Aus der

Klinik für Gynäkologie und Gynäkologische Onkologie

des Universitätsklinikums Bonn

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Früherkennung und Biomarker bei HPV-assoziierten gynäkologischen Malignomen

Habilitationsschrift

zur Erlangung der *venia legendi*

der Hohen Medizinischen Fakultät

der Rheinischen Friedrich-Wilhelms-Universität Bonn

für das Lehrgebiet

„Frauenheilkunde und Geburtshilfe“

vorgelegt von

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2024

1. Inhaltsverzeichnis

Datum der Antrittsvorlesung: 07.10.2024

2. Grundlage der kumulativen Habilitationsschrift

Der vorliegenden kumulativen Habilitationsschrift liegen vier Originalarbeiten zu Grunde. Die wissenschaftlichen Ergebnisse dieser vier Originalarbeiten werden in dieser Habilitationsschrift in einem zusammenhängenden Kontext subsumiert und diskutiert:

I) Condic M*, Neidhöfer C*, Ralser DJ, Wetzig N, Thiele R, Sieber M, Otten LA, Warwas LK, Hierauf A, Mustea A, Parčina M. Analysis of the cervical microbiome in women from the German national cervical cancer screening program. J Cancer Res Clin Oncol. 2023 Feb 13. doi: 10.1007/s00432-023-04599-0.

II) Condic M*, Ralser DJ*, Klümper N, Ellinger J, Qureischi M, Egger EK, Kristiansen G, Mustea A, Thiesler T. Comprehensive Analysis of N6-Methyladenosine (m6A) Writers, Erasers, and Readers in Cervical Cancer. Int J Mol Sci. 2022 Jun 28;23(13):7165. doi: 10.3390/ijms23137165

III) Condic M*, Thiesler T*, Staerk C, Klümper N, Ellinger J, Egger EK, Kübler K, Kristiansen G, Mustea A, Ralser DJ. N6-methyladenosine RNA modification (m6A) is of prognostic value in HPV-dependent vulvar squamous cell carcinoma. BMC Cancer. 2022 Sep 1;22(1):943. doi: 10.1186/s12885-022-10010-x

IV) Condic M, Egger EK, Klümper N, Kristiansen G, Mustea A, Thiesler T, Ralser DJ. TROP-2 is widely expressed in vulvar squamous cell carcinoma and represents a potential new therapeutic target. J Cancer Res Clin Oncol. 2023 Apr 17. doi: 10.1007/s00432-023-04761-8.

***** Geteilte Erstautorenschaft

3. Einleitung

Epidemiologie des Zervix- und Vulvakarzinoms

Das Zervixkarzinom ist weltweit das vierthäufigste Malignom der Frau(1). Jährlich werden mehr als 600.000 Frauen mit einem Zervixkarzinom neu diagnostiziert und 300.000 versterben an der Erkrankung. Ungefähr 90% aller Zervixkarzinome treten in Entwicklungsländern auf, was auf fehlende Vorsorgeprogramme und niedrige Impfraten gegen das humane Papillomavirus (HPV) zurückzuführen ist(2). In den Industrieländern mit etablierten Vorsorgeprogrammen haben sich die Inzidenzraten des Zervixkarzinoms über die letzten 30 Jahre mehr als halbiert. Das mittlere Erkrankungsalter liegt in Deutschland bei 55 Jahren und die relative 5-Jahres-Überlebensrate bei 67%(3).

Zu den häufigsten histologischen Subtypen gehören das Plattenepithelkarzinom mit einem Anteil von 80% und das Adenokarzinom mit 20% der Fälle (4) . Andere Tumorentitäten wie das neuroendokrine oder klarzellige Zervixkarzinom sind äußerst selten.

Hauptursächlich für die Entstehung eines Zervixkarzinoms ist die Infektion mit *high risk* HPV-Viren, vor allem der Typen 16 und 18(5). HPV-Viren werden durch Geschlechtsverkehr oder Hautkontakt übertragen. Man geht davon aus, dass sich 80% aller Frauen und Männer im Laufe ihres Lebens mit HPV infizieren. Meist wird die Infektion durch das Immunsystem bekämpft, bei jedoch 5-10% der Patientinnen zeigt sich eine persistierende Infektion(6). Diese führt zunächst zur Entwicklung von Vorläuferläsionen, den cervikalen intraepithelialen Neoplasien (CIN), die ebenfalls in den meisten Fällen durch das Immunsystem zum Ausheilen gebracht werden. Nur etwas 3% der Frauen, die mit high risk HPV Typen infiziert sind, erkranken tatsächlich am Zervixkarzinom(7). Zu den weiteren Risikofaktoren zählen Nikotinabusus, immunsupressive Erkrankungen, häufig wechselnde Geschlechtspartner und niedriger sozioökonomischen Status(8, 9).

Die Therapie des Zervixkarzinoms hängt maßgeblich vom Stadium der Erkrankung ab. Für die Frühstadien ist die radikale Hysterektomie die Therapie der Wahl. Abhängig von Risikofaktoren und dem Alter und Wunsch der Patientin sind auch fertilitätserhaltende Operationen möglich. In den fortgeschrittenen Stadien ist eine Radiochemotherapie indiziert. Die Prognose ist ebenfalls stadienabhängig, mit 5-Jahres-Überlebensraten von über 95% für FIGO I Karzinome und 21% für FIGO IV Karzinome(10).

Im Falle eines Rezidives oder einer metastasierten Erkrankung sind die Therapieoptionen weiterhin eingeschränkt. In den letzten Jahren haben sich zusätzlich zur konventionellen Chemotherapie Antikörper gegen VEGF und immuntherapeutische Ansätze etabliert. Auch wenn diese neuen Therapiemodalitäten zu verbesserten Ansprechraten führen, sind weitere Forschungsansätze erforderlich um zielgerichtete individualisierte Therapiestrategien zu entwickeln.

Im Gegensatz zum Zervixkarzinom ist das Vulvakarzinom mit 3% aller gynäkologischen Malignome weltweit eine sehr seltene Tumorentität(11).

Das Plattenepithelkarzinom ist das histologisch häufigste Vulvakarzinom. Es wird in zwei ätiologische Subtypen unterteilt(12): (i) HPV-abhängige Karzinome die ungefähr 34% der investiven Karzinome betreffen(13) und (ii) HPV-unabhängige Karzinome die auf dem Boden des Lichen *sclerosus et atrophicus* entstehen, einer chronischen Autoimmundermatose(14). Über die letzten Jahre zeigt sich ein stetiger Anstieg der Inzidenzraten(15), sowohl bei den HPV-abhängigen als auch bei den HPV-unabhängigen Karzinomen. Dies ist auf das zunehmende Alter der Bevölkerung und vermehrte Exposition mit high risk HPV Typen zurückzuführen(16). Ein gezieltes Screening ist beim Vulvakarzinom nicht vorhanden.

Ist die Erkrankung lokalisiert, wird eine Tumorexzision der Vulva mit Entfernung des Wächterlymphknotens oder je nach Stadium der Leistenlymphknoten durchgeführt. Bei Risikofaktoren wird postoperativ eine Strahlentherapie ergänzt. Die 5-Jahres-Überlebensraten sind mit über 85% in den Frühstadien sehr gut, wobei HPV-abhängige karzinome eine bessere Prognose haben als HPV-unabhängige(17).

Wie auch beim Zervixkarzinom sind die Therapieoptionen bei lokal fortgeschrittenen, rezidivierenden oder metastasierten Erkrankungen sehr eingeschränkt. Hier liegen die 5-Jahres-Überlebensraten bei nur 10-15%(18).

Ebenfalls analog zum Zervixkarzinom nehmen Immuntherapien und Antikörpertherapien einen zunehmenden Stellenwert ein. Aufgrund der geringen Fallzahl sind randomisierte klinische Studien zum Vulvakarzinom eine Rarität. Ein besseres Verständnis der Tumorbiologie, insbesondere bezogen auf die zwei unterschiedlichen Ätiologien, ist dringend erforderlich, um in Zukunft neue zielgerichtete Therapien zu entwickeln.

Pathogenese der HPV-assoziierten gynäkologischen Malignome

Humane Papillomaviren sind die weltweit häufigste Ursache sexuell übertragbarer Erkrankungen. Global betrachtet erkranken jährlich 610.000 Frauen und Männer an bösartigen Erkrankungen, die durch HPV-Viren bedingt sind, was zu 265.653 Todesfällen pro Jahr führt(19). HPV Viren verursachen nicht nur gynäkologische Malignome der Frau wie das Zervix-, Vulva- oder Vaginalkarzinom, sondern sind auch an der Entwicklung von Analkarzinomen, Peniskarzinomen und Oropharynxkarzinomen beteiligt(20).

Humane Papillomaviren sind unbehüllte, doppelsträngige DNA Viren. Sie gehören zur Gruppe der *Papillomaviridae*. Sie infizieren die Zellen der Haut und die Keratinozyten der Schleimhaut. Insgesamt sind mehr als 150 HPV-Typen bekannt, wobei nur 40 die Haut/Schleimhaut Zellen nachweislich infizieren. Man unterteilt die HPV Viren in *„low risk“* und *„high risk“* Typen. Zu den *„low risk“* zählen vor allem HPV 6 und HPV 11, die für die Entstehung von anogenitalen Warzen ursächlich sind. Unter den *„high risk“* Typen sind vor allem HPV 16 und HPV 18 für die Entwicklung von bösartigen Erkrankungen verantwortlich(21).

Das Genom der HPV Viren ist eine ringförmige Doppelstrang-DNA mit 6800-8000 Basenpaaren und kodiert für 8 frühe Virusproteine E1, E2, E4, E5, E6 und E7 sowie die späten Strukturproteine L1 und L2, welche das Viruskapsid bilden (22). Das Virus tritt durch Mikroverletzungen der Haut/Schleimhaut in den Körper ein. Dabei ist die Transformationszone der Zervix, wo Platten- in Zylinderepithel übergeht, ein vor allem während dem Geschlechtsverkehr besonders vulnerabler Bereich(23). Das Immunsystem ist in der Lage, die meisten HPV Infektionen zum Abheilen zu bringen, wobei das Virus in den Basalzellen des Plattenepithels verbleibt und im Falle einer Schwäche des Immunsystems reaktiviert und in das Genom der Wirtszelle integriert wird. Dies wird als entscheidender Schritt in der Karzinogenes angesehen (24). Histologisch zeigen sich persistierende HPV-Infektionen durch die Ausbildung von cervikalen intrapithelialen Dysplasien (CIN), die sich mikroskopisch durch nukleare Atypien und den Verlust der normalen Schichtung des Epithels auszeichnen. Low grade Läsionen (LSIL oder CIN I) aber auch ein Teil der *high grade* Läsionen (HSIL oder CIN II/CIN III) heilen in der Regel ohne Therapie aus(25). Die beschriebene Integration der Virus DNA in das Genom der Wirtszelle führt über zwei Wege zur Tumorentstehung: Durch (i) Blockierung der Apoptose und (ii) Blockierung der Synthese von Regulatorproteinen, was zu einer unkontrollierten Zellteilung führt(26). Die zwei wesentlichen Gene des HPV Genom sind E6 und E7, die zu einer Inaktivierung von p53 und dem Retinoblastomprotein (pRb)

führen. Dabei sind nur high risk HPV Viren in der Lage, an p53 oder pRb zu binden(27). Das E6 des HPV führt zu strukturellen Veränderungen am p53 der Wirtszelle, diese neue Variante von p53 ist nicht in der Lage an geschädigte DNA zu binden(28). In weiterer Folge wird die Kaskade über das Protein p21 nicht aktiviert, welche normalerweise zum Zellzyklusarrest in der G1 Phase und somit zur Apoptose von mutierten Zellen führt. Das Retinoblastomprotein ist ein Tumorsupressor, der ebenfalls die Replikation von geschädigter DNA verhindert. Ist E7 an pRb gebunden, ist pRb nicht mehr funktionsfähig und die Zellproliferation läuft ohne Gegensteuerung(29). Diese beiden Hauptmechanismen über Inaktivierung von p53 und pRb der Wirtszelle durch E6/E7 des HPV führt schließlich zur Entstehung der intraepithelialen Neoplasien und in weiterer Folge zu Karzinomen, wenn die Vorstufen nicht erkannt und therapiert werden.

Früherkennung und Prävention

Eine Primärprävention der HPV-assoziierten Malignome ist durch eine Impfung gegen *„high risk“* HPV Genotypen möglich. Die Impfstoffe basieren auf „virus-like particles“ (VLP), welche inaktive L1 Proteine und keine aktive DNA enthalten, und somit keine akute Infektion jedoch eine Stimulation des Immunsystems zur Bildung spezifischer Antiköper verursachen(30). Die Impfung induziert Serumantikörper, deren Titer über hundertfach höher als nach natürlicher Infektion liegen und langfristig erhalten bleiben. Diese Antikörper verhindern durch Bindung an die Viruskapside eine Infektion der Epithelzellen.

Im Jahr 2006 erfolgte die erste FDA Zulassung des Vierfachimpfstoffes Gardasil-4 ®, welcher die Typen 6,11, 16 und 18 abdeckte(31). Drei Jahre später wurde ein weiterer bivalenter Impfstoff, Cervarix ®, gegen die HPV Typen 16 und 18 zugelassen(32). Gardasil-4 ® wurde 2015 durch die Zulassung von Gardasil-9 ®, einen nonavalenten Impfstoff abgelöst. In die Zulassungsstudie wurden 14.000 Frauen, Mädchen und Jungen aufgenommen. Der nonavalente Impfstoff enthält zusätzlich die HPV-Typen 31, 33, 45, 52 und 58. Bei den Endpunkten der impftypspezifischen intraepithelialen Neoplasien von Zervix, Vulva und Vagina bestätigte sich eine sehr hohe Wirksamkeit von über 95% in der HPV-naiven Gruppe(33).

Die STIKO empfiehlt eine Impfung für alle Mädchen und Jungen zwischen dem 9. und 14. Lebensjahr mit zwei Impfdosen. Wurde die Impfung verpasst, so kann eine Nachholimpfungen bis zum 17. Lebensjahr erfolgen, dann aber mit drei Impfdosen. Auch ältere Frauen, außerhalb des empfohlenen Altersrahmens der STIKO, können von der Impfung profitieren(34). Alle HPV Impfstoffe zeigten in den Studien ein hervorragendes Sicherheitsprofil, zu

den häufigsten Nebenwirkungen zählten lokale Reaktionen an der Einstichstelle, allergische Reaktionen waren sehr selten (1.7 Fälle auf 1 Mio. Impfdosen)(35).

Populationsbasierte Daten aus Ländern mit hoher HPV Impfrate zeigen eine deutliche Reduktion an anogenitalen Warzen und auch Präkanzerosen. In Australien konnte in der geimpften Population ein Rückgang von Genitalwarzen von über 90% verzeichnet werden, verglichen mit der nicht geimpften > 30-jährigen Population(36). Ebenso wurde ein Rückgang an HPV16/HPV18 high-grade intraepithelialen Neoplasien festgestellt(37). In England konnte ebenfalls ein deutlicher Rückgang der CIN III und der invasiven Karzinome nach Einführung eines Impfprogrammes verzeichnet werden(38).

Die Sekundärprävention basiert auf Untersuchungen zur Zervixkarzinomfrüherkennung. In Deutschland wurde im Jahre 1971 der jährliche zytologische Abstrich (PAP-Abstrich) ab dem 20. Lebensjahr eingeführt. Die Inzidenzraten des Zervixkarzinoms konnten in den ersten Jahrzehnten nach Einführung um 75% gesenkt werden, stagnierten aber in den letzten Jahren. Der zytologische Abstrich hat eine niedrige Sensitivität von 60-80% für das Erkennen einer high-grade Dysplasie oder eines Karzinoms, wobei die falsch-positive Rate bei 15-50% und die falsch-negative Rate bei 30% liegt(39). Basierend auf diesen Zahlen und durch Erkenntnisse aus Kohortenstudien/randomisiert-kontrollierten Studien zur Verwendung von Tests auf high-risk HPV Typen im Vergleich zur klassischen Zytologie wurde die Zervixkarzinomvorsorge in Deutschland weiterentwickelt(40-42). In einigen europäischen Ländern wurde bereits auf eine HPV-basierte Testung gewechselt, da der HPV Test hoch sensitiv ist und im Falle eines negativen Ergebnisses von einem sehr niedrigen Risiko für high-grade Dysplasien oder einem Zervixkarzinom auszugehen ist(43). Die Integration eines HPV Tests in das Zervixkarzinomscreening hat in Studien zu einer zusätzlichen Senkung der Inzidenzraten geführt, was vor alle auch an der besseren Erkennung von glandulären Vorstufen liegt, die durch die konventionelle Zytologie häufiger verpasst werden(44). Das reformierte organisierte Screening startete in Deutschland am 01.01.2020. Für Frauen zwischen dem 20. und 34. Lebensjahr ist es unverändert bei einer jährlichen zytologischen Untersuchung geblieben. Ab dem 35. Lebensjahr wird eine Ko-Testung durchgeführt, bestehend aus einem HPV Test und der zytologischen Diagnostik. Sind beide Ergebnisse unauffällig, erfolgt die nächste Untersuchung in drei Jahren. Bei Auffälligkeiten erfolgt die Überweisung in eine Dysplasiesprechstunde zur Kolposkopie(45).

Nach Einführung des neuen Screenings werden die Daten gesammelt und nach sechs Jahren evaluiert, ob eine Änderung der Screeningstrategie erforderlich ist.

Für das Vulvakarzinom gibt es kein etabliertes Screeningprogramm. Optische Veränderungen, die im Rahmen der Zervixkarzinomvorsorge bei der Inspektion auffallen, werden in einer Dypslasiesprechstunde weiter abgeklärt.

Ein Ziel dieser kumulativen Habilitationsschrift war es, das neue organisierte Screeningprogramm in Deutschland am Patientinnenkollektiv aus der Dysplasiesprechstunde zu evaluieren.

Die Rate an histologisch gesicherten Dysplasien in Abhängigkeit vom HPV Status und der Zytologie wurden ausgewertet, um den zusätzlichen Nutze der HPV Testung zu beurteilen.

Die Rolle des Mikrobioms

Das menschliche Mikrobiom, bestehend aus Bakterien, Pilzen und Viren ist in den letzten Jahren immer mehr in den wissenschaftlichen Fokus gerückt. Die Anzahl der Mikroorganismen im Körper übersteigt die der somatischen Zellen und die Interaktion mit dem Immunsystem scheint mit der Entstehung vieler Erkrankungen zusammenzuhängen, unter anderem auch Präkanzerosen und bösartiger Tumore(46, 47).

Mit der Entwicklung molekularer Diagnostik, vor allem der 16S rRNA Sequenzierung, konnte das vaginale Mikrobiom besser erforscht werden. Anhand der identifizierten bakteriellen Spezies kann das vaginale Mikrobiom in fünf Gruppen, sogenannte *„community state types“ (CST)* unterteilt werden(48). Hierbei sind sind CST I-III und CST V durch eine Dominanz von *Lactobacillus crispatus*, *L. gasseri*, *L. iners*, und *L. jensenii* gekennzeichnet, wohingegen CST IV eine niedrige Anzahl an Laktobazillen und eine Dominanz an fakultativen Anaerobiern zeigt. Mit der Produktion von Milchsäure, wodurch der vaginale pH auf unter 4.5 gesenkt wird, und weiterer antimikrobieller Substanzen erfüllen Laktobazillen eine wesentliche Funktion in der Immunabwehr gegen pathogene Keime(49). Hohe Östrogenspiegel und das Glykogen des vaginalen Epithels, welches östrogenabhängig akkumuliert, führen zu einer vaginalen Flora dominiert durch *L. crispatus, L. gasseri* und *L. jensenii.* Während der Menstruation, nach Geschlechtsverkehr und vor allem mit dem Eintritt in die Menopause kommt es zu einer Reduktion an Laktobazillen und Überbesiedlung der vaginalen Flora mit

Anaerobiern(50, 51). In den Wechseljahren wird dadurch das urogenitale Menopausensyndrom verursacht, welches sich klinisch mit Scheidentrockenheit, Dyspareunie und Dysurie äußert(52).

Eine Dysbiose der vaginalen Flora steigert das Risiko für Infektionen mit sexuell übertragbaren Erkrankungen, und Infektionsraten mit HIV oder HSV 2 sind ebenfalls bei Laktobazillenmangel erhöht(53). Es gibt ausreichend Daten, dass das vaginale Mikrobiom einen bedeutenden Einfluss auf viele gynäkologische und geburtshilfliche Erkrankungen hat, wie Frühgeburt, Fehlgeburt, Endomyometritis aber auch die Entwicklung von cervikalen intraepithelialen Neoplasien (54). Das Mikrobiom scheint auch eine wesentliche Rolle in der Karzinogenese vieler Tumorerkrankungen zu spielen. CST IV ist assoziiert mit der Entstehung von dysplastischen Veränderungen und geringerer Eliminierung von HPV Infektionen. Vergleicht man low grade und high grade Dysplasien, so zeigt sich eine zunehmende Besiedlung mit *Sneathia sanguinegens, Anaerococcus tetradius* und *Peptostreptococcus anaerobius*(55, 56).

In dieser kumulativen Habilitationsschrift wurde das vaginale Mikrobiom von Frauen, die sich in der Dysplasiesprechstunde vorstellten, analysiert und mit HPV Status, Zytologie und dem histologischem Nachweis von Dysplasien korreliert.

Biomarker - N6-Methyladenosin Ribonukleinsäure-Modifikation

Biomarker haben im letzten Jahrzehnt einen zentralen Stellenwert in der Onkologie eingenommen. Ihre prognostische und prädikative Wertigkeit ist Gegenstand aktueller Forschungen. In der Gynäkologie galt die Entdeckung des HER2 Rezeptors („human epidermal growth factor receptor 2“) als *practice changing* und hat die Therapie des Mammakarzinoms revolutioniert. Aktuelle und zukünftige Studien in der gynäkologischen Onkologie fokussieren sich auf biomarkerbasierte Therapien(57). Biomarker sollen in der klinischen Routine fest integriert werden, um das Ansprechen und Resistenzentwicklung gegenüber gewählten Therapien vorhersagen zu können, was ein bedeutender Schritt in Richtung personalisierter Medizin ist(58). Trotz zahlreicher Forschungsansätze haben sich in der Behandlung des Vulva- und Zervixkarzinomes individualisierte Therapiestrategien nicht etablieren können und es bedarf weiterer Studien, um die Zusammenhänge zwischen Immunologie und Onkologie besser verstehen zu können und zielgerichtete Therapien auf den Markt bringen zu können.

Epigenetische Veränderungen spielen eine zentrale Rolle in der Biomarker-Forschung. Unter dem Begriff der Epigenetik werden Prozesse zusammengefasst, die die Expression von Genen regulieren, wie DNA/RNA Methylierung(59), Histon Modifikation und Neuordnung von Chromatin. Diese Prozesse sind potentiell reversibel, nicht vererbbar und abzugrenzen von DNA/RNA Sequenzveränderungen. Hierüber wird gesteuert, welche Gene in einem bestimmten Zelltyp für die Transkription zur Verfügung stehen(60). Dabei galt RNA lange Zeit als unbedeutendes Zwischenprodukt zwischen DNA und dem Protein, was in zahlreichen Studien widerlegt werden konnte, und heutzutage ist klar, dass RNA einen bedeutende Rolle in der post-transkriptionelen Genregulation spielt(61). Unter den mehr als 100 bekannten RNA Modifikationen ist N6-Methyladenosin (m6A), welches durch Methylierung von Adenosin an Position 6 entsteht, die am häufigsten vorkommende RNA-Modifikation(62). In jüngster Zeit hat die Identifizierung von Methyltransferase- , Demethylase- und Bindungsproteinen, die m6A einbauen, entfernen oder erkennen, bisher unbekannte Rollen von m6A in fast allen Aspekten des RNA-Stoffwechsels sowie in verschiedenen physiologischen und pathologischen Prozessen offenbart(63, 64). Jüngste Studien haben gezeigt, dass die m6A-Modifikation die Aktivierung von Immunzellen und die Infiltration in die Mikroumgebung des Tumors moduliert und somit die Wirksamkeit der Immuntherapie beeinflussen kann(65). Daher ist die m6A-Modifikation ein potenzielles Ziel für die Krebsimmuntherapie. Der Prozess der m6A-Modifikation wird dynamisch und reversibel durch drei Arten von Enzymen reguliert: m6A-Methyltransferasen (*"writers"*), m6A-Demethylasen (*"erasers"*) und m6A-Bindeproteine (*„readers“*)(66).

Die *"writer"* von m6A bestehen hauptsächlich aus METTL3, METTL14 und ihrem Kofaktor WTAP. METTL3 und METTL14 weisen ein S-Adenosylmethionin-Bindungsmotiv auf. Diese beiden Gene befinden sich gemeinsam im Kernbereich und bilden einen stabilen Heterodimerkomplex. Als Pseudo-Methyltransferase spielt METTL14 eine wichtige Rolle bei der Stabilisierung von METTL3 und der Erkennung von Ziel-RNAs(67). Als wichtigstes Regulierungs- und Komponentenmolekül des m6A-Methylierungskomplexes kann WTAP dazu beitragen, dass sich METTL3 und METTL14 in den nukleären Plaques ansiedeln(68). Zu den *"writer"* gehört außerdem KIAA1429 und dient der m6A-Methylierung in der Nähe des Stoppcodons. METTL3 spielt eine Rolle als Onkogen und Tumorsuppressor-Gen bei verschiedenen Krebsarten, darunter Leberzell-, Magen-, Kolorektal-, und Blasenkarzinom(69, 70). Eine abnorme Expression von METTL3 in Tumorzellen beeinträchtigt die Infiltration von Immunzellen. Im Zervixkarzinom wurde METTL3 in Tumorgeweben wesentlich stärker exprimiert als in den tumorumgebenden Zellen, und die Konzentration stand in positivem Zu-

sammenhang mit der Dichte von CD33+ myeloiden Suppressorzellen, die wiederum mit einer schlechten Überlebensrate der Patienten in Verbindung gebracht wurde(71). Die Expressionswerte von METTL3 und METTL14 im Mammakarzinom korrelierten negativ mit der Überlebensrate und der Anzahl an CD8+ T-Zellen, T-Helferzellen und aktivierten NK-Zellen(72, 73).

Die Demethylierung erfolgt hauptsächlich durch die *„eraser“* FTO und ALKBH5. Als reversibler Schritt der m6A-Methylierung kann die Demethylase FTO die Fettproduktion und Energiehomöostase regulieren. Die hochregulierte Expression von FTO ist mit Fortschreiten des Tumors bei akuter melodischer Leukämie und dem Glioblastom beschrieben(74, 75). In therapeutischen Ansätzen konnte ein niedermolekularer FTO-Inhibitor die FTO-vermittelte Immunmodulation beeinflussen und synergistisch mit der PD-1/PD-L1-Checkpoint-Blockade wirken(76). Bei Melanompatienten konnte die Deletion von ALKBH5 die Wirksamkeit der Anti-PD-1-Therapie erhöhen(77).

Neben den *„writer“* und *„eraser“*, gibt es eine weitere unverzichtbare Gruppe in der m6A-Modifikation, die *„reader“*. Sie können Veränderungen erkennen, sich mit ihnen verbinden und erfüllen unterschiedlichste biologische Prozesse. YTHDF1 und YTHDF2 sind die am meisten untersuchten *„reader“*. Unter normalen oder Stressbedingungen fördert sie den Abbau von m6A-abhängiger RNA. Eine Überexpression von YTHDF1 ist mit dem Fortschreiten einiger Krebserkrankungen, wie dem nicht-kleinzelligen Bronchialkarzinom oder dem Ovarialkarzinom assoziiert(78, 79). Dabei scheinen *„reader“* mit der Expression von Immuncheckpoint-Rezeptoren zu korrelieren.

Zusammenfassend zeigten die oben genannten Studien, dass m6A abnormal im Tumor exprimiert wird und eine immunsuppressive Mikroumgebung schafft. Die Modifikation von m6A scheint ein möglicher Ansatz in der Immuntherapie von verschiedensten Krebsarten zu sein. Ein Ziel dieser kumulativen Habilitationsschrift war es, die Expression der m6A „reader“, „eraser“, und „reader“ auf ihre prognostische Aussagekraft im Zervix- und Vulvakarzinom zu untersuchen.

Zielgerichtete Therapiestrategien bei gynäkologischen Tumoren

Antikörper-Wirkstoff-Konjugate (engl. antibody-drug-conjugates ADC) werden bei vielen Tumorentitäten bereits erfolgreich in der Therapie eingesetzt. Der Wirkmechanismus der ADCs basiert auf der Wirksamkeit eines Zytostatikums kombiniert mit der Selektivität eines Antikörpers. Alle ADCs bestehen aus insgesamt drei Komponenten: einem Antikörper, der

gegen ein Zelloberflächenprotein gerichtet ist, einem zytotoxischen Payload sowie einem Linker, der das Zytostatikum an den Antikörper bindet. Ein großer Vorteil ist, dass der zytotoxische Wirkstoff direkt an den Zielort gebracht wird. Nach Bindung das ADCs an die Zelloberfläche der Zielzelle wird über Endozytose der gesamte ADC-Komplex in das Zellinnere gebracht. Nach Verschmelzung mit Lysosomen wird der Komplex abgebaut und der zytotoxische Wirkstoff wird freigesetzt. Der programmierte Zelltod wird abhängig vom Payload durch Inhibition der Mikrotubulisynthese oder durch direkte DNA-Schäden ausgelöst(80). Unter den gynäkologischen Tumorentitäten wurden die größten Therapieerfolge beim Mammakarzinom verzeichnet. Hier sind ADCs, basierend auf Anti-Her-2-Antikörpern, seit Jahren in erfolgreicher Anwendung(81). Für die Subgruppe der metastasierten triple-negativen Mammakarzinome wurde kürzlich ein neues ADC zugelassen, Sacituzumab Govitecan, welches gegen das transmembrane Glycoprotein TROP-2 ("trophoblast cell-surface antigen 2“) berichtet ist(82). TROP-2 wurde initial als Onkogen eingestuft, da es regulatorische Prozesse in der Karzinogenese steuert. Jedoch zeigten neuere Ergebnisse, das TROP-2, abhängig von Zellart und Lokalisation, sowohl Tumorprogression als auch Tumorsuppression verursachen kann(83). Die genauen Zusammenhänge müssen auf molekularer Ebene weiter untersucht werden. Fest steht jedoch, dass TROP-2 aufgrund der vorhanden hohen Expressionslevel im Tumorgewebe ein potentielles therapeutisches Target für viele Tumorentitäten darstellt. In der Behandlung des Zervix-und Vulvakarzinoms besteht dringender Bedarf nach innovativen, zielgerichteten Therapiestrategien, da für fortgeschrittene, rezidivierende oder metastasierte Erkrankungen die Optionen sehr limitiert sind(84).

Im Zervixkarzinom konnte gezeigt werden, dass TROP-2 tumorsupressive Eigenschaften zeigt und ein gradueller Verlust eine Rolle in der Progression von intraepithelialen Neoplasien zum invasivem Tumor spielt(85). Ebenso gibt es bereits erste Ergebnisse, dass eine gegen TROP-2 gerichtete Therapie Erfolge in der Behandlung des Zervixkarzinoms zeigt(86). Zum Vulvakarzinom, einem ätiologisch ähnlichen Tumor zum Zervixkarzinom, gibt es im Hinblick auf TROP-2 bisher keine Daten.

Ein weiteres Ziel dieser kumulativen Habilitationsschrift war es, die TROP-2 Expression im Vulvakarzinom zu analysieren. Ein besonderer Schwerpunkt lag hier auf den Expressionsunterschieden von HPV-abhängigen/HPV-unabhängigen Tumoren und das Überleben der Patientinnen. Die übergeordnete Zielsetzung der kumulativen Habilitationsarbeit war die

Analyse von Biomarkern, neuen potentiellen Therapiestrategien und der Einfluss des Mikrobioms auf die Entstehung und das Fortschreiten des Vulva- und Zervixkarzinoms und ihrer Vorstufen.

4. Ergebnisse

4.1 Condic M*, Neidhöfer C*, Ralser DJ, Wetzig N, Thiele R, Sieber M, Otten LA, Warwas LK, Hierauf A, Mustea A, Parčina M. Analysis of the cervical microbiome in women from the German national cervical cancer screening program. J Cancer Res Clin Oncol. 2023 Feb 13. doi: 10.1007/s00432-023-04599-0.

Zielsetzung - In dieser Studie wurde das neue Zervixkarzinom-Vorsorgeprogramm im Hinblick auf die Entdeckungsrate höhergradiger Dysplasien untersucht. Gleichzeitig wurde die Rolle des vaginalen Mikrobioms analysiert.

Methodik und Ergebnisse - Die Kohorte umfasste *N*=310 Patientinnen, die gemäß der neuen Vorsorgeleitlinie aufgrund auffälliger PAP und/oder HPV Abstriche zur weiteren Abklärung in die Dysplasiesprechstunde überwiesen wurden. Es erfolgte eine Biopsieentnahme zur Beurteilung ob eine Dysplasie vorliegt durchgeführt. Zudem wurde das Mikrobiom der Patientinnen sequenziert um eine Korrelation mit klinischen und pathologischen Parametern zu ermitteln. 52.3% der Patientinnen erhielten eine Kolposkopie aufgrund eines positiven HPV Abstriches in zwei aufeinanderfolgenden Jahren bei unauffälliger Zytologie. In diesem Kollektiv zeigte sich bei nur 2.1% eine HSIL/CIN III. Die wiederholte HPV Diagnostik zeigte eine Diskrepanz in 40 Fällen des Gesamtkollektivs, was auf unterschiedliche HPV Assays oder den zeitlichen Abstand der Analyse zurückzuführen ist. Den größten Einfluss auf das Mikrobiom hatte der Menopausenstatus. Es zeigte sich eine negative Korrelation mit *Lactobacillus crispatus* (*p*=<.001) sowie eine positive Korrelation mit *Pseudomonas* (*p*=.001), *Prevotella* (*p*= .011), *Cutibacterium* (*p*= .015), *Atobium* (*p*=.027), *Staphylococcus (p*=.014), *Dialister* (*p*=.008), *Acinetobacter* (*p*=.017), *Oscillospirales* (*p*=.001) und *Fusobacterium* (*p*=.008). Eine Kolonisierung mit *Ureaplasma parvum* (*p*=0.15) war mit dem histologischen Nachweis von dysplastischen Vorstufen der Zervix assoziiert.

Zusammenfassung - Das neue Screeningprogramm führt zu einer gesteigerten Anzahl an durchgeführten Kolposkopien, aber die Entdeckungsrate an höhergradigen Vorstufen ist niedrig. Dies betrifft vor allem Patientinnen mit HPV Persistenz aber unauffälliger Zytologie. Die HPV Diagnostik scheint sehr variabel vom Assay und dem Zeitpunkt der Testung abzuhängen. Nur die Besiedlung mit *Ureaplasma parvum* war mit dysplastischen Läsionen assoziiert, was aktuell zahlreich auf dem Markt vorhandene Medikamente zur Optimierung des Mikrobioms um zervikale Vorstufen zu verhindern, kritisch hinterfragt.

Journal of Cancer Research and Clinical Oncology
https://doi.org/10.1007/s00432-023-04599-0

RESEARCH

Analysis of the cervical microbiome in women from the German national cervical cancer screening program

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Received: 28 December 2022 / Accepted: 25 January 2023

Abstract

Purpose Cervical cancer (CC) is caused by a persistent high-risk human papillomavirus (hrHPV) infection. The cervico-vaginal microbiome may influence the development of (pre)cancer lesions. Aim of the study was (i) to evaluate the new CC screening program in Germany for the detection of high-grade CC precursor lesions, and (ii) to elucidate the role of the cervico-vaginal microbiome and its potential impact on cervical dysplasia.

Methods The microbiome of 310 patients referred to colposcopy was determined by amplicon sequencing and correlated with clinicopathological parameters.

Results Most patients were referred for colposcopy due to a positive hrHPV result in two consecutive years combined with a normal PAP smear. In 2.1% of these cases, a CIN III lesion was detected. There was a significant positive association between the PAP stage and *Lactobacillus vaginalis* colonization and between the severity of CC precursor lesions and *Ureaplasma parvum*.

Conclusion In our cohort, the new cervical cancer screening program resulted in a low rate of additional CIN III detected. It is questionable whether these cases were only identified earlier with additional HPV testing before the appearance of cytological abnormalities, or the new screening program will truly increase the detection rate of CIN III in the long run. Colonization with *U. parvum* was associated with histological dysplastic lesions. Whether targeted therapy of this pathogen or optimization of the microbiome prevents dysplasia remains speculative.

Keywords Cervicovaginal microbiome · Cervical cancer screening · HPV diagnostic · Colposcopy

Introduction

In recent years, the human microbiome has increasingly become the focus of scientific interest. The colonization of our body with microbiota is at least as diverse and complex as our somatic cell physiology (Sender et al. 2016). It is estimated that about 500–1000 different microorganisms simultaneously colonize our body (Turnbaugh et al. 2007). Alterations in the human microbiome, as well as interactions with the immune, endocrine, and nervous systems, have been linked to a variety of health changes and diseases, including cancer and their precursor lesions (Kostic et al. 2013; Helmink et al. 2019). The precise manner in which the microbiome influences the maintenance of health or the development of disease, however, is still far from being answered.

CC is predominantly caused by infection with human papillomavirus (HPV), in ≥ 99% with the high-risk (hr) HPV types 16 and 18 (Walboomers et al. 1999). About 90% of women are exposed to HPV infections during the course of their lives, and in only 10% of the cases the infection persists with a high risk of developing precancerous cervical intraepithelial lesions and CC (Shulzhenko et al. 2014). HPV persistence is co-induced by impaired immune reactions, and adverse accompanying effects exerted by the

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Published online: 13 February 2023

cervico-vaginal microbiome (Garrett 2015). There is growing scientific evidence for a relationship between a cervico-vaginal microbiome dominated by species other than lactobacilli, and a higher risk of HPV infection, HPV persistence and the development of CC and its precursor lesions (Mitra et al. 2015; Laniewski et al. 2020; Lin et al. 2020; Norenhag et al. 2020).

The development and improvement of molecular methods, in particular represented by bacterial 16S ribosomal RNA gene sequencing, has led to a deeper understanding of the cervico-vaginal microbiome (van de Wijgert et al. 2014). According to the presence of distinct bacterial species that are identified by 16S RNA sequencing, the cervico-vaginal microbiome is sometimes classified into five groups, designated as community state types (CST). In detail, CST I–III and CST V are characterized by an abundance of *Lactobacillus crispatus*, *L. gasseri*, *L. iners*, and *L. jensenii*, respectively, whereas, in contrast, CST IV shows a combination of diverse facultative anaerobes with low abundances of lactobacilli (Ravel et al. 2011). In reproductive-aged women, shifts from the *Lactobacillus*-dominated microenvironment are commonly observed during menses and sexual activity caused by a reduction of lactobacilli (Gajer et al. 2012). With increasing age and the decrease of estrogen and glycogen levels, *Lactobacillus* species are replaced by diverse anaerobes (Gliniewicz et al. 2019). This transformation of the cervico-vaginal site flora is associated with the genitourinary syndrome of menopause (Hummelen et al. 2011).

Dysbiosis of the lower female reproductive tract increases the risk for infections with STD (Martin et al. 1999). Further, the absence of Lactobacilli is associated with the increase risk of HIV and HSV transmission (Cherpes et al. 2003). Recent studies confirm that changes of the human microbiome can impair the symbiotic relationship between microorganisms and host, leading to the development of different cancer types and suggesting a role for microbiota in genesis of various malignancies (Bhatt et al. 2017; Lin et al. 2020; Norenhag et al. 2020).

For early detection of CC, an annual cytological examination program (PAP smear) has been introduced in Germany in 1971. Since then, incidence rates of CC dropped remarkably by 75% in the first decades but, however, incidence rates have stagnated in recent years. The PAP smear has a low sensitivity (60–80%), a false negative rate of 30% and false-positive rates ranging from 15 to 50% (Yim and Park 2007). Hence, in some European countries, a switch to primary HPV-DNA testing was established recently. HPV testing is a highly sensitive approach and the absence of hrHPV infection indicates a low risk for CC precursor lesions and CC development (Dillner et al. 2008). As part of the German National Cancer Plan, the Federal Joint Committee (G-BA) implemented an updated organized cervical cancer screening program starting in January 2020. Annual cytology screening remained unchanged for women between 20 and 34 years. For women of 35 years and older, a co-testing, comprising a Pap smear and an HPV test was introduced. In case of positive findings, women are referred for colposcopy (Bujan Rivera and Klug 2018). The aim of the present study was to evaluate the new screening program for the detection of high-grade precursor lesions and to investigate whether microbiome analyses could have a potential role in this screening.

Methods

Study design and population

The study cohort included women who were referred for colposcopy to the certified Colposcopy Centre at the Department of Gynecology and Gynecological Oncology of the University Hospital Bonn from November 2021 until February 2022. Colposcopy was indicated according to the guidelines of the new national cancer screening program (abnormal PAP smear finding and/or a positive result for hrHPV).

Routine colposcopy was performed including the application of acetic acid. In cases of TZ type 1 or TZ type 2 (Quaas et al. 2013), a targeted biopsy was performed from the most conspicuous lesion. In case of a TZ type 3 with no visible lesion on the ecto-cervix, an endo-cervical curettage was performed.

Clinical data regarding nicotine abuse, menopause status, HPV vaccination, the application of local suppositories, the intake of hormonal contraceptives or hormone replacement therapy, the presence of an intrauterine device (IUD-copper or hormonal) and the last sexual intercourse were obtained from patient questionnaires and the clinical database.

Histopathological analysis and HPV diagnostics

The taken biopsies were histopathologically classified into benign, low squamous intraepithelial lesions (LSIL/CIN I), and high squamous intraepithelial lesions (HSIL) according to the 2014 WHO classification. HSIL lesions were further sub-classified into CIN II and CIN III lesions according to Richart (Richart 1973).

In women above 35 years, HPV status was available as a part of the new cancer screening program. Due to the use of different HPV molecular detection assays and, therefore, inconsistent data for specific HPV types, analyses with respect to HPV were limited to low risk (lr) and hrHPV. In the presence of hrHPV, it was differentiated whether hrHPV types 16 and/or 18 were present.

HPV diagnostics were repeated from all samples with the Anyplex II HPV28 Detection (Seoul, South Korea) that detects 19 hrHPV: 16, 18, 26, 31, 33, 35, 39, 45,51, 52, 53,

56, 58, 59, 66, 68, 69, 73, 82; and nine low carcinogenic risk HPV types: 6, 11, 40, 42, 43, 44, 54, 61, 70. It was performed strictly following the manufacturer's instructions. DNA was extracted on a Seegene NIMBUS (Seoul, South Korea) and analyzed on a CFX96 real-time PCR instrument (Bio-Rad Laboratories, Inc., Hercules, California, USA). Data recording and analysis were automated using the Seegene Viewer software.

Women, in which no biopsy was taken for histological analysis and women with pathologies of the vulva were excluded from the study.

Sample collection and preparation for sequencing

During the colposcopic examination, before the application of acetic acid, a flocked swab (eNAT® system, Copan Italia, Brescia, Italy) was taken by three experienced gynecologists from the cervical canal. The swabs were stored at 4 °C and subsequently processed within 2–9 days.

Highly purified DNA was extracted from all samples using the column-based ZymoBIOMICS DNA Miniprep Kit (Zymo Research Europe GmbH, Freiburg, Germany). The isolation was performed strictly according to the manufacturer's instructions. The crucial mechanical lysis step of the samples was performed by Precellys® Evolution homogenizer from Bertin Technologies SAS (Bretonneux, France). At the end of the extraction process, the DNA was eluted to 100 uL volume and qualitatively and quantitatively evaluated using the NanoDrop OneC, Thermo Fisher Scientific Inc. (Waltham, MA, USA).

16S rRNA gene sequencing libraries were constructed from each sample using the Quick-16S NGS Library Prep Kit (Zymo Research Europe GmbH, Freiburg, Germany) with its included V1–V2 primer pairs. Each run included 94 samples, the positive control included in the kit, and a negative control. For quantitative PCR, quality control, and normalization purposes, the Bio-Rad CFX96 Real-Time PCR Detection System (Bio-Rad Laboratories, Inc., Hercules, California, USA) was utilized.

After pooling, the DNA was quantified with the QuantiFluor® dsDNA System on the Quantus™ Fluorometer (both: Promega GmbH, Walldorf, Germany) and diluted strictly according to the Illumina-protocol for MiSeq sample preparation. For the final library, a loading concentration of 10 pm was chosen and a 10% Illumina v3 PhiX spike-in control was added before running it on the Illumina MiSeq platform with a 500cycle v2 Illumina MiSeq Reagent Kit (all three: Illumina, San Diego, CA, USA).

Bioinformatic analysis

The bioinformatic analysis included three main parts, starting with the preprocessing of raw paired end reads. Following the preprocessing, the sequences were assigned to taxonomies. Finally, a statistical and graphical evaluation was performed on the resulting taxa.

QIIME2 (Bolyen et al. 2019) was used for both preprocessing and classification of the data. With the plugin tool DADA2 (Callahan et al. 2016), forward and reverse reads were trimmed from the 3' end at position 249, while shorter reads as well as low-quality reads got discarded. DADA2 was also used to perform error correction, merging of forward and reverse reads if there was an overlap of at least 12 base pairs, and chimera removal.

The processed sequences were clustered into OTUs (operational taxonomic units) of 100% sequence identity and assigned to taxa, using a classifier trained on full-length sequences of SILVA (Quast et al. 2013). The trained classifier was provided by QIIME2 using scikit-learn 0.24.1 and the plugin tool q2-feature-classifier (Bokulich et al. 2018; Robeson et al. 2021).

Statistical analysis

Statistical analysis was performed using Stata version 14 for the clinical data and Datatab version 1.12.1 for taxa frequency comparisons and correlation with clinical parameters. P values less than 0.05 were considered statistically significant.

Ethics statement

The study was approved by the Ethics Committee of the Medical Faculty of the University of Bonn (vote: 128/21). All methods were carried out in accordance with relevant guidelines and regulations. Informed consent was obtained from all subjects.

Results

Participant characteristics and clinical results

The study cohort included 310 women. All relevant clinicopathological parameters are summarized in Table 1. The mean age of the study cohort was 44.6 years (± standard deviation (SD) 12.4 years). 72.9% of the women were premenopausal, and 27.1% were postmenopausal. 30.3% of the patients were active smokers. Within the whole cohort, 12.3% of the patients had been vaccinated against HPV. Among the subgroup of women ≤ 30 years, 62.2% had been vaccinated against HPV. In the subgroup of premenopausal women, 29.2% used hormonal contraceptives, and 13.3% had an IUD. Of these, 26.7% had a copper, 63.3% a Mirena ® IUD, 6.7% a Jaydess ®, and 3.3% a Kyleena ® IUD. In the subgroup of postmenopausal women, 8.3% received

Table 1 Clinicopathological characteristics of the entire cohort

Clinicopathological parameter	
Age (years)	
Mean (± SD)	44.6 ± 12.4
Min–max	20–82
Menopausal status	
Pre	226 (72.9%)
Post	84 (27.1%)
Smoker	
No	216 (69.7%)
Yes	94 (30.3%)
HPV vaccination	
No	272 (87.7%)
Yes	38 (12.3%)
HPV vaccination < 30 years	
No	14 (37.8%)
Yes	23 (62.2%)
Hormonal contraceptives (premenopausal)	
No	160 (70.8%)
Yes	66 (29.2%)
Intrauterine device (IUD) (premenopausal)	
No	196 (86.7%)
Yes	30 (13.3%)
IUD	
Cooper	8 (26.7%)
Hormonal	22 (73.3%)
Hormonal replacement therapy (postmenopausal)	
No	77 (91.7%)
Yes	7 (8.3%)
Vaginal suppositories (postmenopausal)	
No	66 (78.6%)
Yes	18 (21.4%)
HPV high-risk status	
Negative	31 (10.0%)
Positive	262 (84.5%)
Unknown	17 (5.5%)
HPV 16/18	
Negative	183 (69.8%)
Positive	79 (30.2%)
Clinicopathological parameter	
Pap smear cytology	
I/IIa	162 (52.3%)
IIp	40 (12.9%)
IIg	6 (1.9%)
IIIp	11 (3.5%)
IIIg	4 (1.3%)
IIID1	46 (14.8%)
IIID2	22 (7.1%)
IVa-p	18 (5.8%)
IVa-g	1 (0.3%)
Histological diagnosis	
No CIN	201 (64.8%)
LSIL CIN I	51 (16.5%)
HSIL CIN II	36 (116%)
HSIL CIN III	22 (7.1%)
Histological diagnosis, Pap I/IIa, HPV high-risk pos	
No CIN	112 (77.8%)
LSIL CIN I	20 (139%)
HSIL CIN II	9 (6.3%)
HSIL CIN III	3 (2.1%)
Surgical therapy	
No	263 (84.8%)
Yes	47 (15.2%)
Type of surgical therapy	
LEEP conization	38 (80.9%)
Hysteroscopy/curettage	3 (6.4%)
Hysterectomy	2 (4.3%)
Laser vaporization	4 (8.5%)
Therapy of CIN III	
LEEP conization	19 (86.4%)
Hysterectomy	1 (4.5%)
No surgical therapy	2 (9.1%)
Therapy of CIN II	
LEEP conization	19 (52.8%)
Hysterectomy	1 (2.8%)
No surgical therapy	16 (44.4%)

hormone replacement therapy. The proportion of postmenopausal women who used vaginal suppositories (estriol) was 21.4%. Our analysis included five pregnant women.

84.5% of the whole study cohort were positive for a hrHPV type. The most prevalent subtypes were HPV types 16 und 18 in 30.2% of the cases. In 5.5% of the study cohort, HPV status was not available and 10.0% were negative for hrHPV types.

Most interestingly, 52.3% of the study cohort had a regular Pap smear (I or IIa, according to the Munich III classification). They were referred to a colposcopy due to a positive status for hrHPV in two consecutive years. This approach corresponds to the new guidelines. Among the subgroup with a positive hrHPV status and normal cytology, 8.4% of the women had an HSIL. A CIN III was detected in only 2.1% (3/144).

In the entire study cohort, histological examination revealed in 16.5% of the cases a CIN I, in 11.6% a CIN II, and in 7.1% a CIN III. 15.2% of all patients received surgical therapy due to precursor lesions of the cervix, with 80.9% receiving a LEEP conization, 6.4% a hysteroscopy with curettage, 4.3% a hysterectomy, and 8.5% laser vaporization. Among the 22 patients diagnosed with a CIN III lesion, 19 received conization and one a hysterectomy. Two patients

with CIN III did not receive surgical treatment: one woman was pregnant at the time of diagnosis, and one was 20 years old. In this case, a close surveillance every three months was scheduled, which is in line with guidelines. Among the CIN II subgroup, 52.8% received a LEEP conization and one woman a hysterectomy. In 44.4% of the cases, a follow-up was scheduled in 6 months according to the guidelines. In cases with LSIL/CIN I, no surgical therapy was performed, and a follow-up colposcopy in 6 months was scheduled.

HPV Diagnostic

HPV diagnostic was repeated in all patients. In 252 cases, HPV status determined within the CC screening program was in concordance with our analysis. In 18 cases, the comparison was not possible as these patients did not receive prior HPV testing or our analysis was invalid. In 40 cases, HPV HR diagnostic showed a discrepancy in the results: In 20 cases, that were initially tested negative for HPV HR within the CC screening program, a positive hrHPV status was determined in our analysis. Among these cases, 11 were positive for HPV 16. The medical history showed that 9 patients had cervical dysplasia before. In the actual biopsy, none of the patients had an HSIL. In 20 cases that were initially hrHPV-positive, no hrHPV infection was detected in our analysis. Five of these patients were initially positive for HPV 16/18. As observed for the counterpart subgroup, none of the patients had an HSIL.

In 17 cases, HPV diagnostic was not performed previously, as patients were younger than 35 years. These patients were referred for colposcopy due to an abnormal PAP smear. We found a positive HPV HR status in 16 patients; histology showed in 5 cases an LSIL and in 7 cases an HSIL.

Cervical microbiome profiles

The 310 sequenced cervical samples generated a total of 31,881,480 reads with a mean read count of 102,843 per sample. Of these samples, 293 passed the minimum quality filter (> 1500 reads and > 1000 merged reads).

Cervical microbiota profiles were classified into 5 groups based upon the dominant (or at least > 30% relative abundance) taxa observed within each sample at the genus level (see Fig. 1). Accordingly, 194 microbiomes were predominated by *Lactobacillus* (66.21%), 52 by *Gardnerella* (17.75%), 9 by *Bifidobacterium*, 6 by *Streptococcus*, 2 by *Pseudomonas*, 1 by *Prevotella* and in 29 cases (9.9%) none of these genera predominated.

Nine bacterial species had an average prevalence of > 0,5%, namely *Lactobacillus crispatus* (mean prevalence 33,21%), *Lactobacillus iners* (23,12%), *Gardnerella vaginalis* (5,98%), *Lactobacillus jensenii* (2,84%), *Bifidobacterium breve* (1,47%), *Streptococcus agalactiae* (1,13%), *Bifidobacterium longum* (1,01%), *Streptococcus anginosus* (0.72%), and *Prevotella bivia* (0.58%) (Fig. 1B).

The relative abundance of *L. crispatus* was negatively correlated with *L. iners* ($r = -0.41$, $p < 0.001$), *G. vaginalis* ($r = -0.36$, $p < 0.001$), *L. jensenii* ($r = -0.12$, $p = 0.043$), and *P. bivia* ($r = -0.13$, $p = 0.028$). The relative abundance of *L. iners* was negatively correlated with *L. crispatus and G. vaginalis* ($r = -0.21$, $p < 0.001$). No other significant correlations between these species were observed.

Correlations between clinical and demographic variables and the microbiome

The patient's age was correlated negatively with *L. crispatus* ($r = -0.3$, $p < 0.001$) and positively with *G. vaginalis* ($r = 0.15$, $p = 0.011$) and *B. longum* ($r = 0.14$, $p < 0.016$). However, performing multiple linear regression analysis to examine the influence of the menopausal state, revealed that only the *B. longum* was associated with age ($p < = 0.027$). As depicted in Fig. 2, being postmenopausal correlated significantly negatively with *L. crispatus* (rpb = − 0.32, $n = 293$, $p = < 0.001$) and positively with the genus *Pseudomonas* (rpb = 0.2, $p = 0.001$), *Prevotella* (rpb = 0.15, $p = 0.011$), *Cutibacterium* (rpb = 0.14, $p = 0.015$), *Atobium* (rpb = 0.13, $p = 0.027$), *Staphylococcus* (rpb = 0.14, $p = 0.014$), *Dialister* (rpb = 0.16, $p = 0.008$), *Acinetobacter* (rpb = 0.14, $p = 0.017$), *Oscillospirales* (rpb = 0.19, $p = 0.001$), and *Fusobacterium* (rpb = 0.15, $p = 0.008$) ($n = 293$ for all). The cervical microbiomes of postmenopausal patients displayed higher richness ($t(124.46) = -2.71$, $p = 0.008$, 95% confidence interval [− 40.29, − 6.23]) and higher fisher-alpha diversity ($t(116.25) = -3.13$, $p = 0.002$, 95% confidence interval [− 8.11, − 1.82]).

Premenopausal women with an IUD displayed lower richness in their cervical microbiome ($t(45.87) = 2.27$, $p = 0.028$, 95% confidence interval [2.19, 36.46]) than those without. However, one-factor analysis of variance showed that there was no significant difference between not having an IUD, having a hormonal IUD, or a copper IUD and the variable richness $F = 1.84$, $p = 0.161$ (Fig. A1 in the Appendix displays differences among IUDs that were not statistically significant). Among premenopausal women, intake of oral contraceptives was linked to a higher prevalence of *L. crispatus* ($t(209) = -3.42$, $p = 0.001$, 95% confidence interval [− 35.48, − 9.43]) and a lower prevalence of *L. iners* $t(182.96) = 4.45$, $p = < 0.001$, 95% confidence interval [10.58, 27.58]. Among postmenopausal women taking hormone replacement therapy, no such differences were observed (Fig. A2 in the Appendix displays these differences that were not statistically significant). Smoking was positively correlated with the genus *Veillonella* (rpb = 0.16, $n = 293$, $p = 0.008$).

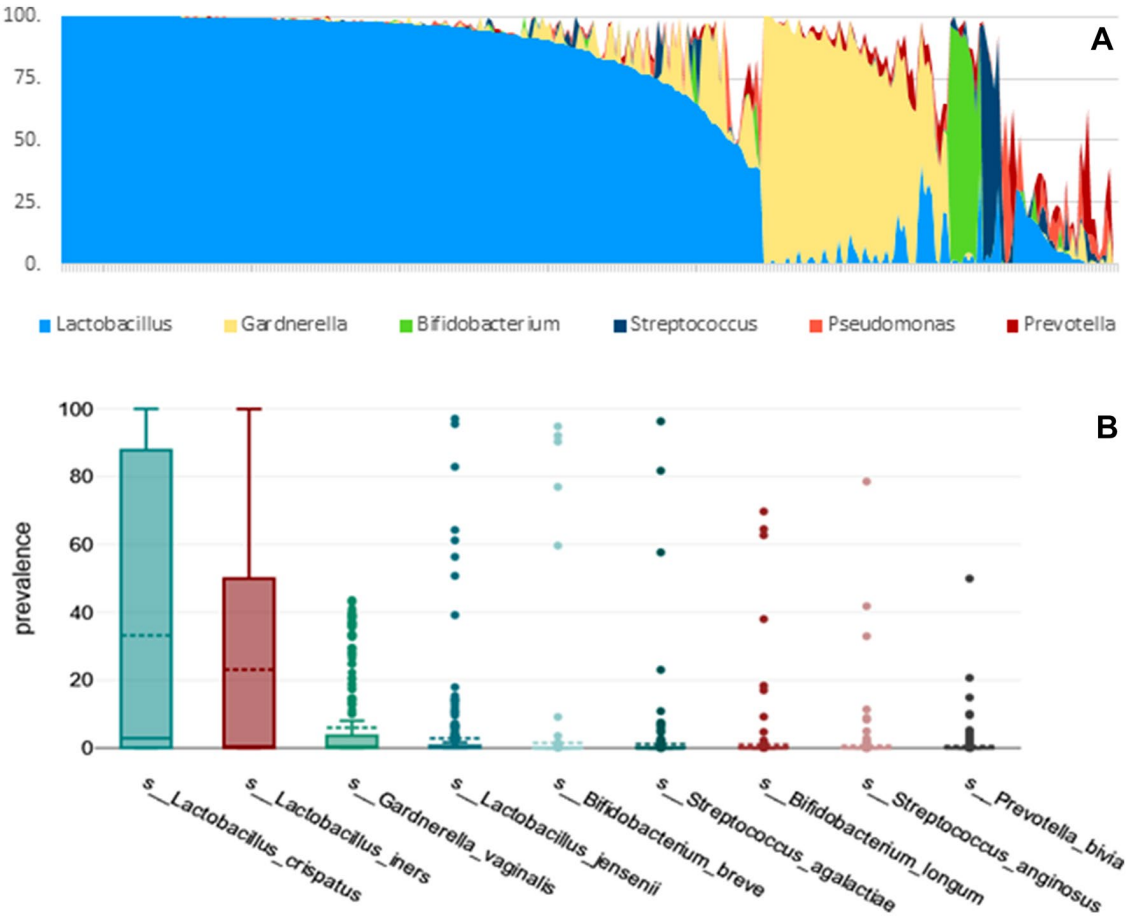

Fig. 1 Genus-level cervical microbiota profiles **A**. Prevalence of species with an average prevalence > 0.5% **B**

Correlations between cytology/histology and the microbiome

The result of the Pearson correlation showed that there was a significant low positive association between Pap stage and the order *Lactobacillales* (r(291) = 0.15, $p = 0.008$), the genus *Lactobacillus* (r(291) = 0.15, $p = 0.008$), the genus *Bacillus* (r(291) = 0.21, $p = < 0.001$) and with *Lactobacillus vaginalis* (r(291) = 0.22, $p = < 0.001$). When excluding postmenopausal patients from the analysis, only the positive associations with the genus *Bacillus* (r(209) = 0.24, $p = < 0.001$) and with *Lactobacillus vaginalis* (r(209) = 0.24, $p = < 0.001$) remained significant.

Further, there was a significant low positive association between histological stage and the genus *Lactobacillus* (r(291) = 0.12, $p = 0.038$), the genus *Bacillus* (r(291) = 0.17, $p = 0.004$),and *L. vaginalis* (r(291) = 0.16, $p = 0.006$). In addition, there was a low, positive correlation between histological stage and *Sneathia sanguinegens* (r(291) = 0.14, $p = 0.02$), the order *Mycoplasmatales* (r(291) = 0.19, $p = 0.001$), the genus *Ureaplasma* (r(291) = 0.18, $p = 0.002$), and *Ureaplasma parvum* (r(291) = 0.17, $p = 0.004$). Multiple linear regression analysis to examine the influence of age, menopausal state, smoking, and IUD in addition to Pap stage and histology, revealed that Pap stage remained associated with the genus *Bacillus* ($p = 0.033$), and *L. vaginalis* ($p = 0.021$). Histological stage remained associated with the order *Mycoplasmatales* ($p = 0.015$), the genus *Ureaplasma* ($p = 0.016$), and *U. parvum* ($p = 0.15$) (see Fig. 3).

Discussion

This study was conducted to evaluate the newly implemented German national CC screening program combined with an analysis of the cervical microbiome in patients referred to colposcopy according to the new guidelines.

There is broad scientific evidence from randomized controlled trials and meta-analyses that screening for HPV is more sensitive in the detection of cervical intraepithelial neoplasia grade III (CIN III) and CC than conventional cervical cytology (Naucler et al. 2007; Anttila et al. 2010;

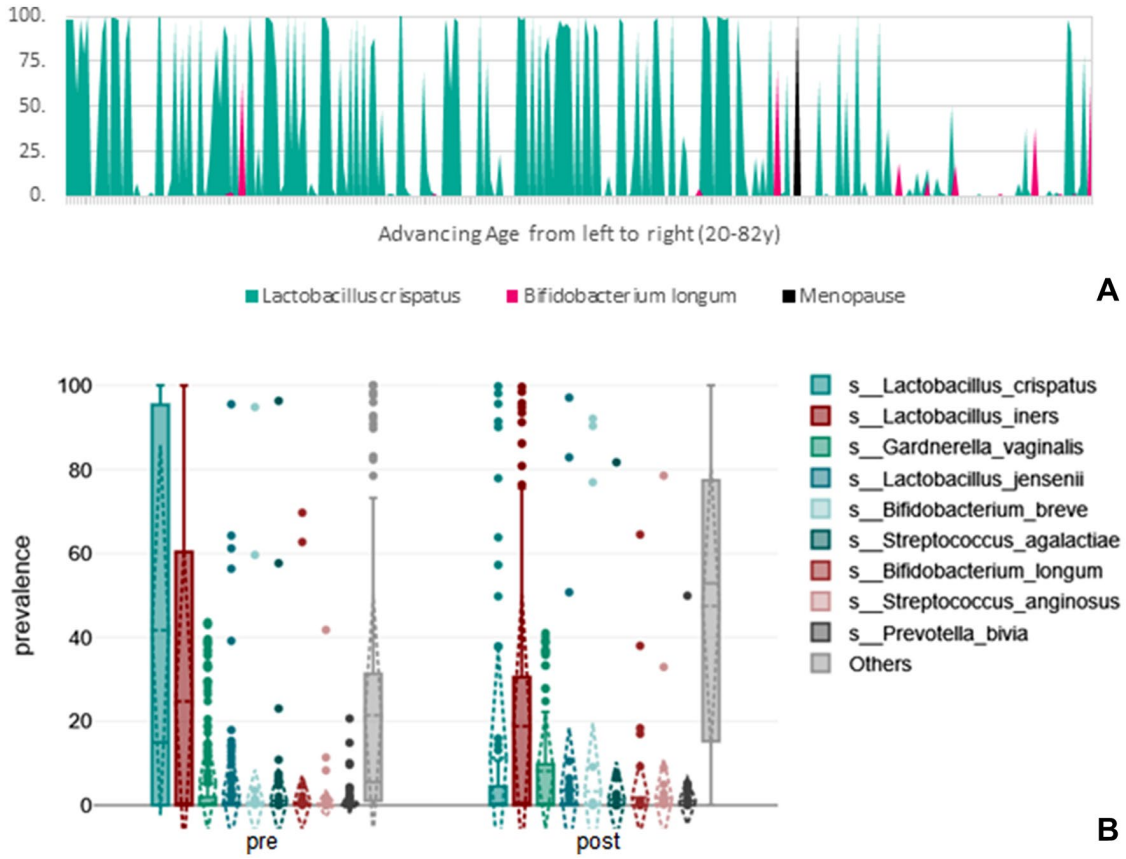

Fig. 2 Visualization of the influence of menopause and age on *L. crispatus* and *B. longum*, respectively (A), and the displacement of *L. crispatus* by other species **B**

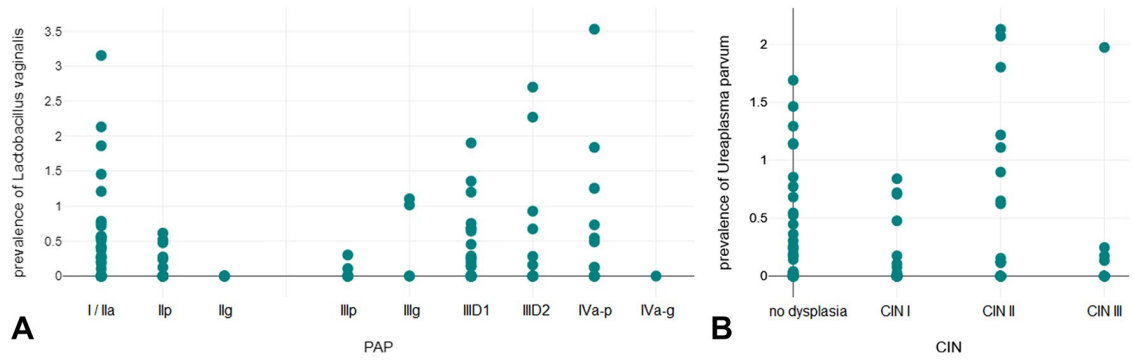

Fig. 3 Scatter diagram on the prevalence of *L. vaginalis* by cytological stage (**A**) and *U. parvum* by histological stage (**B**)

Ronco et al. 2010; Rijkaart et al. 2012). Integration of HPV testing into CC screening programs led to a decrease in CC incidence (Kjaer et al. 2010). This is, in particular, attributable to higher detection rates of cervical adenocarcinoma and its precursor lesions, as this subgroup is often underdiagnosed by cytological methods (Castle et al. 2010; Katki et al. 2011). Trials showed that women that are negative for hrHPV display a very low risk for the development of CIN III CC precursor lesions or CC (Dillner et al. 2008; Mesher et al. 2010). Based on these data, the new national CC screening program was set up in Germany with the implementation of a Pap smear/HPV co-testing for women aged 35 years and older.

Our study showed that 52.3% of the patients were referred for colposcopy due to an hrHPV-positive result in two consecutive years and normal cytology in both years. Among this subgroup, histological examination revealed only 3 cases of CIN III (2.1%) and 9 cases of CIN II (6.3%). Data from other studies reported a CIN III incidence range of 3–7% in women with normal cytology and a positive high-risk HPV test (Petry et al. 2003; Thrall et al. 2010). Another study from Germany that evaluated co-testing in women older than 30 years showed CIN III lesions in 9.2% of hrHPV-positive/normal-cytologic cases (Luyten et al. 2014). The implementation of colposcopy for Pap-normal/hrHPV-positive women in two consecutive years had the goal of diagnosing approximately 10% CIN III detected lesions. One finding is that the new screening program leads to an increased need for colposcopies and histological examinations performed. In our study, the supplementary HPV testing identified 3 cases of CIN III that would not have received histological assessment in the old screening program. Whether the number of CIN III remains the same in the long term, and additional HPV testing only detects them earlier before cytological abnormalities are detectable, must be clarified in further studies.

In 17 cases, the HPV status was not available as women were younger than 35 years and referred for colposcopy due to an abnormal Pap smear. Histological examination revealed in 5 cases a LSIL and 7 cases an HSIL lesion. HPV analyses showed a positive result for hrHPV in 16/17 cases. As all precursor lesions among these patients were detected by Pap smear, there was no additional benefit of HPV testing. However, this must be interpreted with caution as women < 35 with unremarkable PAP smears but positive hrHPV status were not included in the study and are not referred for a colposcopy within the current screening algorithm.

In our study, we repeated the HPV diagnostic with a test based on the Anyplex II HPV28 Detection for all enrolled patients. In 40 cases, there were deviating results from the initial testing for hrHPV. In 20 cases, that initially tested positive, the new negative result can be explained by spontaneous regression, as the time difference between the two analyses was 3–5 months. In 20 initial hrHPV negative cases, we found a positive result for hrHPV, with even 11 cases being positive for HPV 16. Divergence of these results can be explained by different sensitivity of HPV tests, a new infection with hrHPV, or a reactivation of a hrHPV infection in the meantime. A crucial step in an HPV-based cervical cancer screening program is the selection of an appropriate HPV test (Arbyn et al. 2015). As HPV infections are very common, with a high tendency for spontaneous regression, the positive predictive value for all HPV tests is relatively low. On the global market, 82% of the HPV tests lack any published analytical and/or clinical evaluation (Poljak et al. 2016). In the case of this study, Anyplex II HPV28 Detection was chosen as the broadest CE/IVD PCR assay, and extensively validated in the Vigilant Framework settings (Bonde et al. 2018).

Most of the available HPV tests are DNA-based and can only discriminate between the presence and absence of HPV-specific DNA. Hence, these tests are not able to discriminate between an active or inactive infection (Benevolo et al. 2011). Tests that use E6/E7 mRNA detection demonstrated higher clinical specificities than DNA-based tests, as E6/E7 mRNA is only found in actively infected cells (Ratnam et al. 2010; Arbyn et al. 2013). Currently, there are a variety of approved HPV tests available in Germany for screening, both DNA- and mRNA-based, with most using DNA test kits. Caution is needed when interpreting HPV results, as there are many different assays and positive HPV-DNA does not necessarily mean that an active infection is present. Future studies evaluating the new cancer screening program will need to clarify whether supplemental HPV testing improves the detection rate of CIN III in the long term and not just increases the number of examinations performed (colposcopies and histologic assessments). In future, more emphasis should be given to the selection of HPV assays, as the conclusions from mRNA and DNA assays differ significantly.

The production of lactic acid leading to a pH below 4,5 and antimicrobial substances such as bacteriocins, the competition for nutrients to counteract the overgrowth by other microorganisms, and the modulation of the local immune response are the main mechanisms of the protective role of lactobacilli (Aroutcheva et al. 2001). High estrogen levels and especially the glycogen content of the vaginal epithelium (Mirmonsef et al. 2016) lead to an environment dominated primarily by *L. crispatus*, *L. gasseri*, and *L. jensenii* (Gajer et al. 2012). The production of lactic acid is one central mechanism by which microorganisms protect themselves from viruses and competitors (Mitra et al. 2016). Further, *L. iners* dominance over *L. crispatus* has been associated with a higher risk for intraepithelial squamous lesions and cancer (Norenhag et al. 2020). A study analyzing the microbiota of HPV-positive and negative women demonstrated that *L. gasseri* is associated with a higher HPV elimination (Brotman et al. 2014). Accordingly, CST IV is associated with cervical abnormalities, low-grade squamous intraepithelial lesions (LSIL), high-grade SIL (HSIL), and cervical cancer. Comparing LSIL and HSIL samples, there was a microbiome shift to a greater abundance of *Sneathia sanguinegens*, *Anaerococcus tetradius*, and *Peptostreptococcus anaerobius* and a lower abundance of *L. jensenii* with HSIL (Mitra et al. 2015). These data suggest a major role of the vaginal and cervical microbiome in the development of precancerous lesions of the cervix. Nevertheless, the most important previously published papers on the role of the cervical microbiome in the development of cervical carcinoma were

limited to 169 (Mitra et al. 2015), 137 (Zhang et al. 2018), 126 (Seo et al. 2016), 120 (Oh et al. 2015), 94 (Wu et al. 2021), 92 (Tango et al. 2020), 47 (Kwon et al. 2019) and 32 (Audirac-Chalifour et al. 2016) patients, respectively. While despite all limitations in comparing results of microbiome studies, the relative patterns in various conditions would be expected to be reasonably consistent (Berman et al. 2020). This does not necessarily hold true if different primer pairs targeting 16 s-rDNA are used. On the one hand, universal V3/V4 Primer pairs do, for example, allow for better vaginal community state types assignment than universal V1/V2-based primers, detect more taxa, and generally present a higher abundance of *Gardnerella vaginalis* (Graspeuntner et al. 2018). In silico, on the other hand, V1/V3 primers seemed to perform at least as good as V3/V4 (Hugerth et al. 2020), and optimized V1/V2 primers, such as those used in our study, cover *Bifidobacteria* and disease-associated taxa, such as *G. vaginalis* and *Chlamydia trachomatis* (Frank et al. 2008; Zhang et al. 2019), while better differentiating among *Lactobacilli* (Zhang et al. 2019). In our study, we not only see more *G. vaginalis* than would be expected with universal V1/V2 primers, but also similar proportions of *Lactobacillus*-, *Gardnerella*-, and mixed-flora-dominated microbiomes to those observed in the largest shotgun metagenomics study performed to date of the cervical microbiome (Jie et al. 2021).

Primers previously used included, most importantly, universal V1/V2 (Mitra et al. 2015), V1/V3 (Oh et al. 2015; Seo et al. 2016), V3/V4 (Audirac-Chalifour et al. 2016; Zhang et al. 2018), and such targeting the V4 region (Wu et al. 2021). Nevertheless, even among studies using the same primer pairs, results substantially differed. While some argued that anaerobes, greater alpha diversity, and consequently lower levels of *Lactobacilli* seemed associated with a bad prognosis (Mitra et al. 2015; Audirac-Chalifour et al. 2016; Wu et al. 2021), others did not find differences linked to *Lactobacilli* or associations with anaerobes found in earlier studies (Oh et al. 2015; Seo et al. 2016; Zhang et al. 2018). Both studies targeting the V1/V3 region found excessively large proportions of *Fannyhessea (Atopobium) vaginae* and, respectively, little *G. vaginalis.* All studies agreed, however, that more studies involving larger sample sizes are needed, given the possible bias occurring with smaller sample sizes.

We are skeptical of differences linked to rare taxa in previous studies, also due to partially small study populations. Despite the V3/V4 primers being described as detecting more taxa (Graspeuntner et al. 2018), meta-transcriptome analyses have shown that only a couple of dominant genera contribute to most of the bacterial transcripts (Arroyo Muhr et al. 2021).

Rather than with neoplasia, we see the most remarkable differences in terms of alpha diversity and *L. crispatus* associated with menopause. Seeing that the study that most closely reflected our findings was a meta-genomic analysis of 516 women to evaluate the effect of lifestyle on the cervical microbiome (Jie et al. 2021), we find confirmation on the one hand and point out on the other hand that microbiome-based studies need to be conducted on large sample sizes. Moreover, just as in mentioned study, we find a significantly larger prevalence of *L. crispatus* among premenopausal women on oral contraceptives and no linkage between *G. vaginalis* and a disturbed microenvironment. In the same study, *L. vaginalis* was positively correlated with irregular menstruation, while in ours, with increasing PAP-score.

Regarding the role of *U. parvum* in the progression of neoplasia, we found three reports in the literature on the possible association between *U. parvum*, HPV, and intraepithelial neoplasia of the cervix (Biernat-Sudolska et al. 2011; Szostek et al. 2014; Drago et al. 2016), indicating the need for further investigation, as the detection of *U. parvum* is currently not indicative for therapy (Patel and Nyirjesy 2010; Kokkayil and Dhawan 2015).

In our study, only a few additional CIN III cases were identified. Results from other centers must be awaited to determine whether the addition of HPV testing will improve cervical cancer screening in Germany. In contrast to many other studies, dyplastic changes were only associated with *U. parvum*. We believe that there is currently insufficient data to support modulation of the vaginal microbiome, which is currently heavily marketed to counter dysplastic changes.

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s00432-023-04599-0.

Author contributions MC, CN and MP conceived and designed the study. MC, CN, MP, NW, RT and MS performed data analysis and interpretation. MC, LW and LO collected data. CN and MP performed lab experiments. AM, AH and DJR provided scientific insight and/or contributed to the interpretation of parts of the data. All authors read, edited, and reviewed the manuscript.

Funding Open Access funding enabled and organized by Projekt DEAL.

Data availability The datasets generated during the current study are available from the corresponding author on reasonable request.

Code availability Software packages are published, available to the public, and sources are cited in the Methods section.

Declarations

Conflict of interests The authors declare no financial or commercial conflict of interest.

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4.2 Condic M*, Ralser DJ*, Klümper N, Ellinger J, Qureischi M, Egger EK, Kristiansen G, Mustea A, Thiesler T. Comprehensive Analysis of N6-Methyladenosine (m6A) Writers, Erasers, and Readers in Cervical Cancer. Int J Mol Sci. 2022 Jun 28;23(13):7165. doi: 10.3390/ijms23137165

Zielsetzung - In zwei unabhängigen Zervixkarzinom-Kohorten (CC) wurden die Protein- und mRNA Expressionlevel der m6A „writer" (METTL3, METTL4, METTL14, WTAP, KIAA1429), „eraser" (FTO, ALKBH5), und „reader" (HNRNPA2B1, HNRNPC, YTHDC1, YTHDC1, YTHDF1-3) im Hinblick auf das Gesamtüberleben analysiert.

Methodik und Ergebnisse - Eine Kohorte umfasste *N*=118 Patientinnen mit CC, die am Universitätsklinikum Bonn (UKB) behandelt wurde. Die Proteinexpressionlevel wurden nach immunhistochemischer Färbung ermittelt. Die zweite Kohorte für die Analyse der mRNA Expression umfasste *N*=307 Patientinnen, die Daten wurden über das *The Cancer Genome Atlas* (TCGA) *Research Network* bezogen. Hohe Proteinexpressionswerte von METTL14 (HR: 2,592 (95%KI: 1,154-5,825), log-rank *p*=0.021), WTAP (HR: 2,387 (95%KI: 1,239-5,825), log-rank *p* 0.009), KIAA1439 (HR: 5,838 (95%KI: 2,886-11,812), log-rank *p* <0.001), ALKBH5 (HR: 3,603 (95%KI: 1,837-7,068), log-rank *p* <0.001), HNRNPC (HR: 2,506 (95%KI: 1,196-5,254), log-rank *p* =0.015), YTHDC1 (HR: 3,284 (95%KI: 1,758-6,134), log-rank *p* <0.001) und YTHDF3 (HR: 2,422 (95%KI: 1,289-4,550), log-rank *p*=0.006) waren in der UHB Kohorte mit einem schlechteren Gesamtüberleben assoziiert. Betrachtet man die mRNA Expressionswerte der TCGA Kohorte, so zeigten METTL14 (HR: 1,814 (95%KI: 1,129-2,915), log-rank *p* 0.012), WTAP (HR: 1,625 (95%KI: 1,016-2,600), log-rank *p*= 0.041), KIAA1429 (HR: 1,760 (95%KI: 1,106-2,800), log-rank *p*= 0.016) und YTHDC1 (HR: 0,592 (95%KI: 0,371-0,944), log-rank *p*=0.026) einen prognostischen Wert. Bis auf YTHDC1 waren die Daten zwischen beiden Kohorten übereinstimmend. Ob YTHDC1 mit einem besseren oder schlechteren Outcome assoziiert ist, muss in Folgestudien geklärt werden. Nach der Korrektur für multiples Testen (*q*<0.1) blieb die statische Signifikanz nur für die m6A-Proteinexpression aufrecht. Darüber hinaus ergab die Korrelation der m6A-Protein-Expressionswerte mit dem Grading und dem Lymphknotenbefall keine statistisch signifikanten Werte. Die Expressionsniveaus der m6A-Proteine wiesen hohe positive Korrelationskoeffizienten zueinander auf, was auf eine Koexpression der Proteine hinweist, die an der m6A-RNA-Modifikation im CC beteiligt sind.

Zusammenfassung - Unsere Studie deutet auf eine dysregulierte m6A-Modifikation im Zervixkarzinom hin. Daher könnte m6A als vielversprechender prognostischer Biomarker und therapeutisches Ziel dienen.

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Comprehensive Analysis of N6-Methyladenosine (m6A) Writers, Erasers, and Readers in Cervical Cancer

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Abstract: There is growing scientific evidence for the crucial role of post-transcriptional RNA modifications in carcinogenesis, progression, metastasis, and drug resistance across various cancer entities. N6-methyladenosine (m6A) is the most abundant type of RNA modification. m6A is coordinated by a dynamic interplay of 'writers' (METTL3, METTL4, METTL14, WTAP, KIAA1429), 'erasers' (FTO, ALKBH5), and 'readers' (HNRNPA2B1, HNRNPC, YTHDC1, YTHDC1, YTHDF1-3). In this study, we comprehensively examined protein and mRNA expression levels of m6A writers, readers, and erasers in two cervical cancer (CC) cohorts (UHB CC cohort, $N = 118$; TCGA CC cohort, $N = 307$) with regard to clinical outcomes. In the UHB CC cohort, high protein expression levels of METTL14 ($p = 0.016$), WTAP ($p = 0.007$), KIAA1439 ($p < 0.001$), ALKBH5 ($p < 0.001$), HNRNPC ($p = 0.012$), YTHDC1 ($p < 0.001$), and YTHDF3 ($p = 0.004$) were significantly associated with a shorter overall survival (OS). In the TCGA CC cohort, mRNA expression levels of *METTL14* ($p = 0.012$), *WTAP* ($p = 0.041$), *KIAA1429* ($p = 0.016$), and *YTHDC1* ($p = 0.026$) showed prognostic values. However, after correction for multiple testing, statistical significance remained only for m6A protein expression levels ($q < 0.1$). Our study points towards dysregulated m6A modification in CC. Hence, m6A might serve as a promising prognostic biomarker and therapeutical target in CC.

Keywords: cervical cancer; m6a; RNA modification; biomarker

Citation: Condic, M.; Ralser, D.J.; Klümper, N.; Ellinger, J.; Qureischi, M.; Egger, E.K.; Kristiansen, G.; Mustea, A.; Thiesler, T. Comprehensive Analysis of N6-Methyladenosine (m6A) Writers, Erasers, and Readers in Cervical Cancer. *Int. J. Mol. Sci.* **2022**, *23*, 7165. https://doi.org/10.3390/ijms23137165

Academic Editor: Attila A. Seyhan

Received: 21 May 2022
Accepted: 25 June 2022
Published: 28 June 2022

1. Introduction

Cervical cancer (CC) represents the fourth most common malignancy diagnosed in women worldwide [1]. CC incidence varies substantially depending on the availability of an effective screening program, causing significantly higher incidence and cancer-related deaths in developing countries [2]. The predominant histologic CC subtype is squamous cell carcinoma, accounting for over 80% of all cases. The remaining 20% are mainly attributable to adenocarcinomas and less common histologic subtypes [3,4]. Research has identified infection with human papilloma virus (HPV) as an obligatory cofactor for the development of CC [5]. The use of cervical cytology and HPV co-testing has significantly improved the detection of preinvasive cervical lesions and resulted in the significant decrease in invasive CC incidence [6] HPV vaccination, implemented since the mid-2000s, is expected to lead to further reductions in CC disease rates [7]. For early-stage CC, standard surgical treatment consists of radical hysterectomy. In patients with advanced local disease or presence of histopathologic risk factors, concurrent chemoradiotherapy is an equivalent therapy approach [8]. The prognosis of CC is stage-dependent. While early carcinomas

display excellent 5-year survival rates, the prognosis of advanced disease stages is extremely poor. In particular, the treatment of recurrent or metastatic CC is challenging due to a lack of effective therapeutic strategies. In this context, a deeper understanding of CC carcinogenesis, in particular epigenetic regulation mechanisms of oncogenic drivers, might help to discover potential targets for individualized therapy.

Research has implicated post-transcriptional messenger RNA (mRNA) modification to be involved in tumorigenesis, proliferation, angiogenesis, and tumor immunity across different cancer entities [9–11]. In this context, N6-methyladenosine (m6A) has been identified as the most common type of mRNA modification. The biological importance of m6A underlines its great potential to be used for diagnostic and therapeutic purposes. The process of m6A is coordinated by three different enzyme groups, designated as 'writers' (methylases; METTL 3, METTL 4, METTL 14, WTAP, KIAA1429), 'erasers' (demethylases; FTO, ALKBH5), and 'readers' (HNRNPA2B1, HNRNPC, YTHDC1, YTHDF1-3). Writers and erasers have opposite functions: while writers transfer S-adenosyl methionine to the RNA base adenine, erasers undo this process. These m6A RNA modifications are recognized by readers to mediate downstream effects [12].

However, little is known about the expression levels of m6A writers, erasers, and readers in CC. In this study, we comprehensively examined protein and mRNA expression levels of m6A writers, readers, and erasers in CC with regard to clinical outcomes.

2. Results

Immunohistochemical staining was performed in the UHB CC cohort comprising 118 patients. The mean age of the study cohort was 51.3 (+/− standard deviation (SD) 13.9) years. In total, 83.1% of the patients had squamous histology, and 16.9% were cervical adenocarcinomas. The median follow-up was 77.6 months. Clinicopathologic characteristics of the UHB CC cohort (grading, lymph node involvement, tumor stage according to FIGO, HPV status) are shown in Table 1.

In the UHB CC cohort, expression of all different m6A writers, readers, and erasers was identified (Supplementary Figures S2–S8). The proteins involved in m6A functions were present in different cell compartments, reflecting the diversity of RNA metabolism. Writers were typically observed in the nucleus. Congruently, METTL3, METTL14, WTAP, and KIAA1429 displayed strong nuclear staining. Likewise, immunohistochemical analysis revealed a strong nuclear staining for the eraser FTO and the two readers HNRNPC und HNRNPA2B1. In contrast, the readers YTHDF1, YTHDF2, and YTHDF3, as well as the writer METTL4, were detected in the cytoplasm (Table 2).

In Kaplan–Meier survival analysis, high expression levels of METTL14 ($p = 0.016$, Figure 1A–C), WTAP ($p = 0.007$, Figure 1D–F), KIAA1439 ($p < 0.001$, Figure 1G–I), ALKBH5 ($p < 0.001$, Figure 2A–C), HNRNPC ($p = 0.012$, Figure 2D–F), YTHDC1 ($p < 0.001$, Figure 3A–C), and YTHDF3 ($p = 0.004$, Figure 3D–F) correlated significantly with a shorter overall survival (OS). For the remaining proteins, there was a trend towards a shorter OS in patients with higher m6A protein expression levels, however, without reaching statistical significance (Table 2, Supplementary Figure S1). To correct for multiple hypothesis testing, the Benjamini and Hochberg method was applied with a significance threshold of $q < 0.1$. Prognostic significance remained after correction for multiple testing ($q < 0.1$) for the respective seven m6A proteins (Table 2). The prognostic value of METTL14, WTAP, KIAA1429, ALKBH5, HNRNPC, YTHDC1, and YTHDF3 was confirmed in univariate Cox regression analysis (Table 2). However, this prognostic value could not be observed in multivariate Cox regression analysis including established clinicopathological prognostic markers (age, grading, lymph node involvement, and FIGO stage).

Table 1. Clinicopathological characteristics of the UHB CC cohort (*N* = 118). SD = standard deviation. IHC = immunohistochemistry.

Cervical Cancer Cohort	
Age (years)	
Mean (±SD)	51.3 ± 13.9
Min–max	21–88
Histology	
Squamous cell carcinoma	98 (83.1%)
Adenocarcinoma	20 (16.9%)
Follow-up (months)	
Mean (±SD)	77.58 ± 58.3
Min–max	0–199
FIGO classification	
IA	5 (4.2%)
IB	56 (47.5%)
IIA	11 (9.3%)
IIB	18 (15.3%)
III	13 (11.0%)
IVA	15 (12.7%)
Lymph node involvement	
Yes	26 (22.0%)
No	54 (45.8%)
Unkown	38 (32.2%)
Grading	
G1	2 (1.7%)
G2	76 (64.4%)
G3	39 (33.1%)
Unknown	1 (0.8%)
HPV-Status (p16 IHC positive)	
Positive	107 (90.7%)
Negative	5 (4.2%)
Unknown	6 (5.1%)

Table 2. Summary of analyzed proteins and their correlation with OS in the UHB CC cohort. *p*-values for the group comparison (low vs. high expression) are based on log-rank tests, significance threshold $p < 0.5$, and estimated hazard ratios (HR) with 95% confidence intervals are based on univariate Cox regression analyses, significance threshold $p < 0.5$. *Q*-values are based on multiple hypotheses testing using the method of Benjamini and Hochberg with a significance threshold of $q < 0.1$. Significant values are highlighted in bold.

Proteins	Localization	Staining Intensity	N (Low/High)	*p*-Value (log)	*q*-Value	Hazard Ratio	95% CI	*p*-Value (cox)
Writer								
METTL3	Nuclear	0–1+/2–3+	15/92	0.128	0.166	2.416	0.745–7.830	0.142
METTL4	Cytoplasmatic	0–1+/2–3+	49/61	0.448	0.448	1.264	0.689–2.319	0.450
METTL14	Nuclear	0–1+/2–3+	31/82	**0.016**	**0.030**	2.592	1.154–5.825	**0.021**
WTAP	Nuclear	0–2+/3+	90/20	**0.007**	**0.018**	2.387	1.239–4.598	**0.009**
KIAA1429	Nuclear	0–1+/2–3+	96/13	**<0.001**	**0.013**	5.838	2.886–11.812	**<0.001**
Eraser								
FTO	Nuclear	0–1+/2–3+	36/75	0.061	0.100	2.060	0.951–4.462	0.067
ALKBH5	Cytoplasmatic/ nuclear	0–1+/2–3+	60/51	**<0.001**	**0.004**	3.603	1.837–7.068	**<0.001**
Reader								
HNRNPA2B1	Nuclear	0–2+/3+	75/37	0.108	0.156	1.628	0.892–2.972	0.112
HNRNPC	Nuclear	0–1+/2–3+	41/66	**0.012**	**0.026**	2.506	1.196–5.254	**0.015**
YTHDC1	Membraneous/ cytoplasmatic/ nuclear	0–2+/3+	82/26	**<0.001**	**0.007**	3.284	1.758–6.134	**<0.001**
YTHDF1	Cytoplasmatic	0–2+/3+	76/33	0.206	0.243	1.522	0.789–2.936	0.210
YTHDF2	Cytoplasmatic	0–2+/3+	89/21	0.260	0.281	1.499	0.737–3.051	0.264
YTHDF3	Cytoplasmatic	0–1+/2–3+	63/40	**0.004**	**0.013**	2.422	**1.289–4.550**	**0.006**

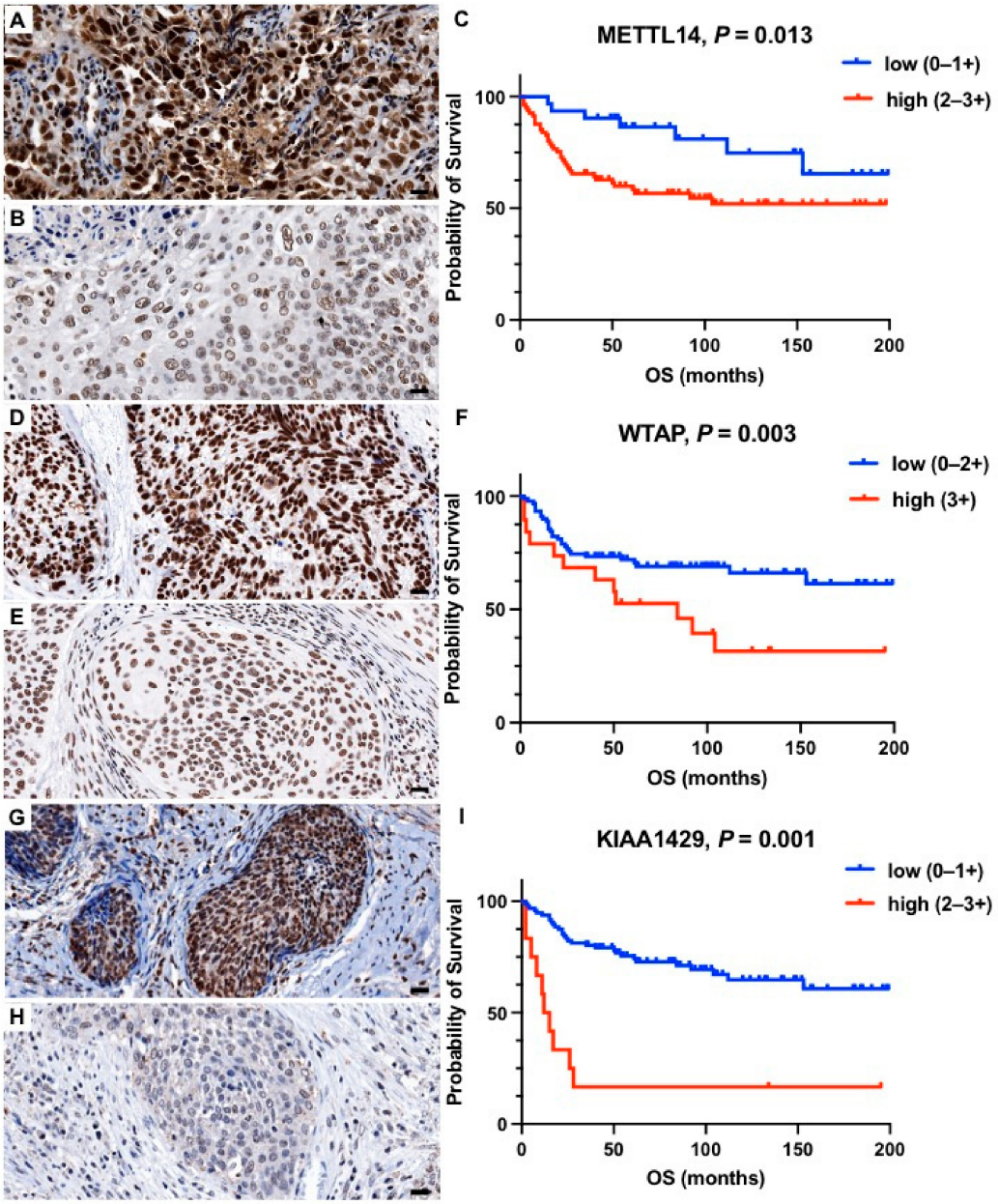

Figure 1. Representative histology sections show high (**A**,**D**,**G**) and low (**B**,**E**,**H**) expression levels of METTL14, WTAP, and KIAA1429 visualized by immunohistochemistry; hematoxylin (blue) was used for nuclear staining (bright field image, 400× magnification). Kaplan–Meier estimates show a significantly shorter overall survival ($p < 0.05$) in patients with high expression of (**C**) METTL14, (**F**) WTAP, and (**I**) KIAA1429. Scale bar = 20 µm.

Furthermore, correlation of m6A protein expression levels with respect to grading and lymph node involvement showed no statistically significant values.

Of note, m6A protein expression levels showed high positive correlation coefficients towards each other, indicating a co-expression of proteins involved in m6A RNA modification in CC (Figure 4).

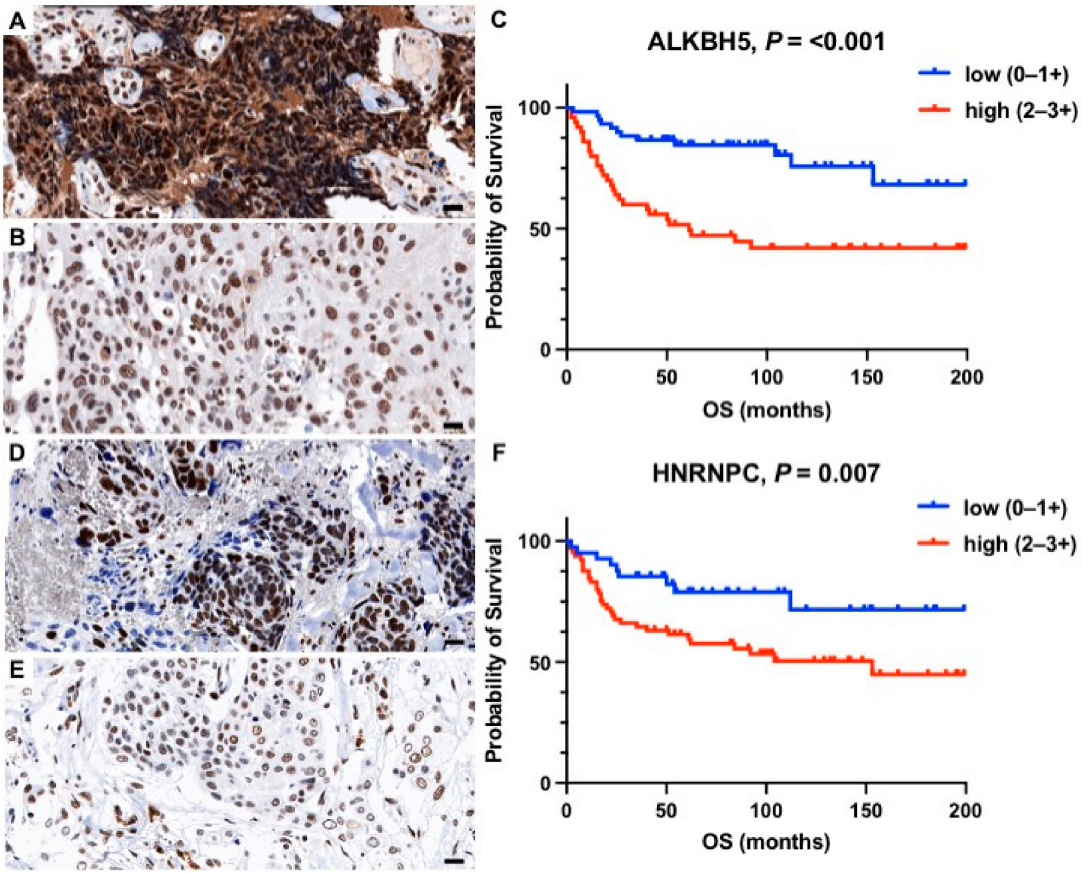

Figure 2. Representative histology sections show high (**A**,**D**) and low (**B**,**E**) expression levels of ALKBH5 and HNRNPC visualized by immunohistochemistry; hematoxylin (blue) was used for nuclear staining (bright field image, 400× magnification). Kaplan–Meier estimates show a significantly shorter overall survival ($p < 0.05$) in patients with high expression of (**C**) ALKBH5 and (**F**) HNRNPC. Scale bar = 20 µm.

In the TCGA cohort, mRNA expression levels of *METTL14*, *WTAP*, *KIAA1429*, and *YTHDC1* were significantly associated with OS (*METTL14*: $p = 0.012$; *WTAP*: $p = 0.041$; *KIAA1429*: $p = 0.016$; *YTHDC1*: $p = 0.026$; Supplementary Table S2). In line with protein expression data obtained from the UHB CC cohort, enhanced mRNA expression levels of *METTL14*, *WTAP*, and *KIAA1429* were associated with a shorter OS. In contrast, enhanced *YTHDC1* mRNA expression was associated with a prolonged OS. However, after correction for multiple testing, the prognostic value of m6A mRNA expression did not reach statistical significance.

In summary, our results show that high protein expression levels of METTL14, WTAP, KIAA1429, ALKBH5, HNRNPC, YTHDC1, and YTHDF3 are associated with a shorter OS independent of their function (writer, reader, or eraser).

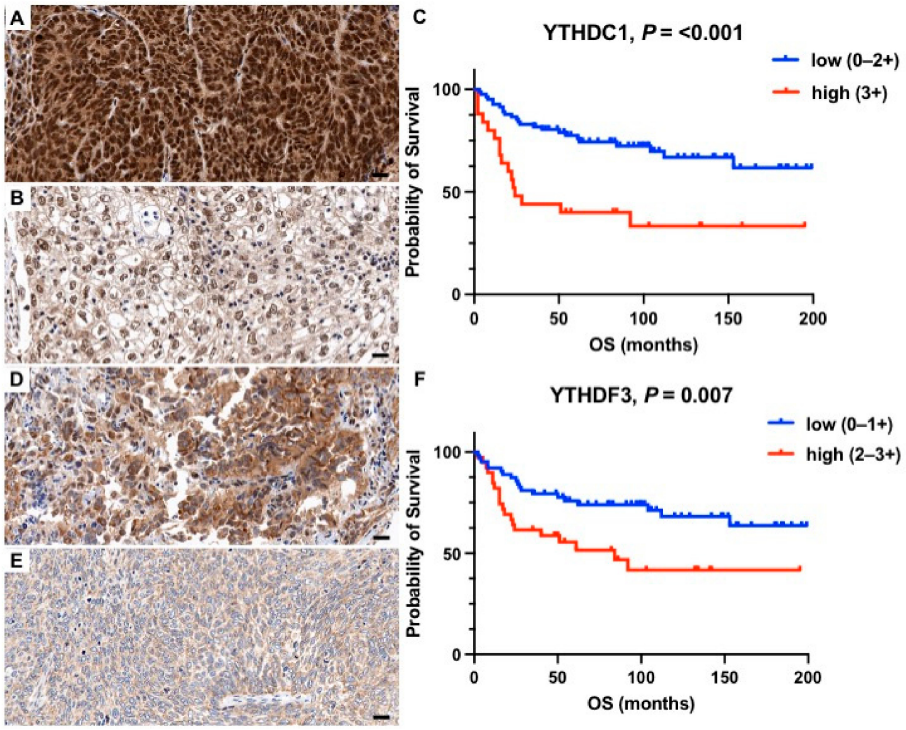

Figure 3. Representative histology sections show high (**A**,**D**) and low (**B**,**E**) expression levels of YTHDC1 and YTHDF3 visualized by immunohistochemistry; hematoxylin (blue) was used for nuclear staining (bright field image, 400× magnification). Kaplan–Meier estimates show a significantly shorter overall survival ($p < 0.05$) in patients with high expression of (**C**) YTHDC1 and (**F**) YTHDF3. Scale bar = 20 µm.

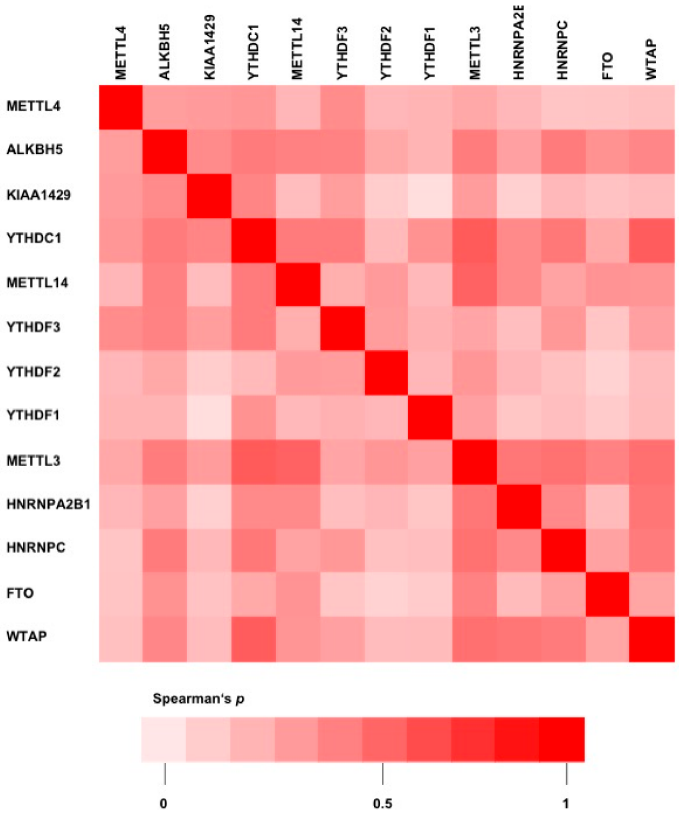

Figure 4. Correlation heatmap visualizing Spearman's *p* correlation coefficients of m6A protein expression in the UHB CC cohort.

3. Discussion

In the present study, protein and mRNA expression levels of m6A writers, erasers, and readers were determined in two independent CC cohorts. mRNA and protein expression data were further analyzed with regard to clinical outcomes. On the protein level, we demonstrated that seven m6A proteins, namely METTL14, WTAP, KIAA1429, ALKBH5, HNRNPC, YTHDC1, and YTHDF3, were significantly associated with a poor OS in CC (UHB CC cohort; Table 2). In particular, higher expression levels of these respective proteins were linked to a shorter OS. Of note, this prognostic value was independent of lymph node involvement and tumor stage. These findings were substantiated by analyzing mRNA expression data obtained from an independent CC cohort (TCGA CC cohort). On the transcriptional level, we detected significant prognostic values for METTL14, WTAP, KIAA1429, and YTHDC1. In line with immunohistochemical data for METTL14, WTAP, and KIAA1429, higher mRNA levels were associated with a shortened OS. Contrasting results, however, were obtained for YTHDC1. Within the TCGA CC cohort, higher YTHDC1 mRNA expression levels were linked to prolonged OS, whereas in the UHB CC cohort, higher YTHDC1 protein expression levels were associated with a worse OS. Discordant expression data on the transcriptional and protein level are frequently reported in the literature and might be attributable to analytical issues and spatial tumor heterogeneity [13–15]. In the biological context, protein expression might be more relevant. However, caution is warranted when interpreting incongruent results obtained from two different cohorts. With regard to YTHDC1 and its prognostic value on OS in CC, further studies need to be conducted to clarify this issue.

There is broad scientific evidence that abnormal m6A modification plays an essential role in tumor proliferation, angiogenesis, and metastasis across various cancer types. In CC, m6A dysregulation was linked to chemo- and radiotherapy-resistance and a more progressive phenotype. Zhou et al. [16] reported enriched FTO expression in CC tumor tissue compared to normal adjacent tissue (NAT). Higher FTO expression levels were associated with enhanced resistance to chemo- and radiotherapy caused by decreased beta-catenin and increased ERCC1 expression levels. Another study indicated FTO as an important oncogenic driver in CC by regulating proliferation and migration of CC cells [17]. These findings are in line with data from our present study. In the UHB CC cohort, higher FTO protein expression showed a trend to a shortened OS in Kaplan–Meier survival analysis but without statistical significance ($p = 0.061$, Supplementary Figure S1). METTL3, in its function as a writer, was previously shown to be upregulated in CC cells. High METTL3 protein expression was correlated with a poor prognosis [18]. In our analyses, however, METTL3 expression levels had no distinct effect on OS (Table 2). Within the m6A writer subgroup, METTL14 is crucial for recognizing substrate RNAs and stabilizing the catalytic function of METTL3 [19,20]. A recent study on m6A in hepatocellular carcinoma (HCC) showed involvement of METTL4 in HCC tumor progression. In downstream analyses, this effect was attributed to m6A-dependent regulation of cysteine sulfidic acid decarboxylase (CSAD), glutamic-oxaloacetic transaminase 2 (GOT2), and suppressor of cytokine signaling 2 (SOCS2) [21]. In the UHB and TCGACC cohorts, METTL14 overexpression was identified to be associated with shortened OS. Analogous to METTL14, WTAP upregulation in HCC promoted liver cancer development [22]. Likewise, the same might be applicable to CC carcinogenesis. In both studied cohorts, the presence of WTAP overexpression was associated with worse OS. In our analyses, HNRNPC protein expression was identified among the m6A enzymes associated with poor OS. However, little is known regarding its role in carcinogenesis. Writers and erasers accomplish opposite functions. Hence, our finding of co-expression of these two enzyme groups appears to be counterintuitive (Figure 4). However, research has demonstrated a dual role for m6A in cancer biology comprising both cancer promotion and cancer suppression. Its specific role is dependent on the cell context and the downstream target RNA and its function (tumor promoter vs. tumor suppressor) [20,23,24]. In the literature, contradictory phenomena have been described for different tumor entities. While in breast cancer high FTO expression levels

are associated with increased tumor cell proliferation, increased FTO levels in clear cell renal cell carcinoma resulted in tumor cell growth inhibition [25,26].

The YTH domain-containing proteins, including YTHDF1-3 and YTHDC1-2, participate in mRNA splicing, nuclear export, and translation. Due to post-transcriptional modifications, they modulate the expression of genes involved in cancer migration, invasion, proliferation, and immunity [27]. Especially unbalanced alternative splicing, which has been found in different kinds of cancer, can be caused by YTH domain dysregulation leading to tumor cell proliferation and invasion [28]. In our CC cohort, YTHDC1 and YTHDF3 overexpression was linked to shortened OS. Research has shown that nearly all YTH proteins, including YTHDF1-3 and YTHDC1-2, are upregulated in most types of cancer. In ovarian cancer, YTHDF1 facilitates tumorigenesis and metastasis by promoting the translation of EIF3C mRNA in an m6A-dependent manner [29]. In breast cancer, the overexpression of YTHDF1, YTHDF3, and KIAA1429 predicted a poor prognosis in terms of OS [30]. Furthermore, expression of YTHDC1, especially its alternative splicing components, was detected in a panel of prostate cell lines that was absent in benign cell lines, indicating that YTHDC1 might act as an oncogene in prostate cancer [31].

As m6A RNA modification is implicated in carcinogenesis, it might display a potential target for anticancer therapy. In dendritic cells, loss of YTHDF1 enhanced the cross-presentation of tumor antigens and the cross-priming of CD8(+) T cells in vivo. Furthermore, the therapeutic efficacy of the PD-L1 checkpoint was enhanced in YTHDF1 $(-/-)$ mice, indicating that YTHDF1 might be a potential therapeutic target in anticancer immunotherapy [32]. In colorectal cancer and melanoma, loss of METTL3 and METTL14 enhanced the sensitivity to anti-PD-1 treatment [33]. ALKBH5 regulates the content of lactic acid and accumulation of tumor immune cells in the tumor microenvironment, so that ALKBH5 might serve as a potential therapeutic target to enhance the effect of immunotherapy in melanoma, colorectal, and potentially other cancer types [34]. The influence of m6A proteins on targeted cancer therapy, especially checkpoint inhibitors, might also have an impact in CC patients, where PD-L1 inhibitors are used for the therapy in the recurrent or metastatic setting [35].

Overall, these findings point towards the potential impact of m6A RNA modification for CC and cancer in general. Limitations of our study are the retrospective design. Protein expression analysis is based on tissue microarray, where tumor heterogeneity might be a potential bias. However, within our study, clinically relevant signals were detected. The dysregulation of m6A proteins might be used as biomarkers and indicators for poor prognosis but also as potential targets for novel therapeutic drugs. There is still a need to conduct further studies to investigate their biological functions and precise corresponding molecular mechanisms in detail.

4. Materials and Methods

4.1. Patients and Specimens

UHB CC cohort: The retrospective study population comprised 118 patients with CC diagnosed at the University Hospital between 2002 and 2016. The collection of tissue was performed within the framework of the Biobank initiative of the University Hospital Bonn. Tissue was obtained from biopsies or surgical specimens. All patients provided written informed consent prior to collection of biomaterials. The study was approved by the Ethics Committee of the University of Bonn (vote: 208/21).

Clinicopathological parameters are summarized in Table 1. Baseline characteristics were obtained from a clinical database. Histopathological diagnosis was deduced based on World Health Organization (WHO) criteria. The International Federation of Gynecology and Obstetrics (FIGO) classification was used to determine the tumor stage.

TCGA CC cohort: mRNA expression data from 307 CC patients were obtained from The Cancer Genome Atlas Research Network [36]. Patients had signed informed consent prior to registration in accordance with the declaration of Helsinki principles. Clinicopathological characteristics of this cohort have been published elsewhere [37].

4.2. Tissue Microarray (TMA) Construction (UHB CC Cohort)

Formalin-fixed paraffin-embedded CC tissue (FFPE) specimens were used to generate TMAs. Staining with hematoxylin and eosin (HE) was performed to identify representative tumor areas. For each case, two 1 mm core biopsies (0.785 mm^2) were taken from different cancer areas and arranged in TMA blocks.

4.3. Immunohistochemistry

Immunostaining of the different writers, erasers, and readers was performed on TMAs. An automated staining system (BenchMark ULTRA; Ventana Medical Systems, Oro Valley, AZ, USA) was applied for deparaffinization, pretreatment with cell conditioning buffer (CC1 buffer, pH8), and primary antibody incubation. Incubation with the primary antibody was performed at 4 °C overnight. For signal detection, the UltraView DAB IHC Detection Kit (Ventana) was used. A detailed overview of the antibodies and dilutions is presented in Supplementary Table S1.

For immunohistochemical analyses, an Olympus BX51 microscope (Olympus, Tokio, Japan) and the Panoramic Viewer 3DHistech (3DHISTECH Kft., Budapest, Hungary) were used. Staining intensities were evaluated by two different investigators on technical duplicates. Briefly, a four-tier scoring system was applied to categorize staining intensities (0: no staining, 1: low staining, 2: moderate staining, 3: high staining). The obtained staining intensities were divided into two groups (low and high) with the median protein expression as a cut-off. For ALKBH5 and YTHDC1, immunohistochemical staining of multiple subcellular compartments was observed. Here, the predominant subcellular localization (nuclear) was considered for statistical analysis. Classification of the groups, low vs. high depending on the staining intensity, is provided for each antibody in Table 2.

4.4. Statistical Analysis

Statistical analysis (Kaplan–Meier survival analysis, log-rank tests, Cox regression analysis, and non-parametric Spearman's p correlation coefficients) were performed with the Statistical Package for the Social Sciences (SPSS®) version 28 (SPSS INC., IBM Corp., Armonk, NY, USA) and the GraphPad Prism software (GraphPad Software, San Diego, CA, USA). Statistical significance was approved at a two-sided $p < 0.05$. To correct for multiple testing, the Benjamini and Hochberg method was applied. P-values were converted to false discovery rate (FDR) Q-values with a significance threshold of $q < 0.1$.

5. Conclusions

In CC, enhanced expression levels of m6A proteins are associated with unfavorable clinical outcomes. This effect is independent of established clinicopathological prognostic parameters. Hence, our study highlights the potential of m6A as a promising prognostic biomarker in CC. The crucial role of m6A in CC pathogenesis holds the potential for the development of new anticancer therapeutics.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/ijms23137165/s1.

Author Contributions: Conceptualization, M.C. and D.J.R.; methodology, M.C., D.J.R. and T.T.; validation, G.K., formal analysis, M.C., D.J.R. and T.T.; investigation, M.C., D.J.R. and T.T.; resources, J.E., G.K., A.M.; data curation, M.C., D.J.R., M.Q. and T.T.; writing—original draft preparation, M.C. and D.J.R.; writing—review and editing, M.C., D.J.R., N.K., J.E. and E.K.E.; supervision, G.K. and A.M.; project administration, M.C. and D.J.R.; funding acquisition, D.J.R. and N.K. All authors have read and agreed to the published version of the manuscript.

Funding: This research was funded by the BONFOR program of the Medical Faculty of the University of Bonn (D.J.R.: 2021-1A-14; N.K.: 2020-2A-12).

Institutional Review Board Statement: The study was conducted in accordance with the Declaration of Helsinki and approved by the Institutional Ethics Committee of the Medical Faculty of the University of Bonn (vote: 208/21).

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

Data Availability Statement: Raw data are available on request from the authors.

Acknowledgments: The tissue specimens were collected within the framework of the Biobank of the University Hospital Bonn. We thank Susanne Steiner for her technical support.

Conflicts of Interest: The authors declare no conflict of interest.

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Zielsetzung - Ziel dieser Studie war die Analyse der Proteinexpression von m6A „writer“ (METTL3, METTL4, METTL14, WTAP, KIAA1429), „eraser“ (FTO, ALKBH5), und „reader“ (HNRNPA2B1, HNRNPC, YTHDC1, YTHDC1, YTHDF1-3) im Hinblick auf das Gesamtüberleben bei 126 Patientinnen mit primärem Plattenepithelkarzinom der Vulva (VSCC).

Methodik und Ergebnisse - Die Proteinexpression der m6A „writer", „eraser“ und „reader“ wurde mit Immunhistochemie ermittelt. Die Kohorte umfasste *N*=126 Patientinnen mit Vulvakarzinom, wovon *N*=79 Tumore HPV-unabhängig und *N*=23 HPV-abhängig waren. Bei *N*=24 Patientinnen war der HPV Status unbekannt. Die statistische Auswertung erfolgte getrennt für die jeweiligen Subgruppen. Drei Proteine waren signifikant höher exprimiert in HPV-abhängigem VSCC: METTL14 (63% vs 34% in HPV-unabhängig VSCC; p=0.049, Fisher's exact test), FTO (47% vs 13% in HPV-abhängig VSCC; p=0.002) und ALKBH5 (79% vs 59% in HPV-abhängig VSCC; p=0. 040). Betrachtet man das Gesamtüberleben, zeigte sich für die Gesamtkohorte keine Korrelation mit der m6A Expression. Bei den HPV-abhängigen VSCC jedoch korrelierte eine hohe Expression von METTL3 (p=0.010, q=0.08, log-rank test und Benjamini and Hochberg korrigierter log-rank test), METTL 14 (p=0.020, q=0.09) und YTHDC1 (p=0.012, q=0.08) mit einem schlechterem Gesamtüberleben. Eine Überexpression von METTL 4 (p= 0.034) und YTHDF2 (p=0.040) war ebenfalls mit einem schlechteren Gesamtüberleben assoziiert, blieb aber nach der Korrektur für multiples Testen mit q<0.1 nicht signifikant. Die Proteinexpressionlevel von METTL3, METTL4, METTL14, YTDHC1, and YTHDF2 waren unabhängig von klinisch-pathologischen Parametern wie Lymphknotenbefall und Grading. Wir fanden positive Spearman's p Korrelationskoeffizienten für die Expression der prognostischen m6A-Proteine METTL3, METTL14 bzw. YTDHC1 in der HPV-abhängigen Untergruppe. Von diesen drei Proteinen identifizierten wir METTL14 als das Protein, das typischerweise mit den anderen zusammenhängt, im Gegensatz zu METTL3 und YTHDC1, die zusätzliche Informationen zu den anderen beiden lieferten.

Zusammenfassung - Hohe Expressionslevel von Proteinen, die an der m6A-Modifikation beteiligt sind, korrelieren mit einem schlechten Gesamtüberleben bei Patienten mit HPV-abhängigem VSCC. Daher könnte m6A als prognostischer Biomarker bei HPV-abhängigen VSCC dienen.

Condic et al. BMC Cancer (2022) 22:943
https://doi.org/10.1186/s12885-022-10010-x

BMC Cancer

RESEARCH Open Access

N6-methyladenosine RNA modification (m6A) is of prognostic value in HPV-dependent vulvar squamous cell carcinoma

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Abstract

Background: Vulvar squamous cell carcinoma (VSCC) is an uncommon gynecologic malignancy but with an increasing incidence in recent years. Etiologically, VSCC is classified into two subtypes: HPV-dependent and HPV-independent. Localized VSCC is treated surgically and/or with radiation therapy, but for advanced, metastatic or recurrent disease, therapeutic options are still limited.

N6-methyladenosine (m6A) is the most prevalent post-transcriptional messenger RNA (mRNA) modification and involved in many physiological processes. The group of m6A proteins can be further divided into: ‚writers' (METTL3, METTL4, METTL14, WTAP, KIAA1429), ‚erasers' (FTO, ALKBH5), and ‚readers' (HNRNPA2B1, HNRNPC, YTHDC1, YTHDF1-3). Dysregulated m6A modification is implicated in carcinogenesis, progression, metastatic spread, and drug resistance across various cancer entities. Up to date, however, only little is known regarding the role of m6A in VSCC.

Methods: Here, we comprehensively investigated protein expression levels of a diverse set of m6A writers, readers and erasers by applying immunohistochemical staining in 126 patients with primary VSCC.

Results: In the entire study cohort, dominated by HPV-independent tumors, m6A protein expression was not associated with clinical outcome. However, we identified enhanced protein expression levels of the ‚writers' METTL3, METTL14 and the ‚reader' YTHDC1 as poor prognostic markers in the 23 patients with HPV-dependent VSCC.

Conclusion: Our study suggests dysregulated m6A modification in HPV-associated VSCC.

Keywords: m6A, N6-methyladenosine RNA modification, Vulvar squamous cell carcinoma, HPV, Biomarker

Background

Vulvar carcinoma is responsible for 3% of all gynecological malignancies worldwide and represents the fourth most common tumor of the female genital tract [1]. In the last decade, the incidence of human papillomavirus (HPV)-dependent and HPV-independent vulvar carcinoma has increased by more than 20%, likely driven by increased high-risk HPV exposure and a generally aging population [2–4].

Vulvar squamous cell carcinoma (VSCC) is the predominant histological subtype. VSCC can be further subclassified into two etiologic subtypes: (i) HPV-dependent VSCC [5, 6], accounting for 34% of invasive VSCC [7]; and (ii) HPV-independent VSCC arising on the basis of *lichen sclerosus and atrophicus* [8], a chronic vulvar dermatosis affecting mostly elderly patients. Of note, HPV-independent VSCC displays a worse overall prognosis than HPV-dependent VSCC [9].

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In localized disease, tumor excision with inguinofemoral sentinel lymphonodectomy and/or inguinofemoral systematic lymphonodectomy represents the therapeutic mainstay. Additional radiotherapy is applied in the presence of risk factors. With > 85% survival rates, the 5-year overall survival (OS) is excellent in localized disease [10]. However, for patients with locally advanced, metastatic or recurrent disease, there are only limited treatment strategies with an overall poor 5-year OS of only 15–50% [11]. Hence, there is an unmet need for new therapeutic options in this difficult-to-treat patient population [12]. In this context, a deeper understanding of the VSCC tumor biology, in particular for the respective etiologic subtypes, might pave the way to identify novel therapeutic approaches in VSCC.

N6-methyladenosine (m6A) is the most abundant messenger RNA (mRNA) modification. Briefly, three different enzyme groups are involved in m6A modification: (i) methylases ('writers'; METTL 3, METTL 4, METTL 14, WTAP, KIAA1429) that catalyze the transfer of S-adenosyl methionine groups to RNA adenine bases; (ii) demethylases ('erasers'; FTO, ALKBH5) that have the capacity to reverse the methylation process; and (iii) 'readers' (HNRNPA2B1, HNRNPC, YTHDC1, YTHDF1-3) that recognize m6A RNA modification and activate downstream regulatory pathways [13].

m6A modifications were previously identified to be involved in tumorigenesis, proliferation, angiogenesis and tumor immunity across diverse cancer entities [14–20]. This central role of m6A emphasizes its great potential in both diagnostic and therapeutic applicability [21]. Recently, we were able to provide evidence for m6A involvement in CC that bears etiological and tumor biological similarity to VSCC [22]. To the best of our knowledge, there is no data regarding m6A modification in VSCC. In this study, we thus comprehensively analyzed protein expression levels of a diverse set of m6A writers, readers and erasers by immunohistochemistry in a cohort of 126 VSCC patients to understand the effects of RNA modifications on tumorigenesis, especially with regard to the two etiologic subtypes.

Methods

Patients and specimens

The retrospective single-center study population included 126 patients with primary VSCC treated at the University Hospital Bonn between 2002 and 2017. The collection of tissue was within the framework of the Biobank initiative of the University Hospital Bonn. Tissue was obtained from biopsies or surgical specimens. All patients provided written informed consent prior to the collection of biomaterials. The study was approved by the Ethics Committee of the Medical Faculty of the University of Bonn (vote: 208/21).

Clinicopathological characteristics of the entire cohort, the HPV-independent and the HPV-dependent subcohorts, obtained from a clinical database, are presented in Table 1. The histopathological diagnosis was based on the World Health Organization (WHO) criteria. The 2010 revision of the International Federation of Gynecology and Obstetrics (FIGO) system was used to determine the tumor grade. The 7th TNM classification of the Union for International Cancer Control (UICC) allowed to determine the tumor stage.

Tissue microarray (TMA) construction

The TMA was generated from formalin-fixed paraffin (FFPE)-embedded VSCC tissue specimens. Hematoxylin and eosin (HE) stained sections were applied to identify representative tumor areas. Subsequently, a 1 mm core biopsy (0.785mm^2) was taken from the selected cancer areal and arranged in TMA blocks.

DNA extraction und HPV analysis

Tumor tissue was deparaffinized and macrodissected from unstained slides. The tumor tissue was then lysed with proteinase K overnight. DNA extraction from FFPE-embedded tissue was performed with the BioRobot M48 Robotic workstation and the corresponding MagAttract DNA Mini M48 Kit (Qiagen, Germany). Determination of HPV subtypes was performed applying the HPV Type 3.5 LCD-Array Kit (Chipron, Germany) according to the manufacturer's instructions as described previously [23]. With this assay the detection of 32 different HPV subtypes is possible (HPV types 06,11,16,18,31,33,35,39, 42,44,45,51,52,53,54,56, 58,59,61,62,66,67,68,70, 72,73,81,82,83,84,90 and 91).

Immunohistochemistry

Immunostaining of METTL3, METTL4, METTL14, WTAP, KIAA1429, FTO, ALKBH5, HNRNPA2B1, HNRNPC, YTHDC1, YTHDF1,YTHDF2, and YTHDF3 was performed on the TMAs using an automated staining system (BenchMark ULTRA; Ventana Medical Systems) which performed deparaffinization, pretreatment with cell conditioning buffer (CC1 buffer, pH8), and incubation with primary antibodies (FTO (1:50; Atlas Antibodies #HPA041086), ALKBH5 (1:200; Novus #NBP1-82,188), METTL3 (1:1000; Biorbyt #orb374082), METTL4 (1:40; Atlas Antibodies #HPA040061), METTL14 (1:100; Atlas Antibodies #HPA038002), WTAP (1:100; Atlas Antibodies #HPA010550), KIAA1429 (1:25; Atlas Antibodies #HPA031530), HNRNPC (1:25; Atlas Antibodies #HPA051075), HNRNPA2B1 (1:100; Atlas Antibodies #HPA001666), YTHDC1 (1:25; Atlas Antibodies

Table 1 Clinicopathological characteristics of the entire VSCC cohort, HPV-independent cohort, and HPV-dependent sub-cohorts. No HPV status was available for 24 patients. SD = standard deviation

Clinicopathological parameters	All (*N* = 126)	HPV-independent (*N* = 79)	HPV-dependent (*N* = 23)
Age (years)			
Mean (± SD)	64.1 ± 14.4	65.1 ± 14.0	57.5 ± 15.2
Min–max	25—93	33—93	25—84
Overall survival (months)			
Mean *(± SD)*	54.0 ± 42	58.8 ± 44.7	53.7 ± 39.5
Median	46.0	58.0	48.0
TNM classification			
T1	102 (81.0%)	65 (82.3%)	16 (69.6%)
T2	17 (13.5%)	12 (15.2%)	4 (17.4%)
T3	3 (2.4%)	1 (1.3%)	2 (8.7%)
Tx	4 (3.2%)	1 (1.3%)	1 (4.3%)
N0	48 (38.1%)	33 (41.8%)	4 (17.4%)
N1	10 (7.9%)	6 (7.6%)	4 (17.4%)
N2	21 (16.7%)	16 (20.3%)	4 (17.4%)
N3	1 (0.8%)	0	0
Nx	46 (36.5%)	24 (30.4%)	11 (47.8%)
Grading			
G1	11 (8.7%)	5 (6.3%)	2 (8.7%)
G2	82 (65.1%)	52 (65.8%)	16 (69.6%)
G3	29 (23.0%)	19 (24.1%)	4 (17.4%)
not determined	4 (3.2%)	3 (3.8%)	1 (4.3%)
HPV-subtypes			
16			18 (76%)
33			3 (12%)
33 + 16			3 (12%)

#HPA036462), YTHDF1 (1:10; Biorbyt #orb179018), YTHDF2 (1:200; Biorbyt #orb39199), YTHDF3 (1:200; Biorbyt #orb374095) at 4 °C overnight. Signal detection was performed with the UltraView DAB IHC Detection Kit (Ventana).

Immunostained cells were analyzed with an Olympus BX51 microscope and the Panoramic Viewer 3DHistech. Staining intensities were evaluated for all m6A proteins separately by MC and DJR. In case of discordance between these two investigators, TT was consulted as a board-certified gynecopathologist. In addition, random reviews of the staining intensities were conducted by TT. In detail, a four-tier scoring system was applied to categorize staining intensities (0: no staining, 1: low staining, 2: moderate staining, 3: high staining). Staining intensities were divided into two groups (low and high expression) based on the median protein expression in the entire study cohort.

Statistical analysis

Kaplan–Meier survival analyses and log-rank tests allowed to compare OS between the two groups (low vs. high expression) for each analyzed protein. Correlation analyses were performed applying the nonparametric Mann–Whitney U test. In addition, the two-sided Fisher's exact test was used for the evaluation of statistical significance; a significance threshold was considered at a p-value of < 0.05. We performed multiple hypotheses testing using the method of Benjamini and Hochberg and converted p-values to false discovery rate (FDR) q-values with a significance threshold of $q < 0.1$. Non-parametric Spearman's p correlation coefficients were calculated for co-expression analysis. Statistical analysis was performed with the Statistical Package for the Social Sciences (SPSS ®) version 28 (SPSS Inc., IBM Corp.) and the GraphPad Prism software (GraphPad software).

Results

Proteins of m6A are frequently expressed in VSCC

Across the cohort of 126 primary VSCC samples (Table 1) we identified protein expression of all distinct m6A writers, readers and erasers. The proteins involved in the different m6A functions were present in the different cell compartments reflecting the diversity of RNA metabolism. Writers were typically observed in the nucleus including METTL3, METTL14, WTAP and KIAA1429. Likewise, immunohistochemical analysis revealed a strong nuclear staining for the eraser FTO, and the two readers HNRNPC und HNRNPA2B1. In contrast, the readers YTHDF1, YTHDF2 and YTHDF3 as well as the writer METTL4 showed a strong cytoplasmic staining (Table 2).

Proteins of m6A are differently expressed in VSCC subtypes

Given the two etiologically distinct VSCC subgroups, namely HPV-dependent and HPV-independent VSCC, each subgroup was next examined separately. In the HPV-dependent subgroup of 23 patients, 76% of cases were positive for HPV type 16, 12% for HPV type 33, and 12% displayed a co-infection with both HPV types 16 and 33. The HPV-independent cohort comprised 79 patients. For 24 patients, HPV status was unknown (Table 1).

First, we analyzed m6A proteins for their different expression regarding to the VSCC subtypes. For most m6A proteins (10/13), we did not find a differential expression between the two etiologic subtypes (Fig. 1A-B, D-E, H-M). However, we observed differences for 3 proteins that were all significantly enriched in HPV-dependent VSCC: the writer METTL14 (63% vs 34% in HPV-independent VSCC; $p=0.049$, Fisher's exact test; Fig. 1C), and the erasers FTO (47% vs 13% in HPV-independent VSCC; $p=0.002$, Fisher's exact test; Fig. 1F), and ALKBH5 (79% vs 59% in HPV-independent VSCC; $p=0.040$, Fisher's exact test; Fig. 1G).

Proteins of m6A indicate poor outcome in HPV-dependent but not HPV-independent VSCC

In the entire cohort, none of m6A proteins analyzed was associated with OS (Table 2). Likewise, when focusing our analysis on HPV-independent VSCCs only, we also did not find an association with outcome (Table 2). However, when evaluating the subgroup of HPV-dependent VSCC, high expression levels of the writers METTL3 ($p=0.010$, $q=0.08$, log-rank test and Benjamini and Hochberg corrected log-rank test; Fig. 2A-C; Table 2), METTL 14 ($p=0.020$, $q=0.09$, Fig. 2D-F) and the reader YTHDC1 ($p=0.012$, $q=0.08$, F ig. 2G-I) were significantly correlated with shorter OS. Increased expression of the writer METTL4 ($p=0.034$, Supplementary Fig. 1A-C) and the reader YTHDF2 ($p=0.040$, Supplementary Fig. 1D-F) were also associated with poor outcome but did not remain significant when correcting for multiple hypothesis testing at a significance threshold of $q<0.1$. Protein expression levels of METTL3, METTL4, METTL14, YTDHC1, and YTHDF2 were not associated with the clinicopathological parameters nodal stage and histomorphological grading in the entire study cohort and the two subgroups, respectively (Supplementary Table 1).

We found high positive Spearman's p correlation coefficients for the expression of the prognostic m6A proteins METTL3, METTL14, and YTDHC1, respectively in the HPV-dependent subgroup (Fig. 3). In 6/15 patients, high levels of the writers METTL3 and METTL14 were cooccurring (Spearman's $p=0.797$; two-sided t-test $p=<0.001$). Likewise, in 5/15 patients the writer METTL3 and the reader YTHDC1 (Spearman's $p=0.036$; two-sided t-test $p=0.872$) and in 7/15 patients the writer METTL14 and YTHDC1 (Spearman's $p=0.443$; two-sided t-test $p=0.034$) were at high levels. Of these 3 proteins, we identified METTL14 to be the protein that typically cooccurred with the others in contrast to METTL3 and YTHDC1 that gave additional information to the other two.

Discussion

In the present study, our analysis suggests that expression levels of the 'writers' METTL3 and METTL14 and the 'reader' YTHDC1 are involved in HPV-dependent VSCC tumorigenesis, but not HPV-independent tumor development. HPV is a small DNA virus that is usually transmitted sexually. Sexually active individuals carry a lifetime risk for HPV infection of around 80–90% [24]. It is estimated, that 5% of human cancers are caused by a persistent infection with high risk HPV types [25] including not only VSCC but also cervical, penile, and head and neck SCC [26]. HPV-dependent VSCC account for 30% of all VSCC cases and exhibit a more favorable prognosis compared to the HPV-independent VSCC subtype. Although HPV-dependent and HPV-independent VSCC represent etiologically different subtypes, both are treated equally in current clinical practice [27].

Previous research has shown that m6A modification is implicated in viral infection by modulating the interaction between the virus and the host. Thus, m6A can influence both, the susceptibility of the host cells to viral infection, and the replication of the virus in the host cell [28, 29]. There is only sparse known regarding m6A modification in the context of HPV infection and cancer. In cervical cancer (CC), that is predominantly caused by infection with high-risk HPV, there is broad scientific evidence, that abnormal m6A modification plays an essential role in tumor proliferation, angiogenesis and

Table 2 Summary of the analyzed m6A proteins as indicated and their correlation with overall survival (indicated as %alive) for the entire study cohort, HPV-independent, and HPV-dependent VSCC. The HPV-status was not available for 24 patients. Samples were grouped according to high and low expression based on the staining intensities. *p*-values for the group comparisons are based on log-rank tests (significance threshold $p < 0.5$). *q*-values are based on multiple hypotheses testing using the method of Benjamini and Hochberg with a significance threshold of $q < 0.1$

			All			HPV-independent			HPV-dependent			
Proteins	**Localisation**	**Staining intensity (low/high)**	**N (low/high)**	**%alive (low/high)**	***p*-value**	**N (low/high)**	**%alive (low/high)**	***p*-value**	**N (low/high)**	**%alive (low/high)**	***p*-value**	***q*-value**
Writer												
METTL3	nuclear	0–2 + /3 +	77/27	62.3/40.7	0.104	51/18	56.9/38.9	0.435	11/7	81.8/28.6	**0.010**	**0.08**
METTL4	cytoplasmatic	0–1 + /2–3 +	60/36	58.3/50.0	0.113	44/24	54.5/45.8	0.128	8/7	87.5/42.9	**0.034**	0.10
METTL14	nuclear	0–1 + /2–3 +	54/45	55.6/55.6	0.586	44/23	47.7/60.9	0.541	6/10	100/55.6	**0.020**	**0.09**
WTAP	nuclear	0–2 + /3 +	90/16	54.4/68.8	0.311	63/6	49.2/83.3	0.132	16/4	68.8/75.0	0.694	0.74
KIAA1429	nuclear	0–1 + /2–3 +	66/35	56.1/60.0	0.782	47/22	53.2/59.1	0.661	10/5	60.0/40.0	0.482	0.70
Eraser												
FTO	nuclear	0–2 + /3 +	83/21	55.4/76.2	0.113	61/9	54.1/66.7	0.361	10/9	50.0/77.8	0.257	0.48
ALKBH5	cytoplasmatic/ nuclear	0–1 + /2–3 +	44/63	50.0/65.1	0.231	35/35	45.7/62.9	0.161	4/15	50.0/66.7	0.699	0.74
Reader												
HNRNPA2B1	nuclear	0–2 + /3 +	85/23	57.6/73.9	0.429	62/10	54.8/70.0	0.602	17/2	58.8/100	0.297	0.48
HNRNPC	nuclear	0–1 + /2–3 +	54/48	51.9/62.5	0.162	34/35	50.0/54.3	0.518	11/7	63.6/71.4	0.736	0.74
YTHDC1	membraneous/ cytoplasmatic/ nuclear	0–2 + /3 +	78/23	61.5/43.5	0.111	58/12	58.6/41.7	0.496	12/7	83.3/28.6	**0.012**	**0.08**
YTHDF1	cytoplasmatic	0–2 + /3 +	70/26	57.1/50.0	0.893	52/15	55.8/33.3	0,409	10/4	60.0/75.0	0.551	0.72
YTHDF2	cytoplasmatic	0–1 + /2–3 +	31/73	58.1/54.8	0.422	21/48	52.4/52.1	0.665	5/13	100/46.2	**0.040**	0.10
YTHDF3	cytoplasmatic	0–1 + /2–3 +	45/51	55.6/58.8	0.725	28/37	50.0/56.8	0.554	6/7	66.7/57.1	0.084	0.18

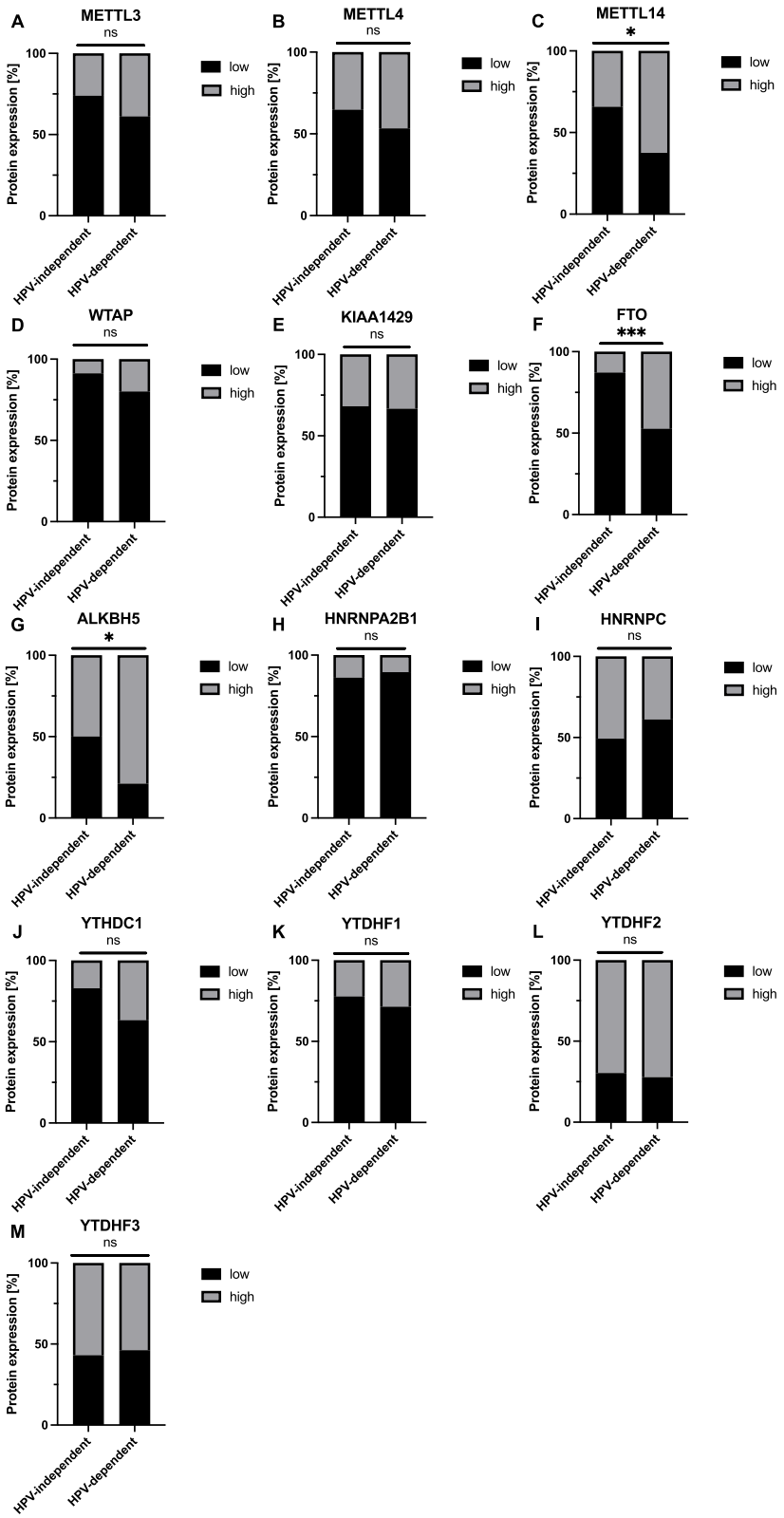

Fig. 1 Differential expression (high vs. low) of m6A protein depending on the VSCC subtype. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$ (Fisher's exact test)

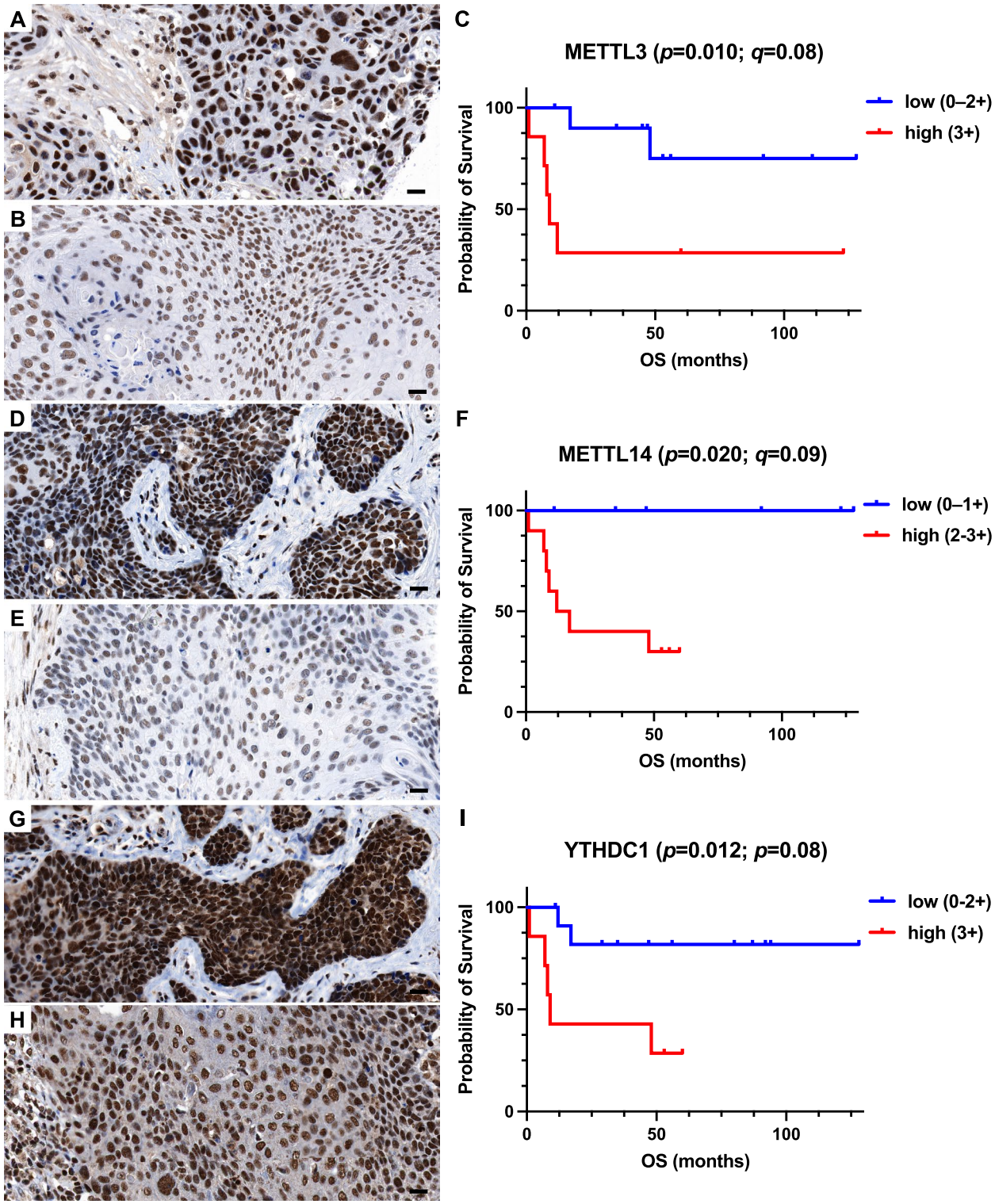

Fig. 2 Representative histology sections show high (**A**, **D**, **G**) and low (**B**, **E**, **H**) expression levels of METTL3, METTL14 and YTHDC1 visualized by immunohistochemistry; hematoxylin (blue) was used for nuclear staining (bright field image, 400xmagnification). Kaplan–Meier estimates show a significantly shorter 5-year survival ($p < 0.05$) in patients with high expression of METTL3, (**F**) METTL14, and (I) YTHDC1. Prognostic significance remained after correction for multiple testing ($q < 0.1$). Scale bar = 20 um

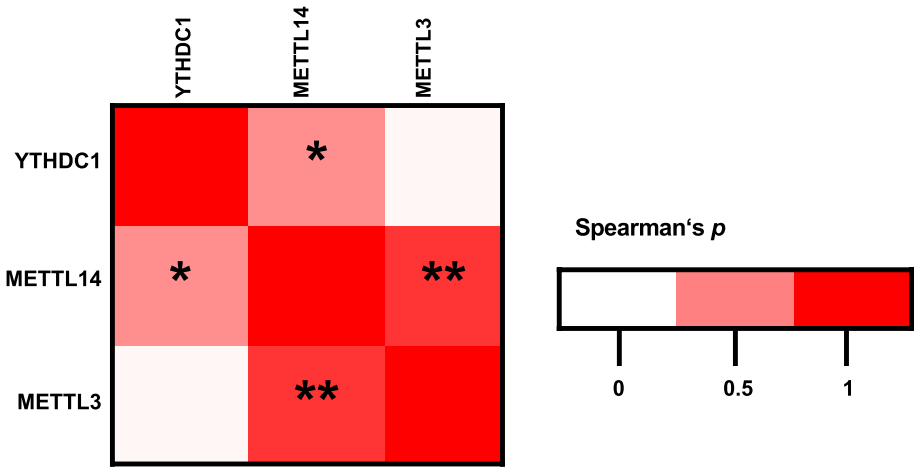

Fig. 3 Correlation heatmap visualizes Spearman's *p* correlation coefficients of METTL3, METTL14, and YTDHC1 in the HPV-dependent VSCC cohort. * $P < 0.05$; ** $P < 0.01$ (two-sided t-test)

metastatic spread. METTL3 was shown to be upregulated in CC cells and linked to lymph node metastasis and unfavorable outcomes [30]. Further, m6A dysregulation is linked to chemo- and radiotherapy-resistance and a more progressive CC phenotype [17]. In this context, especially the demethylase FTO was identified to be an important oncogenic driver by regulating proliferation and migration of CC cells [31]. Another study confirmed the importance of METTL14 in CC. Silencing METTL14 induced a cell cycle arrest in CC cells via the the PI3K/AKT/mTOR signaling pathway [32]. The interaction between m6A and the PI3K/AKT/mTor signaling pathway has also been described for endometrial cancer and further entities [16, 33]. The etiologic resemblance of CC and VSCC suggests dysregulated m6A modification to be involved in VSCC tumorigenesis. In head and neck SCC, which are frequently associated with HPV-infection, overexpression of METTL3 and METTL14 correlated with advanced T stage and poor OS [34]. Further, enhanced METTL3 expression was observed in oral SCC, that is also linked to HPV infection [35].

There is no data available regarding the precise biological mechanism of m6A modification and HPV-driven tumorigenesis. However, there is data on other oncogenic viruses like Kaposi's sarcoma-associated herpesvirus (KSHV): Research has shown, that depletion of METTL3 and YTHDF2 lead to lower expression levels of the lytic genes ORF50 and ORF57 as well as decreased virion production [36]. Lytic genes are required to enter the viral lytic replication cycle. These findings suggest m6A to promote a pro-viral environment for KSHV infection. Comparable data were obtained for simian virus 40. Here, overexpression of YTHDF2 was found to be associated with enhanced viral replication in BSC40 cells whereas depletion of YTHDF2 or METTLL3 lead to contrary effects [37].

Besides METTL3 and METTL14, our analysis also showed significant data for the reader YTHDC1 in the HPV-dependent VSCC subgroup. As YTHDF2, YTHDC1, is involved in mRNA splicing, nuclear export and translation. In the context of viral infection, research has shown, that YTHDC1 is involved in splicing of genes important for the lytic replication [36]. Given the involvement of m6A in HPV-dependent VSCC harbors the potential to be used therapeutically. 3-deazaadenosine (DAA) inhibits m6A modification and has exhibit antiviral effects in both, cell culture and mouse models of viral infection [38]. To date, it has not been studied whether there is also cytotoxic potential of DAA in HPV-dependent malignancies. In addition to direct drug targeting of methylation, inhibition of the PIK3/AKT/mTOR signaling pathway might be a promising therapeutic option, in particular due to the described interaction between m6A and this pathway. There are various therapeutic agents that could be considered, such as everolimus or the PIK3 inhibitor alpelisib. So far, these therapeutics have not been investigated in VSCC, but, however, might be of potential interest.

Our findings point towards the important role of m6A RNA modification in cancer and especially in HPV-dependent tumors. This is the first study implicating the relationship between HPV infection, m6A RNA modification, and carcinogenesis in VSCC. However, as a limitation of the present study, the relatively small cohort size of 23 HPV-dependent VSCC has to be mentioned. Consecutively, multivariate statistical analyses could not be

performed. A further limitation is the retrospective study design and the determination of protein expression based on a tissue microarray with sigle cores per sample. Hence, tumor heterogeneity might not be adequately reflected by our method approach. However, to the best of our knowledge, there is no evidence for intratumoral heterogeneity regarding m6A protein expression analysis. Of note this is also reflected by our own data regarding m6A protein expression in endometrial and cervical cancer [22, 39].

Dysregulation of m6A proteins might be used as biomarkers and indicators for poor prognosis but also as potential targets for novel therapeutic drugs. However, the specific mechanisms explaining the interaction of m6A modification and HPV infection remains to be elucidated in further studies.

Conclusion

High expression levels of proteins involved in m6A modification correlate with a poor OS in patients with HPV-dependent VSCC. Hence, m6A might serve as a prognostic biomarker in HPV-dependent VSCC.

Abbreviations

CC: Cervical cancer; FDR: False discovery rate; FFPE: Formalin-fixed paraffin-embedded; FIGO: International Federation of Gynecology and Obstetrics; HE: Hematoxylin and eosin; HPV: Human papillomavirus; KSHV: Kaposi's sarcoma-associated herpesvirus; m6A: N6-methyladenosine (m6A); mRNA: Messenger RNA; OS: Overall survival; SCC: Squamous cell carcinoma; TMA: Tissue microarray; UICC: Union for International Cancer Control; VSCC: Vulvar squamous cell carcinoma; WHO: World Health Organization (WHO).

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s12885-022-10010-x.

Additional file 1.

Additional file 2.

Acknowledgements

The tissue examples were collected within the framework of the Biobank of the CIO Cologne-Bonn at the University Hospital Bonn. We thank Susanne Steiner for technical support.

Authors' contributions

M.C., T.T. and D.J.R. were involved in the study design and concept. M.C. and D.J.R. drafted the manuscript. M.C., T.T., C.S. and D.J.R. performed the experiments and statistical analysis. N.K., J.E., E.E., K.K., G.K. and A.M. revised the manuscript for critical intellectual content. All authors read and approved the final version of the manuscript.

Funding

Open Access funding enabled and organized by Projekt DEAL. D.J.R is supported by the BONFOR program of the Medical Faculty of the University of Bonn (grant ID 2021-1A-14). N.K. is supported by junior research group funding by the BONFOR program of the Medical Faculty of the University of Bonn (grant ID 2020-2A-12). K.K. is supported by the Private Excellence Initiative Johanna Quandt of the Stiftung Charité at BIH.

Availability of data and materials

The datasets generated and/or analyzed during the current study are available on request from the authors.

Declarations

Ethics approval and consent to participate

Tissue collection was performed within the framework of the Biobank initiative of the University Hospital Bonn. All patients provided written informed consent prior to the collection of biomaterials. The study was approved by the Ethics Committee of the Medical Faculty of the University of Bonn (vote: 208/21) and conducted in accordance with the Declaration of Helsinki.

Consent for publication

Not applicable.

Competing interests

The authors declare that there are no conflicts of interests.

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Received: 4 June 2022 Accepted: 16 August 2022
Published online: 01 September 2022

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Publisher's Note

4.4 Condic M, Egger EK, Klümper N, Kristiansen G, Mustea A, Thiesler T, Ralser DJ. TROP-2 is widely expressed in vulvar squamous cell carcinoma and represents a potential new therapeutic target. J Cancer Res Clin Oncol. 2023 Apr 17. doi: 10.1007/s00432-023-04761-8.

Zielsetzung - Studienziel war es zu analysieren, ob TROP-2 beim primärem Plattenepithelkarzinom der Vulva (VSCC) exprimiert wird und welchen prognostischen Wert die Expression auf das Überleben der Patientinnen hat.

Methodik und Ergebnisse - VSCC-Proben von *N*=103 Patientinnen wurden immunhistochemisch auf das Vorhandensein von TROP-2 untersucht. Es wurde eine Korrelationsanalyse mit klinisch-pathologischen Parametern durchgeführt, getrennt für die zwei Subkohorten der HPV-abhängigen und HPV-unabhängigen Tumore. In dem Kollektiv waren *N*=70 Tumore HPV-unabhängig, *N*=18 HPV-abhängig und für *N*=15 Fälle war kein HPV Ergebnis vorhanden. Eine TROP-2 Expression konnte in 97,1% der Fälle (*N*=100) nachgewiesen werden. Dabei zeigten 74,8% aller Fälle eine moderate bis hohe TROP-2 Expression. In der Subgruppe der HPV-abhängigen Tumore zeigten alle Proben ein moderate bis hohe TROP-2 Expression.

Betrachtet man den Einfluss der TROP-2 Expression auf das Überleben, so zeigte sich im Gesamtkollektiv ein Trend zur besseren Prognose bei hoher TROP-2 Expression (log rank *p*=0.058). Für die Subkohorte der HPV-unabhängigen Karzinome war eine moderat bis hohe TROP-2 Expression statistisch signifikant mit einem längerem Gesamtüberleben assoziiert (log rank *p*=0.048). In der univariaten Cox-Analyse bestätigte sich TROP-2 als prognostischer Marker im VSCC.

Zusammenfassung - Zusammengefasst zeigen unsere Ergebnisse, dass TROP-2 im VSCC weitgehend exprimiert wird und eine hohe Expression ein prognostischer Marker für ein besseres Gesamtüberleben ist. Somit könnte sich Sacituzumab-Govitecan, ein TROP-2 gerichtete ADC, für die Behandlung des Vulvakarzinoms eignen. Die hohe Expression in HPV-abhängigen Tumoren deutet auf einen mechanistischen Zusammenhang zwischen HPV-Infektion und TROP-2 hin.

Journal of Cancer Research and Clinical Oncology
https://doi.org/10.1007/s00432-023-04761-8

RESEARCH

TROP-2 is widely expressed in vulvar squamous cell carcinoma and represents a potential new therapeutic target

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Received: 13 March 2023 / Accepted: 8 April 2023

Abstract

Purpose Vulvar squamous cell carcinoma (VSCC) is a rare malignancy of the female genital tract with increasing incidence rates. Etiologically, HPV-dependent and HPV-independent VSCC are distinguished. Surgical treatment and/or radiotherapy represent the therapeutic mainstay for localized disease. For recurrent or metastatic VSCC, treatment options are limited. Research has identified trophoblast cell surface antigen 2 (TROP-2) to be broadly expressed across different tumor entities. The aim of the present study was to systematically investigate the expression of TROP-2 in VSCC.

Methods TROP-2 protein expression was investigated by immunohistochemistry in a cohort comprising $n = 103$ patients with primary VSCC. A four-tier scoring system (0: no staining, 1+: low staining, 2+: moderate staining, 3+: high staining) was applied for quantification of protein expression. For further analyses, two groups (low TROP-2 expression: 0/1+; high TROP-2 expression: 2+/3+) were generated. The entire study cohort, as well as HPV-dependent and HPV-independent VSCC were considered separately.

Results In the entire VSCC study cohort, TROP-2 expression was present in 97.1% of all cases ($n = 100$) with 74.8% displaying high TROP-2 expression (2+/3+). Only 2.9% of tumors showed absent TROP-2 expression. Of note, all HPV-dependent VSCC ($n = 18$) demonstrated high TROP-2 expression (2+/3+). In the subgroup of HPV-independent VSCC ($n = 70$), high TROP-2 expression was associated with favorable clinical outcomes based on log rank test and univariate cox analysis.

Conclusion TROP-2 protein expression is of prognostic value in HPV-independent VSCC. The broad expression of TROP-2 in VSCC indicates the TROP-2 directed ADC Sacituzumab govitecan as a potential new therapeutic strategy for VSCC patients.

Keywords Vulvar squamous cell carcinoma · TROP-2 · Antibody-drug conjugate · HPV

Introduction

Vulvar cancer (VC) is the fourth most common genital tumor in women, accounting for 3% of all gynecological cancers worldwide (Sankaranarayanan and Ferlay 2006). Albeit VC is considered a rare tumor entity, its incidence has increased over the recent decade by 20%. This is mainly attributed to the increased prevalence of human papillomavirus (HPV) infections and the overall demographic aging of the population (Schuurman et al. 2013; Kang et al. 2017).

The predominant histological VC-subtype is vulvar squamous cell carcinoma (VSCC). Etiologically, HPV-dependent VSCC, which account for one-third of the cases (Zhang et al. 2018), and HPV-independent VSCC, which develop on the basis of *lichen sclerosus et atrophicus* (Bleeker et al. 2016), are distinguished. Besides the different etiology, the prognosis of

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Published online: 17 April 2023

these two VSCC subtypes also differs with HPV-independent VSCC exhibiting a comparably worse overall prognosis. The therapeutic mainstay for localized VSCC is surgical resection of the tumor with bilateral sentinel inguinofemoral lymphonodectomy or systematic bilateral inguinofemoral lymphonodectomy, followed by radiotherapy if appropriate risk factors are present. Whereas localized VSCC has an 5-year overall survival (OS) rate of over 85% (Schnürch et al. 2015), recurrent, advanced or metastatic disease has a limited 5-year OS rate of only 15–50% (Nooij et al. 2016). This is predominantly attributed to the limited therapeutic options. Hence, for this population, there is an unmet need for new therapeutic options (Clancy et al. 2016).

Trophoblast cell surface antigen 2 (TROP-2) was initially described as a transmembrane protein on the surface of trophoblast cells (Ripani et al. 1998). In the last decade, research has shown that TROP-2 plays an essential role in regulatory processes of carcinogenesis and cancer progression thereby accomplishing the function of an oncogene. Recently, however, there is growing scientific evidence that TROP-2 is capable to promote both, tumor progression and tumor suppression, depending on the cell context and localization (Zhang et al. 2014; Zeng et al. 2016). The precise pathomechanism for these conflicting functions remains to be elucidated. Regardless of its function, TROP-2 represents a promising therapeutic target, especially since TROP-2 is widely overexpressed across different tumor entities (Goldenberg et al. 2018). Sacituzumab govitecan (SG) is an antibody–drug conjugate (ADC) consisting of an antibody targeting TROP-2 that is linked to the cytotoxic payload SN-38, a topoisomerase I inhibitor. SG has recently been approved for the treatment of metastatic triple-negative breast cancer and is considered for further indications with plethora of clinical trials ongoing (Bardia et al. 2019). However, up to now, SG is not considered in the treatment of VSCC. For cervical carcinoma (CC), a gynecological malignancy with etiological resemblance to VSCC, research has shown that gradual loss of TROP-2 plays a role in the progression of intraepithelial neoplasia to invasive carcinoma and exhibits tumor suppressive functions (Wang et al. 2014; Sin et al. 2019). Further, there is preclinical evidence that therapy directed against TROP-2 is effective in CC (Zeybek et al. 2020). To the best of our knowledge, there are no data on TROP-2 expression in VSCC. The aim of the present study was (i) to investigate the expression of TROP-2 in VSCC, (ii) to determine its impact on clinical outcomes, and (iii) to evaluate its potential to serve as a therapeutic target.

Methods

Patients and specimens

The retrospective single-center study cohort included $n = 103$ patients with primary VSCC treated at the University Hospital Bonn between 2002 and 2017. Tissue was obtained from biopsies or surgical specimens that were collected within the framework of the Biobank initiative of the University Hospital Bonn. The Ethics Committee of the Medical Faculty of the University of Bonn approved the study (vote: 208/21).

Clinicopathological characteristics of the entire cohort, and the HPV-independent and the HPV-dependent subcohorts were obtained from a clinical database. Details are depicted in Table 1. Histopathological diagnosis was based on the World Health Organization (WHO) criteria. Pathological grading was determined applying the International

Table 1 Clinicopathological characteristics of the entire VSCC cohort, HPV-independent cohort, and HPV-dependent cohort

Clinicopathological parameters	All ($n = 103$)	HPV-independent ($n = 70$)	HPV-dependent ($n = 18$)
Age (years)			
Mean (± SD)	64.8 ± 14.4	65.7 ± 14.0	56.9 ± 14.1
Min–max	25–93	33–93	25–77
Overall survival (months)			
Mean (± *SD*)	53.0 ± 43.5	57.6 ± 46.0	47.9 ± 41.3
Median	44.0	55.5	46.0
TNM classification			
T1	80 (77.7%)	56 (80.0%)	11 (61.1%)
T2	16 (15.5%)	12 (17.2%)	4 (22.2%)
T3	3 (2.9%)	1 (1.4%)	2 (11.1%)
Tx	4 (3.9%)	1 (1.4%)	1 (5.6%)
N0	37 (35.9%)	29 (41.4%)	2 (11.2%)
N1	10 (9.7%)	6 (8.6%)	4 (22.2%)
N2	20 (19.4%)	16 (22.9%)	4 (22.2%)
N3	1 (1.0%)	0	0
Nx	35 (34.0%)	19 (27.1%)	8 (44.4%)
Grading			
G1	8 (7.7%)	4 (5.7%)	1 (5.6%)
G2	66 (64.3%)	46 (65.7%)	12 (66.7%)
G3	26 (25.1%)	18 (25.7%)	4 (22.2%)
Not determined	3 (2.9%)	2 (2.9%)	1 (5.5%)
HPV-subtypes			
16			14 (77.8%)
33			3 (16.7%)
33 + 16			1 (5.5%)

For $n = 15$ patients, no HPV status was available

SD standard deviation

Federation of Gynecology and Obstetrics (FIGO) that was revised in 2010. Tumor stage was classified according to the 7th TNM classification of the Union for International Cancer Control (UICC).

Tissue microarray (TMA) construction

The TMA was generated from formalin-fixed paraffin (FFPE)-embedded VSCC tissue specimens. Representative tumor areas in hematoxylin and eosin (HE) stained sections were identified. 1 mm core biopsies (0.785 mm^2) were taken from the selected cancer areal and arranged in TMA blocks.

HPV analysis

Determination of HPV subtypes was performed applying the HPV Type 3.5 LCD-Array Kit (Chipron, Germany) according to the manufacturer's instructions and as described previously (Hecking et al. 2017).

Immunohistochemistry

Immunostaining of TROP-2 was performed on VSCC-TMAs using an automated staining system (BenchMark ULTRA; Ventana Medical Systems, Tucson, AZ, USA) which performed deparaffinization, pretreatment with cell conditioning buffer (CC1 buffer, pH8), and incubation with the primary TROP-2 antibody (1:1500, Enzo Life Sciences Inc, Farmingdale NY, USA, Clon-01 IgG1, mouse) at 4 °C overnight. Signal detection was obtained with the UltraView DAB IHC Detection Kit (Ventana Medical Systems, Tucson, AZ, USA). Analysis of immunostained cells was carried out with an Olympus BX51 microscope and the Panoramic Viewer 3DHistech. All staining intensities were evaluated separately by MC and DJR. TT was consulted as a board-certified gynecopathologist in case of discordance between these two investigators. Staining intensities were categorized in a four-tier scoring system (0: no staining, 1 + : low staining, 2 + : moderate staining, 3 + : high staining). Two groups, low (0/1 +) and high (2 + /3 +) expression, were generated for survival analysis.

Statistical analysis

Kaplan–Meier survival analyses, log-rank tests and univariate cox analyses were conducted to compare OS between the two groups (low vs. high expression of TROP-2) for the entire study cohort and the HPV-independent subgroup. Significance threshold was considered at a p-value of < 0.05. Statistical analysis was performed with the Statistical Package for the Social Sciences (SPSS ®) version 29 (SPSS Inc., IBM Corp.) and the GraphPad Prism software (GraphPad software).

Results

TROP-2 is frequently expressed in VSCC

Immunohistochemical staining of TROP-2 revealed strong membranous expression. In the entire study cohort, TROP-2 expression was present in 97.1% of all cases ($n = 100$). Only 2.9% of tumors showed absent TROP-2 expression (0, Fig. 1A). Low expression (1 +, Fig. 1B) was detected in 22.3% of the cases. The majority of all samples (74.8%, $n = 77$) exhibit moderate (2 +, Fig. 1C) to high (3 +, Fig. 1D) TROP-2 expression. The distribution of the different expression intensities across the entire study cohort and the individual sub-cohorts (HPV-independent/dependent tumors) is depicted in Fig. 1E.

HPV-dependent VSCC displays exclusively moderate to high TROP-2 expression

The sub-cohort of HPV-dependent VSCC comprised $n = 18$ cases. $n = 70$ cases were HPV negative and for $n = 15$ cases, HPV testing was inconclusive. Whereas TROP-2 expression covered the whole expression intensity spectrum in the HPV-independent VSCC population, HPV-dependent VSCC exhibit exclusively high TROP-2 membranous staining pattern (Fig. 1 E).

High expression of TROP-2 correlates with favorable outcomes in HPV-independent tumors

In the entire study cohort, the median OS was 44 months. In the entire cohort, TROP-2 expression was not significantly associated with OS (Fig. 2A, log rank $p = 0.058$). However, there was a trend indicating that tumors with high TROP-2 expression levels exhibit a better prognosis. In the subgroup of HPV-independent VSCC, TROP-2 expression was significantly linked to OS. In particular, low TROP-2 expression (0/1 +) was linked to a shorter OS compared to high TROP-2 expression (2 + /3 +; p(log) = 0.048; HR 0.5 (95%CI: 0.248–1.009), p(cox) = 0.05; Fig. 2B). TROP-2 protein expression was not associated with known clinicopathological prognostic parameters like nodal stage and histomorphological grading.

Discussion

The present study is, to the best of our knowledge, the first systematic investigation of TROP-2 protein expression in VSCC tissue. TROP-2 was found to be broadly expressed in VSCC which is highly relevant from a therapeutic point of

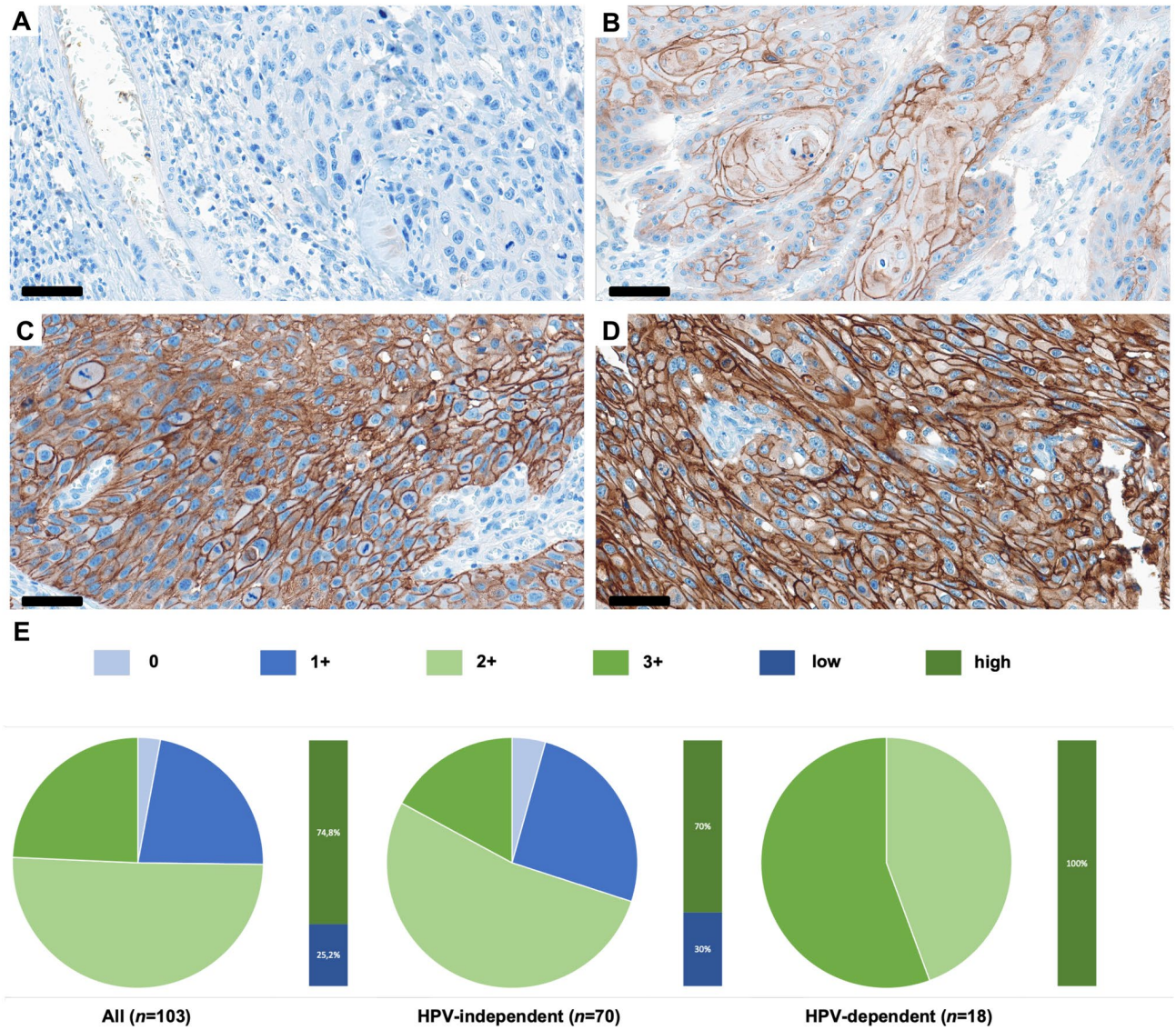

Fig. 1 Representative histology sections showing absent (0; A), low (1+; B), moderate (2+, C), and high (3+, D) TROP-2 protein expression. (E) Pie charts illustrating the distribution of expression intensities in the entire VSCC cohort, the HPV-independent VSCC cohort, and the HPV-dependent VSCC cohort. Bar graphs showing differentiation into low (0, 1+) and high (2, 3+) TROP-2 protein expression. Scale bar = 60 µm

view as there is an approved TROP-2 directed therapy with the ADC SG available and further TROP-2 ADC are currently in preclinical and clinical testing. Further, we provide evidence for the prognostic value of TROP-2 protein expression in HPV-independent VSCC. In our HPV-independent VSCC study cohort, higher TROP-2 expression levels were linked to favorable clinical outcomes.

TROP-2 was previously classified as an oncogene and high TROP-2 expression levels were linked to poor prognosis across various tumor entities including breast cancer (Lin et al. 2014), prostate cancer (Trerotola et al. 2013), and colon cancer (Zhao et al. 2015). However, contrasting tumor suppressive properties for TROP-2 have been described in head and neck carcinoma and lung carcinoma (Zhang et al. 2014; Erber et al. 2021). A recent study showed epithelial-mesenchymal transition of keratinocytes and consecutive skin tumor formation in TROP-2 knockout ($ARF^{-/-}$ C57BL/6) mice (Wang et al. 2011), suggesting that in the context of squamous cell carcinoma, TROP-2 fulfills the function of a tumor suppressor. Another study investigated TROP-2 expression in normal tissue and in squamous cell carcinoma (SCC) of the cervix, esophagus, and head and neck. The authors found that a gradual loss of TROP-2 was associated with a stepwise progression from precursor lesions to invasive SCC. Additionally, TROP-2 expression affected treatment response (Wang et al. 2014). Further evidence

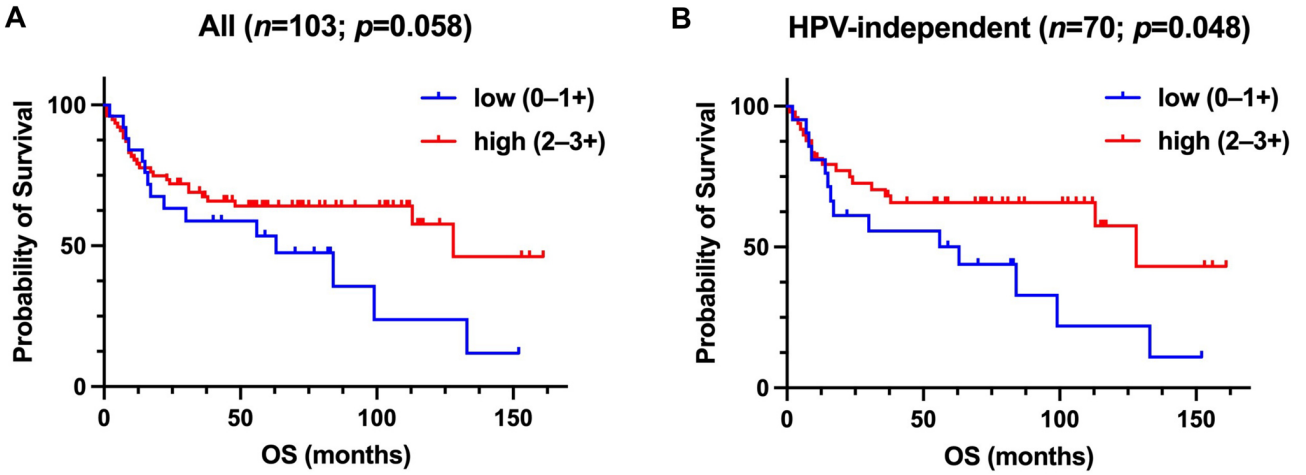

Fig. 2 Kaplan–Meier estimates show a trend towards a shorter overall survival (OS; $p=0.058$) in patients with low expression of TROP-2 in the entire VSCC cohort (A). In HPV-independent VSCC, high TROP-2 expression was linked to favorable OS ($p=0.048$; B). *P*-values for the group comparison (low vs., high expression) are based on log-rank tests, significance threshold $p<0.5$

for the tumor suppressive function of TROP-2 is provided by *Sin* et al. who demonstrated that TROP-2 reduced oncogenicity of CC cells (Sin et al. 2019). Congruent with these results, TROP-2 was broadly expressed in our VSCC-cohort and low expression levels were associated with unfavorable outcomes. Thus, TROP-2 could potentially exert a tumor suppressive function in VSCC, with loss of TROP-2 appearing as a sign of increasing dedifferentiation in aggressive VSCC. Of particular note, we found that all HPV-dependent VSCC samples showed moderate to high TROP-2 expression which indicates a connection between HPV-infection, TROP-2 expression and the etiology of HPV-dependent VSCC. Large proteomic analyses have identified more than 100 signaling pathways that are modulated by TROP-2, including the PI3K/AKT/mTOR pathway (Guerra et al. 2016). In the context of HPV-dependent SCC, alterations in the PI3K pathway are also described (Cochicho et al. 2022). The specific interaction between TROP-2 expression and HPV needs to be further elucidated. Besides the identification of TROP-2 protein expression to serve as a prognostic biomarker in HPV-independent VSCC, the broad expression of TROP-2 in VSCC provides a strong rationale to evaluate the anti TROP-2 ADC SG in clinical VSCC trials. Additional supporting data is available from Zeybek et al. (Zeybek et al. 2020), who found a moderate to strong TROP-2 staining in 95% of squamous cell cervical carcinoma by immunohistochemistry. TROP-2 positive cervical cancer cell lines were highly sensitive to SG in vitro (Zeybek et al. 2020). From a mechanistic point of view, tumorous TROP-2 expression represents the biological prerequisite for effective SG treatment. However, data from the approval relevant ASCENT study showed, that for triple negative breast cancer, the level of TROP-2 expression is not predictive for SG treatment response (Bardia et al. 2021). However, we have previously shown that target protein expression is relevant for the response to other ADCs, such as Enfortumab vedotin for patients with metastatic urothelial cancer (Klumper et al. 2022). Considering that in our study only 2.9% of VSCC were TROP-2 negative and 75% expressed moderate to high levels of TROP-2, is seems reasonable to propose SG as a novel targeted therapy option in VSCC. However, this needs to be further investigated in ideally biomarker-driven clinical trials.

Conclusion

In this study, we demonstrate that TROP-2 is broadly expressed in VSCC. In our study cohort, HPV-dependent VSCC showed exclusively high expression levels of TROP-2, indicating a relationship between TROP-2 and HPV-dependent VSCC etiology. In HPV-independent VSCC, TROP-2 expression was of prognostic value and appears to have tumor suppressive function. Considering the broad expression of TROP-2 in VSCC, our study provides the rationale to evaluate TROP-2 directed therapeutic approaches in VSCC.

Acknowledgements The tissue examples were collected within the framework of the Biobank of the CIO Cologne-Bonn at the University Hospital Bonn. We thank Susanne Steiner for technical support.

Author contributions MC, TT and DJR: were involved in the study design and concept. MC and DJR: drafted the manuscript. MC, TT and DJR: performed the experiments and statistical analysis. EKE, NK, GK and AM: revised the manuscript for critical intellectual content. All authors read and approved the final version of the manuscript.

Funding Open Access funding enabled and organized by Projekt DEAL. D.J.R is supported by the BONFOR program of the Medical Faculty of the University of Bonn (grant ID 2021-1A-14). N.K. is supported by junior research group funding by the BONFOR program of the Medical Faculty of the University of Bonn (grant ID 2020-2A-12).

Availability of data and materials The datasets generated and/or analyzed during the current study are available on request from the authors.

Declarations

Conflict of interest The authors declare that there are no conflicts of interests.

Ethical approval and consent to participate Tissue collection was performed within the framework of the Biobank initiative of the University Hospital Bonn. All patients provided written informed consent prior to the collection of biomaterials. The study was approved by the Ethics Committee of the Medical Faculty of the University of Bonn (vote: 208/21) and conducted in accordance with the Declaration of Helsinki.

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5. Diskussion

Die oben aufgeführten Arbeiten beschäftigen sich mit der Früherkennung, den Risikofaktoren, Biomarkern und der Tumorimmunologie bei HPV-assoziierten gynäkologischen Tumoren, insbesondere dem Vulva- und Zervixkarzinom.

Wie bereits in der Einleitung erwähnt, ist eine HPV Infektion obligat für die Entstehung eines Zervixkarzinoms, beim Vulvakarzinom sind ungefähr 34% HPV bedingt.

Daten aus randomisierten kontrollierten Studien und Meta-Analyse belegen, dass ein Screening auf HPV bei der Erkennung höhergradiger intraepithelialer Neoplasien der Zervix und des Zervixkarzinoms sensitiver ist als die herkömmliche Zytologie(39, 43, 87). Die Integration von HPV-Testungen in ein Vorsorgeprogramm führte zu einem Rückgang der Zervixkarzinom Inzidenz(88). Dies ist zum Teil auf höhere Erkennungsraten von Adenokarzinomen des Gebärmutterhalses und seiner Vorläuferläsionen zurückzuführen, da diese Untergruppe bei zytologischen Methoden oft unterdiagnostiziert wird. Außerdem zeigten Studien, dass Frauen, die negativ auf HPV high risk Typen getestet wurden, ein sehr geringes Risiko für die Entwicklung von CIN III oder des Zervixkarzinoms aufweisen(89). Basierend auf diesen Daten wurde in Deutschland das neue nationale Zervixkarzinom Screening-Programm mit der Einführung eines PAP-Abstrichs/HPV-Ko-Testung für Frauen ab dem 35. Lebensjahr eingeführt. Bereits nach kurzer Zeit wurde deutlich, dass mit diesem neuen Leitfaden der Bedarf an Kolposkopien in Deutschland massiv ansteigen wird. Dies beruht vor allem an der Vorgabe, dass Frauen mit unauffälliger Zytologie, aber einem positiven Test auf HPV high risk Typen in zwei aufeinanderfolgenden Jahren, verpflichtend zu einer Abklärungskolposkopie geschickt werden müssen. Daten aus Studien zeigen für dieses Kollektiv eine CIN III Rate von 3-7%(90). Eine weitere Studie aus Deutschland bei Frauen über dem 30. Lebensjahr, die zytologisch unauffälligen aber high risk HPV positiv waren, ergab eine CIN III Rate von 9.2%(91). In unserem Kollektiv wurden 52.3% der Patientinnen aufgrund einer positiven high risk HPV Testung aber unauffälligen Zytologie zur weiteren Abklärung in die Dysplasiesprechstunde überwiesen. Nach histologischer Abklärung zeigte sich in nur 2.1% der Fälle eine CIN III und in 6.3% der Fälle eine CIN II. Es bleibt abzuwarten, welche Zahlen aus anderen Zentren gemeldet werden. Nach unseren Daten zu urteilen ist der Benefit einer Kolposkopie bei zytologisch unauffälligen Frauen mit einer HPV Persistenz fraglich, eher wird eine große Anzahl an vermeidbaren Untersuchungen verursacht. Außerdem ist in unserer Analyse aufgefallen, dass sich in 13,7% der Fälle die HPV Ergebnisse aus dem Scree-

ning mit der in domo durchgeführten zweiten Testung nicht deckten. Alle verwendeten Testkits basierten auf einer DNA-Testung. Diese können nur zwischen dem Vorhandensein und dem Fehlen von HPV-spezifischer DNA unterschieden, aber nicht zwischen einer aktiven oder inaktiven Infektion(92). Tests, die E6/E7-mRNA nachweisen, zeigen eine höhere klinische Spezifität als DNA-basierte Tests, da E6/E7-mRNA nur in aktiv infizierten Zellen gefunden wird(93, 94), diese werden in Deutschland aber aktuell nicht häufig angewendet. Ob die veränderte Vorsorge langfristig zu einem Rückgang der Zervixkarzinom Inzidenz in Deutschland oder nur zu einer Zunahme von vermeidbaren Untersuchungen führen wird, bleibt abzuwarten.

Die Analyse des menschlichen Mikrobioms ist über die letzten Jahre in den wissenschaftlichen Fokus gerückt. Durch die Anwendung neuer Methoden, insbesondere der 16S rRNA-Sequenzierung, wurden tiefgründigere Analysen möglich gemacht und mit der Entstehung vieler Erkrankungen und auch unterschiedlicher Tumore in Verbindung gebracht. Einzelne Studien zeigten auch einen Zusammenhang zwischen dem vaginalen Mikrobiom und der Entstehung von zervikalen Dysplasien. So konnte man im Vergleich zwischen HPV-negativen und HPV-positiven Frauen eine erhöhte Rate an HPV-Eliminierung nachweisen, wenn das Mikrobiom durch Laktobazillen dominiert war(95). Passend hierzu wiesen Frauen mit vermehrter Anzahl an Gardnerellen auch höhere Raten an CIN-CIN III auf. Aufgrund solcher Studien wurden auch immer mehr Produkte zur Optimierung des vaginalen Mikrobioms eingeführt. Dabei handelt es sich meist um Zäpfchen mit unterschiedlichen Laktobazillen als Inhaltsstoff, die bei regelmäßiger Anwendung das vaginale Mikrobiom optimieren und so die HPV-Eliminierung unterstützen sollen.

Bei allen Studien gilt zu beachten, dass diese auf einer geringen Fallzahl beruhen. Das größte Kollektiv bestand aus 169 Patientinnen(54). Außerdem hängt die Mikrobiom Analyse stark von den verwendeten Primern ab, eine Verfälschung der Genus/Species Auflösung kann bei der Amplifikation nicht repräsentativer Genomregionen auftreten(96).

Die in dieser Arbeit aufgeführte Studie analysierte das Mikrobiom von 310 Patientinnen. Wir sahen bemerkenswertesten Unterschiede in Bezug auf die Alpha-Diversität und *Lactobacillus crispatus* in Abhängigkeit von der Menopause. Nur die Besiedlung mit *Ureaplasma parvum* korrelierte mit histologisch gesicherten Dysplasien der Zervix. In der Literatur sind drei Studien über einen möglichen Zusammenhang zwischen U. parvum, HPV und intraepithelialen Neoplasien des Gebärmutterhalses publiziert(97-99). Hierzu sind weitere Studien erforderlich, da Ureaplasa parvum gemäß der Leitlinie keine zwingende Therapieindikation

darstellt. Außerdem müssen mikrobiombasierte Studien an einer größeren Anzahl von Probanden durchgeführt werden, um einen eindeutigen Zusammenhang zwischen HPV-Persistenz, Mikrobiom und der Entstehung von Dysplasien festzustellen.

Um der Fragestellung nach Biomarkern in HPV-assoziierten Tumoren nachzugehen, wurden in der vorliegenden kumulativen Habilitationsschrift Prozesse der m6A RNA-Modifikation hinsichtlich der Prognose im Vulva- und Zervixkarzinom analysiert. Wir konnten in beiden Studien zeigen, dass eine erhöhte Expression einzelner m6A Proteine signifikant mit einem schlechteren Gesamtüberleben assoziiert war, unabhängig von gängigen Risikofaktoren wie Grading, Tumorstadium oder Lymphknotenbefall.

Im Vulvakarzinom korrelierte die erhöhte Expression der „writer“ METTL3 und METTL14 sowie der „reader“ YTHDC1 mit einer schlechteren Prognose, allerdings nur in der HPV-abhängigen Subkohorte. Dieser Effekt konnte in der Subgruppe der HPV-unabhängigen Tumore nicht gezeigt werde. Auch hier wird nochmal deutlich, wie unterschiedlich sich die zwei Ätiologien des Vulvakarzinomes auf molekularer Ebene verhalten. Allerdings werden beide in der Klinik identisch behandelt(100), was deutlich macht wie wichtig neue Erkenntnisse über diese beiden Ätiologien sind, um zielgerichtete Therapien etablieren zu können. Studien, welche den Zusammenhang zwischen HPV Infektion, m6A Modifikation und Karzinogenese analysierten gibt es kaum. Von anderen Virusinfektionen ist bekannt, dass die m6A Modifikation die Interaktion zwischen dem Virus und dem Wirt moduliert(101). Daten für andere onkogene Viren, wie Herpesviren, die ursächlich für das Kaposi-Sarkom sind, zeigen dass die Expression von METTL3 und YTHDF2 in den mit dem DNA Virus infizierten Zellen signifikant erhöht war. Für den „reader“ YTHDC1, der auch in unserer Studie signifikante Ergebnisse zeigte, ist im Zusammenhang mit Virusinfektionen bekannt, das dieser am Spleißen von Genen beteiligt ist, die für die lytische Replikation verantwortlich sind(102). Unsere Studie zeigte als erste einen Zusammenhang zwischen HPV Infektion, m6A-RNA-Modifikation und Karzinogenese im Vulvakarzinom. Dabei scheint die m6A Modifikation vor allem bei den HPV-abhängigen Tumoren eine wichtige Rolle zu spielen. Somit könnte man m6A Proteine als Biomarker, Indikator für eine schlechte Prognose, aber auch als potentielle Angriffspunkte für neue therapeutische Medikamente nutzen, wobei die spezifischen Wechselwirkungen zwischen m6A-Modifikation und HPV-Infektion in der Zukunft geklärt werden müssen.

Den Zusammenhang zwischen HPV Infektion und m6A Modifikation in der Karzinogenese bestätigen Daten aus dem Zervixkarzinom, einem Tumor der wie bereits erwähnt fast ausschließlich durch eine HPV Infektion verursacht wird.

Auch in unserer Studie war die erhöhte Proteinexpression von METTL14, WTAP, KIAA1439, ALKBH5, HNRNPC, YTHDC1, und YTHDF3 mit einem kürzeren Gesamtüberleben assoziiert. Dies konnte auf mRNA Ebene anhand von TCGA (The Cancer Genome Atlas) Daten für METTL14, WTAP, und KIAA1429 bestätigt werden. Nur für YTHDC1 zeigten sich diskrepante Daten zwischen mRNA und Proteinexpression. Während auf Proteinebene erhöhte Expression von YTHDC1 mit einem schlechterem Gesamtüberleben assoziiert war, war ein hohes mRNA Expressionslevel ein Indikator für eine gute Prognose. Diskordante Expressionsdaten auf Transkriptions- und Proteinebene werden in der Literatur häufig berichtet und können durch Tumorheterogenitäten oder unterschiedliche analytische Techniken erklärt werden, wobei im biologischen Kontext die Proteinexpression relevanter ist(103).

Aus vorherigen Studien wurden m6A Modifikationen mit Chemo- und Strahlentherapieresistenz sowie einem progressiveren Phänotyp im Zervixkarzinom in Verbindung gebracht. Für FTO, einem m6A „eraser“, zeigte sich eine verstärkte Expression in Zervixkarzinom Tumorzellen als in Normalgewebe. Höhere FTO-Expressionswerte waren mit einer erhöhten Resistenz gegenüber Chemo- und Strahlentherapie assoziiert, verursacht durch vermindertes Beta-Catenin und erhöhte ERCC1-Expressionswerte(104). In einer anderen Studie wurde FTO als wichtiger onkogener Faktor durch die Regulierung der Proliferation und Migration der Tumorzellen beschrieben(105). Unsere Daten bestätigten diesen Trend mit einem schlechteren Gesamtüberleben bei erhöhter FTO Expression, allerdings war die Kaplan-Meier Analyse nicht signifikant.

Für die Gruppe der „writer“ gibt es Daten für METTL 3, dieses zeigte sich in den Tumorzellen erhöht exprimiert und war mit einer schlechteren Prognose assoziiert(106). METTL 14, ein weiterer „writer“, ist an der Erkennung der Substrat-RNAs und an der Stabilisierung der katalytischen Funktionen von METTL 3 beteiligt. Im hepatozellulären Karzinom zeigte sich für METTL14 eine zentrale Rolle in der Tumorprogression(107). Unsere Daten bestätigten dies für das Zervixkarzinom sowohl auf Protein als auch auf mRNA Expressionsebene. Kongruente Daten zeigten sich auch für WTAP, ebenfalls einen „writer“. Im hepatozellulären Karzinom waren hohe Expressionslevel mit einer schlechteren Prognose assoziiert, und in vitro Experimente bestätigten das WTAP die Proliferationsaktivität der Tumorzellen beeinflussen

kann(108). Neben dem Zervix- und hepatozellulären Karzinom gibt es auch Daten für andere Karzinome, wo erhöhte WTAP Expressionen zu schlechterem Gesamtüberleben führen(109).

Die YTH-Domäne enthaltenden Proteine, darunter YTHDF1-3 und YTHDC1-2, sind am mRNA-Spleißen, am Kernexport und an der Translation beteiligt. Durch post-transkriptionelle Modifikationen modulieren sie die Expression von Genen, die an der Migration, Invasion, und Proliferation von Tumoren beteiligt sind(110). Vor allem alternatives Spleißen, das bei verschiedenen Krebsarten gefunden wurde, kann durch eine Dysregulation der YTH-Domäne verursacht werden und zu einer Proliferation und Invasion von Tumorzellen führen(111). Frühere Studien zeigten, dass fast alle YTH-Proteine, einschließlich YTHDF1-3 und YTHDC1-2, bei den meisten Krebsarten hochreguliert sind. Im Eierstockkrebs fördert YTHDF1 die Tumorentstehung und Metastasierung(78), und im Brustkrebs ist die Überexpression von YTHDF1, YTHDF3 und KIAA1429 ein schlechter prognostischer Faktor für das Gesamtüberleben(112). In unserer Zervixkarzinomkohorte war eine Überexpression von YTHDC1 und YTHDF3 mit einem verkürzten Gesamtüberleben assoziiert.

Zusammenfassend zeigt sich, dass dysregulierte m6A RNA Modifikationen, hervorgerufen durch abberante Expression diverser an der m6A Modifikation beteiligter Proteine, eine zentrale Rolle im Zervixkarzinom als auch im HPV-bedingten Vulvakarzinom spielen. Daher eignen sie sich sowohl als Biomarker als auch als potentielles target für zielgerichtete Therapien. Im Kolonkarzinom und Melanom erhöhte der Verlust von METTL3 und METTL14 die Empfindlichkeit gegenüber einer Anti-PD-1-Behandlung(113). Die Unterdrückung von METTL14 induzierte in Zervixkarzinomzellen einen Zellzyklusarrest über den den PI3K/AKT/mTOR-Signalweg(114). Die Interaktion zwischen m6A und dem PI3K/AKT/mTor-Signalweg wurde auch für andere Tumorentitäten beschrieben(115). Neben der direkten medikamentösen Beeinflussung der Methylierung könnte die Hemmung des PIK3/AKT/mTOR-Signalwegs eine vielversprechende therapeutische Option sein, insbesondere aufgrund der beschriebenen Wechselwirkung zwischen m6A und diesem Signalweg. Es gibt verschiedene therapeutische Wirkstoffe wie Everolimus oder der PIK3-Inhibitor Alpelisib, die in Frage kommen würden. Bislang wurde diese Therapeutika im Vulvakarzinom nicht untersucht, könnten aber von potenziellem Interesse sein. Ebenso können m6A Modifikationen insbesondere die Therapie mit Checkpoint-Inhibitoren beeinflussen, die im Zervixkarzinom in der Rezidivsituation oder bei Metastasierung bereits eingesetzt werden(116).

Für die Zukunft sind weitere Studien erforderlich, um die genauen Mechanismen zwischen m6A-Modifikation und HPV-Infektion zu erklären. Die vorliegenden Arbeiten zeigen, dass Prozesse der m6A Modifikation großes Potential als Biomarker, Indikatoren für eine schlechte Prognose, aber auch als potenzielle Angriffspunkte für neue Therapeutika in gynäkologischen Tumoren haben.

Um der Frage nach neuen therapeutischen Optionen im Vulvakarzinom nachzugehen, wurde in der vorliegenden Arbeit erstmalig die TROP-2 Expression in diesem Tumor untersucht. Die nachgewiesene, in den meisten Fällen moderate bis hohe Expression, ist von großer therapeutischer Relevanz, da mit Sacituzumab-Govitecan ein gegen TROP-2 gerichtetes ADC bereits erfolgreich in der Therapie des Mammakarzinoms eingesetzt wird und somit auch für die Behandlung des Vulvakarzinoms denkbar wäre.

Für TROP-2 liegen Daten als Onkogen mit schlechter Prognose im Falle einer erhöhten Expression im Mammakarzinom(117) oder Darmkrebs(118) vor. Jedoch scheint bei Plattenepithelkarzinomen TROP-2 eine tumorsupressive Wirkung zu entfalten. Dies konnte im Lungenkarzinom und bei Kopf-Hals-Tumoren gezeigt werden(119). Ein TROP-2 knockout ($ARF^{-/-}$ C57BL/6) in Mäusen führte zu einer epithelial-mesenchymalen Transition von Keratinozyten und in weitere Folge zu einer Tumorbildung der Haut(120). In einer weiteren Studie konnte gezeigt werden, dass ein gradueller TROP-2 Verlust im Plattenepithel des Halses, der Speiseröhre und der Cervix zu einer Progression von intraepithelial Vorstufen und schließlich zur invasive Tumorbildung führt(85). Unsere Daten bestätigten im Plattenepithelkarzinom der Vulva ebenfalls die tumorsupressive Wirkung von TROP-2. Eine erhöhte Expression war signifikant mit einem besseren Gesamtüberleben assoziiert.

Interessanterweise zeigten alle HPV-abhängigen Tumore eine moderate bis hohe TROP-2 Expression, was auf eine Assoziation zwischen HPV Infektion, Karzinogenese und TROP-2 hindeutet. TROP-2 beeinflusst mehr als 100 Signalwege, unter anderem auch PI3K/AKTmTOR, welcher ebenfalls durch eine Infektion mit HPV Viren beeinflusst wird(121). Der genaue Zusammenhand zwischen TROP-2 und HPV Infektion muss jedoch in weiteren Untersuchungen geklärt werden.

In TROP-2 positiven Cervixkarzinom Zelllinien konnte ein hohes Ansprechen auf eine Therapie mit Sacitzumab-Govitecan gezeigt werden(122). Unsere Ergebnisse zeigen, dass mit nur 2,9% TROP-2 negativen Fällen und 75% moderat bis hoher Expression, eine Therapie mit Sacituzumab-Govitacan auch im Vulvakarzinom eine mögliche Therapieoption darstellt.

Außerdem eignet sich TROP-2, wie es bereits für andere Plattenepithelkarzinome gezeigt werden konnte, als prognostischer Marker auch im Vulvakarzinom.

6. Zusammenfassung

Das übergeordnete Ziel dieser Habilitationsschrift war die Analyse von Biomarkern und Risikofaktoren in der Karzinogenese bei HPV-assoziierten gynäkologischen Malignomen, insbesondere dem Vulva- und dem Zervixkarzinom.

Über die letzten Jahre hat die Untersuchung des menschlichen Mikrobioms in der Medizin zunehmend an Bedeutung gewonnen. Für viele bösartige Erkrankungen zeigte sich, dass die Besiedlung mit Keimen einen Einfluss auf die Karzinogenese hat. Das Mikrobiom scheint ebenfalls eine wichtige Rolle bei vielen gynäkologischen Erkrankungen zu spielen, wie Frühgeburtlichkeit, Sterilität und auch der Entstehung und Progression von zervikalen intraepithelialen Dysplasien. Aufgrund dieser Daten finden sich auf dem Markt zunehmend Produkte zur Optimierung des vaginalen Mikrobioms, umso die Entstehung von Dysplasien zu verhindern und auch bereits vorhanden zu therapieren. Unsere Arbeit zeigte, dass das Mikrobiom vor allem vom Menopausenstatus und somit auch von der Östrogenexpostion abhängt. Eine Korrelation mit zervikalen Dysplasien konnte mit dem Keim *Ureaplasma parvum* festgestellt werden. Inwiefern eine gezielte Behandlung und Optimierung des Mikrombioms Krebsvorstufen der Zervix therapieren und vorbeugen kann, muss in weiteren Studien geklärt werden.

Als Biomarker zur Risikostratifizierung im Vulva- und Zervixkarzinom wurde die Expression der Proteine, die am Prozess der m6A RNA Modifikation beteiligt sind, untersucht. Für beide Tumorentitäten konnte gezeigt werden, das eine erhöhte Expression einzelner „writer“, „eraser“ und „reader“ mit einem schlechteren Gesamtüberleben assoziiert ist, unabhängig von Risikofaktoren wie Grading oder Lymphknotenstatus.

Im Vulvakarzinom zeigte sich dieser Zusammenhang nur bei den HPV-assoziierten Tumoren. Somit ist die Bestimmung von Proteinen, die an der m6A Modifikation beteiligt sind, bei HPV-assoziierten Tumoren ein potentieller Biomarker und auch mögliches therapeutisches Ziel, was in weiteren Studien untersucht werden sollte.

Des weiteren wurde in dieser Arbeit die TROP-2 Expression im Vulvakarzinom untersucht. Die meisten Tumore zeigten eine moderat bis hohe Expression, so dass Sacituzumab-Govitecan beim Vulvakarzinom als zielgerichtetes Therapeutikum in Frage kommen würde. Außerdem war eine erhöhte TROP-2 Expression mit einem besseren Gesamtüberleben assoziiert, so dass TROP-2 auch ein prognostischer Marker im Vulvakarzinom ist.

7. Erklärung zur kumulativen Habilitationsschrift

Die vorliegende Habilitationsschrift hat vier publizierte Originalarbeiten zur Grundlage.
In dieser kumulativen Habilitationsarbeit fließen Forschungsarbeiten ein, in denen der Habilitand als geteilter Erstautor gelistet ist. Die unter 4.2 aufgeführte Publikation ist Grundlage der kumulativen Habilitationsschrift von Herrn Dr. med. Damian Ralser. Die thematischen Schwerpunkte der beiden Habilitationsschriften unterscheiden sich wesentlich, da Herr Dr. med. Damian Ralser epigenetische Regulationen betrachtet hat und in der vorliegenden Arbeit Früherkennung und Biomarker bei HPV-assoziierten Tumoren bearbeitet wurden. Eine Überlappung mit weiteren Habilitationsschriften ist nicht gegeben.

Die jeweiligen Eigenbeiträge der geteilten Erstautorenschaften sind im Folgenden für die unter 2. Grundlage der kumulativen Habilitationsschrift aufgeführten Originalarbeiten in Anlehnung zu dem *authors contributions* Teil dargestellt:

I) Condic M*, Neidhöfer C*, Ralser DJ, Wetzig N, Thiele R, Sieber M, Otten LA, Warwas LK, Hierauf A, Mustea A, Parčina M. Analysis of the cervical microbiome in women from the German national cervical cancer screening program.

Dr. Condic (MC) und Dr. Neidhöfer (CN) sind geteilte Erstautoren:

MC: Planung und Konzeption, Datenerhebung, Durchführung der Experimente, Statistische Auswertung, Schreiben des Manuskriptes.

CN: Datenerhebung, Durchführung der Experimente, Statistische Auswertung, Schreiben des Manuskriptes.

II) Condic M*, Ralser DJ*, Klümper N, Ellinger J, Qureischi M, Egger EK, Kristiansen G, Mustea A, Thiesler T. Comprehensive Analysis of N6-Methyladenosine (m6A) Writers, Erasers, and Readers in Cervical Cancer. Int J Mol Sci. 2022 Jun 28;23(13):7165.

Dr. Condic (MC) und Dr. Ralser (DJR) sind geteilte Erstautoren:

MC: Planung und Konzeption, Datenerhebung, Durchführung der Experimente, Statistische Auswertung, Schreiben des Manuskriptes.

DJR: Planung und Konzeption, Datenerhebung, Durchführung der Experimente, Statistische Auswertung, Schreiben des Manuskriptes.

III) Condic M*, Thiesler T*, Staerk C, Klümper N, Ellinger J, Egger EK, Kübler K, Kristiansen G, Mustea A, Ralser DJ. N6-methyladenosine RNA modification (m6A) is of prognostic value in HPV-dependent vulvar squamous cell carcinoma. BMC Cancer. 2022 Sep 1;22(1):943.

Dr. Condic (MC) und Hr. Thiesler (TT) sind geteilte Erstautoren:

MC: Planung und Konzeption, Datenerhebung, Durchführung der Experimente, Statistische Auswertung, Schreiben des Manuskriptes.

TT: Datenerhebung, Durchführung der Experimente, Statistische Auswertung

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9. Danksagung

Mein erster Dank gilt Herrn Professor Dr. Dr. Alexander Mustea. Seine fortwährende Unterstützung sowohl in der Klinik als auch in der Wissenschaft hat mir ermöglicht, klinisch operativ ausgebildet zu werden und mich gleichzeitig Forschungsprojekten zu widmen. Seine stetige Förderung hat einen wesentlichen Anteil an der Fertigstellung dieses Werkes.

Ein besonderer Dank gilt meinem Doktorvater PD Dr Juan Manuel Maler und meinem Betreuer PD Dr. Philipp Spitzer. Beide haben maßgeblich zur Erweiterung meines wissenschaftlichen Horizontes beigetragen und waren exzellente Mentoren zu Beginn meines wissenschaftlichen Werdeganges.

Außerdem möchte ich meinen engen Kooperationspartnerinnen und -partnern danken, wobei ich insbesondere Herrn Dr. Damian Ralser, Herrn PD Dr. Marijo Parčina und Herrn T. Thiesler hervorheben möchte. Nur durch ihre Unterstützung und wissenschaftliche Zusammenarbeit war diese Umsetzung der Projekte möglich, die Grundlage für diese kumulative Habilitationsschrift sind.

Dank gilt allen Freunden und Kollegen, die den bisherigen beruflichen und privaten Weg ganz oder teilweise mitbestritten haben.

Abschließend gilt mein Dank meiner Familie, denen diese Arbeit gewidmet ist, für ihre liebevolle Unterstützung: meinen Eltern Zlata und Krešimir sowie meinem Bruder Karlo.