutational burden, and programmed death ligand 1 (PD-L1) expression levels, are prognostic factors for treatment response and outcome [8–11]. In HNSCC, the TME is composed of tumor-infiltrating immune cells such as CD4⁺ and CD8⁺ T cells, myeloid-derived suppressor cells, and tumor-associated M1- or M2-polarized macrophages (TAMs) that interact with tumor cells to promote or suppress growth [12]. PD-L1 is present on both tumor and tumor-infiltrating immune cells and can be used as a biomarker to select patients eligible for immunotherapy. In most cases, PD-L1 abundance is calculated using the combined positive score (CPS), where expression ≥ 1 is considered positive [13, 14]. In HNSCC, confounding factors, such as intratumor heterogeneity, may affect the validity [14]. Accordingly, the predictive value of PD-L1-CPS is moderate, and objective responses to PD1 blockade are sometimes independent of PD-L1 status [15, 16]. Therefore, PD-L1 alone is not sufficient to predict patient outcomes, and additional reliable markers are required.

A very interesting candidate is CKLF-like MARVEL transmembrane domain-containing 6 (CMTM6) [17]. CMTM6 stabilizes PD-L1 through physical interactions and co-localization on the cell surface and induces CD163⁺ M2-like macrophage polarization [18]. Depending on the tumor site, CMTM6 is associated with either improved or worsened treatment responses and outcomes [19, 20]. Another recently identified function of CMTM6 is the induction of chemoresistance, particularly to cisplatin, via activation of the *Wnt* signaling pathway [21]. Therefore, CMTM6 is an emerging target for refractory HNSCC, especially in those selected for second-line immunotherapy. Before implementing CMTM6 in routine pathological diagnosis, a deeper understanding of its spatial distribution and prognostic value is required.

In this study, we investigated the expression patterns of CMTM6 and several immune-related biomarkers in treatment-naïve patients with advanced HNSCC from different anatomical sites. In addition, we correlated our findings with the expression levels of PD-L1 and the pattern of immune cell infiltration within tumors. Our study identified CMTM6 as a critical survival factor in HPV^{pos} and HPV^{neg} HNSCC that should be actively considered when making therapeutic decisions.

2 Material and methods

2.1 Patient and tissue selection

Tumor tissues of patients with histologically proven primary HNSCC diagnosed from 06/2014 to 01/2021 were selected from the archival database of the Department of Pathology, and clinical data were obtained from patients' archives at the clinical database (both SAP®), Rostock University Medical Center. Follow-up data were obtained from the regional Cancer Registries. Patients included from 01/2018 and later were additionally included in our HNSCC biobank [22]. The selection criteria were (1) primary tumor site (oral cavity, oropharynx, hypopharynx, larynx, and/ or homologous neck lymph node metastases); (2) tumor size ≥ 1.0 cm to guarantee sufficient tumor material; (3) > 18 years of age. When suitable, tissue samples from resected specimens rather than biopsy material were preferred for the analysis. Cancers diagnosed before 2017 were re-classified according to the 8th edition of the Union for International Cancer Control (UICC) tumor, lymph node, and metastasis (TNM) classification (published in 2017 [23, 24]) using the latest edition of the World Health Organization (WHO) classification. Therefore, p16-positive squamous cell cancers of the oropharynx that were diagnosed before 2017 met the criterion of the newly established subtype of "p16-positive oropharynx carcinomas" according to the latest TNM classification. With respect to the clinical impact, these tumors were re-classified, resulting in downstaging of the nodal status in five patients (pN2b $\rightarrow$ pN1). Patient consent was obtained for each sample. The Institutional Ethics Committee of University Hospital Rostock approved this study (A2018-0003; A2022-0120). The study was performed in accordance with the principles of the Declaration of Helsinki.

2.2 Immunohistochemistry

Formalin-fixed paraffin-embedded 4 µm tissue slides were used. Antigen retrieval was performed using a buffer at pH9 (20 min at 97 °C). The following steps were performed in an Autostainer link 48 instrument (Leica Biosystems, Wetzlar, Germany): 5 min of incubation in peroxidase-blocking buffer followed by incubation with primary antibody (anti-p16: 1:100, G175-405, BD Bioscience, San Jose, CA, USA; anti-CMTM6: 1:1000, EPR23015-45, Abcam, Cambridge, UK; anti-Dickkopf-1 (DKK1): 1:100, SC06-86, GeneTex, Alton Pkwy Irvine, CA, USA; anti-CD163: 1:100, GHI/61, Invitrogen, Waltham, MA, USA; anti-p53: 1:100, Do-7; anti-cd56: 1:50, NCAM1; anti-CD68: 1:100, D4B9C; anti-PD-L1: 1:100, 22C3; anti-ki67: 1:500, Mib-1; anti-CD8: 1:100, c8/144B; all Dako, Glostrup, Denmark) and detection of 3,3'-diaminobenzidine (DAB) using the Dako-kit K8000 according to the manufacturer's instructions. For bone-containing specimens, soft decalcification was performed using ethylenediaminetetraacetic acid to preserve protein structures. The slides were counterstained with hematoxylin. All staining was performed on whole slide sections. Appropriate positive and negative controls were used.

2.3 Microscopic evaluation

Hematoxylin and eosin (H&E)-stained slides were checked for invasive squamous cell carcinoma and were graded. For the assessment of tumor-infiltrating leukocytes (TILs) per section and location (invasive front, tumor center), five areas with the highest density of TILs were identified at 40× magnification (4× objective lens, 10× ocular lens), and the average count within these hot-spots at 200× magnification (20× objective lens, 10× ocular lens, 0.237 mm² per field) defined its final categorization into one of the five ranges (varying from absent/0; minimal/1; low/2; intermediate/3 to high/4; Supplementary Fig. 1). In small-sized samples, the entire tumor area was evaluated. Analogous to this schema, the cytoplasmic and/or membranous reactivity of DKK1, CD8, CD68, CD163, and CD56 in TILs (invasive front, tumor center) was evaluated, whereby the minimum and maximum expression per marker within all samples defined the ranges of the five subgroups. DKK1 scoring on tumor cells (TCs) was performed semi-quantitatively using the percentage of moderately to strongly positive cells. PD-L1 and CMTM6 expression was scored analogous to the tumor proportion score (TPS: percentage of TCs with marker expression compared with all TC), immune cell score (ICS: proportion of tumor area occupied by PD-L1-expressing TILs), and CPS, defined as the ratio of all marker-positive TCs, lymphocytes, and macrophages to TCs in the corresponding area multiplied by 100 (therefore containing no unit), as previously described [25]. All scores were calculated within an area of at least 100 viable tumor cells. Any membranous immunoreactivity (independent of concomitant cytoplasmic/nuclear staining) with at least weak intensity was set as positive. All samples were categorized as PD-L1 *positive vs. negative* (CPS ≥ 1: positive) and CMTM6 *high vs. low* (CPS ≥ 10: high). Nuclear Ki67 positivity on TC defined its proliferation index, and nuclear p53 reactivity defined its p53-status (weak expression: wild type; strong expression > 50% of TC: mutant; absent expression: "null pattern" = deleted). Strong continuous nuclear and cytoplasmic staining for p16 in > 60% of TC ("block staining") was considered HPV-positive. In samples with questionable and positive p16-pattern, HPV status was analyzed molecularly. All histological scores were assessed twice by two independent, experienced, and board-certified pathologists (ASB, AZ) and in cases of divergent results, the final score was determined through an internal discussion (mainly the samples that were initially grouped as "intermediate" were subsequently redefined as low or high).

2.4 HPV status based on molecular pathology

Human papilloma virus (HPV) testing was performed using a commercially available kit (VisionArray HPV Chip 1.0, Zyto-Vision, Bremerhaven, Germany) according to the manufacturer's instructions.

2.5 RNA isolation, cDNA synthesis, and quantitative real-time PCR

Total RNA was isolated with RNeasy Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. RNA was reverse-transcribed into cDNA from 0.5 µg RNA using 1 µl dNTP mix (10 mM), oligo (dT)₁₅ primer (50 ng/µl), 1 µl reverse transcriptase (100 U) and 4 µl 5×RT buffer complete (all purchased from Bioron GmbH, Ludwigshafen, Germany). cDNA synthesis conditions were as follows: 70°C for 10 min, 45°C for 120 min, 70°C for 10 min. Target cDNA levels were analyzed by quantitative real-time PCR using TaqMan Universal PCR Master Mix (Thermo Fisher Scientific, Darmstadt, Germany) and predesigned CMTM6 and PD-L1 TaqMan gene expression assay labeled with 6-FAM-3' BHQ-1: Hs00608023_m1 (Thermo Fisher Scientific). GAPDH was used as housekeeping gene. All probes were from ThermoFisher

Table 1 Clinico-pathological characteristics of the HNSCC cohort

Group characteristics	Σ n = 129
Female n [%]	22 [82.9]
Male n [%]	107 [17.1]
Median age [years ± SD]	64.0 ± 8.2
ECOG performance status	0.8
Noxae	
Smoking [> 10 py in %]	53.1
Alcohol [> 1 drink/d in %]	33.1
Localization [n]	
Oral cavity	42
Oropharynx	46
Hypopharynx	16
Larynx	22
Lymph node	3
p16/HPV status [n = 107/129]	
Positive [%]	33.6
HPV type 16/26/16 & 33/33/35/negative/N/A [%]	69.3/2.8/5.6/5.6/2.8/2.8/11.1
Negative [%]	66.4
Grading [only p16 negative, n = 101/129]	
G1/G2/G3 [%]	7.9/67.3/24.8
TNM classification	
T1/T2/T3/T4 [%]	10.9/35.2/21.1/32.8
N0/N1/N2/N3 [%]	28.3/18.1/40.9/12.6
M0/M1/Mx [%]	93.0/5.4/1.6
CPS for PD-L1 and CMTM6 [%]	
PD-L1: ≥ 1	74.4
CMTM6: ≥ 10	67.2
Adjuvant treatment [n = 109/129]	
RT/RCT/RIT	26/21/6
CTX/ICI	55/1

Values are given as absolute/relative numbers and mean ± SD. ECOG Eastern Cooperative Oncology Group, py pack years, d day, HPV human papilloma virus, G1/2/3 grading, CPS combined positive score, RCT radio-chemotherapy, RIT radio-immunotherapy, RT radiotherapy, CTX chemotherapy (not specified), ICI immune-checkpoint inhibition (Nivolumab)

Scientific. Reaction was performed with 12.5 ng cDNA and the following PCR conditions: 95°C for 10 min, 40 cycles of 15 s at 95°C, and 1 min at 60°C. All reactions were run in triplicates. The mRNA levels of target genes were normalized to GAPDH ($2^{-\Delta CT}$ method).

2.6 Complementary analysis using the The Cancer Genome Atlas (TCGA) database

CMTM6 and *PD-L1* (CD274) RNA-seq expression and the survival data of The Cancer Genome Atlas (TCGA) -HNSCC cohort were downloaded from UCSC Xena website (<https://xenabrowser.net/datapages/>). Expression profile data were classified into high and low expression groups according to the median *CMTM6* expression value. Correlations between marker expression and survival with respect to median mRNA levels of *CMTM6* and *PD-L1* were calculated using the public Xena platform (University of California Santa Cruz).

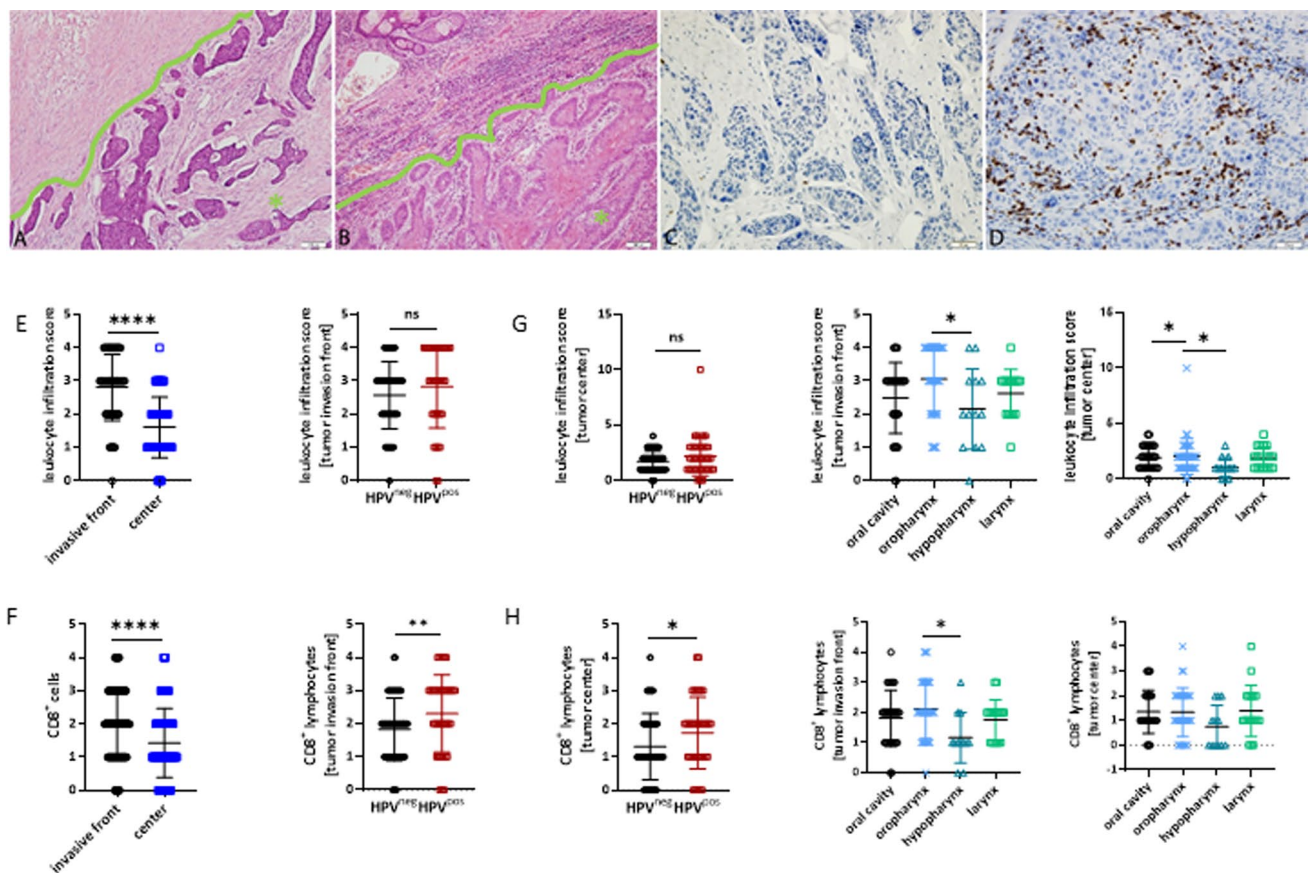


Fig. 1 Assessment of leukocyte infiltration score by morphology and CD8⁺ T cells by immunohistochemistry for the invasive front and the tumor center. Representative slides showing different infiltration patterns in individual tumors with absent (A) leukocyte infiltration (=Score 0; HE, 100x), moderate (B) leukocyte infiltration (=Score 3; HE, 100x), minimal (C) CD8⁺ cells (=Score 1, 200x) and high (D) CD8⁺ cells (=Score 4, 200x). Asterisk=tumor center, green line=invasive front. **E** TILs at the invasive front and the tumor center (left, $n=63$ invasive front; $n=66$ center; **** $p<0.0001$, Unpaired t-test (two-tailed)), as well as between HPV^{neg} and HPV^{pos} HNSCCs ($n=88$ HPV^{neg}; $n=36$ HPV^{pos}, ns=not significant). **F** CD8⁺ cells at the invasive front (left; * $p<0.05$, one-way ANOVA (Tukey's multiple comparisons test)) and the tumor center (right; * $p<0.05$, one-way ANOVA (Tukey's multiple comparisons test)), as well as between HPV^{neg} and HPV^{pos} HNSCCs ($n=88$ HPV^{neg}; $n=36$ HPV^{pos}, ns=not significant) (G, H) TILs and CD8⁺ cells in different anatomical sites. $n=35$ oral cavity; $n=36$ oropharynx; $n=13$ hypopharynx; $n=16$ larynx; * $p<0.05$, one-way ANOVA (Tukey's multiple comparisons test))

2.7 Statistics

Statistical evaluation was performed using GraphPad PRISM software, version 8.0.2 (GraphPad, San Diego, USA). Values are either reported individually or as mean $\pm$ standard deviation (SD). After proving the assumption of normality, the differences between individual groups were calculated using unpaired Student's t-test. If normality failed, the nonparametric Mann–Whitney U test was applied. Multiple comparisons were performed using one-way analysis of variance (ANOVA) on ranks (Tukey's multiple comparisons test) or the Kruskal–Wallis test (Bonferroni's multiple comparison test). Correlation analysis was performed using the Pearson correlation, or in the case of a nonparametric distribution, the Spearman's correlation (two-tailed p-value). Kaplan–Meier survival curves were analyzed using the log-rank (Mantel Cox) test. Cox proportional hazards regression (Cox regression) analysis was performed to analyze the covariates with a potential influence on OS. Multivariable regression models were fitted using complete cases, imputation procedures for missing values were not carried out. Significant parameters in univariate analysis were introduced into the multivariate Cox regression model to determine the adjusted risk ratio. Statistical significance was set at $p<0.05$.

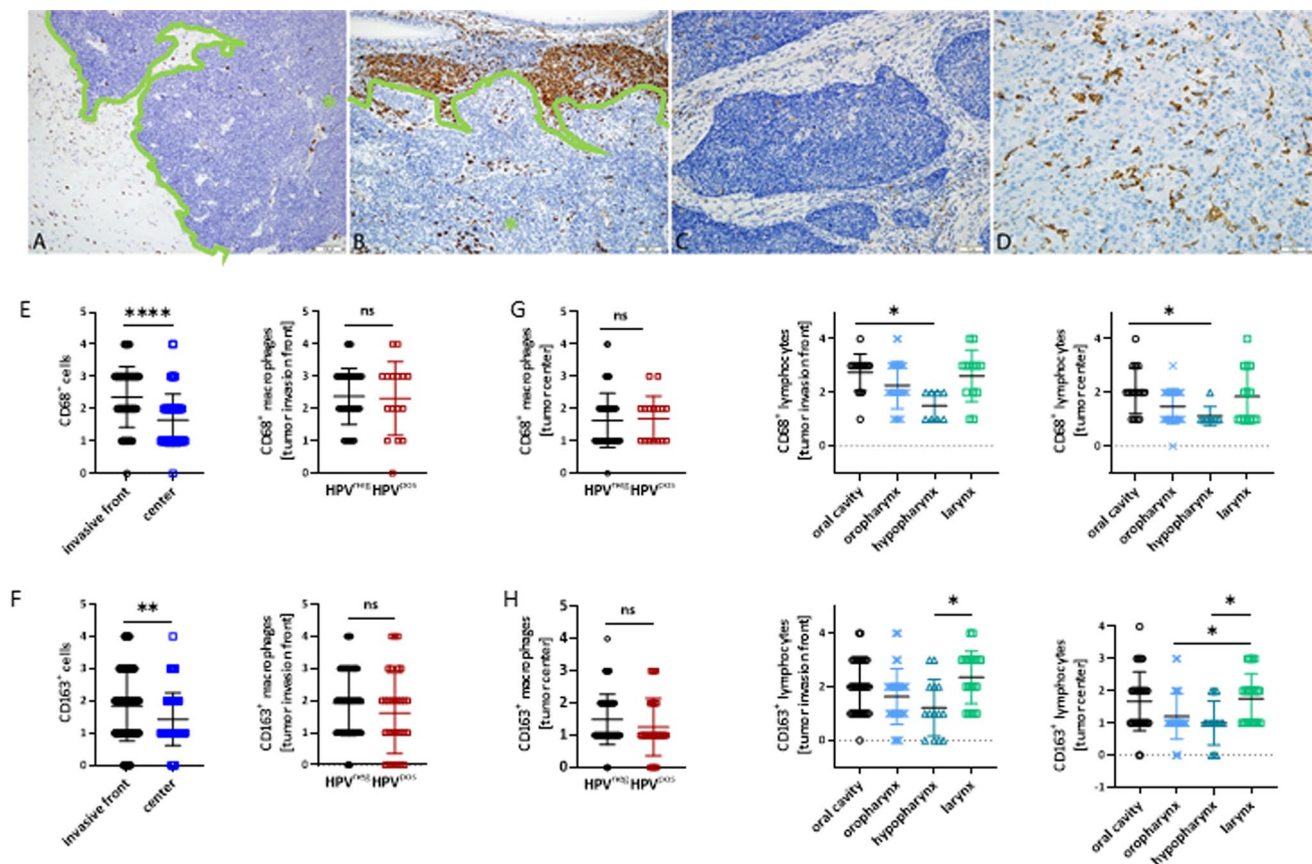


Fig. 2 Assessment of CD68⁺ and CD163⁺ cells by immunohistochemistry for the invasive front and the tumor center. **A–D** Representative slides showing macrophage infiltration patterns (CD68⁺, CD163⁺) in individual tumors with minimal (**A**) CD68⁺ TAMs (=Score 1; 100x), high (**B**) CD68⁺ TAMs (=Score 4; 100x), minimal (**C**) CD163⁺ TAMs (=Score 1, 200x) and high (**D**) CD163⁺ TAMs (=Score 4, 200x). Asterisk=tumor center, green line=invasive front. **E** CD68⁺ macrophages at the invasive front and the tumor center (left, n=66 invasive front; n=68 center; ****p<0.0001, U-test (two-tailed)), as well as between HPV^{neg} and HPV^{pos} HNSCCs (n=52 HPV^{neg}; n=16 HPV^{pos}, ns=not significant). **F** CD163⁺ macrophages at the invasive front and the tumor center (left, n=63 invasive front; n=66 center; **p<0.01, U-test (two-tailed)), as well as between HPV^{neg} and HPV^{pos} HNSCCs (n=84 HPV^{neg}; n=31 HPV^{pos}, ns=not significant). **G** CD68⁺ and **H** CD163⁺ macrophages at the invasive front (left; *p<0.05, Kruskal Wallis test (Dunn's multiple comparisons test)) and the tumor center (right; *p<0.05, one-way ANOVA (Tukey's multiple comparisons test)) in different anatomical sites. CD68: n=16 oral cavity; n=27 oropharynx; n=8 hypopharynx; n=14 larynx; CD163: n=34 oral cavity; n=41 oropharynx; n=14 hypopharynx; n=20 larynx

3 Results

3.1 Patient and tumor characteristics

The study cohort consisted of 129 patients, all diagnosed with primary HNSCC, with surgical resection specimens available in 98% prior to adjuvant therapy. The clinicopathological characteristics are presented in Table 1. Most tumors were localized in the oral cavity and oropharynx (n=42 and n=46, respectively). Sixteen patients had hypopharyngeal cancer, 22 had laryngeal cancer, and regional lymph node metastases were observed in 3 cases. Approximately half of the patients were smokers (53.1%, ≥ 10 py), and one-third did not have critical alcohol consumption (33.1%). One third of the tumors (32.7%) were p16⁺ owing to previous HPV infection. In 11 p16⁺ cases, molecular HPV testing could not be performed because of poor DNA quality. These cases were classified as "HPV-like-p16⁺" because they were localized in the oropharynx (tonsils) and had any patient history of noxae. One case was finally classified as p16⁺/HPV^{neg}. Tumors were obtained from all stages (T1–T4), with metastases in 7% of the cases. For three patients with available tumor material from primary and meta/synchronous metastatic/recurrent disease, the staining results from primary carcinomas were included for further analyses.

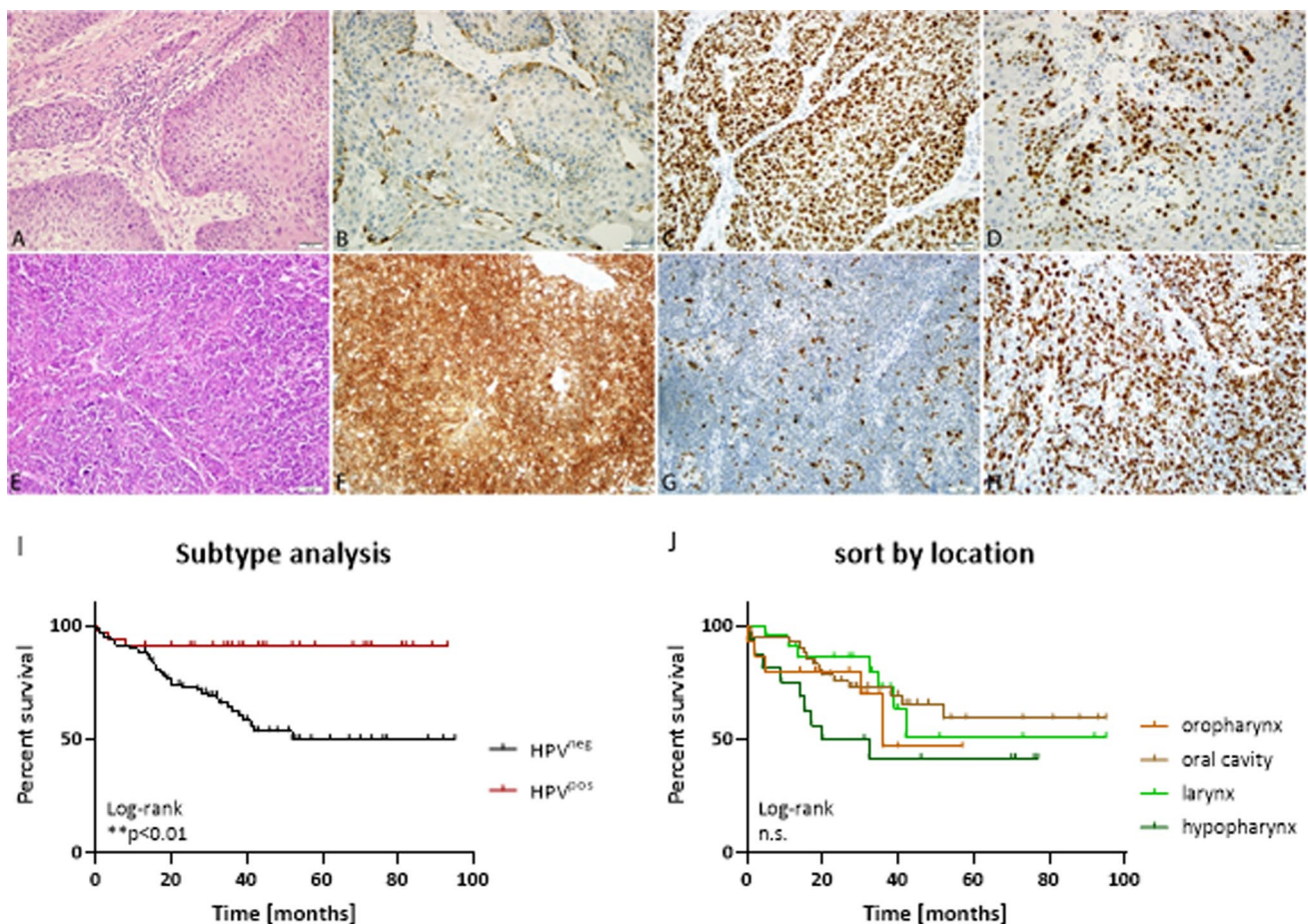


Fig. 3 Morphology, immunophenotyping and prognostic impact of HPV-negative and HPV-positive cancer. **A** moderate differentiated squamous cell carcinoma of the larynx (HE staining), p16^{INK4A}-negative (**B**), p53-mutant (**C**) with moderate proliferation index (**D**). **E** Poorly differentiated squamous cell carcinoma of the right tonsil, **F** p16^{INK4A}-positive with diffuse nuclear and cytoplasmic staining, **G** p53 wildtype and **H** higher proliferation index (200× magnification). **I** Kaplan–Meier curves show a significant longer OS for HPV⁺ cases, n = 129; ** p < 0.01 Log-rank analysis. **J** Kaplan Meier survival curve of HNSCC patients depending on anatomical location revealed best OS for tumors located in the oral cavity; n = 126; Log-rank analysis, n.s.–not significant

3.2 Intra- and inter-individual heterogeneity of the TME

The TME was first examined using H&E-stained whole-mount sections to estimate the number and distribution of tumor-infiltrating leukocytes (TILs) (Figs. 1A, B). The TIL score at the invasion front was significantly higher than that at the tumor center ($p < 0.0001$; Fig. 1E, left). Subtype analysis of HPV^{pos} and HPV^{neg} cases revealed higher infiltration levels in the former, especially at the invasion front and tumor center (Fig. 1E, middle and right). Specific immunological subpopulations (T cells, macrophages, and natural killer [NK] cells) were then quantified (Figs. 1, 2, and Supplementary Fig. 2). The number of CD8⁺ cytotoxic T cells, as well as CD68⁺ and CD163⁺ macrophages, differed significantly between the invasive front and the tumor center (Fig. 1F, left; Figs. 2E, F, left). The number of CD8⁺ T cells was higher in HPV-associated cases than that in their HPV-unrelated counterparts ($p < 0.01$ (invasion front); $p < 0.05$ (tumor center); Fig. 1F, middle and right). In contrast, the number of macrophages (CD68⁺ and CD163⁺) was similar between the two subtypes (Figs. 2E, F, middle, and right). The number of CD56⁺ natural killer (NK) cells was low and showed a similar pattern in each compartment (Supplementary Figs. 2A, B, E, and G).

Hypopharyngeal tumors had fewer TILs (HE, CD8⁺), both at the invasion front and tumor center ($p < 0.05$ vs. oropharynx; Figs. 1G, H). Conversely, oropharyngeal carcinomas showed the highest levels of TILs and CD8⁺ cells at the invasion front. The oral and laryngeal tumors had comparable TIL levels (Figs. 1G, H). The number of CD68⁺ and CD163⁺ macrophages differed significantly between the different anatomical sites. Again, hypopharyngeal cancers

Table 2 Uni- and Multivariate Cox regression analysis of clinic-pathological variables

Variable	Univariate Cox regression	Multivariate Cox regression		
	HR (95% CI)	p value	HR (95% CI)	p value
Gender	1.032 (0.455–2.343)	0.939		
Male				
Females§				
Grade	0.437 (0.159–10.198)	0.0076	0.377 (0.148–0.960)	0.041
TNM	1.138 (0.842–1.539)			
T	0.969 (0.701–1.340)	0.399		
N	1.309 (0.315–5.444)	0.85		
M		0.711		
UICC		0.249		
§1 vs. 2	0.675 (0.151–3.020)	0.608		
§1 vs. 3	0.602 (0.135–2.688)	0.506		
§1 vs. 4	1.424 (0.432–4.687)	0.561		
ECOG	1.407 (0.944–2.099)	0.094	1.518 (0.912–2.528)	0.108
0 vs. 1 vs. 2 vs. 3 vs. 4				
p16 Status positive vs. negative	0.263 (0.069–0.804)	0.001	0.271 (0.079–0.932)	0.038
CD8 ⁺ T cells	0.601 (0.427–0.874)	0.004	0.667 (0.462–0.963)	0.031
Tumor center high vs. low§				
PD-L1 CPS positive vs. negative§	0.279 (0.146–0.532)	<0.001	0.380 (0.156–0.923)	0.033
CMTM6 high vs. low§	0.385 (0.197–0.753)	0.005	0.391 (0.160–0.955)	0.039

§Reference category

HR hazard ratio, CI confidence interval. For selecting OS influencing factors for the multivariable regression approach, the cut-off was set to $\alpha=0.05$, bold values in the regression analysis indicate significant differences between the test samples

had the lowest number of infiltrating CD68⁺ and CD163⁺ macrophages in each compartment (CD68: $p < 0.05$ vs. oral cavity and CD163: $p < 0.05$ vs. larynx; Figs. 2G, H).

3.3 Impact of HPV status, anatomical site, TILs, and CD8⁺ T cells on overall survival

Morphological examination of HPV^{pos} and HPV^{neg} cancers revealed characteristic patterns in both subtypes with respect to differentiation, p53 staining, and Ki-67 proliferation index (Figs. 3A–H). We then focused on the prognostic impact of the TME on the overall survival (OS) of patients at the initial diagnosis. This analysis revealed a significant survival benefit for patients with HPV-associated HNSCC compared with their HPV-unrelated counterparts ($p < 0.01$; Fig. 3i; Table 2). In the HPV-related cohort, tobacco smoking ($n = 8$) and alcohol consumption ($n = 4$) did not adversely affect patient survival ($p = 0.3$). In this subgroup, 89% of the patients received adjuvant therapy (i.e., cisplatin, cetuximab, and/or radiotherapy). Four patients underwent surgery without the administration of adjuvant therapy. In this small group, two patients were still alive, and two died. The latter presented with a worse overall health status at diagnosis (ECOG 2 vs. ECOG 0) and advanced-stage disease. In the HPV-unrelated group, the choice of treatment had little effect on OS. When analyzing the impact of the anatomical site, oral cancer was found to have the best OS, while hypopharyngeal cancer had the worst outcome (Fig. 3J). Similarly, TILs and CD8⁺ T cells were positively associated with OS, independently of the treatment (Fig. 4). For TILs, the difference was statistically significant (invasion front). For CD8⁺ T cells, the survival benefit was independent of the tumor location according to the univariate analysis of Kaplan–Meier curves (invasion front: $p < 0.001$; center: $p < 0.01$, log-rank) and multivariate Cox regression (Table 2).

3.4 PD-L1 and CMTM6 status and its prognostic impact

Although PD-L1 is an approved biomarker, we tested whether CMTM6 might have a comparable prognostic value (Figs. 5, 6). First, a consensus cutoff was established to determine positivity. All samples were categorized as CMTM6

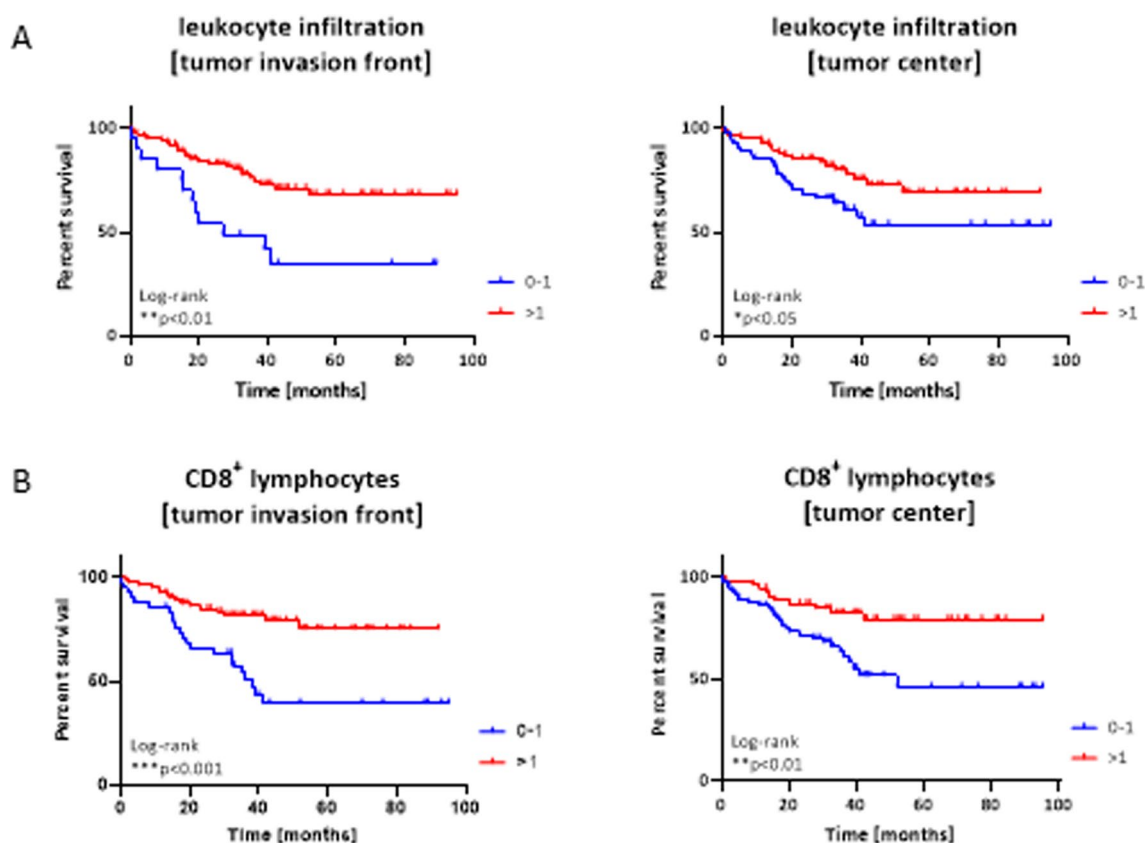


Fig. 4 Impact of TILs and CD8⁺ T cells on overall survival of HNSCC patients. **A** Prognostic relevance of TILs depending on the location, i.e. tumor invasion front or tumor center. Categorization: 0–1; > 1; n = 121 and n = 124, respectively; ** p < 0.01, * p < 0.05, Log-rank analysis. **B** Prognostic relevance of CD8⁺ cytotoxic T cells depending on the location, i.e. tumor invasion front or tumor center. Categorization: 0–1; > 1; n = 124 and n = 126, respectively; ** p < 0.01; *** p < 0.001, Log-rank analysis

high vs. low, with CPS ≥ 10 defined as high, according to the results of the receiver operating characteristic (ROC) curve analysis (area under the curve (AUC): 0.72; specificity > 65%). Using this cutoff, 67.2% of all samples were classified as CMTM6^{high}, which is slightly below the PD-L1 positivity in this cohort (CPS ≥ 1 : 74.4%, Table 1). However, the median CPS of CMTM6 was higher than the median CPS of PD-L1 (25 vs. 5). Representative images of individual HNSCC cases are shown in Figs. 5C–F.

A closer look at PD-L1 and its spatial distribution revealed a strong correlation between CPS and TPS (Spearman's $r = 0.779$, $p < 0.001$), CPS and ICS (Spearman's $r = 0.851$, $p < 0.001$), and between ICS and TPS (Spearman's $r = 0.616$, $p < 0.001$). For CMTM6, there was a strong correlation between CPS and TPS (Spearman's $r = 0.730$, $p < 0.0001$). While CPS correlated weakly with ICS (Spearman's $r = 0.242$, $p < 0.01$), there was no correlation between ICS and TPS (Spearman's $r = -0.041$).

A weak correlation was observed between the CPS of both markers (Spearman's $r = 0.217$; Supplementary Fig. 3A). Most samples with a CMTM6 CPS ≥ 10 were PD-L1 positive. For both markers, the CPS did not differ significantly between HPV-associated and HPV-unrelated HNSCCs (Figs. 5A, B, middle panel). Among the HPV-associated specimens, high-risk HPV subtypes (Table 1) were evenly distributed between the CMTM6^{low/high} and PD-L1^{pos/neg} groups. Neither UICC stage, smoking or alcohol consumption affected CMTM6 or PD-L1 status.

Likewise, anatomical location had little impact on PD-L1 or CMTM6 positivity (Figs. 5A, B, right). An exception was observed for PD-L1 CPS, which was generally lower in hypopharyngeal cancers than that in other sites (Fig. 5A, right).

To validate the immunohistochemical observations at the molecular level, PD-L1 and CMTM6 mRNA expression levels were detected in 29 tumors (Fig. 7). In this subgroup, PD-L1 and CMTM6 mRNA expression levels showed high similarity (Fig. 7A) and a weak correlation (Pearson's $r = 0.22$; $p = 0.12$, Fig. 7B). For PD-L1, mRNA levels correlated with protein status in 22 of 29 samples analyzed. For CMTM6, mRNA levels correlated with protein status in 21 of 26 samples analyzed (Fig. 7C). Using TCGA data from 612 primary HNSCC samples, the correlation between CMTM6 and PD-L1 mRNA expression

Fig. 5 Combined Positive Score, Tumor Proportion Score and Immune Cell Score for PD-L1 and CMTM6. **A** Overall PD-L1 score (CPS, TPS, ICS), and CPS in HPV^{neg} and HPV^{pos} as well as in different anatomical sites. $n = 125$; * $p < 0.05$; **** $p < 0.0001$ Kruskal Wallis test (Dunn's multiple comparisons test), ns—not significant). **B** Overall CMTM6 score (CPS, TPS, ICS), and CPS in HPV^{neg} and HPV^{pos} as well as in different anatomical sites. $n = 119$; **** $p < 0.0001$ Kruskal Wallis test (Dunn's multiple comparisons test), ns—not significant). **C** Squamous cell cancer specimen with negativity for PD-L1 on the tumor cells showing one positive tumor infiltrating lymphocyte (=PD-L1 negative), **D** membranous positivity for PD-L1 on the tumor cells and abundant positive tumor infiltrating lymphocytes (=PD-L1 positive), **E** negativity for CMTM6 on the tumor cells and few positive tumor infiltrating lymphocytes (=CMTM6 low), **F** membranous positivity for CMTM6 on the tumor cells and abundant positive tumor infiltrating lymphocytes (=CMTM6 high; all 200x). For PD-L1, CPS ≥ 1 was set positive, for CMTM6, CPS ≥ 5 was set positive. CPS, Combined Positive Score; TPS, Tumor Proportion Score; ICS, Immune Cell Score

was positive (Spearman's $\rho = 0.09845$; $p = 0.02141$; supplementary Fig. 4A). Using either the median or interquartile range of expression, none of the markers had a significant impact on survival in the 250 analyzed samples (supplementary Fig. 4B, C), which were, however, not stratified according to p16 status.

In our cohort, patients with PD-L1 CPS ≥ 1 and the subgroup with CMTM6 CPS ≥ 10 had a significantly longer OS ($p < 0.0001$ and $p < 0.001$, respectively; log-rank test, Figs. 6A, B, E, F). The Cox regression analysis revealed sex, grade, TNM, ECOG, HPV status, and CD8⁺ T cells as prognostic predictors. Multivariable Cox regression analysis also indicated a significantly better OS of the CMTM6 high versus CMTM6 low groups ($p = 0.032$; Table 2). Besides, tumor grade, p16 status, CD8⁺ T cells, and PD-L1, were statistically significant parameters for OS (Table 2). Notably, the significance of CMTM6 was comparable to that of PD-L1, confirming its prognostic relevance. The combination of both markers showed prolonged survival in the subgroup with PD-L1 expression ≥ 1 and synchronous CMTM6 expression ≥ 10 ($p < 0.0001$; log-rank test, Figs. 6C, E, F). In contrast, the CMTM6^{high}/PD-L1^{neg} and CMTM6^{low}/PD-L1^{neg} subgroups showed a shorter OS (median OS, 40 months; Figs. 6C, G–J).

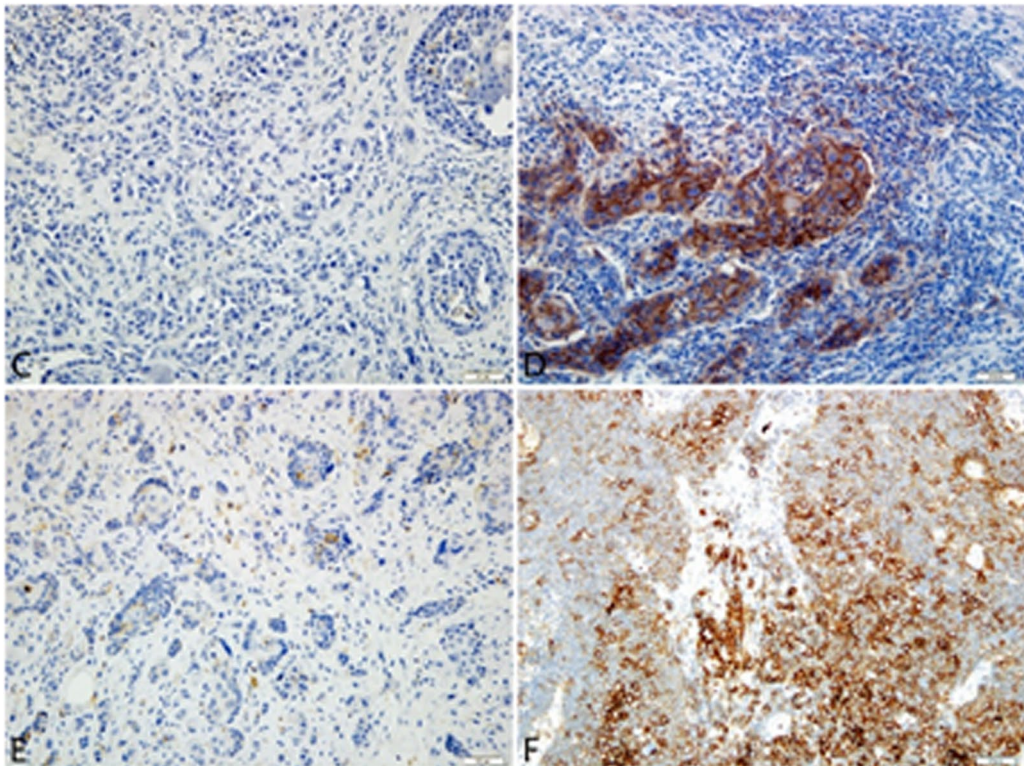
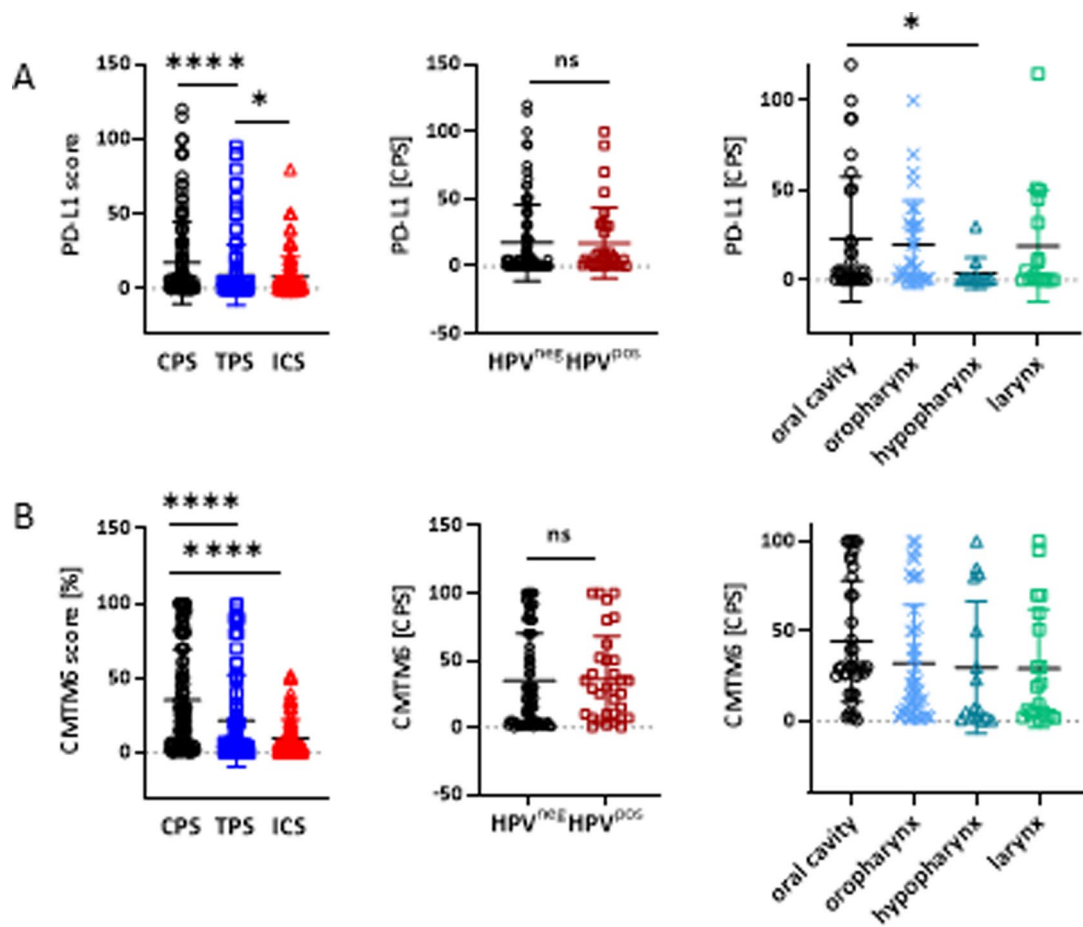
The best outcome was seen for patients with PD-L1^{pos}/HPV^{pos} cancers with a 100% survival rate in a follow-up duration of > 80 months (Fig. 6D). Contrastingly, patients with PD-L1^{neg}/HPV^{neg} HNSCC had the worst outcomes (median OS, 39 months). A comparable outcome was observed with CMTM6 (Supplementary Fig. 3B). Notably, HPV^{pos} patients without CMTM6 expression showed poor outcomes. Noteworthy, however, is the high variation between individual patient samples, identified by nested analysis for CMTM6, PD-L1 CPS, and CD8 positivity in HPV^{neg} and HPV^{pos} HNSCCs (supplementary Fig. 3C).

Additional prognostic markers were examined to obtain a more holistic view of the relationship between CMTM6 and PD-L1 expression. The number of central TILs showed a moderate correlation with the CPS of CMTM6 (Spearman's $r = 0.34$, $p < 0.0001$). For PD-L1, a significant correlation was found between CPS and TILs at the invasive front (Spearman's $r = 0.266$, $p < 0.01$). Both parameters correlated with the CD8⁺ T cell score (Spearman's $r = 0.22$ – 0.34). There were no significant differences in PD-L1 or CMTM6 expression between HPV^{pos} and HPV^{neg} cases, although the latter tended to be more PD-L1 positive ($p = 0.07$; Fisher's exact test).

Notably, patients with CMTM6^{high} tumors and a CD8⁺ T-cell score > 1 showed the best OS (Supplementary Fig. 5A). The same positive prognostic effect was observed when CD8⁺ T-cells and PD-L1 CPS were included in the analysis (Supplementary Fig. 5B). Hence, CMTM6, in addition to PD-L1, is indicative of an inflammatory tumor microenvironment and predicts better patient outcomes.

3.5 Significance of TAMs, NK cells, and DKK1 expression

Neither the number of CD163⁺ and CD68⁺ macrophages nor the number of CD56⁺ NK cells had a significant influence on OS (Fig. 8, Supplementary Fig. 2). There was a highly significant correlation between CD68⁺ and CD163⁺ TAMs (Spearman's $r > 0.80$ for both sites, $p < 0.0001$), with analogous staining patterns in the examined samples. Twelve patients with a CD68/CD163 ratio > 1 in the tumor center showed significantly longer OS than those without dominant M1-polarized macrophages (Fig. 8C). In the CD56-stained samples, 10% and 5% had a score ≥ 3 in each compartment. In the DKK1-stained subgroup, our scoring method showed that 71% of samples with DKK1-positive tumor cells had no clinicopathological relevance (Supplementary Figs. 2F, H). DKK1-positive carcinomas showed significantly more DKK1-positive cells in the surrounding stroma than DKK1-negative carcinomas ($p = 0.0002$; Fisher's exact test).



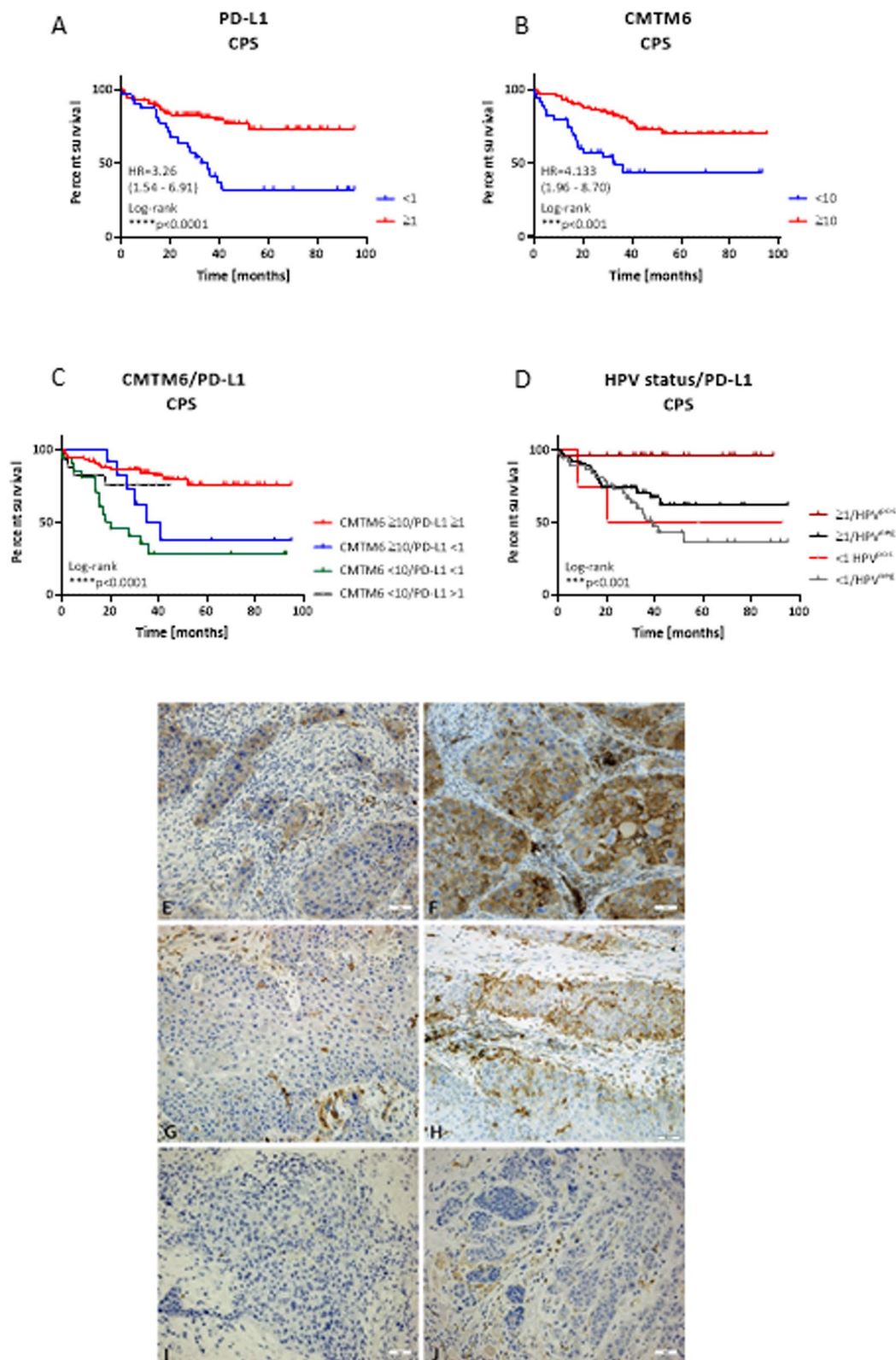


Fig. 6 Impact of PD-L1 and CMTM6 on overall survival of HNSCC patients. **A, B** Prognostic relevance of PD-L1 and CMTM6 within HPV^{neg} and HPV^{pos} cases. CPS: ratio of all marker positive TC, lymphocytes, macrophages to TC in the corresponding area multiplied by 100 (therefore containing no unit). n=128; ** p<0.01; ***p<0.001 Log-rank analysis. **C** Prognostic relevance of PD-L1 and CMTM6 within HPV^{neg} and HPV^{pos} cases depending on the CPS. n=128; * p<0.05 Log-rank analysis. **D** Prognostic relevance of PD-L1 CPS according to HPV status. n=128; *** p<0.001 Log-rank analysis. **E–J** Immunohistochemistry. Paired Examples of cases being PD-L1-positive (**E**)/ CMTM6 high (**F**), PD-L1-negative (**G**, here with positive stained TILs to show reactivity)/CMTM6 high (**H**) and PD-L1- negative (**I**)/CMTM6 low (**J**). There were no tumors with high PD-L1 but low CMTM6-status

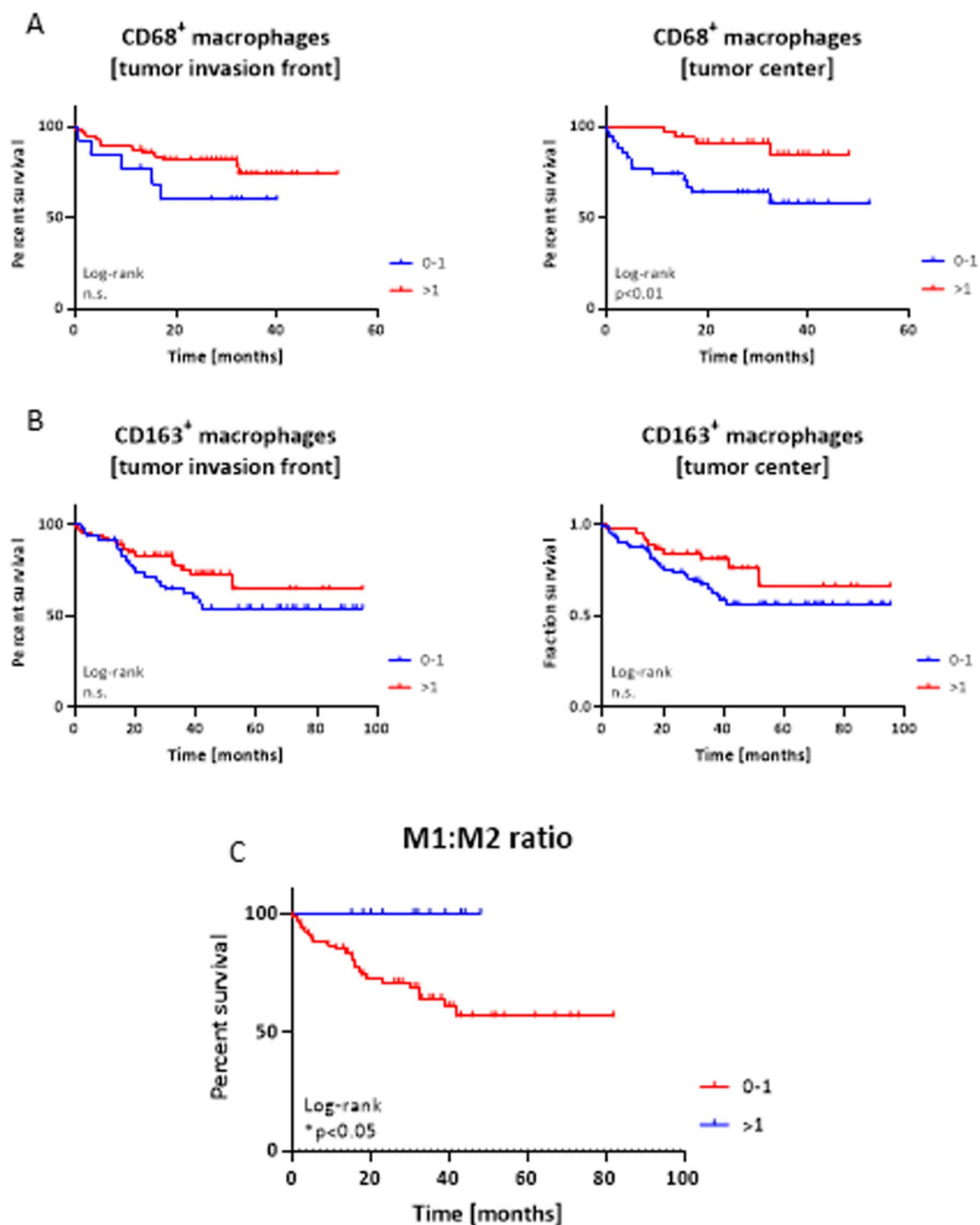
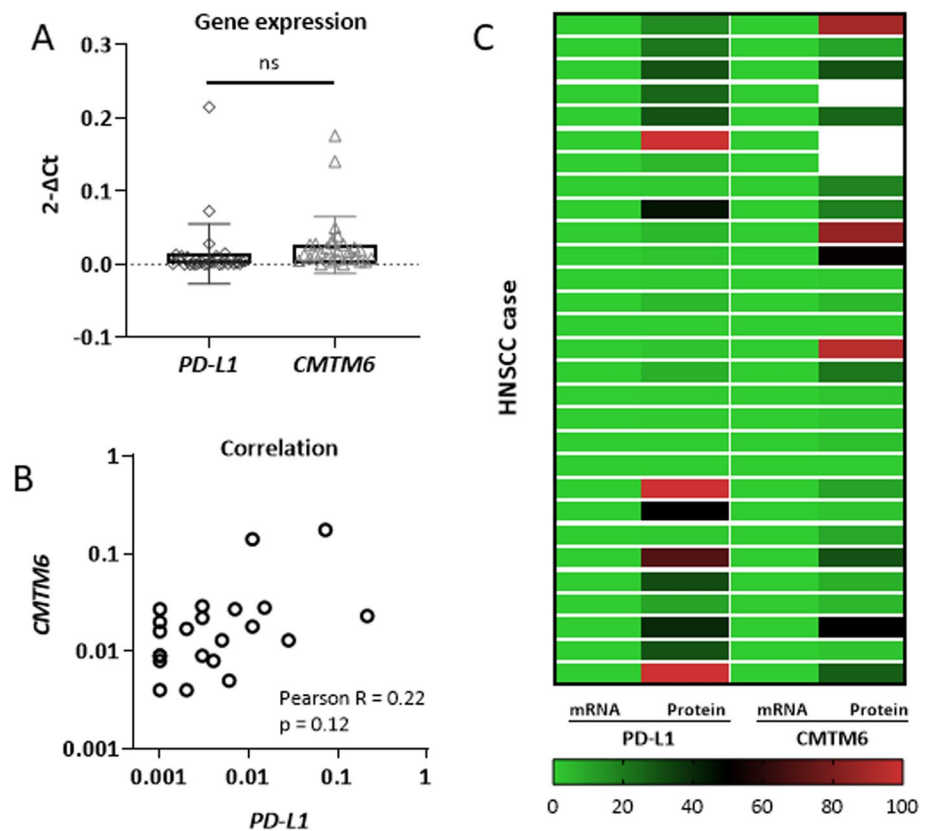


Fig. 7 mRNA expression levels of *PD-L1* and *CMTM6*. **A** Analysis was done on 29 tumor samples, from which mRNA was available. **B** Pearson correlation for *PD-L1* and *CMTM6* mRNA expression levels showed a weak correlation. **C** Heatmap depicting correlation of mRNA levels with protein status in 21 of 26 samples analyzed

Fig. 8 Impact of macrophages on overall survival of HNSCC patients. Prognostic relevance of A CD68⁺ and B CD163⁺ TAMs depending on the location, i.e. tumor invasion front or tumor center. Categorization: 0–1; > 1; n = 100; ** p < 0.01 Log-rank analysis. Categorization: 0–1; > 1; CD68: n = 68; CD163: n = 112; ** p < 0.01, Log-rank analysis. C The M1:M2 macrophage ratio was calculated by dividing the score of CD68⁺ M1 macrophages by the score of CD163⁺ M2 macrophages. Categorization: 0–1 n = 67; > 1; n = 12; * p < 0.05, Log-rank analysis



4 Discussion

In the present study, we identified CMTM6 as a prognostic biomarker in patients with HNSCC. CMTM6 is a regulator of PD-L1 expression and plays an important role in the TME via its expression in both tumor and immune cells. These findings broaden the spectrum of markers with potential relevance to prognostic and therapeutic decisions.

We investigated the prognostic impact of the TME, including TILs, CD8⁺ T cells, NK cells, macrophages, DKK1, PD-L1, and CMTM6 positivity, on primary HNSCCs located at different anatomical sites. Multivariate analysis showed that only the immune markers PD-L1, CD8⁺ T cells, and CMTM6 were independent predictors of OS. Thus, CMTM6 has the potential to become an additional biomarker. The independent prognostic value of TILs for patient OS is well established [26, 27] and in HNSCC, high CD8⁺ lymphocyte counts are also associated with a better outcome [28, 29]. In our study, we confirmed the positive prognostic effects of TILs and CD8⁺ cells. In contrast, NK cells and DKK1, which are regulators of the immune response via the *Wnt* pathway, had insignificant effects on OS. This finding contradicts previous reports describing unfavorable OS in HNSCC dependent on the DKK1 expression status [30]. In contrast, CD68⁺ M1-polarized macrophages alone and the M1:M2 ratio (CD68:CD163) were associated with prolonged OS, as previously described for other tumor entities [31, 32]. In HNSCC, M2-like polarization is thought to be driven by the receptor for activated C kinase 1 via NF- κ B suppression to promote tumor development [33]. A direct correlation is expected between M2-macrophage infiltration and disease stage.

To our knowledge, this is the first study to investigate PD-L1 and CMTM6 status in combination with the TME using a purely histological approach in HNSCC. We found that HPV^{pos} HNSCC harbored more CD8⁺ T cells than HPV^{neg} cases, which is consistent with recent literature [34, 35]. HPV status did not affect PD-L1 or CMTM6 status; however, patients with either PD-L1^{pos} or CMTM6^{high} tumors had good outcomes comparable to that in the HPV^{pos} subgroup, regardless of HPV status. Although the impact of PD-L1 on OS has been reported to be the opposite in a previous study [36], we propose a direct association between PD-L1 and high TIL infiltration as an explanation for the positive prognostic impact. For CMTM6, a positive association similar to that recently reported for triple-negative breast cancer is likely [37].

In our cohort, nine patients with PD-L1^{neg}/CMTM6^{high} tumors had significantly worse OS than those in the PD-L1^{pos}/CMTM6^{high} subgroup. In contrast, the CPS of PD-L1 and CMTM6 correlated with CD8⁺ T cells, and patients with CMTM6^{high}

tumors and a CD8⁺ T cell score > 1 showed excellent OS. This highlights the immuno-oncological crosstalk between tumor cells and TILs, mediated not only by the druggable target PD-L1 but also by CMTM6. Patients whose tumors are positive for CMTM6 might therefore be good candidates for ICI treatment. However, before CMTM6 can be introduced in routine pathological diagnostics, further studies are required to gain a detailed understanding of this molecule and its regulation, which hopefully will help improve the prognostic value of PD-L1 [38]. Standardization of the antibody clones, scoring systems, and cutoffs is also essential to obtain homogeneous datasets [39]. To date, there is no consensus regarding CMTM6 positivity, which is mostly classified as either “low” or “high” [40–42]. This remains a source of uncertainty for oncologists and pathologists. We propose defining ‘high’ positivity as exceeding a 10% cut-off for both tumor cells and TILs. The rationale for choosing 10%, instead of 1% similar to that for PD-L1, is the higher overall expression of CMTM6 in HNSCC. In our cohort, this resulted in CMTM6 positivity of ~67%, which is slightly less than PD-L1 positivity (74.4%, CPS ≥ 1). Using this scoring system, CMTM6 was identified as a reliable and clinically relevant prognostic biomarker, and its validity was increased by adding PD-L1 and CD8⁺ T cells to this marker panel.

Recently, a direct link between PD-L1 and CMTM6 was described in triple-negative breast cancer patients undergoing epithelial-mesenchymal transition (EMT) [43]. Similar mechanisms have been identified for HNSCC [44]. Thus, CMTM6 may play a role in the acquisition of cancer stem cell-like properties and in the regulation of EMT through the *Wnt/β-catenin* pathway, ultimately leading to cisplatin resistance [44]. This finding is of particular importance; however, it does not fit in with the prognostic relevance of PD-L1 and CMTM6 in our study and warrants further investigation. PD-L1/CMTM6 colocalization maintains PD-L1 surface expression by preventing lysosome-mediated degradation. The lack of correlation for PD-L1 and CMTM6 mRNA expression levels is consistent with this post-translational influence of CMTM6, although mRNA data were only available for a subset of samples. In the subset of our samples with available mRNA data, CMTM6 and PD-L1 expression showed a weak positive correlation. Furthermore, an even weaker, yet statistically significant, positive correlation was observed in HNSCCs from the TCGA datasets. In the latter case, the samples were not stratified according to HPV status, which limits the interpretability of the results [17]. This validates our correlation findings, which have now been confirmed at the protein level in a larger Caucasian HNSCC cohort [45]. Zhao’s group [17] found a strong negative correlation between activated NK cells and a strong positive correlation between activated CD4-positive memory cells and CMTM6 in HNSCC. While they found no statistically significant difference in CMTM6 mRNA levels between HNSCC samples and normal tissue, another group [46] observed increased CMTM6 mRNA expression levels with UICC stage. In agreement with us, Zhao et al. did not observe an effect of CMTM6 mRNA levels on survival. However, in their cohort of 210 oral squamous cell carcinoma patients, Chen et al. showed that elevated CMTM6 protein status is associated with shorter overall survival. Taken together, CMTM6 and PD-L1 mRNA TCGA samples show a weak positive correlation. Indeed, the divergent survival findings demonstrate that complex post-translational modifications such as methylation interact with final protein levels in cancer samples.

A similar favorable prognostic role for CMTM6 as shown here for HNSCC has been found in ovarian and colorectal cancer, which has been attributed to an “immunologically hot” TME [19, 20]. Such beneficial effects can be expected in HNSCC, and a positive correlation was found between CMTM6, PD-L1, and CD8⁺ T cells, which were ultimately independent of EMT.

A good biomarker must be easily detected or quantified using affordable and robust assays, be uniquely expressed within tumors, and provide comparable results between preoperative biopsies and surgically resected specimens when available. The applicability of PD-L1 as a biomarker in preoperative biopsies has recently been confirmed [47, 48]. Neoadjuvant immunotherapy with nivolumab alone or in combination with ipilimumab is safe and effective for patients with locoregionally advanced HNSCC [49]. This finding is clinically relevant, as many patients with HNSCC present at a locally advanced and unresectable stage. Therefore, it will be interesting to investigate whether accurate determination of CPS of PD-L1 and CMTM6 will improve screening for immunotherapy eligibility, and ultimately improve treatment response. The occurrence of a comparably consistent expression pattern at different anatomical sites indicates the validity of CMTM6 as a predictive biomarker for HNSCC.

This study was performed with a fairly homogeneous cohort of patients with primarily advanced HNSCC and without prior chemotherapy. Confounding factors, such as intratumoral heterogeneity, were addressed by either evaluating the entire tumor area or scoring different areas within the specimen. Furthermore, the use of CPS ≥ 1 as a cutoff for PD-L1 positivity negates potential differences in staining intensity between fresh samples and those stored long-term due to reduced antigenicity in the latter [50].

Nevertheless, our study has some limitations: Methodologically, the scoring of immunohistochemistry, including for PD-L1 and CMTM6, and general TIL scoring were performed in the “classical” way using the eyes, which incorporates tumor heterogeneity but may not be as reliable as computational pathology. To maintain high reproducibility

of our data, all scores were generated twice by two independent experienced pathologists. The complexity of TME scoring was balanced by analyzing five well-selected areas per sample. Owing to the limited number of cases in our well-documented patient cohort, the validation of the (highly significant) monitoring of the prognostic role of PD-L1 and CMTM6 is reasonable. If verified, the possible associations should be addressed using a mechanistic approach. The mechanistically hypothesized function of CMTM6 in post-translational PD-L1 stabilization and the empirically observed comparability of the staining patterns of both markers in HNSCC emphasize the need for a more precise assessment via co-localization in the membranous tumor compartment, for example, using double immunofluorescence, as recently proposed [51].

In conclusion, we found that CMTM6 is a reliable prognostic marker for HNSCC, with even greater power when co-expressed with PD-L1. Both proteins showed a strong correlation with positivity in TILs, tumor cells, and CD8⁺ T cells. Therefore, we propose that CMTM6 may serve as an additional predictive biomarker for future research on ICIs.

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Author contributions ASB: performed development of methodology, data acquisition and interpretation of data, writing and revision of the paper; NW: performed TCGA analysis; SZ: assisted in data acquisition and critically reviewed the paper; AZ: performed data acquisition and critically reviewed the paper; BS: performed molecular HPV testing and analyzed data; DFS: recruited patients, provided material support and critically reviewed the paper; MK and A-SB: assisted in data acquisition; CM: performed study concept and design, analysis and interpretation of data, and writing and revision of the paper. AE and CJ critically revised the paper. All authors read and approved the final paper.

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Data availability “The datasets generated during and/or analysed during the current study are available from the corresponding author on reasonable request.”

Declarations

Ethics approval The institutional ethic committee at the University Hospital Rostock approved the study (A2018-0003; A2022-0120). The study was performed following the Declaration of Helsinki.

Consent to participate “Informed consent was obtained from all individual participants included in the study.”

Consent to publish “Informed consent was obtained from all individual participants included in the study.”

Competing interests The authors declare no competing interests.

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Article

Identifying Predictive Biomarkers for Head and Neck Squamous Cell Carcinoma Response

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Simple Summary: Head and neck cancer patients have a poor prognosis, even with the best therapeutic options. This is partly due to the lack of reliable prognostic biomarkers. In this study, we aimed to unravel the prognostic and predictive value of immune-related biomarkers in the tumor microenvironment. We identified the PD-L1-stabilizing and membrane-associated protein CMTM6 (CKLF-like MARVEL transmembrane domain-containing protein) as beneficial for patients receiving radiotherapy. Co-expression with the regulatory T cell transcription factor FOXP3 was predictive of a response to radiotherapy. However, no association with survival was seen for other treatment regimens, including chemotherapy, radio-chemotherapy, and immunotherapy. In the latter, CTLA-4 was associated with worse outcomes, whereas PD-L1 was not predictive. Therefore, this study describes a strategy to identify predictive markers for treatment response in head and neck cancer patients and highlights the need for treatment-specific biomarker screening.

Abstract: The 5-year survival rate for head and neck squamous cell carcinoma (HNSCC) is approximately 65%. In addition to radio-chemotherapy, immunotherapy is an approach in the treatment of advanced HNSCC. A better understanding of the immune context would allow personalized treatment by identifying patients who are best suited for different treatment options. In our discovery cohort, we evaluated the expression profiles of CMTM6, PD-L1, CTLA-4, and FOXP3 in 177 HNSCCs from Caucasian patients of all tumor stages and different treatment regimens, correlating marker expression in tumor and immune cells with outcomes. Patients with CMTM6^{high}-expressing tumors had a longer overall survival regardless of treatment. This prognostic benefit of CMTM6 in HNSCC was validated in an independent cohort. Focusing on the in the discovery cohort (n = 177), a good predictive effect of CMTM6^{high} expression was seen in patients receiving radiotherapy ($p = 0.07$; log rank), but not in others. CMTM6 correlated with PD-L1, CTLA-4 and FOXP3 positivity, with patients possessing CMTM6^{high}/FOXP3^{high} tumors showing the longest survival regardless of treatment. In chemotherapy-treated patients, PD-L1 positivity was associated with longer progression-free survival ($p < 0.05$). In the 27 patients who received immunotherapy, gene expression analysis revealed lower levels of CTLA-4 and FOXP3 with either partial or complete response to this treatment, while no effect was observed for CMTM6 or PD-L1. The combination of these immunomodulatory markers seems to be an interesting prognostic and predictive signature for HNSCC patients with the ability to optimize individualized treatments.



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Keywords: HNSCC; CMTM6; PD-L1; immune-checkpoint inhibitor; cisplatin; radiotherapy; tumor microenvironment

1. Introduction

Head and neck squamous cell carcinoma (HNSCC) is a highly heterogeneous and common malignancy, with an increasing prevalence in the Western world. The majority of HNSCCs diagnosed worldwide are in men [1–3]. The major risk factors include chronic alcohol and tobacco use and high-risk human papillomavirus (HPV) infection [4,5]. The standard of care includes surgery, chemotherapy, and radiotherapy either alone or in combination with EGFR-targeting and/or immunotherapy. Although many patients initially respond well to platinum-based radio-chemotherapy, resistance and thus treatment refractoriness frequently occur. The heterogeneity of the disease and the associated individual patient response also make it difficult to standardize treatment protocols and estimate prognosis.

To date, HPV status is the most reliable prognostic factor [6,7], but other aspects, such as molecular and pathological characteristics, tumor location, and clinical stage, additionally influence patient outcomes. The introduction of the PD1-targeting antibodies pembrolizumab and nivolumab into the first- and second-line treatment of patients with recurrent and/or metastatic (r/m) HNSCC has raised hopes for improved outcomes, but objective response rates have been reported to be less than 20%. As a result, the prognosis has not improved significantly over the decades, and the five-year overall survival rate remains at 50–60% [8–11].

Positivity for programmed death-ligand 1 (PD-L1) is the only approved biomarker for immune checkpoint inhibition [12–14]. However, the limited predictive value is a major pitfall limiting immunotherapy success. We have recently identified CKLF-like MARVEL transmembrane domain 6 (CMTM6) as a novel prognostic biomarker with, so far, unknown predictive value. CMTM6 stabilizes PD-L1 on the tumor cells' surface by inhibiting its ubiquitination and subsequent degradation via lysosomes, exhibiting opposing functions across tumors [15–19]. In colorectal cancer, high CMTM6 levels are associated with an inflamed tumor microenvironment, while in hepatocellular carcinoma, CMTM6 inhibits cell proliferation by preventing p21 ubiquitination [15,20]. In HNSCC, CMTM6 was previously reported to mediate cisplatin resistance and regulate AKT-mTORC1-dependent ribosome biogenesis [21,22]. Furthermore, tumor-secreted exosomal CMTM6 was reported to induce M2-like macrophage polarization, indicating an active crosstalk between tumor and immune cells [23]. To our knowledge, studies examining the impact of CMTM6 expression in HNSCC patients receiving immunotherapy are lacking.

Given this, and the fact that the mainstay of treatment for HNSCC patients includes platinum-based (radio-)therapy or immune checkpoint inhibition, the identification of reliable predictive biomarkers remains an unmet need. Increasing evidence suggests that immune status may differ between responders and non-responders. In a very recent report, a gene signature related to the inflammatory response was described, and *OLR1* (a key receptor for oxidized low-density lipoprotein) and *INHBA* (a member of the transforming growth factor- β superfamily) were strongly associated with a poor prognosis of HNSCC patients [24]. In addition, a risk prognostic signature for predicting radiotherapy response based on the expression levels of therapeutic response-related genes has just been reported by Lin et al. [25]. In this study, we performed a detailed analysis using RNA and protein profiling of the tumor microenvironment obtained from primary and recurrent and/or metastatic HNSCC cases. We identified this marker as beneficial for patients receiving radiotherapy. Additionally, we confirmed the prognostic value of CMTM6 in an independent cohort.

2. Materials and Methods

2.1. Patient Cohort and Multi Tumor Tissue Blocks

Pretreatment samples from locally advanced and/or r/m HNSCC patients, partly treated with immune checkpoint inhibitors (ICI) (pembrolizumab, nivolumab), diagnosed between 2017 and 2021, were collected from the Rostock Pathology archives to establish our discovery cohort. If available, additional pre/post ICI treatment tumor biopsies were included. The patients' clinicopathological characteristics, including response to treatment according to the Response Evaluation Criteria in Solid Tumors (RECIST) version 1.1 and progression-free survival (PFS), were obtained from clinical records. Using pseudonyms, follow-up data were obtained from the regional cancer registries. Tumor tissue was obtained from formalin-fixed, paraffin-embedded (FFPE) specimens. Tissue microarrays (TMAs) of three representative areas were constructed using 5 mm cores for samples from ICI-treated patients and 1 mm cores for all non-ICI-treated patients using a tissue chip microarrayer (Beecher Instruments, Silver Spring, MD, USA). The assembly of 5 mm cores preserved the tumor center and the invasion front, including infiltrating cells, for analysis and was performed with biopsy punches (Stiefel Laboratorium, Burgdorf, Germany). Tissues were collected with the patients' consent. The institutional ethic committee at the University Hospital Rostock approved the study (number A2022-0120), which was conducted in accordance with the Declaration of Helsinki of 1975. An independent, well-described cohort from primary HNSCCs was used as a control group to validate our prognostic findings [26–28].

2.2. Gene Expression Analysis

For RNA extraction, after reviewing all original tissue slides, FFPE tissue blocks were selected and recut for hematoxylin–eosin-stained sections to determine tumor surface area. After deparaffinization, RNA was extracted from unstained FFPE tumor sections using the RNeasy FFPE kit (Qiagen, Hilden, Germany). Microdissection was performed in cases showing <50% tumor content or inked borders. mRNA expression profiling was performed using the NanoString nCounter gene expression platform (NanoString Technologies, Seattle, WA, USA). To evaluate cancer-related genes, we applied the Nanostring™ PanCancer IO 360™ panel comprising 770 targets according to manufacturer's instructions. In short, a hybridization reaction was performed using 100 ng of RNA per sample followed by further proceeding in the fully automated Prep Station with data acquisition conducted using the Digital Analyzer (performed at the Department of Internal Medicine B, Cardiology, University Medicine Greifswald, Germany). The data were exported as reporter code count (RCC) files and imported to the NanoString nSolver™ analysis software v4.0 for further analysis. Automatic quality control of mRNA was performed according to the software's instructions.

2.3. Immunohistochemistry and Microscopic Evaluation

Sections of 4 µm were used. Heat-induced antigen retrieval was performed with a high pH buffer (20 min at 97 °C). The following steps were performed in an Autostainer link 48 instrument (Dako, Hamburg, Germany): 5 min of incubation in peroxidase-blocking buffer followed by 20 min of incubation with primary antibody (dilution and clone per antigen—AKT: 1:500; EPR1798, p-AKT (phosphor-S473): 1:50; EP2109Y, CMTM6: 1:1000; EPR23015-45, FOXP3: 1:200; 20034, all Abcam (Cambridge, UK); cytotoxic T-lymphocyte-associated protein 4 (CTLA-4): 1:200; UMAB249 (Rockville, MD, USA), D4B9C, PDL1: 1:100; 22C3, β2-M: 1:800, A0072; all Dako) and 3,3'-diaminobenzidine (DAB) detection using the Dako-kit K8000 according to the manufacturer's instructions. Slides were counterstained with hematoxylin. Appropriate positive and negative controls were used.

For assessment of immune cell distribution status (Figure S1), cytoplasmic and/or membranous reactivity of CTLA-4 and β2 m as well as nuclear staining for FOXP3 were evaluated. Minimum and maximum expression per marker within all samples defined the ranges of the four applied categories (0: absent; 1: minimal; 2: moderate; 3: abundant),

with 0/1 defined as low and 2/3 as high. Cytoplasmic and/or membranous reactivity for AKT and pAKT (S473) was scored semi-quantitatively by the percentage of positive cells and the staining intensity resulting in no/low- vs. high-expressing cases. Scores were built counting 3–5 high-power fields in the tumor center with an Axio-Cam 205 color microscope (field diameter 0.5 mm, Zeiss, Oberkochen, Germany).

PD-L1 and CMTM6 expression were scored analogous to the combined positive score (CPS) as described previously [29]. All scores were calculated within an area showing at least 100 viable tumor cells. Depending on PD-L1 and CMTM6 expression, samples were categorized as PD-L1-positive vs. -negative (cut off ≥ 1 : positive) and CMTM6-low vs. -high (cut off ≥ 10 : high).

2.4. Statistics

Based on D’Agostino, Pearson and Shapiro–Wilk tests for normality, Pearson’s or Spearman’s correlation coefficients (r) were used to analyze the linear association between two continuous variables and used the non-parametric t -test Mann–Whitney tests to compare groups. Overall survival (OS) and PFS curves were constructed using the Kaplan–Meier analysis, and statistical significance was determined using the log-rank test. Unadjusted univariable analysis was conducted. All statistical tests were two-sided, and p values below 0.05 were considered statistically significant. All statistical analyses were performed using GraphPadTM Prism[®] v7.0 software (supplier) and nSolver analysis software (version 4.0). After removing samples with the quality control (QC) flag, mRNA count normalization and $\log_2$ Fold Change (FC) calculation with Welch’s t -test p values were performed. Significant DEGs were defined by $p < 0.05$ and $|\log_2\text{FC}| > 1$ as the threshold.

3. Results

3.1. Patient and Tumor Characteristics

The discovery cohort included a total of 177 patients diagnosed with r/m HNSCC with a median age of 66 years (± 9.8). Clinicopathologic characteristics are shown in Table 1, where stage refers to the time of treatment initiation. Approximately 45% of all patients were smokers (≥ 10 py) and $>32\%$ reported regular alcohol consumption. Thus, most of the cases included here were noxae-associated, either localized in the oral cavity or oropharynx. A total of 18 patients suffered from hypopharyngeal cancer and 15 from laryngeal cancer. One regional lymph node metastasis as well as four distant metastases were obtained, while the remaining samples in the cohort consisted of locally resected primary/recurrent tumors. A total of 20% of all cases were due to a previous high-risk HPV infection.

After diagnosis, patients received either cisplatin-based chemotherapy ($n = 66$), radiotherapy ($n = 30$), combined radio-chemotherapy ($n = 13$), ICI ($n = 27$), or ICI plus chemotherapy ($n = 3$). A small subset of patients were treated with other agents ($n = 16$) or received best supportive care only due to reduced general conditions ($n = 22$). Median PFS on treatment was 21 months and the OS was 29 months. PFS in the ICI-treated subgroup ranged from 1 to 18 months (median 4). Using the RECIST criteria, 1 patient showed a complete response, and 10 patients showed a partial response (=responders), while 5 patients were classified as mixed response/stable disease and the remaining 10 developed tumor progression.

A validation cohort of 286 treatment-naïve primary HNSCCs derived from Caucasian patients with comparable clinicopathological parameters was included in the initial survival analysis. Anonymized clinical information on this patient cohort was obtained from the cooperating institute (University of Lübeck) (Figure 1). Ref. [28] provides precise information about the validation cohort. Kaplan–Meier plots for the validation cohort stratified by CMTM6 are shown in Supplementary Figure S2.

Table 1. Clinico-pathological characteristics of the discovery cohort.

Group Characteristics	Σ n = 177
Female n (%)	34 (19.2)
Male n (%)	143 (80.8)
Median age (years $\pm$ SD)	66 $\pm$ 9.8
Noxae	
smoking (>10 py in %): yes/no	51.9/34.5
alcohol (>1 drink/d in %): yes/no	36.7/63.3
no information (smoking/alcohol)	11.3/2.3
p16/HPV status (%)	
positive	21
negative	79
Tumor stage (UICC stage 8th edition) (n)	
1/2/3/4	37/56/29/55
Localization (n)	
oral cavity	79
oropharynx	65
hypopharynx	18
larynx	15
Treatment (n)	
C/RT/RCT/ICI/ICI + C	66/33/13/27/3
Other/no adjuvant therapy	16/19
Median survival (month)	
PFS	21.0 $\pm$ 20.6
OS	29.0 $\pm$ 21.4

Values are given as absolute/relative numbers and mean $\pm$ SD unless otherwise stated. Abbreviations: py—pack years; d—day; HPV—human papilloma virus; RT—radiotherapy; RCT—radio-chemotherapy; C—chemotherapy (not specified); ICI—immune checkpoint inhibition (nivolumab or pembrolizumab); PFS—progression-free survival; OS—overall survival.

3.2. CMTM6 Status Predicts Response to RCT but Not to Anti-PD1 Treatment

In a total of 463 patients from both cohorts combined, the mean CMTM6 CPS was 33.7 ± 32.3 . PD-L1 staining was only available for the discovery cohort ($n = 177$), and mean PD-L1 CPS was 14.9 ± 26.2 (Table 2). Notably, 56 cases (31%) were either classified as “negative” (PD-L1 CPS < 1) or “low” (CMTM6 CPS < 10). In >40% of these cases, PD-L1 negativity correlated with low CMTM6 status.

Both markers had prognostic value, i.e., patients with PD-L1 positivity or high CMTM6 status showed a significantly better overall survival (Figure 2A,B left, $p < 0.05$). Hence, the prognostic value of PD-L1 and CMTM6 was formally confirmed in both patient cohorts. Stratification by HPV status additionally confirmed a significant prognostic impact for CMTM6 but not for PD-L1 (Figure 2A,B, middle and right).

We then examined the predictive value of these immunologic markers in $n = 177$ patients from the discovery cohort (Figure 2C,D). We focused on cisplatin, RT, RCT, and ICI to cover the most relevant treatment regimens. A predictive value for PD-L1 was only seen in the cisplatin subgroup. Here, patients with a CPS ≥ 1 showed a significantly better OS than their counterparts with CPS < 1 (Figure 2B). No statistically significant benefit was seen for the other regimens. For CMTM6, there was a trend towards a better outcome when the tumor was classified as high and the patient received RT ($p = 0.07$). Likewise, a slightly improved overall survival was seen in the other treatment groups, i.e., cisplatin or RCT. However, patients with high PD-L1 and/or CMTM6 tumors who received anti-PD1 ICI treatment had a comparable OS to their counterparts (Figure 2B,C). Hence, no predictive impact of either marker was seen in this subgroup.

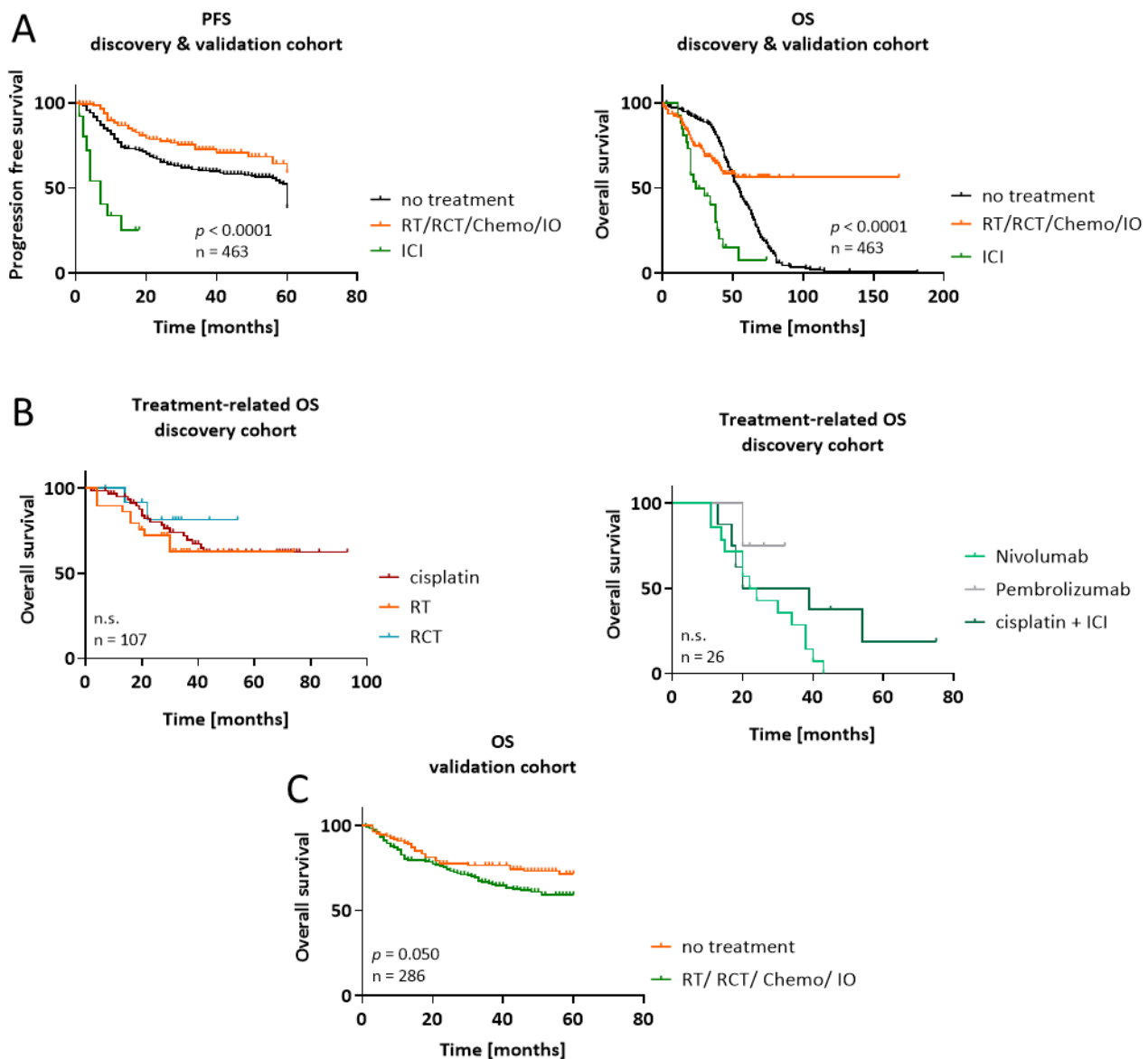


Figure 1. Kaplan–Meier survival curves of the HNSCC patients. The discovery ($n = 177$) and validation cohort ($n = 286$) included HNSCC patients with comparable clinicopathological parameters receiving first- or second-line treatment or best supportive care. **(A)** Progression-free survival (PFS) and overall survival (OS) for the patients from both cohorts. Treatment regimens included radiotherapy (RT), radio-chemotherapy (RCT), chemotherapy, immune-oncology (IO), or ICI (immune checkpoint inhibition). **(B)** Treatment-related OS in the discovery cohort ($n = 177$) stratified according to the specific regimen. (left) Cisplatin-based chemotherapy ($n = 64$), RT ($n = 29$) or RCT ($n = 13$). (right) ICI mono ($n = 14$ and 4 , respectively) or ICI-based combination regimens ($n = 8$). **(C)** Treatment-related OS in the validation cohort ($n = 286$). Log-rank analysis was performed to study differences between the individual regimens. n.s.—not significant.

Table 2. Immunohistochemistry staining results for the markers in the discovery group.

Defined Criteria for Evaluation				
	Cut-Off *	Low/Negative	High/Positive	Median
n = 177	n (%)	n (%)	n (%)	n (%)
CMTM6	(CPS) 10	57 (32.2)	120 (67.8)	33.7
PD-L1	(CPS) 1	58 (32.8)	119 (67.2)	14.9
CTLA-4	(density of positive cells) <i>low</i> vs. <i>high</i>	93 (52.5)	84 (47.5)	-
FOXP3	(density of positive cells) <i>low</i> vs. <i>high</i>	77 (43.5)	100 (56.5)	-
AKT	(% positive cells × staining intensity) <i>no/low</i> vs. <i>high</i>	119 (67.2)	58 (32.8)	-
pAKT	(% positive cells × staining intensity) <i>no/low</i> vs. <i>high</i>	152 (85.9)	25 (14.1)	67.2

* in (brackets): scheme for evaluation. Abbreviations: CPS, combined positive score.

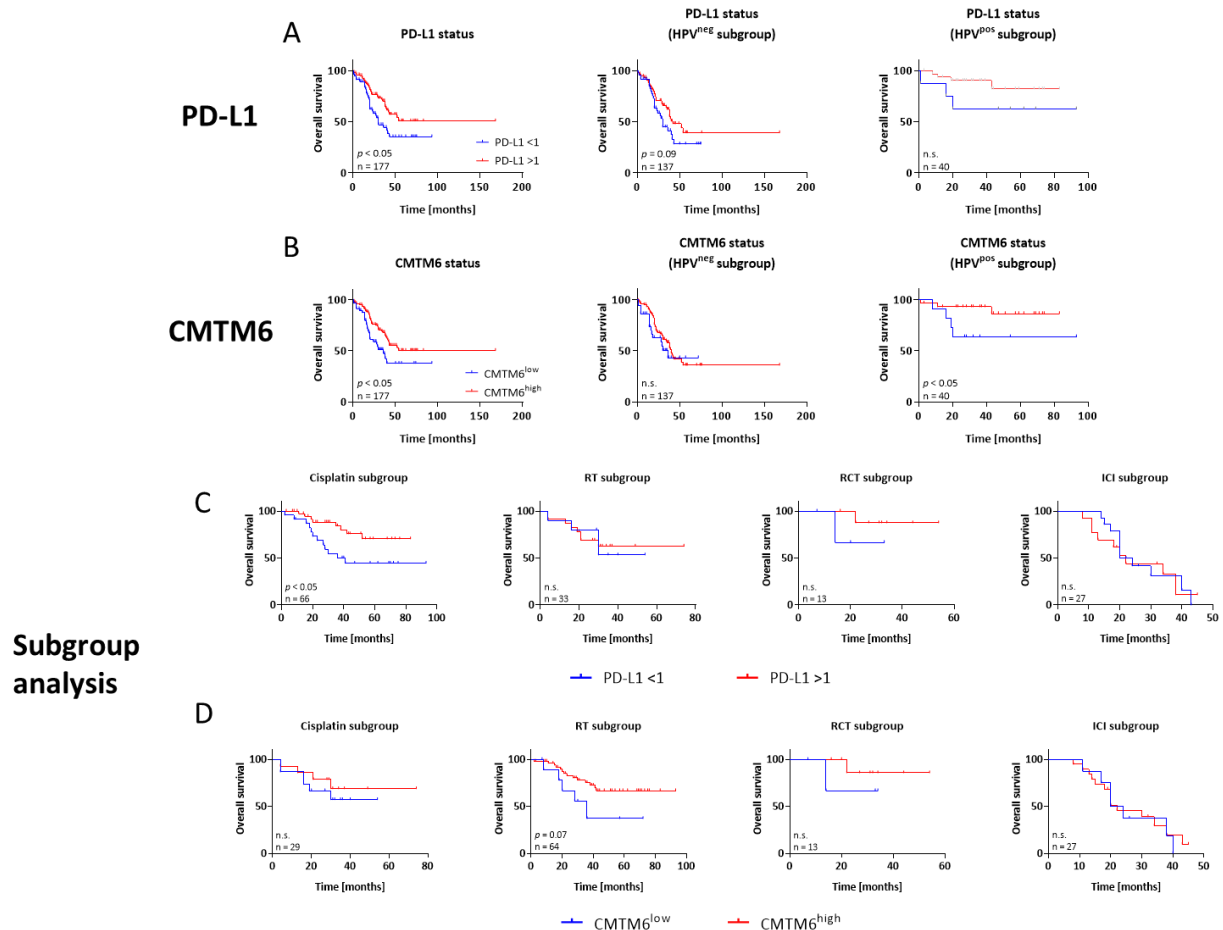


Figure 2. Kaplan–Meier survival curves of HNSCC patients. For the biomarker-driven and treatment-related prognosis, analysis was performed on n = 177 patients (discovery cohort) receiving first- or second-line treatment. Treatment regimens included chemotherapy, radiotherapy (RT), radio-chemotherapy (RCT), or ICI (immune checkpoint inhibition). (A,B) Overall survival (OS) according to PD-L1 or CMTM6 in all patients (left) and stratified according to HPV status (middle and right). (C) Treatment-related OS stratified according to the specific regimen and PD-L1 status. (D) Treatment-related OS stratified according to the specific regimen and CMTM6 status. Numbers of patients for each treatment group, i.e., cisplatin, RT, RCT, or ICI are given in their respective graphs. Log-rank analysis was performed to study any differences between the individual regimens. n.s.—not significant.

3.3. Correlation of CMTM6 Status with Other Immune Biomarkers

The interplay between different immune cell subtypes within the tumor microenvironment (TME) prompted us to check for additional biomarkers with potential prognostic and predictive value (Spearman correlation) in the discovery cohort. For this analysis, treatment-unrelated (=prognostic) and treatment-related (=predictive) correlations were conducted (Table 3). For the latter, only patients who received cisplatin, RT, or RCT were included. CMTM6 was analyzed for correlation with PD-L1, CTLA-4, FOXP3, AKT, and pAKT. Tables 2 and 3 show the staining results and the correlations between the markers. Table 4 refers to staining pattern depending on the clinical parameters.

Table 3. Spearman correlation for markers in the discovery cohort including samples from patients receiving no adjuvant or either chemotherapy/radiation/chemoradiation.

CMTM6 vs.		Biomarker				
		PD-L1	CTLA-4	FOXP3	AKT	pAKT
Treatment Unrelated n = 151 #	r	0.26	0.63	0.85	0.31	0.22
	95% CI	0.098 to 0.41	0.52 to 0.72	0.80 to 0.89	0.15 to 0.46	0.047 to 0.38
	p (two-tailed)	0.0014	<0.0001	<0.0001	0.0002	0.0110
	p value summary	**	****	****	***	*
RT n = 33	r	0.32	0.76	0.89	0.19	0.16
	95% CI	−0.061 to 0.62	0.54 to 0.88	0.78 to 0.95	−0.20 to 0.54	−0.26 to 0.53
	p (two-tailed)	0.0877	<0.0001	<0.0001	0.3221	0.4350
	p value summary	ns	****	****	ns	ns
Cisplatin n = 66	r	0.36	0.55	0.85	0.03	0.13
	95% CI	−0.12 to 0.56	0.34 to 0.70	0.76 to 0.91	−0.25 to 0.31	−0.16 to 0.40
	p (two-tailed)	0.0037	<0.0001	<0.0001	0.8311	0.3636
	p value summary	**	****	****	ns	ns
RCT n = 13	r	0.51	0.84	0.71	0.29	0.31
	95% CI	−0.10 to 0.85	0.53 to 0.95	0.25 to 0.91	−0.39 to 0.77	−0.38 to 0.77
	p (two-tailed)	0.089	0.0006	0.0078	0.4182	0.3549
	p value summary	ns	***	**	ns	ns

Interpretation: ≥ 0.70 : very strong relationship; 0.40–0.69: strong relationship; 0.30–0.39: moderate relationship; 0.20–0.29: weak relationship; 0.01–0.19: no relationship. ns—not significant. # PD-L1 n = 148; AKT n = 134; pAKT n = 130. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$.

Independent of treatment, CMTM6 expression strongly correlated with FOXP3, a specific transcriptional regulator in Tregs. Moderate correlations were observed between CMTM6 and PD-L1 and CTLA-4. Notably, a strong correlation between CMTM6 and FOXP3 was seen across treatments. In all subgroups, CMTM6 correlated more strongly with CTLA-4 status than PD-L1. No association was found between CMTM6 and AKT or pAKT.

Then, the prognostic role of these immune markers was analyzed (Figure 3). High FOXP3 expression levels were associated with significantly improved overall survival in both HPV^{pos} and HPV^{neg} cases (Figure 3A, $p < 0.05$). To study if FOXP3 has comparable prognostic relevance to CMTM6, the overall survival of patients was analyzed in additional subgroups. Therefore, tumors were classified into FOXP3^{low}/CMTM6^{low} (n = 51), FOXP3^{high}/CMTM6^{low} (n = 6), FOXP3^{low}/CMTM6^{high} (n = 17), and FOXP3^{high}/CMTM6^{high} (n = 99). Patients in the latter group and those with FOXP3^{high}/CMTM6^{low} tumors had the best prognosis with survival rates exceeding 50% at the longest time point. This underlines the prognostic impact of both markers in HNSCC.

Table 4. Contingency table for the markers in the discovery group.

n = 177	Biomarker											
	CMTM6 (n)			PD-L1 (n)			FOXP3 (n)			CTLA-4 (n)		
	Low	High	<i>p</i>	Negative	Positive	<i>p</i>	Low	High	<i>p</i>	Low	High	<i>p</i>
Gender			ns			ns			ns			ns
Male	38	105		48	95		60	83		75	68	
Female	9	25		10	24		17	17		18	16	
Smoking [#]			ns			ns			ns			ns
Yes	33	59		38	54		37	55		56	36	
No	29	36		20	45		35	30		33	32	
Alcohol ^{##}			ns			ns			ns			ns
Yes	30	31		28	33		24	37		28	33	
No	43	69		41	71		49	63		65	47	
HPV												
Pos.	10	28		9	29		13	26		15	24	
Neg.	47	92		48	91		64	74		69	69	
TNM			ns			ns			ns			*
T1	9	28		12	25		18	19		10	27	
T2	18	38		15	41		26	30		25	31	
T3	11	18		13	16		9	20		15	14	
T4	19	36		18	37		24	31		34	21	
N−/N+	23/34	66/54	ns	23/35	66/53	ns	37/40	48/52	ns	40/44	49/44	ns
M0/M1	43/14	102/17	ns	43/15	103/16	ns	63/14	83/17	ns	65/19	81/12	ns
Recurrence												
No	34	92	*	24	91	****	48	91	****	25	100	**
Yes	23	28		34	28		27	9		21	31	

[#] for n = 157; ^{##} for n = 173; * *p* < 0.05; ** *p* < 0.01; **** *p* < 0.0001. Abbreviations: *p*—*p* value; HPV—human papilloma virus; ns—not significant.

To see if this prognostic role could be transferred to the predictive model, we performed a subgroup analysis with respect to the individual treatments. A trend towards a better outcome was observed for all treatment regimens (Figure 3B), reaching statistical significance for patients receiving RCT (*p* < 0.05 vs. FOXP3^{low}/CMTM6^{low}). A closer look at the survival curve of ICI-treated patients revealed a delayed favorable response with a median OS of 43 months in the FOXP3^{high}/CMTM6^{high} subgroup (vs. 22 months FOXP3^{low}/CMTM6^{low}). Thus, although statistical significance was not achieved in this small patient cohort, we confirmed the predictive value by doubling the median OS of patients with r/m HNSCC.

CTLA-4 had no effect on median OS. Finally, the relevance of serine/threonine protein kinase AKT and its active phosphorylated form (pAKT) was analyzed, because AKT is a downstream target of CMTM6-mediated Wnt signaling and may be involved in cisplatin resistance. Hence, AKT is considered as a proto-oncogene. In our study, we identified a slightly improved median OS in the AKT high subgroup (vs. AKT low) and vice versa when pAKT was considered. Still, no significant survival benefit was seen in either group.

Further treatment-related analysis was only performed for the cisplatin subgroup but did not yield any prognostic value. Hence, the prognostic value of AKT/pAKT in our patient cohort is rather low.

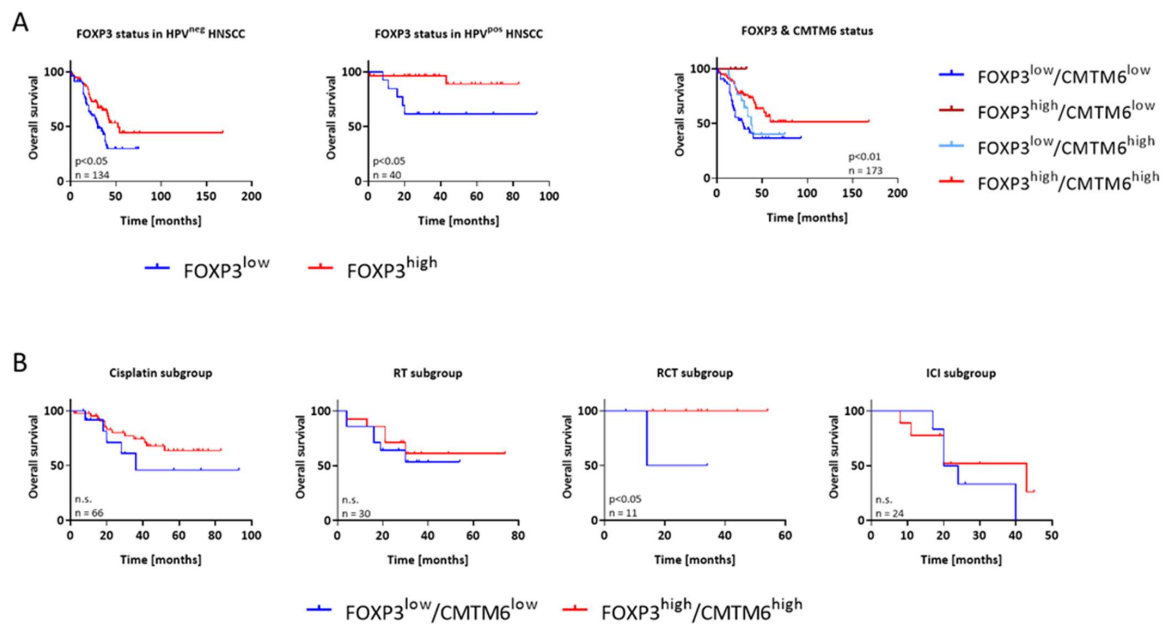


Figure 3. Kaplan–Meier survival curves of HNSCC patients from the discovery cohort. For biomarker-driven and treatment-related prognosis, analysis was performed on $n = 177$ patients receiving first- or second-line treatment. Treatment regimens included chemotherapy, radiotherapy (RT), radio-chemotherapy (RCT), or ICI (immune checkpoint inhibition). (A) Overall survival (OS) of patients according to FOXP3 and combined FOXP3/CMTM6 status. (B) Treatment-related OS stratified according to the specific regimen and FOXP3/CMTM6 status. Cisplatin-based chemotherapy, RT, RCT, or ICI. Log-rank analysis was performed to study any differences between the individual regimens. n.s.—not significant.

3.4. Immunotherapy Response in r/m HNSCC Gene Signatures in Responders

Transcriptomic and immunological profiling was performed on pre- and post-treatment samples of r/m HNSCC patients receiving anti-PD1 immunotherapy. These samples were from the discovery cohort and only included one patient with high-risk oncogenic HPV infection; the remaining samples were HPV-negative. Gene expression analysis for anti-tumor immune activity showed two distinct subgroups (Figure 4A). Concerning the profiled gene signatures, responders had significantly lower mRNA expression levels of CTLA-4 than non-responders (Figure 4B). By plotting the receiver operator characteristics (ROCs), CTLA-4 had the highest capacity to predict response (area under the curve 0.753; PD-L1: 0.7) of 43 signatures. While the expression levels of genes associated with T cells, including exhausted CD8⁺ cytotoxic cells, as well as levels of the immune checkpoint receptor T cell immunoglobulin and ITIM domain (TIGIT) were lower in responders, this failed to be statistically significant in the multivariate analysis (Figure 4C). In line with our findings described above, the abundance of Tregs as measured by gene expression of FOXP3 was lower in these immunologically cold tumors, here showing a better treatment response to ICI, and vice versa. Patients benefiting longer from ICI had lower expression genes levels in tumors involved in signatures of CD8 T cells, TIGIT, and CTLA-4 compared to patients with shorter progression times (Figure 4D). Interestingly, we found no differences in PD-L1 expression between responders and non-responders. Genes involved in the pathway downstream of the IFN gamma axis were significantly higher in samples defined as “PD-L1-positive” by IHC (Figure 4E). In three patients with re-biopsy material at the time point of progression during ICI treatment, neither CMTM6 nor PD-L1 status by IHC changed according to the applied cut-offs, and Beta-2-microglobulin expression was preserved. Focusing on the profiled immune cell population in these patients (one classified as a responder, two as non-responders), the tumor inflammation signature (TIS), which predicts a response to pembrolizumab, decreased in the two non-responders (Figure 4F). On the other hand,

CTLA-4 levels increased in the third patient, who progressed after 8 months of a partial response (Figure 4G). We also focused on the seven included patients with available tumor material (a) either before or (b) at disease progression during ICI treatment. Using IHC, PD-L1, CMTM6, FOXP3 and CTLA-4 status was stable in two patients with mixed changes in the remaining patients. Immune profiling generally revealed inhibition of immune cell abundance and antitumor immune activity; however, an increase in inhibitory immune signaling was observed when primary tumors were compared with the material from r/m disease, as previously described [28].

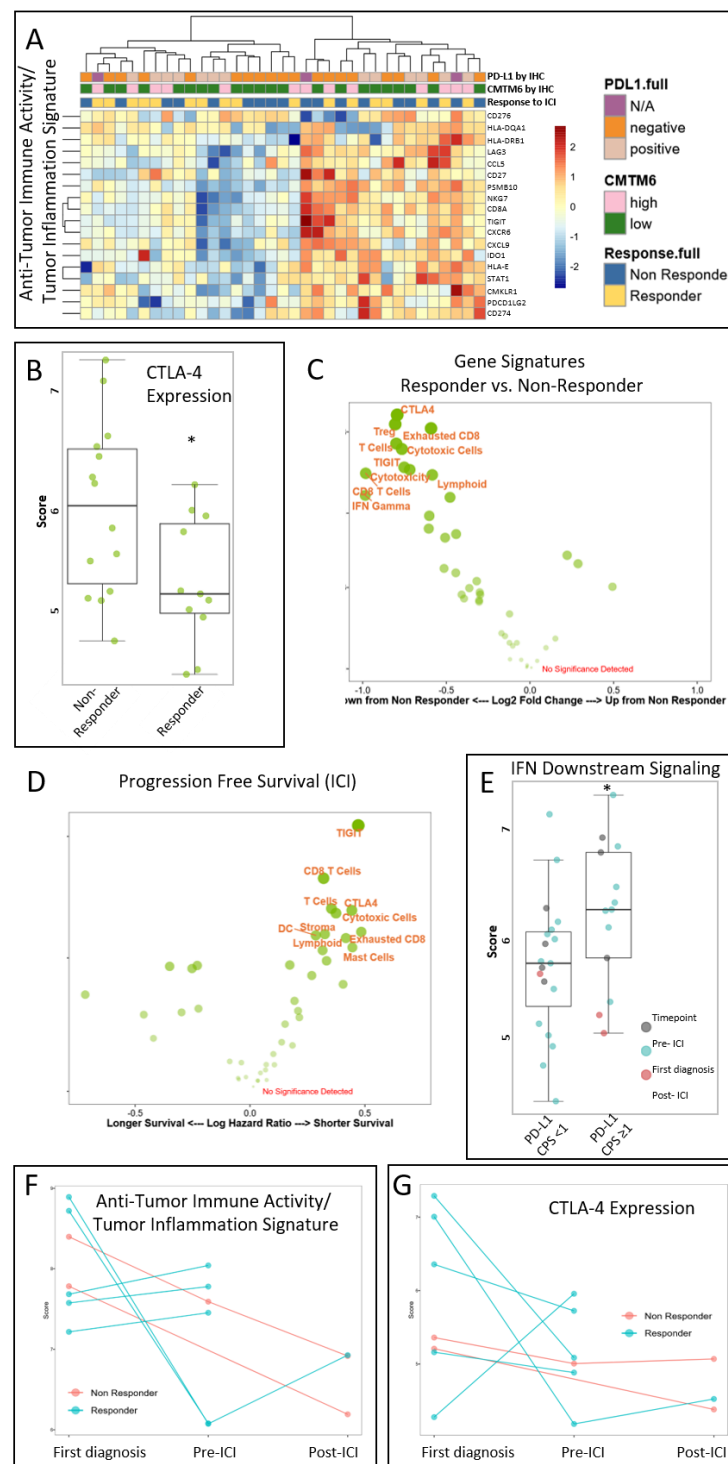


Figure 4. Gene signatures in HNSCC patients from the discovery group with their response to immune checkpoint inhibitors. (A) Heatmap representing the down- and upregulated genes involved

in antitumor immune activity by unsupervised hierarchical clustering. Scores are scaled to have a mean of zero and a standard deviation of one. The standardized signature scores are truncated at ± 3 standard deviations (99% of the data should fall within ± 3 standard deviations of the mean). (B) Box plot indicating a significantly lower CTLA-4 signature in patients with a response to ICI as assessed by univariate analysis ($p = 0.02$). (C) Volcano plot of all gene signatures displays no significant difference between the ICI responders and non-responders in a multivariate analysis. Up indicates upregulated and down indicates downregulated genes. (D) Survival volcano plot of all gene signatures displays no significant difference between the signature's hazard ratios in a multivariate analysis. Signatures further to the right are associated with a decreased risk of an event relative to the baseline. (E) Box plot indicating a significantly higher IFN downstream signature in patients whose tumors showed a CPS ≥ 1 for PD-L1 immunohistochemistry as assessed by univariate analysis ($p = 0.04$). (F) Spaghetti plot displaying a decrease in antitumor immune activity from initial diagnosis to r/m disease with start of ICI until the time point of progression under ICI in non-responders, and (G) an increase in CTLA-4 in recurrent tumor material from a patient who initially had a partial response to ICI. * $p < 0.05$. CPS—combined positive score; Pre-ICI—sample taken just before the start of ICI; Post-ICI—sample taken just after progression under ICI.

4. Discussion

The identification of reliable biomarkers for HNSCC is ongoing. In this study, we applied a classical pathological approach and examined the TME of HPV-related and -unrelated cases to elucidate the biological relevance of several known and novel biomarkers. We included cases from different sites and treatment regimens to cover the clinical heterogeneity and related challenges. The overall outcome was comparable to reports in the literature and confirmed the beneficial response to combined radio-chemotherapy compared to either monotherapy in the first-line treatment of HNSCC. However, in the r/m setting, the use of ICIs has little impact on PFS or OS with frequent relapse in all patients.

In our discovery cohort, we observed that CMTM6 positivity correlates with an improved OS, independently of adjuvant treatment modalities. We observed this beneficial prognostic impact in an independent validation cohort from treatment-naïve HNSCC patients with comparable clinical characteristics. CMTM6 is now well known to stabilize PD-L1 protein expression on the tumor cells' surface [19,30]. It is widely expressed on both tumor and immune cells and plays an important role in regulating T cell activation and antitumor response. The prognostic impact of CMTM6 is comparable to PD-L1, underlying its overlapping biological functions. However, the predictive value of this novel biomarker is largely unknown [31]. By stratifying patients from the discovery cohort according to the most frequent treatment regimens, i.e., chemotherapy, radiotherapy (RT), radio-chemotherapy, or immune checkpoint inhibition, a predictive value was only seen in the RT group. Patients with high CMTM6 expression on tumor cells and/or in the TME showed a survival benefit compared to their CMTM6-low counterparts. While HPV is an approved biomarker, the prognostic impact was even higher here when tumors were also CMTM6-high. The prognostic impact of CMTM6 is in accordance with reports on colorectal (CRC) and hepatocellular carcinomas [15,20] but in contrast to the findings of Chen et al. [32,33]. In their cohort of 210 primary HNSCC samples collected from the School and Hospital of Stomatology, Wuhan University, China, the authors identified CMTM6 overexpression by IHC as a poor prognostic marker. Using digital image analysis, membranous and/or cytoplasmic CMTM6 positivity in tumor cells predicted a shorter OS independent of other clinicopathological parameters. Their scoring system for CMTM6, analogous to the tumor proportion score (TPS), excluded immune cells surrounding the tumor. In our study, we quantified CMTM6 positivity using the CPS. This score includes cells within the TME, which regulates tumor cell survival and confers anticancer effects [3,32]. Although the group describes similar staining patterns for PD-L1 and CMTM6 as seen here, the results are not comparable due to the different scoring methods. Furthermore, Chen et al.

performed their study in an Asian cohort, where risk factors and outcomes differ from those in Caucasians [33]. With this in mind, the authors reviewed The Cancer Genome Atlas (TCGA) [34] to support their findings. While they do not provide information on the impact of *CMTM6* RNA expression in the TCGA cohort, they show that both *CMTM6* RNA expression and protein levels, when analyzed analogously to TPS, increased with higher pathological grade. Our cohort includes 21% HPV-related and therefore conventionally ungraded tumors. We did not see any correlation between tumor grade and *CMTM6*. However, another Chinese group investigated *CMTM6* RNA levels in CRC samples and found a higher *CMTM6* expression in early clinical stages (UICC I/II vs. III/IV) [15] (ref. Peng). Although CRC as an adenocarcinoma is not comparable to HNSCC, this underlies the importance of future studies to elucidate the impact of *CMTM6* in solid cancers.

Still, no such correlation was seen in the other subgroups. The most unexpected finding was the missing predictive value for the ICI subgroup, which is described for lung carcinoma [35]. The same missing correlation was seen for PD-L1. Here, a beneficial effect was only obtained when patients received cisplatin but not with other therapies. Although this finding is somehow unexpected, it fits with recent reports in which ICI-based treatments yielded mixed responses and sometimes failed to improve the outcome of r/m HNSCC patients [36–38]. Hence, PD-L1 and/or *CMTM6* alone may be insufficient to predict ICI responses in this patient cohort.

Here, we performed a more global approach by combining protein and gene expression-based analysis in patients from the discovery cohort. Firstly, we identified a strong correlation between *CMTM6* and *FOXP3*. The latter is a specific transcription factor in regulatory T cells with known immunosuppressive effects and a correspondingly conflicting role in patient prognosis. In HNSCC, high *FOXP3* levels have previously been reported to correlate with better outcomes [39,40] regardless of treatment [41]. Here, we further confirmed the prognostic impact independent of HPV status. Comparable results were shown in gastric cancer, where co-expression of *FOXP3* and *CMTM6* was related to a favorable prognosis [42]. *FOXP3* was found to co-localize with tumor-infiltrating CD20⁺CD27⁺ B cells to promote effector and memory T cell differentiation and enhance B cell and NK cell activation and function. Therefore, the dual role of *FOXP3* in cancer is advantageous for HNSCC patients, and this positive impact is probably enhanced by *CMTM6*. Adding to this, Jing et al. recently described an inflammatory response-related gene profile to predict the prognosis in HNSCC [24]. In a parallel study, a risk model was developed to predict therapy response and prognosis in response gene-defined subgroups of HNSCC [25]. With our study, we suggest additional predictive biomarkers that may be potential targets in the near future.

Another novel finding of our study was the strong correlation between *CMTM6* and *CTLA-4*, which was even stronger than with PD-L1. This is intriguing and describes a new, previously unknown interaction of *CMTM6* with immunoregulatory molecules [19]. Unlike *CMTM6*, *CTLA-4* was not expressed in tumor cells, as reported recently by Hoffmann et al. [43]. In this study, lower *CTLA4* promoter methylation correlated with a response to ICI and longer progression-free survival, but these findings were not confirmed at the protein level. This adds to Sholl's conclusion that not only protein but also genomic and transcriptomic levels mediate the variable response to ICIs [44].

The interaction between the tumor and host immune-specific genetic and epigenetic factors is complex and it is now clear that PD-L1 protein expression by immunohistochemistry is neither sensitive nor specific enough to serve as a reliable predictive biomarker. Our study adds to this by showing that protein and gene expression levels of PD-L1 were insufficient predictors of a response to immunotherapy. Beyond PD-L1, the transcriptomic profile of this entity can identify possible correlations between an ICI response, TME, and *CMTM6*. By means of gene expression analysis, we found that the mRNA levels of *CTLA-4* and *FOXP3* were lower in the responder group of our patients treated with ICI. Since *CTLA-4* is mainly expressed in tumor-draining lymph nodes, established tissue-based biomarkers for predicting its inhibitory capacity are lacking. In melanoma patients, the

anti-CTLA-4 inhibitor ipilimumab is the standard of care regardless of PD-L1 status, and the combination with an anti-PD1 ICI improves its efficacy. Although some studies have failed to show a beneficial effect of the Nivo/Ipi combination, several phase II/III trials are currently investigating the efficacy of anti-CTLA-4 antibodies as a monotherapy or in combination with anti-PD1 antibodies in HNSCC ([ClinicalTrials.gov](https://clinicaltrials.gov) Identifiers: NCT04080804, NCT04326257, NCT03624231, NCT03212469, NCT03799445). In addition, a lower CD8 profile indicated a trend towards an improved ICI response, which is in contrast to the data from Ayers et al. [45]. Using mRNA expression profiling, they showed that a T cell-inflamed microenvironment characterized by active IFN- γ signaling, cytotoxic effector molecules, and T cell-active cytokines is a common feature of the biology of tumors that respond to PD1 checkpoint blockade. With this knowledge, the authors established a focused set of genes to identify this PD1 checkpoint blockade-responsive biology. Although the genes analyzed were congruent with those studied here, possible explanations for the different findings may be that (1) our study cohort included less patients ($n = 26$ here vs. $n = 43$ by Ayers et al.), (2) only $n = 8$ patients received ICI as a first-line treatment, (3) the PFS in our cohort was shorter, and (4) PD-L1 CPS does not predict patients' responsiveness to treatment.

Combination therapies targeting PD1 and CTLA-4 may also fail due to primary or secondary resistance mechanisms. Beta-2-microglobulin ($\beta 2M$) is a major histocompatibility complex class I component and is essential for antigen presentation. Its loss, either by mutation or degradation, leads to ICI treatment failure [46,47]. Similarly, all tumors in the responder group expressed $\beta 2M$, whereas three non-responders lacked this protein on the tumor cell surface prior to treatment initiation. The three samples with re-biopsy material at the time of progression during ICI treatment had all retained $\beta 2M$ expression, supporting alternative resistance mechanisms.

Finally, the relevance of the serine/threonine kinase AKT and its active form, pAKT, was studied. In oral cancer, pAKT is overexpressed because of reduced protein degradation and contributes to tumor growth, lymph node metastasis, and shorter survival time [48,49]. Other studies related pAKT expression to radio-resistance [50] or HPV positivity [51]. In our study, neither AKT nor its active form were associated with a significant survival benefit. This finding is rather expected and hence additional studies on larger patient cohorts will have to clarify the relevance of AKT/pAKT in HNSCC.

A limitation of our study was the small sample size of only $n = 26$ HNSCCs in the ICI cohort for gene expression profiling. However, as anti-PD1 treatment had only recently been approved for the treatment of HNSCC, the present cohort is of reasonable size, including eight patients without prior systemic therapy. Second, the TMA design weakens our results because separation into the tumor center, invasion front, and stromal compartment is more difficult than on whole mount sections. To address this, we used 1 mm cores in the construction of the TMA and punched tumor tissue multiple times to capture the heterogeneity of the TME. In addition, the biomarkers CMTM6 and PD-L1 were examined on whole slides. We also extracted mRNA from whole tumors, including immune cells. To increase the tumor cell content and decrease bystander cells, tumor microdissection was performed. In challenging cases, we could have used laser microdissection to separate tumor and immune cells, but the risk of losing mRNA-containing cells by focusing on the small amount of tumor in some of our cases seemed too high. Next, the measurement of CMTM6 was not well defined. To include CMTM6 expression on tumor and associated immune cells, we scored CMTM6 analogously to PD-L1 by calculating its CPS, which itself has its weaknesses. Receiver operating curve analysis revealed a cut-off of ≥ 10 for CMTM6. Future studies in independent cohorts will show whether another scoring system for CMTM6 protein expression is more valuable. Additionally, fluorescent labeling of the examined molecules, including immune cells, would prove the co-expression of CMTM6 with other markers in more detail. While membranous co-localization of CMTM6 and PD-L1 was shown via high-resolution fluorescence several times [35,52], the association with other co-stimulatory molecules, including FOXP3 and CTLA-4, especially in HNSCC, is

less clear. Therefore, this question should be addressed in a future project, maybe even incorporating other co-stimulatory surface molecules like CD28.

5. Conclusions

In Caucasian HNSCC patients, CMTM6^{high} tumors showed a longer OS in independent cohorts. CMTM6 correlates with PD-L1-, CTLA-4-, and FOXP3-positive Treg infiltration in HNSCCs with a better response to radiotherapy in CMTM6^{high} tumors. While neither PD-L1 nor CMTM6 status predicted the treatment response to immune checkpoint inhibitors, and low CTLA-4 expression was associated with a good response. This argues for the inclusion of CTLA-4 inhibitors to target the antitumor immunity in HNSCC patients.

Supplementary Materials: The following supporting information can be downloaded at: <https://www.mdpi.com/article/10.3390/cancers15235597/s1>, Figure S1: Staining for immune checkpoint molecules and immune cells in high vs. low expression cases. Figure S2: Progression-free survival and overall survival in the validation cohort stratified by CMTM6 independent of treatment and for subgroups receiving either chemotherapy, radiation or chemoradiation.

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Informed Consent Statement: Informed consent was obtained from all subjects involved in the study.

Data Availability Statement: The data presented in this study are available on request from the corresponding author.

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Abbreviations

C	chemotherapy
CMTM6	CKLF-like MARVEL transmembrane domain 6
CPS	combined positive score
d	day
HNSCC	head and neck squamous cell carcinoma
HPV	human papillomavirus
PD-L1	programmed death-ligand 1
ICI	immune checkpoint inhibitors
OS	overall survival
PFS	progression-free survival
py	pack years
RCT	radio-chemotherapy
RT	radiotherapy
r/m	recurrent and/or metastatic
TIGIT	T cell immunoglobulin and ITIM domain

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Correction

Correction: Becker et al. Identifying Predictive Biomarkers for Head and Neck Squamous Cell Carcinoma Response. *Cancers* 2023, 15, 5597

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Error in Figure

In the original publication [1], there was a mistake in Figure 3A, as published. The included legend of the survival curve on the right had a wrongly named second-line caption. The correct labeling of the subgroup (dark red line) is “FOXP3 high/CMTM6 low” instead of “FOXP3 low/CMTM6 low” (which is the caption for the royal blue line).

The corrected Figure 3A appears below. The authors apologize for any inconvenience caused and state that the scientific conclusions are unaffected. This correction was approved by the Academic Editor. The original publication has also been updated.



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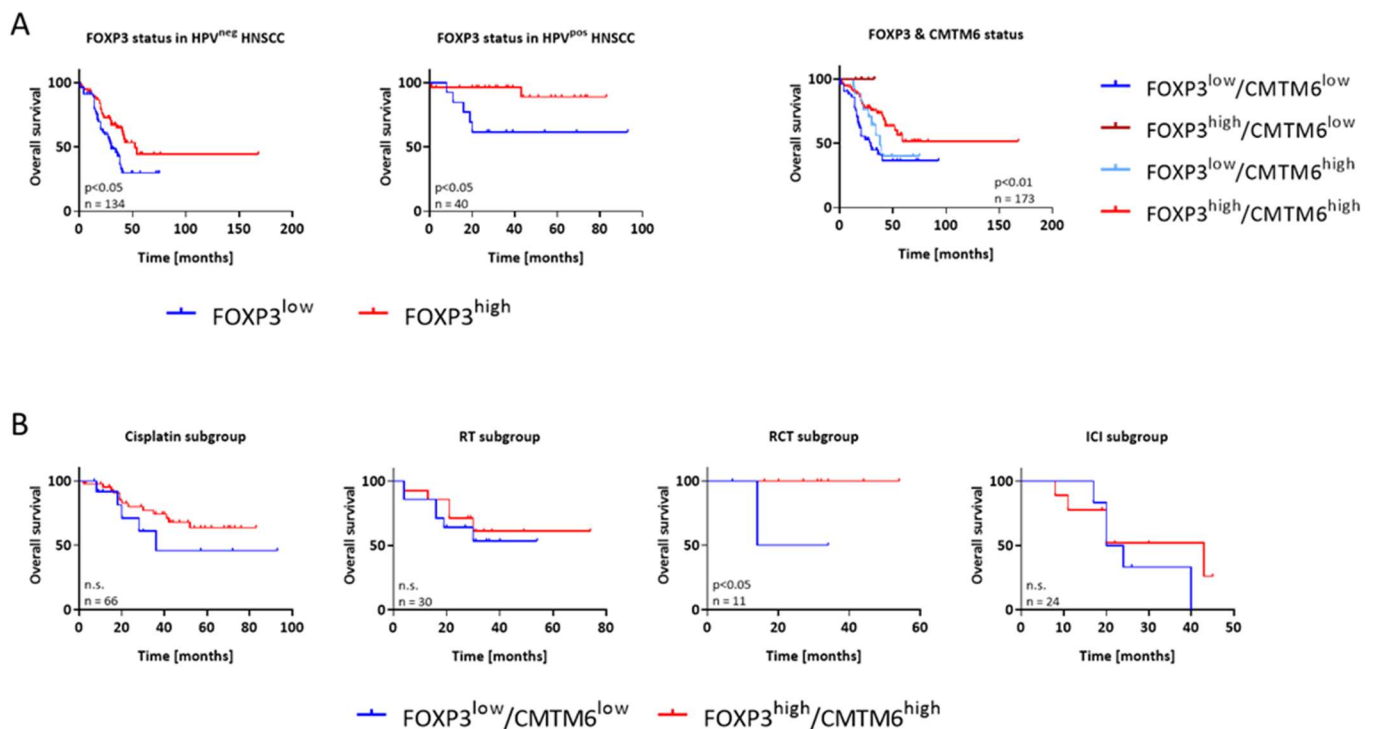


Figure 3. Kaplan–Meier survival curves of HNSCC patients from the discovery cohort. For biomarker-driven and treatment-related prognosis, analysis was performed on $n = 177$ patients receiving first- or second-line treatment. Treatment regimens included chemotherapy, radiotherapy (RT), radio-chemotherapy (RCT), or ICI (immune checkpoint inhibition). **(A)** Overall survival (OS) of patients according to FOXP3 and combined FOXP3/CMTM6 status. **(B)** Treatment-related OS stratified according to the specific regimen and FOXP3/CMTM6 status. Cisplatin-based chemotherapy, RT, RCT, or ICI. Log-rank analysis was performed to study any differences between the individual regimens. n.s.—not significant.

Reference

1. Becker, A.-S.; Kluge, C.; Schofeld, C.; Zimpfer, A.H.; Schneider, B.; Strüder, D.; Redwanz, C.; Ribbat-Idel, J.; Idel, C.; Maletzki, C. Identifying Predictive Biomarkers for Head and Neck Squamous Cell Carcinoma Response. *Cancers* **2023**, *15*, 5597. [[CrossRef](#)] [[PubMed](#)]

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