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Das HPV-assoziierte Zervixkarzinom – Analyse molekularer Prognosefaktoren

Habilitationsschrift

zum Erwerb der Venia Legendi

für das Fach

Frauenheilkunde und Geburtshilfe

vorgelegt von

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2021


SIGILL: UNIVERSI TAT: LUDOVIC: MAXIM:

Vorwort

Neben klinischen Faktoren sind für diverse Tumorentitäten molekulare Prognosefaktoren untersucht, aus denen neben einer Risikoklassifizierung auch weiterführende Therapieoptionen abgeleitet werden können. Im vorliegenden Forschungsprojekt sollen spezifische Prognosefaktoren des HPV-assoziierten Zervixkarzinoms auf molekularer Ebene analysiert werden.

Zunächst wurde eine klinische Prävalenzerhebung von Humanem Papillomavirus (HPV) im Ano-Genitalbereich unterschiedlicher Risikopopulationen für anogenitale Dysplasie bzw. anogenitales Karzinom durchgeführt.

In einem weiteren Schritt dieser Arbeit wurde mittels Immunhistochemie ein einfaches und kostengünstiges Analyseverfahren für spezifische Marker des HPV-assoziierten Zervixkarzinoms etabliert.

Basierend auf bekannten molekularen Mechanismen in der Karzinogenese des HPV-assoziierten Zervixkarzinoms wurde die Expression und Interaktion spezifischer Marker des Zervixkarzinoms auf Proteinebene analysiert. Die Daten der molekularen Analyse aus Zervixkarzinomproben wurden mit klinischen Daten der zugehörigen Patientinnen korreliert. Hierdurch sollten mögliche molekulare Prognosefaktoren des HPV-assoziierten Zervixkarzinoms aufgedeckt werden.

Wie bereits bei anderen Tumorentitäten etabliert, soll, basierend auf einer molekularen Risikostratifizierung, der Anreiz für Forschung an weiterführenden Therapieoptionen beim Zervixkarzinom gelegt werden.

1 Einleitung

1.1 Das Zervixkarzinom - Hintergrund

1.1.1 Epidemiologie

Weltweit stellt das Zervixkarzinom die viert-häufigste maligne Tumorerkrankung der Frau, mit etwa 570.000 Neuerkrankungen im Jahr 2018, dar. Über 80% dieser Fälle traten in Ländern mit niedrigem oder mittlerem sozioökonomischen Status in Südamerika, Afrika und Süd- bzw. Zentralasien auf. Es ist für 7,5% aller Krebstodesfälle verantwortlich, wobei hier ebenfalls der Anteil an Frauen in Südamerika, Afrika und Süd- bzw. Zentralasien mit >80% am höchsten war (Munoz et al 2004, WHO GLOBACAN 2018). In Deutschland erkrankten im Jahr 2018 etwa 4400 Frauen an einem Zervixkarzinom, davon verstarben etwa 1600 Patientinnen mit dieser Diagnose (WHO GLOBACAN 2018). Durch Screening-Programme konnte die Inzidenz und Sterblichkeit am Zervixkarzinom unter anderem in Deutschland in den letzten Jahrzehnten reduziert werden (Arbyn et al 2011). Entgegen diesem Trend ist nach Schätzung der WHO vor allem in Ländern mit niedrigem und mittlerem sozioökonomischen Status von einer weiteren Steigerung der Inzidenz und Mortalität bezogen auf die Erkrankung auszugehen (WHO GLOBACAN 2018).

Eine Übersicht über die relativen Überlebensraten beim Zervixkarzinom abhängig vom individuellen Stadium der Union Internationale Contre le Cancer (UICC) ist in Tabelle 1 dargestellt (Tab.1).

UICC-Stadium	0	1	2	3	4
Relative 5-Jahres Überlebensrate	100%	95%	75%	58%	21%
Relative 10-Jahres Überlebensrate	100%	93%	71%	51%	16%

Tab.1.:
Relatives 5- und 10-Jahres-Überleben abhängig von UICC-Stadium. Krebsregister Bayern 1998-2011. UICC-Stadien nach TNM-Klassifikation: UICC 0 = Tis N0 M0; UICC I = T1 N0 M0; UICC II = T2 N0 M0; UICC III = T3 N0 M0 oderT1-3 N1 M0; UICC IV = T4 N0 M0 oder T4 N1 M0 oder jedes T jedes N M1
UICC: Union Internationale Contre le Cancer, TNM: T= Tumor, N= Nodus, M= Metastase

1.1.2 Das Humane Papillomavirus (HPV)

Eine Infektion mit dem Humanen Papillomavirus (HPV) ist sehr wahrscheinlich und weit verbreitet. Humane Papillomaviren können über Mikroverletzungen der Mukosa des Ano-Genitalbereichs Epithelzellen der Basalzellschicht infizieren (Bodily et al 2011). Meist handelt es sich bei einer solchen Infektion um ein transientes Geschehen über ein bis zwei Jahre, mit einer mittleren Infektionsdauer für sog. Low-Risk-Typen von 8 Monaten und High-Risk-Typen von 13,5 Monaten (Franco et al 1999). Die weltweite Prävalenz des Humanen Papillomavirus (im Mittel 10,5%) unterscheidet sich, wobei die niedrigste Rate in Europa (5,2%), gefolgt von Asien (8,7%), Südamerika (14,3%) und Afrika (25,6%) gemessen werden kann (Clifford et al 2005).

1.1.3 Aufbau HPV

Humane Papillomaviren sind kleine doppelsträngige ringförmige DNA-Viren, bestehend aus etwa 8000 Basenpaaren. Diese besitzen 6 sogenannte frühe Gene (E6, E7, E1, E2, E4, E5), zwei sogenannte späte Gene (L1 und L2) und eine sog. Long Control Region (LCR), welche die Replikation und Genexpression reguliert (Münger et al 2004). Da HPV keine Gene besitzt, die für Enzyme der Replikation kodieren, ist es zur Replikation auf eine Wirtszelle angewiesen (Assmann et al 2011).

Derzeit sind etwa 200 verschiedene HPV-Typen bekannt, welche in 5 Gruppen unterteilt werden (Alpha-HPV, Beta-HPV, Gamma-HPV, Mu-HPV, Nu-HPV). Für eine Infektion der

Mukosa entscheidend ist eine Infektion mit einem Papillomavirus-Typ der Gruppe Alpha-HPV (Doorbar et al 2012). In Abhängigkeit ihrer individuellen karzinogenen Potenz unterscheidet man in dieser Gruppe sog. Low-Risk-Typen (LR-HPV: 6, 11, 40, 42, 43, 44, 54, 61, 70, 72 und 81) von High-Risk-Typen. Eine Infektion mit einem der 15 sogenannten karzinogenen oder High-Risk-HPV-Subtypen (HR-HPV: 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 68, 73 und 82) kann in Malignität münden (Munoz et al 2003). Eine ursächliche Korrelation einer persistierenden Infektion mit HR-HPV-Subtypen und der Entstehung eines Zervixkarzinoms wird in nahezu 100% der Zervixkarzinomfälle angenommen. Hierbei nimmt eine persistierende Infektion mit den HPV-Subtypen 16 und 18 etwa 70% aller Zervixkarzinomfälle ein (Schiffman et al 2007). In Folge einer persistierenden Infektion mit HR-HPV-Typen können zunächst Dysplasien des Epithels der Zervix entstehen, die in einer Zeitspanne von mehreren Jahren in ein invasives Karzinom münden können. Das Protein p16, das bei der Regulation des Zellzyklus eine Rolle spielt, wird bei einer Infektion der Zelle mit HPV verstärkt exprimiert und kann als Marker für die Diagnose eines HPV-assoziierten Karzinoms verwendet werden (Melkane et al 2013).

1.1.4 Zellzyklus HPV

Humane Papillomaviren sind für ihre Replikation auf eine Wirtszelle angewiesen (Assmann et al 2011). Nach Bindung an sog. α-Integrinrezeptoren und Aufnahme der Viren in die Basalzellen der Mukosa wird das Genom zeitgleich zur Replikation der zellulären DNA bis zu 100-fach repliziert (Moody et al 2010, Evander et al 1997, Doorbar et al 2005). Bei der folgenden Teilung der infizierten Wirtszelle in eine sich nicht mehr teilende differenzierende Zelle und eine in der Basalzellschicht verbleibende, sich teilende Tochterzelle, wird die HPV-spezifische DNA aufgeteilt (Stubenrauch et al 1999). Da eine weitere Vermehrung von HPV mangels Polymerasen und Replikationsfaktoren in differenzierenden Zellen ohne Teilung nicht möglich ist, kommt es in diesen Zellen zu einer gesteigerten Expression der viralen Onkogene, so dass weiterhin eine Replikation der viralen DNA mit >1000 Kopien pro Zelle möglich bleibt

(Moody et al 2010). Bei der Wanderung der infizierten, differenzierten Zelle durch die Schichten des Epithels werden hierbei die frühen Gene (E6, E7, E1, E2, E4, E5) exprimiert, gefolgt von einer Expression der späten Gene. Diese sog. späten Proteine L1 und L2 bilden ein Kapsid um das virale Genom. Die neugebildeten Viren werden schließlich über die äußerste Epithelschicht freigesetzt (Moody et al 2010).

1.2 Das Zervixkarzinom – Karzinogenese

1.2.1 Molekulare Grundlagen

Die Pathogenese des Zervixkarzinoms auf molekularer Ebene ist komplex. Im Folgenden sollen molekulare Faktoren, welche für diese Arbeit eine wichtige Rolle spielen, näher erläutert werden.

Eine bedeutende Funktion nehmen die HPV-spezifischen Onkoproteine E6 und E7 ein. Die Replikation der viralen Gene E6 und E7 führt zu zellulärer Freisetzung von Onkoproteinen, die mit dem Zellzyklus interferieren (Gupta et al 2003). Das E6 Onkoprotein kann auf unterschiedliche Weise antiapoptotisch einwirken (Narisawa-Saito et al 2007). E6 kann mit dem sogenannten E6-assoziierten Protein (E6-AP) einen Komplex bilden, der selektiv an p53 binden kann und durch Einwirkung in das Ubiquitin-abhängige Proteasesystem zu Proteolyse des p53 Proteins führt (Moody et al 2010). p53 fungiert als Tumorsuppressor, der im Falle von DNA-Schäden zu Zellzyklusarrest oder Apoptose führt (Tang et al 2002). Durch die Degeneration von p53 durch das E6 Onkoprotein ist die Funktion des bedeutenden Proteins zur Regulierung des Zellzyklus gestört (Mao et al 2007).

In gesunden Zellen wird die Steuerung von p53 durch das sogenannte MDM2 Proto-Onkogen (MDM2) durch einen negativen Feedback-Mechanismus reguliert. MDM2 fördert hierbei die Degeneration von p53 (Assmann et al 2011). Im Falle eines DNA-Schadens wird p53 unter anderem durch Kinasen phosphoryliert. Dadurch kann es nicht mehr mit MDM2 interagieren und wird nicht abgebaut (Vogelstein et al 2000). Eine Verbindung zwischen dem MDM2 Proto-Onkogen, dem p53 Polymorphismus und dem Verlauf eines Zervixkarzinoms ist bekannt (Adams et al 2014). In diversen Krebsarten sind Mutationen im p53-Protein häufig zu finden. Abhängig von Tumortyp und Stadium findet man in mehr als 50% der malignen Tumore eine Mutation oder Deletion, die p53 deaktivieren kann. Mutationen treten hierbei vor allem in der zentralen DNA-Bindungsdomäne auf (Petijean et al 2007).

Neben p53 sind auch weitere Tumorsuppressoren durch HPV-spezifische Onkoproteine beeinflusst. Als Beispiel ist hierbei das Retinoblastom-Protein (Rb) zu nennen, welches die Progression des Zellzyklus von der G1- zur S-Phase blockieren kann. Hierzu bindet es den sog. Transkriptionsfaktor E2F. Dieser wird dadurch inaktiviert (Dyson et al 1998). Das Onkoprotein E7 kann den Zellzyklus auf unterschiedliche Arten stören (Kim et al 2000). Das Retinoblastom-Protein kann durch das E7-Onkoprotein direkt beeinflusst werden (Boyer et al 1996). E2F kann hierdurch nicht mehr vom Retinoblastom-Protein gebunden werden und liegt in seiner aktiven Form vor. Insgesamt kommt es konsekutiv zu einer Überexpression von p16 (Klussmann et al 2009, Khleif et al 1996).

Ein weiteres Protein, das in Zervixkarzinomzellen analysiert werden kann, stellt Galectin-3 (gal-3) dar. Galectin-3 spielt eine wichtige Rolle bei immunologischen und entzündlichen Prozessen und ist an der Angiogenese beteiligt (Kuwabara et al1996, Sano et al 2000, Nangia-Makkar et al 2000). Auch in der Pathogenese von Malignomen ist Galectin-3 ein wichtiger Faktor. Gal-3 kann den gefäßständigen, endothelialen Wachstumsfaktor-Rezeptor-3 aktivieren und somit die Kapazität von Zervixkarzinomzellen zur Invasion ins Gewebe erhöhen (Zengel et al 2012). Es beeinflusst den Zellzyklus, reguliert die Tumorzell-Apoptose und ist an der Tumormetastasierung beteiligt (Liu et al 2005, Ochieng et al 1998, Matarrese et al 2000).

Durch die verschiedenen Stadien der HPV-assoziierten Karzinogenese des Zervixkarzinoms, kommt es zu einer Anhäufung von epigenetischen Veränderungen. Es kann hierbei zu DNA-Methylierung oder posttranslationaler Modifikation von Histonproteinen kommen (Huang et al 2005). In Zellkulturanalysen konnten Histonveränderungen mittels Acetylierung und Methylierung, hervorgerufen durch HPV, nachgewiesen werden (Wooldridge et al 2008). Dabei kommt es zu einer Histonmodifikation am Histon 3 der HPV-assoziierten Proteine E6 und E7 mittels Aceylierung an Lysin 9 (H3K9ac) oder einer Methylierung an Lysin 4 (H3Kme3). Durch diese Effekte wird unter anderem die Transkription sowie die Karzinogenese negativ beeinflusst (Jiang et al 2010). Bezogen auf eine Metastasierung scheinen beide Effekte ein hohes malignes Potential zu zeigen (Jiang et al 2010).

In der Onkologie sind Auswirkungen von Steroidhormonen auf Tumore sehr unterschiedlich. Dies schließt die Expression ihres jeweiligen Rezeptors ein (Lu te al 2005). Prinzipiell kann durch Glukokortikoide durch Bindung an ihren spezifischen Rezeptor die Transkription direkt beeinflusst werden. Ebenso sind Modifikationen an Histonen bekannt (Beck et al 2009). Steroidhormonezeptoren, wie der Glukokortikoidrezepor, scheinen ebenfalls eine Rolle in der Pathogenese des HPV-assoziierten Zervixkarzinoms zu spielen (Pittayakhajonwut et al 2010, Bromberg-White et al 2002). Hierbei ist bekannt, dass HPV-spezifische Sequenzen (long control regions, LCR) an Steroidrezeptoren wie den Glukokortikoidrezeptor binden können (Meng et al 2011, Chan et al 1989). Dadurch kann die Aktivität des sogenannten frühen Promotors in HPV 16 und 18 erhöht werden. Dies beeinflusst die Kontrolle der Transkription der viralen Onkogene E6 und E7 (Fonsenca-Moutinho et al 2004, Chen et al 1996). Zugrundeliegende Daten deuten darauf hin, dass es eine enge Verknüpfung von Glukokortikoiden, deren Interaktion mit dem Glukokortikoidrezeptor und Humanen Papillomaviren gibt. Inwieweit die Expression der Glukokortikoidrezeptoren in Zervixkarzinomgewebe hierbei eine klinische Bedeutung hat, ist nicht bekannt (Lu te al 2005).

Nukleäre Rezeptoren (NRs) wie der Glukokortikoidrezeptor fungieren als wichtige Transkriptionsfaktoren. Um diese Funktion auszuführen sind sie auf zahlreiche Ko-Regulatoren angewiesen. Diese können zusätzlich die Acetylierung von Histonen beeinflussen (Perissi et al 2005). Ko-Regulatoren können als Repressoren oder Aktivatoren fungieren. Als Repressoren hemmen sie die Transkription (McKenna et al 1999). RIP140 (Receptor Interacting Protein of 140kDa), auch bekannt als NRIP1 (Nuclear Receptor Interacting Protein 1) fungiert als Ko-Regulator zahlreicher Rezeptoren wie beispielsweise Steroidhormonrezeptoren (Caracossa et al 2006). Ein weiterer für die Karzinogenese wichtiger Ko-Repressor stellt LCoR (Ligand dependent Corepressor) dar. RIP140 und LCoR spielen eine wichtige Rolle in der Krebsentstehung und –progression verschiedener Krebsarten wie Ovarialkarzinom, Burstkrebs oder Darmkrebs (Docquier et al 2010,Lapierre et al 2015, Jalaguier et al 2017). Über deren Einfluss in Zervixkarzinomgewebe ist bisher wenig bekannt.

Prostaglandine spielen eine wichtige Rolle in der Infektiologie, Immunologie, bei Schmerz und Fieber und sind am vaskulären Gleichgewicht beteiligt. In der Karzinogenese sind sie am Tumorwachstum und an der Tumor-assoziierten Angiongenese beteiligt (Narumiya et al 1999, Sugita et al 2016, Amano et al 2003). Studien legen nahe, dass einige Tumore durch Produkte der Cyclooxigenase (COX), wie beispielsweise durch Prostaglandine, reguliert werden. Eine Überexpression von COX-2 wurde in Zervixkarzinomzellen beschrieben (Ryu et al 2000). Die Ausprägung von Prostaglandinrezeptoren in Zervixkarzinomzellen und deren mögliche Auswirkung ist bisher wenig untersucht.

1.3 Das Zervixkarzinom - Risikostratifizierung und Therapie

Entsprechend internationaler Leitlinien werden Patientinnen mit Zervixkarzinom in Abhängigkeit des Stagings und individueller Risikostratifizierung primär mittels Operation oder Radiochemotherapie behandelt (Marth et al. 2017, Minoz et al 2004, Serrano-Olvera et al 2014, Horn et al 2015). Um eine für den Verlauf der Erkrankung unnötige primäre Kombination beider Behandlungsmodalitäten zu vermeiden, werden Patientinnen, entsprechend der deutschen Leitlinie, abhängig von der individuellen Ausdehnung der Erkrankung und spezifischen Risikofaktoren, einer Behandlungsoption zugeführt.

Zur Risikoabschätzung erkrankter Patientinnen mit Zervixkarzinom sind wichtige klinische Prognosefaktoren bekannt. Zu den etablierten Faktoren zählen die Stadieneinteilung der *„International Federation of Gynecology and Obstetrics“* (FIGO), der histologische Typ, die Tumorgröße oder eine Lymphknotenmetastasierung in pelvine und/oder paraaortale Lymphknoten. Weitere morphologische Risiko- bzw. Prognosefaktoren werden zur Therapieentscheidung genutzt. Zu diesen zählen das Grading, die Infiltrationstiefe des Zervixkarzinoms, eine mögliche Invasion der Tumorzellen in Blut- und/oder Lymphgefäße sowie die Perineuralscheideninfiltration (S3 Leitlinie Zervixkarzinom 2014).

Eine Übersicht über die derzeit in Deutschland üblichen Risiko- bzw. Prognosefaktoren ist in Tabelle 2 dargestellt (Tab.2).

Neben klinischen Faktoren sind für verschiedene Tumorentitäten molekulare Prognosefaktoren untersucht, aus denen Therapieoptionen abgeleitet werden können (Pauletti et al 2000, S3 Leitlinie Mammakarzinom 2020). Ein Beispiel stellt hierbei das Mammakarzinom dar. Bereits bei der primären histologischen Brustkrebsdiagnose können unter anderem etablierte molekulare Marker, wie der Hormonrezeptorstatus mit der Ausprägung des Östrogen- und Progesteronrezeptorstatus sowie der sogenannte Her-2-

Status, erhoben werden. Neben einer prognostischen Aussage haben diese Marker einen direkten Einfluss auf die Therapie des Mammakarzinoms (Pauletti et al 2000, S3 Leitlinie Mammakarzinom 2020). Weitere gynäkologische Tumore, wie das Endometriumkarzinom, sind derzeit Teil der Analyse. Ziel ist eine weiterführenden molekularen Charakterisierung und damit eine mögliche Prognoseeinschätzung der individuellen Erkrankung (Dellinger et al 2016). Das Zervixkarzinom ist diesbezüglich noch unzureichend erforscht.

Name	Standardfaktor	Risiko -/Prognosefaktor	Therapierelevanz
Tumorstadium	+	+	+
Histologischer Typ	+	+ (neuroendokrin)	+ (neuroendokrin)
Perineuralscheideninfiltration	+	unklar	-
Lymphgefäßinvasion	+	unklar	+
Veneninvasion	+	unklar	+
Lokalisation (endo-/ektozervikal)	-	unklar	-
Resektionsränder	+	+	+
Tiefe Stromainvasion	+	unklar	+
Grading	+	+	+
p16	-	-	-
Ki-67	-	-	-
Invasionstiefe	+	unklar	-
Tumorgröße	+	+	+
Lymphknotenmetastasen	+	+	+

Tab. 2: Übersicht über die wichtigsten Risiko- bzw. Prognosefaktoren des Zervixkarzinoms (modifiziert nach Leitlinie). +: positiv, -: negativ (adaptiert nach S3 Leitlinie Zervixkarzinom 2014).

2 Eigene Studienergebnisse

Zu Beginn des Forschungsprojektes wurde in einer klinischen Arbeit eine Prävalenzerhebung von humanem Papillomavirus im Anogenitaltrakt von Patientinnen durchgeführt. Ziel war die Analyse von HPV der Zervix uteri und des Analkanals bei Patientinnen mit und ohne Risikofaktoren. Hierzu wurden zytologische und HPV-Abstriche der Zervix uteri und des Analkanals bei 287 Patientinnen erhoben. Die Patientinnen wurden in drei Gruppen eingeteilt: HIV-negative Kontrollen (G1) und zwei Risikogruppen, aufgeteilt in HIV-negative Patientinnen mit auffälliger Zervix-Zytologie (G2) und HIV-infizierte Patientinnen (G3). Im Vergleich zur Kontrollgruppe G1 zeigten die Risikogruppen G2 und G3 signifikant häufiger positive Ergebnisse für HPV im Analkanal (G2: 71,03% und G3: 83,15%). Die meisten HPV-Subtypen, die detektiert wurden, gehörten zur Gruppe der HighRisk-HPV-Typen. Es konnte eine signifikante Korrelation der analen HPV-Typen mit den individuell-korrespondierenden Zervix-HPV-Typen in den Gruppen G2 und G3 aufgezeigt werden. Im Gegensatz zu den analen Abstrichen war der Zusammenhang zwischen zervikaler Zytologie und zervikalem HPV signifikant in G2 und G3. Insgesamt zeigte sich eine hohe Prävalenz an HPV-Infektion im Anogenitaltrakt von Risikopatientinnen. Patientinnen mit auffälliger Zytologie der Zervix uteri und Patientinnen mit HIV-Infektion haben hierbei ein erhöhtes Risiko an einer begleitenden Infektion mit HighRisk HPV-Subtypen. *Im Einzelnen siehe Oncology Letters 2017 ab Seite 21: Prevalence of human papillomavirus infection of the anal canal in women: A prospective analysis of high-risk populations. Kost BP, Hofmann J, Stoellnberger S, Bergauer F, Blankenstein T, Alba-Alejandre I, Stein A, Stuckart C, Weizsäcker K, Mylonas I, Mahner S, Gingelmaier A.*

Die Entstehung des Zervixkarzinoms ist vielfach untersucht. Unterschiedliche molekulare Faktoren sind an der HPV-assoziierten Karzinogenese beteiligt. Unter anderem spielen HPV-assoziierte Onkoproteine eine entscheidende Rolle. Diese können mit unterschiedlichen Analyseverfahren detektiert werden. Für die Routineanalyse der viralen Onkoproteine E6 und E7 stehen molekulare Detektionsverfahren wie die in situ Hybridisierung oder PCR zur

Verfügung. Die hierfür verwendeten Antikörper weisen unterschiedliche Färbeverhalten auf, so dass eine einheitliche Analyse erschwert ist. Zur Verbesserung der Analysemethode von molekularen Faktoren in HPV-infiziertem Gewebe, wurde in einer Arbeit unter Testung einer Vielzahl von Antikörpern und Färbeprotokollen ein immunhistochemisches Verfahren erarbeitet. Hierzu wurden Paraffinschnitte von normalem Zervixgewebe von Patientinnen mit schwerer zervikaler Dysplasie (CIN III) und Patientinnen mit Plattenepithelkarzinom immunhistochemisch analysiert und hieraus ein einfaches Färbe- und Analyseverfahren etabliert. *Im Einzelnen siehe Anticancer Res. 2016 ab Seite 28: Immunohistochemical Evaluation of E6/E7 HPV Oncoproteins Staining in Cervical Cancer. Stiasny A, Kuhn C, Mayr D, Alexiou C, Janko C, Wiest I, Jeschke U, Kost B.*

Basierend auf der Etablierung der Analysemethode, wurden molekulare Faktoren, die in der Entstehung und Progression des Zervixkarzinoms eine mögliche Rolle spielen, untersucht. Hierfür wurde die immunhistochemische Expression der Onkoproteine E6 und E7, von p16 und dem Tumporsupressor p53 sowie dem Proto-Onkogens MDM-2 und dem Protein Galectin-3 in Zervixkarzinomproben analysiert. Die molekularen Daten wurden mit klinischen Daten der jeweiligen Patientinnen korreliert. In den untersuchten Zervixkarzinomzellen konnte ein signifikanter Zusammenhang zwischen einer erhöhten immunhistochemischen Färbung von E6 mit dem T-Status und der FIGO-Klassifikation gezeigt werden. Die Expression von E6, p53 und p16 in Plattenepithelkarzinomzellen war signifikant unterschiedlich zur Expression in Adenokarzinomzellen. Eine Mutation von p53 scheint in den untersuchten Zellen sehr häufig. Es konnte ein signifikanter Überlebensvorteil bei Patientinnen mit Expression der mutierten Form von p53 im Kern, zu jenen ohne mutierter Form, gezeigt werden. Es bestand eine negative Korrelation von mutiertem p53 zur Expression von E6. Die immunhistochemische Expression von MDM-2 und Galectin-3 war insgesamt sehr häufig und positiv korreliert. Die Expression von Galectin-3 in den untersuchten Zervixkarzinomzellen war mit einer schlechten Prognose in den zugehörigen Patientinnen assoziiert. *Im Einzelnen siehe Oncol Lett. 2017 ab Seite 32: The involvement of E6, p53, p16, MDM2 and Gal-3 in the clinical outcome of patients with cervical cancer. Stiasny A, Freier CP, Kuhn C, Schulze S, Mayr D,*

Alexiou C, Janko C, Wiest I, Dannecker C, Jeschke U, Kost BP und Anticancer Res 2016 ab Seite 42: Immunohistochemical Evaluation of the Role of p53 Mutation in Cervical Cancer: Ser-20 p53-Mutant Correlates with Better Prognosis. Freier CP, Stiasny A, Kuhn C, Mayr D, Alexiou C, Janko C, Wiest I, Jeschke U, Kost B.

In einer weiteren Arbeit wurden epigenetische Faktoren und insbesondere die Ausprägung einer Histonacetylierung oder Histonmethylierung in Zervixkarzinomproben analysiert. Hierbei wurde die Expression von Histon H3acetylK9 und Histon H3trimethylK9 untersucht und mit klinischen Daten der entsprechenden Patientinnen korreliert. H3acetylK9 Färbung war assoziiert mit niedrigem Grading, niedrigem T-status und FIGO-Status sowie negativem LK-Befall. Die Ausprägung war in Adenokarzinomzelllinien höher als in Plattenepithelkarzinomzellen. Die zytoplasmatische Expression von Histon H3trimethylK4 in Zervixkarzinomzellproben war mit höherem T-Status und schlechterer klinischer Prognose korreliert. Hierbei konnten vor allem Patientinnen mit Rezidiv der Erkrankung korreliert werden. Die nukleäre Ausprägung von HistontrimethylK4 war ebenfalls mit schlechterem Outcome der Patientinnen korreliert, wohingegen diese Patientinnengruppe auch ein schlechteres Überleben aufwiesen*. Im einzelnen siehe Int J Mol Sci. 2017 ab Seite 49: Histone H3 Acetyl K9 and Histone H3 Tri Methyl K4 as Prognostic Markers for Patients with Cervical Cancer. Beyer S, Zhu J, Mayr D, Kuhn C, Schulze S, Hofmann S, Dannecker C, Jeschke U, Kost BP.*

In der Onkologie sind Auswirkungen von Steroidhormonen auf Tumore sehr unterschiedlich. Dies schließt die Expression ihres jeweiligen Rezeptors ein. Um den Einfluss des Glucocorticoidrezeptors in Zervixkarzinomzellen zu untersuchen wurden an Zervixkarzinomproben immunhistochemische Analysen durchgeführt und mit klinischen Parametern der Patientinnen korreliert. Hierbei zeigte sich insgesamt eine gehäufte Expression des Rezeptors in diesen Zellen. Im Vergleich zu Adenokarzinomzellen waren Plattenepithelkarzinomzellen signfikant mehr betroffen. Ebenso zeigte sich eine signifikante Korrelation der Färbung zur FIGO-Klassifikation, wobei eine vermehrte Färbung mit niedrigem FIGO-Stadium assoziiert war. Zudem konnte eine signifikante Korrelation der Färbung des

Rezeptors in den Proben und den klinischen Daten der zugehörigen Patientinnen bezogen auf das Überleben gezeigt werden. *Im Einzelnen siehe Arch Gynecol Obstet. 2019 ab Seite 63: Glucocorticoid receptor in cervical cancer: an immunhistochemical analysis. Kost BP, Beyer S, Schröder L, Zhou J, Mayr D, Kuhn C, Schulze S, Hofmann S, Mahner S, Jeschke U, Heidegger H.*

Der Ko-Regulator RIP 140 (Receptor Interacting Protein) ist an der Regulierung onkologischer Signalwege beteiligt. In einer Analyse an Zervixkarzinomgewebe sollte die Expression des Rezeptors und eines weiteren Co-Repressors LCOR in diesen Zellen untersucht und eine mögliche Korrelation mit dem onkologischen Outcome der Patientinnen aufgezeigt werden. Hierbei war die erhöhte Expression von RIP140 mit signifikant schlechterem Überleben in Patientinnen mit Plattenepithelkarzinom assoziiert und kann in der Analyse als unabhängiger Prognosefaktor in diesen Patientinnen gesehen werden. Patientinnen mit Adenokarzinom waren hiervon nicht betroffen. Niedrige Expresion von RIP140 und LCoR waren mit besserem Überleben korreliert. Ebenso war RIP140 nicht als negativer Prognosefaktor in Zervixkarzinompatientinnen zu werten, wenn die LCoR-Expression in den Zellen niedrig war. *Im Einzelnen siehe Oncotarget. 2017 ab Seite 70: Investigation of RIP140 and LCoR as independent markers for poor prognosis in cervical cancer. Vattai A, Cavailles V, Sixou S, Beyer S, Kuhn C, Peryanova M, Heidegger H, Hermelink K, Mayr D, Mahner S, Dannecker C, Jeschke U, Kost B.*

Prostaglandine und deren Rezeptoren sind für Tumorwachstum und die tumorassoziierte Angioogenese wichtig. Die Expression des Prostaglandin E Rezeptors Typ3 (EP3) und eine mögliche Prostglandin-vermittelte Signalkette in der Karzinogenese von Zervixkarzinomzellen sind bisher wenig untersucht. Beim Vergleich der EP-3 Expression, abhängig vom FIGO-Stadium, zeigten die Stadien FIGO II-IV einem signifikanten Unterschied zum FIGO-Stadium I. Eine erhöhte Expression von EP-3 in Zervixkarzinomzellen war hierbei mit schlechterer Prognose und schlechterem Überleben korreliert. *Im Einzelnen siehe Int J Mol Sci. 2017 ab Seite 86: The Prostaglandin EP3 Receptor Is an Independent Negative Prognostic Factor for*

Cervical Cancer Patients. Heidegger H, Dietlmeier S, Ye Y, Kuhn C, Vattai A, Aberl C, Jeschke U, Mahner S, Kost B.

ONCOLOGY LETTERS 13: 2495-2501, 2017

Prevalence of human papillomavirus infection of the anal canal in women: A prospective analysis of high-risk populations

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Received July 19, 2016; Accepted November 23, 2016

DOI: 10.3892/ol.2017.5714

Abstract. Infection with certain types of human papillomavirus (HPV) has been associated with the development of cervical and anal cancer. Worldwide, the incidence of anal cancer has increased markedly. The present study aimed to evaluate the prevalence of HPV infection of the uterine cervix and anal canal in human immunodeficiency virus (HIV)- and non-HIV-infected risk populations. Cervical and anal HPV swabs and cytology samples were collected from 287 patients at the University Hospital of Munich, Germany between 2011 and 2013. Patients were divided into HIV-negative controls (G1) and two risk groups, including HIV-negative patients with cytological abnormalities of the cervix (G2) and HIV-infected patients (G3). Data, including clinical parameters, were analysed. The risk groups had significantly more positive results for HPV in the anus (71.03 and 83.15% for G2 and G3, respectively), as compared with G1. The predominant HPV genotypes found in the anus were high-risk HPV genotypes, which were significantly correlated with concomittant cervical HPV findings. In the risk groups, a significant association between the cytological findings and HPV detection in the cervix was found, while the results of the anus revealed no significance. The results of the present study suggested that the prevalence of HPV infection in the anal canal of risk populations is high. Furthermore, patients with abnormal cervical cytology results and HIV-infected women, irrespective of their individual cervical findings, may have a risk of concomittant anal high-risk HPV infection. Based on the predominant HPV genotypes found in the study, HPV vaccination could reduce the incidence of anal cancer. Nevertheless, high-risk patients should be intensively screened for anal squamous intraepithelial abnormalities to avoid invasive cancer stages.

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Key words: human papillomavirus, human immunodeficiency virus, anal dysplasia, cancer screening, women

Introduction

Infection with certain types of human papillomavirus (HPV) plays an important role in the development of cervical and anal cancer. In the sexually active population, HPV infection of the anogenital region can be found in >60% of individuals (1). Screening for cervical cancer is well established, and is accompanied by a significant risk reduction in the incidence of that cancer type (2). During the pathogenesis of cervical cancer, precursor lesions, such as anal intraepithelial neoplasia (AIN), can develop into invasive squamous cell carcinoma of the anus (3). The majority of anal malignancies are associated with a persistent infection with HPV (4).

Over the past few decades, the incidence of anal cancer has increased significantly, particularly in women (5,6). In a previous study, coexisting HPV infection of the cervix and anal canal was detected in human immunodeficiency virus (HIV)-negative patients (7). Risk populations include men who have sex with men and transplant recipients. Furthermore, HIV-infected patients showed a higher prevalence of HPV-associated anal dysplasia or anal cancer compared with the HIV-negative population (8). In addition, the overall risk was ~28-fold higher in the female HIV-infected population (8). Whether treatment with combined antiretroviral therapy (cART) is able to reduce the risk of anal dysplasia and anal cancer in this patient group is controversial; however, its ability to reduce the occurrence of cervical and anal cancer has been poor to date (9-11).

The effectiveness of anal cancer screening has not been sufficiently evaluated. Furthermore, the question of whether the development of anal cancer can be prevented by the sufficient treatment of high-grade AIN alone has yet to be answered (2). At present, strategies for anal screening and the treatment of high-grade AINs are under investigation in randomized controlled studies (12,13). At the very least, the identification of risk factors, such as anal HPV infection, and the resulting short-term monitoring of high-risk populations,

may lead to the early detection of the precancer and invasive stages.

Generally the prevalence of intra-anal HPV infection in the general HIV-negative female population, as compared with high-risk populations such as HIV-infected or HIV-negative patients with HPV-associated cervical abnormalities, is unknown. Therefore, the present prospective, cross-sectional study aimed to evaluate the prevalence of cervical and anal HPV infection, as well as clinical risk factors, in general controls, as compared with risk populations for anogenital dysplasia, including HIV-negative patients with abnormal cervical cytology attending the clinic for colposcopy evaluation and HIV-infected women.

Materials and methods

Study population. The prospective study included 287 patients who attended the Cervical Disease Screening and Treatment Unit or the specialized Gynaecological Outpatient Clinic for HIV-infected women at the Department of Gynecology and Obstetrics, Ludwig-Maximilian University of Munich (Munich, Germany) between 2011 and 2013. Patients were divided into three groups, as follows: G1, which included HIV-negative patients without a history of abnormal cytological findings who underwent routine cervical cytological screening (low-risk, n=93); G2, which included HIV-negative patients who were sent to our colposcopy unit with at least one preceding abnormal Papanicolaou-smear result (high-risk, n=90); and G3, which included HIV-infected patients who underwent routine cervical cytological screening at our outpatient department for HIV-infected women (high-risk, n=104). None of the patients had received the anti-HPV vaccination or were diagnosed with condyloma acuminata. All patients completed an anonymous, self-administered questionnaire, which collected information regarding their age, medical history, country of origin, smoking status, history of anal intercourse, number of sexual partners, age of first sexual intercourse and current marital status. According to the HIV-related history Centers for Disease Control and Prevention classification system, current cluster of differentiation (CD)4$^+$ counts and nadir, information related to the viral load and ART were collected. Written informed consent was obtained from all patients. The present study was approved by the Local Ethics Committee of the Ludwig-Maximilian University of Munich (approval no. 273-10).

Specimen collection. Cervical and corresponding intra-anal cytology and HPV samples were obtained from each patient, according to the study protocol. Smears for cytology were performed in-house using a moistened cotton swab and cytobrush for cervical samples, and a moistened cotton swab for intraanal samples; they were fixed with M-Fix® spray fixative at room temperature (Merck KGaA, Darmstadt, Germany), stained according to the Papanicolaou protocol and evaluated using Munich nomenclature II (14) by an experienced cytologist using light microscopy. For further evaluation, the results were transferred to the Bethesda system (15). For HPV detection, a separate swab was used for cervical and intraanal probes.

Detection and genotyping of HPV in clinical specimens. DNA was isolated and purified from the specimens using a commercial kit (QIAamp DNA Mini kit; Qiagen GmbH, Hilden, Germany), according to the manufacturer's protocol. Amplification of the L1-open reading frame and genotyping of the HPVs were performed using the INNO-LiPA HPV Genotyping Extra Amp and the INNO-LiPA HPV Genotyping Extra kit (both from Fujirebio Europe, Gent, Belgium). Known HPV genotypes (16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59 and 68), as well as the genotypes 6, 11, 26, 40, 43, 44, 53, 54, 66, 73, 70 and 82 were covered.

Statistical analysis. Statistical analyses were performed by STAT-UP Statistical Consulting & Services (Munich, Germany) using the R statistical package software, version 2.14.0 for Windows. The threshold of significance was set as P=0.05. Groups were compared using Mann-Whitney U, Kruskal-Wallis, Fisher's exact and χ^2 tests. Significance levels in post hoc tests were Bonferroni-Holm adjusted.

Results

Patient characteristics. A total of 287 patients were included in the study and divided into three groups. The baseline characteristics of the examined cohort are presented in Table I.

Anal HPV findings. The prevalence of anal HPV infection among the three analysed groups (G1-G3) was significantly different; 50, 71 and 83% of G1, G2 and G3, respectively, had a positive result for HPV in the anus. As compared with G1, G2 (P=0.011) and G3 (P<0.001) showed significantly more anal HPV infections of any type, while the difference between the G2 and G3 risk groups was not significantly different (P>0.05).

After dividing HPV genotypes into high-risk and low-risk anal HPV types, high-risk HPV genotypes were found significantly more often in the anal samples from G2 (65%; P<0.001) and G3 (62%; P<0.001), as compared with those from G1 (28%). The difference between G2 and G3 with regard to high-risk anal HPV genotypes, as well as the differences between the three groups with regard to low-risk anal HPV genotypes, were not significantly different.

Cervical and anal HPV findings. Regarding the prevalence of different HPV genotypes in the cervix and anus, 13 high-risk HPV genotypes (16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59 and 68) and 11 low-risk HPV genotypes (6, 11, 44, 53, 54, 66, 69, 70, 71, 74 and 82) were found. Fig. 1 illustrates the distribution of the different cervical and anal high-risk HPV genotypes in the analysed groups. In the risk groups (G2 and G3), the most prevalent anal high-risk HPV genotypes were genotypes 16 (G2, 27%; G3, 14%), 18 (G2, 4%; G3, 9%), 31 (G2, 7%; G3, 12%), 51 (G2, 12%; G3, 15%) and 52 (G2, 16%; G3, 8%).

In all groups, a positive HPV result was associated with significant concomitant cervical and anal HPV infection of any type (all P<0.05). A positive result for HPV in samples from the cervix and anus was found in 29% of the analysed patients in G1, 68% in G2 and 56% in G3. The significant association between cervical and anal samples persisted even after

Table I. Baseline patient characteristics.

Characteristic	Whole cohort (n=287)	G1 (n=93)	G2 (n=90)	G3 (n=104)	P-values
Age, years	37.59±9.04	39.74±9.78	33.61±7.52	39.11±8.51	G1 vs. G2, P<0.05; G2 vs. G3, P<0.05; G1 vs. G3, P>0.05
Origin					P<0.05
Western Europe	61.8	65.6	95.4	37.2	
Eastern Europe	12.3	20.4	3.1	9.3	
Central/South America	4.0/10.9	1.5/4.7	1.5/0	2.3/38.4	
Africa	10.9	7.8	0	12.8	
Smoking					P<0.05
Yes	32.0	29.0	44.3	24.0	
No	68.0	71.0	55.7	76.0	
Anal intercourse					P>0.05
Yes	28.8	35.5	25.3	25.7	
No	71.2	64.5	74.7	74.3	
Age at first sexual intercourse, years					P>0.05
≤15	20.0	18.5	14.9	25.7	
16-19	56.1	53.3	66.7	49.5	
20-24	21.4	25.0	16.1	22.8	
≥25	2.5	3.2	2.3	2.0	
Lifetime sex partners					P>0.05
1	9.0	16.5	4.6	5.9	
2-5	46.2	42.9	46.0	49.5	
6-10	24.4	19.8	39.1	15.8	
>10	20.4	20.9	10.3	28.8	
Marital status/stable partner					P>0.05
Yes	81.4	84.8	86.2	74.3	
No	18.6	15.2	13.8	25.7	
History of worst cytological result of the cervix					P>0.05
Not specified	44.3	92.9	21.4	19.6	
PAP 2	8.6	1.2	1.4	19.6	
PAP 3	3.9	2.4	4.3	4.9	
PAP 3D	33.1	3.5	65.7	35.3	
PAP 4a	0.4	0	0	1.0	
PAP 4b	9.7	0	7.2	19.6	

Data are presented as the mean ± standard deviation or %. G1, HIV-negative patients who underwent routine cervical cytology screening; G2, HIV-negative patients with at least one abnormal cytological (Pap)-smear result of the cervix; G3, HIV-infected patients who underwent routine cervical cytology screening; PAP, Papanicolaou (Munich nomenclature II).

dividing HPV genotypes into high-risk and low-risk groups (both P<0.05).

With regard to individual HPV genotypes, there was a significant association between several high- and low-risk HPV genotypes and cervical and anal findings in the three analysed groups. There was a significant association between the cervical HPV genotype and the anal HPV genotype for 4 of the 5 predominant high-risk HPV genotypes found in G2 and G3 (G2: HPV 16, P<0.001; HPV 18, P<0.001; HPV 31, P<0.001; HPV 51, P=0.006; G3: HPV 16, P<0.001; HPV 18, P<0.001; HPV 31, P=0.012; HPV 52, P=0.001). Overall 36% of the analysed patients had a negative HPV result in the cervix after being tested positive for any HPV in the anus. In G2, 19% of the patients tested positive for any HPV genotype in the anus (high-risk, 33%), while 67% of G3 tested positive for any HPV genotype in the anus (high-risk, 43%). The difference between G2 and G3 was statistically significant (P<0.05).

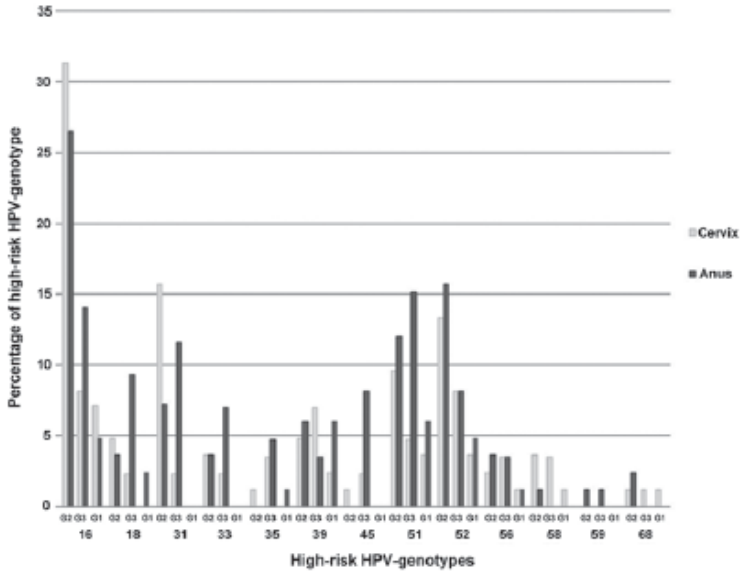

Figure 1. Illustration of the cervical and anal high-risk HPV genotypes in the analyzed groups. G1, HIV-negative patients who underwent routine cervical cytology screening; G2, HIV-negative patients with at least one abnormal cytological (Pap)-smear result of the cervix; G3, HIV-infected patients who underwent routine cervical cytology screening; HPV, human papillomavirus; HIV, human immunodeficiency virus.

Cervical and anal cytology and HPV findings. Analysing the results of the cervical and anal cytological analysis for cervical and anal HPV detection, a significant association between the cervical cytology result and cervical HPV was detected in all groups (all $P<0.05$). The worse the results of the cervical cytology, the more patients in that group tested positive for any HPV genotype in the cervix. Regarding the association between the anal cytology results and anal HPV detection, the analysed groups revealed no significance (Table II).

Anal HPV findings and clinical parameters. A significant association was observed between the detection of anal HPV infection in the present study and the number of lifetime sex partners ($P<0.001$), history of abnormal cervical cytology ($P=0.009$) and the history of cervical HPV ($P<0.001$). None of the other analysed characteristics (age, origin, smoking, anal intercourse, age of first sexual intercourse or marital status) were significantly associated with anal HPV infection. Regarding the HIV-infected patients, only the $CD4^+$ nadir was significantly associated with anal HPV ($P=0.021$). The current $CD4^+$ count, current HIV viral load, and the use of cART were not significantly associated with anal HPV. The clinical aspects related to HIV infection in G3 are summarized in Table III.

Discussion

The incidence of HPV-related cancer of the anus has increased over the past several decades. Risk populations, including women with a history of genital neoplasia or HIV-infected patients, are known. Furthermore, methods for effective anal cancer screening are under investigation (13).

Consistently with a recent review by Stier *et al* (16), both high-risk groups in the present study (G2 and G3) tested positive for any HPV genotype in the anus more often than the controls (G1). Limited data exists regarding anal HPV infection in the non-immunosuppressed general population, with high variance in the prevalence of anal HPV detection itself, and the prevalence of anal HPV in HIV-uninfected high-risk patients compared with HIV-infected women (17,18). All predominant HPV genotypes found in the anus of the two risk groups in the present study were high-risk HPV genotypes. Compared with cervical cancer, the incidence of anal cancer in the general population is low (19). It seems that anal colonisation with HPV is less likely to lead to cell abnormalities than cervical colonisation, although the difference in the carcinogenesis of these mucosal sites has yet to be completely evaluated. At least chronological differences can be assumed (18). Nevertheless, due to high prevalence of anal HPV infection, the detection and possible treatment of anal precancerous lesions could decrease the incidence of anal cancer.

Sufficient cervical screening reduces the incidence of invasive cancer. At the same time, the prophylactic HPV vaccination reduces the incidence of HPV infection, assuming a reduction in the incidence of HPV-associated cancers (20). Prophylactic HPV vaccination is part of the individual immunisation schedule in many countries, although the country-specific performance varies considerably (21). Besides the effect on the uterine cervix, the quadrivalente HPV vaccine was demonstrated to prevent persistent anal HPV infection and anal intraepithelial lesions (22). The five most prevalent anal HPV genotypes found in the present study were HPV types

Table II. Percentage of patients who tested positive for HPV in association with the corresponding cytology results of the cervix and anus.

Cytology classification	n	% HPV	P-value
G1			
Cervix			P<0.05
PAP 2 (NILM)	78	24	
PAP 3 (ASCUS)	12	58	
PAP 3D (LSIL)	2	100	
Anus			P>0.05
PAP 2 (NILM)	64	45	
PAP 3 (ASCUS)	6	100	
PAP 3D (LSIL)	10	60	
G2			
Cervix			P<0.05
PAP 2 (NILM)	15	56	
PAP 3 (ASCUS)	9	82	
PAP 3D (LSIL)	14	93	
PAP 4a (HSIL)	25	96	
Anus			P>0.05
PAP 2 (NILM)	27	73	
PAP 3 (ASCUS)	3	75	
PAP 3D (LSIL)	17	71	
PAP 4a (HSIL)	1	100	
G3			
Cervix			P<0.05
PAP 2 (NILM)	21	42	
PAP 3 (ASCUS)	15	63	
PAP 3D (LSIL)	13	100	
PAP 4a (HSIL)	6	100	
PAP 4b (HSIL/cancer)	1	100	
PAP 5 (cancer)	2	100	
Anus			P>0.05
PAP 2 (NILM)	62	83	
PAP 3 (ASCUS)	4	67	
PAP 3D (LSIL)	5	100	
PAP 4a (HSIL)	1	100	

G1, HIV-negative patients who underwent routine cervical cytology screening; G2, HIV-negative patients with at least one preceding abnormal cytological (PAP)-smear result of the cervix; G3, HIV-infected patients who underwent routine cervical cytology screening; PAP, Papanicolaou using Munich nomenclature II; HPV, human papillomavirus; HIV, human immunodeficiency virus; NILM, negative for intraepithelial lesion or malignancy; ASCUS, atypical squamous cells of undetermined significance; LSIL, low grade squamous intraepithelial lesion; HSIL, high grade squamous intraepithelial lesion.

16, 18, 31, 52 and 51. With the administration of the 9-valent HPV vaccine (types 6, 11, 16, 18, 31, 33, 45, 52 and 58), the rising incidence of anal cancer could be prevented more effectively.

Table III. Specific characteristics of HIV-infected patients.

Characteristic	G3 HIV-infected patients (n=104)
Current detectable viral load (copies/ml)	2,772 (<20->100,000)
Taking cART	
Yes	89
No	11
cART duration (years)	
Range	0-21
Mean ± SD	8.2±5.6
Current CD4 count (cells/μl)	540 (34-1650)
Nadir CD4 count (cells/μl)	258 (1-994)

Data are presented as the mean (interquartile range) or %. G3, HIV-infected patients who underwent routine cervical cytology screening; cART, combined antiretroviral therapy; HIV, human immunodeficiency virus; CD4, cluster of differentiation 4; SD, standard deviation.

It was reported that the detection rate of HPV in simultaneously collected cervical and anal specimens was comparable or even higher in the anus (16). Consistent with the literature, a significant association between the prevalence of concomitant low- and high-risk cervical and anal HPV in all investigated patient groups was detected in the present study. One third of the analysed patients who had a negative result for HPV in the cervix tested positive in the anus. Of the HIV-infected patients with a negative result for HPV in the cervix, 67% tested positive for HPV in the anus. The risk for concomitant HPV infection of the cervix and anus appeared to be likely. However, a negative HPV result in the cervix should not discount the performance of an anal HPV screening, particularly in high-risk patients.

Screening for cervical cancer is well established. The benefits of cytology-based vs. HPV-based cancer screening of different mucosal sites are under discussion (23). A significant association between the cytology findings of the cervix and cervical HPV detection was found in the present study. Controversial results were published concerning the association between anal cytology and anal HPV prevalence. A previous study reported a suspicious anal cytology in <10% of women with lower genital tract dysplasia, while positive anal HPV results were detected in >50% (24). In the present study, a significant association between the cytological results of the cervix and HPV detection in the cervix was observed, while there was no significant association between the anal cytology results and positive anal HPV detection. Compared with the cervical findings, a correlation between anal HPV infection and a suspicious anal cytology is infrequently observed. To date, there have been no valid data concerning the efficacy on any type of anal cancer screening technique. Currently, the primary screening tool for anal HPV-associated disease is cytology. Although the performance of anal cytology is similar to cervical cytology, experience in interpreting anal samples is limited. Other techniques such as high-resolution

anoscopy should be considered to verify the true rate of anal dysplasias, particularly in cytological-negative and HPV-positive anal samples from risk populations. Further studies on the evaluation and implementation of anal cancer screening are required (25).

Consistent with the literature, a significant association between the number of lifetime sex partners and the incidence of anal HPV infection was demonstrated in the present study. Anal intercourse itself was not a significant factor, as demonstrated previously (26,27). The prevalence of concomitant low- and high-risk cervical and anal HPV was significantly associated in all investigated patient groups. It is still unknown if there is a reservoir for HPV in the genito-anal area. Besides sexual transmission, the transfer of HPV between the different mucosal sites may occur as a result of autoinoculation or smear infection (28-30). Irrespective of their individual immune status, the majority of the analysed high-risk patients in the present study showed an comprehensive anogenital HPV colonisation at the time of specimen collection. It was postulated that some type of global immune dysregulation results in the persistence of HPV in the cervix and anus (31). Immunosuppression caused by HIV infection is associated with HPV-related malignancies and contributes to HIV pathogenesis (31). Regarding the HIV-infected patients in the present study, the current CD4 count, as well as the current HIV viral load, did not have a significant influence on anal HPV infection. The impact of CD4 count and HIV viral load was previously discussed controversially. The low number of HIV-infected women analysed in the present study could be an explanation for this discrepancy. However consistent with the findings of Hessol *et al* (17), in which women with lower CD4 cell counts were more likely to have detectable oncogenic and non-oncogenic HPV types, a low CD4 nadir was significantly associated with anal HPV detection in the present study. In a study by Cambou *et al* (32), a significant correlation between a low CD4 nadir and previous CD4 counts was detected, indicating an increased risk for anal dysplasia in HIV-infected women with a history of severe immune devastation. The increased prevalence of HPV infection in HIV-infected individuals seems to be associated with immunosuppression (33). A higher level of HPV replication in women with a compromised immune system due to HIV infection could be the reason for that fact (16). The exact mechanisms of HIV-HPV interactions are still under investigation. A previous study by Palefsky (9) evaluated whether the use of cART has an influence on the prevalence of anal HPV infection by immune recovery, finding that it lead to a limited reduction in HPV prevalence and the regression of cervical intraepithelial neoplasia. Subsequently, it was demonstrated that the risk of persistent HPV infection in HIV-infected women under long-term cART was reduced due to sustained viral suppression and increased CD4 counts (34,35). The relatively short duration of cART use in the present study did not have a significant effect on anal HPV reduction. Due to the increased lifespan of HIV-infected patients, a consistent ART seems to be important to reduce the incidence of acquired immunodeficiency syndrome-defining HPV-associated cancers, such as cervical or anal cancer.

The limitation of this study was the cross-sectional setting. It could not be determined how many of the HPV infections were transient. A type-specific clearance of high- and low-risk HPV genotypes in the majority of women over a 5-year mean follow-up detection period was reported (36). The exception was HPV type 16, and concomitant cervical infection with this genotype, which is found in the majority of cervical high-grade dysplasias, was associated with anal HPV persistence (36). Further studies are required to clear these facts.

In conclusion, the present study demonstrated that the prevalence of anal HPV infection in high-risk populations is high. Non-HIV-infected women with cervical dysplasia and HIV-infected women, irrespective of their individual cervical findings, had a high risk of concomitant anal HPV infection. Due to the increase in lifespan of HIV-infected women receiving cART, these patients should be seen as a lifetime risk population for HPV-associated anal cancer. Based on the predominant HPV genotypes found, the HPV vaccination could reduce the incidence of anal cancer. Concomitant intense screening for cervical and anal dysplasias in high-risk populations should be offered as a matter of routine to avoid invasive stages.

References

1. Nyitray A, Nielson CM, Harris RB, Flores R, Abrahamsen M, Dunne EF and Giuliano AR: Prevalence o fand risk factors for anal human papillomavirus infection in heterosexual men. J Infect Dis 197: 1676-1684, 2008.
2. Melbye M and Sprøgel P: Aetiological parallel between anal cancer and cervical cancer. Lancet 338: 657-659, 1991.
3. Watson AJ, Smith BB, Whitehead MR, Sykes PH and Frizelle FA: Malignant progression of anal intra-epithelial neoplasia. ANZ J Surg 76: 715-717, 2006.
4. Gervaz P, Hirschel B and Morel P: Molecular biology of squamous cell carcinoma oft he anus. Br J Surg 93: 531-538, 2006.
5. Arbyn M, de Sanjosé S, Saraiya M, Sideri M, Palefsky J, Lacey C, Gillison M, Bruni L, Ronco G, Wentzensen N, *et al*: EUROGIN 2011 roadmap on prevention and treatment of HPV-related diesease. Int J Cancer 131: 1969-1982, 2012.
6. American Cancer Society: Cancer facts and figures. American Cancer Society, Atlanta, GA, 2012.
7. Guler T, Uygur D, Uncu M, Yayci E, Atacag T, Bas K, Gunay M and Yakicier C: Coexisting anal human papilloma virus infection in heterosexual women with cervical HPV infection. Arch Gynecol Obstet 288: 667-672, 2013.
8. Frisch M, Biggar RJ and Goedert JJ: Human papillomavirus-associated cancers in patients with human immunodeficiency virus infection and aquired immunodeficiency syndome. J Natl Cancer Inst 92: 1500-1510, 2000.
9. Palefsky JM: Cervical human papillomavirus infection and cervical intraepithelial neoplasia in women positive for human immunodeficiency virus in the era of highly active antiretroviral therapy. Curr Opin Oncol 15: 382-388, 2003.
10. Duncan KC, Chan KJ, Chiu CG, Montaner JS, Coldman AJ, Cescon A, Au-Yeung CG, Wiseman SM, Hogg RS and Press NM: HAART slows progression to anal cancer in HIV-infected MSM. AIDS 29: 305-311, 2015.
11. van der Snoek EM, van der Ende ME, den Hollander JC, Schutten M, Neumann HA and van Doornum GJ: Use of highly active antiretroviral therapy is associated with lower prevalence of anal intraepithelial neoplastic lesions and lower prevalence of human papillomavirus in HIV-infected men who have sex with men. Sex Transm Dis 39: 495-500, 2012.
12. ANCHOR Study: The Anal Cancer/HSIL Outcomes Research Study United States of America. https://anchorstudy.org, 2016.
13. Gosens KC, Richel O and Prins JM: Human papillomavirus as a cause of anal cancer and the role of screening. Curr Opin Infect Dis 30: 87-92, 2017.
14. Langfassung der Leitlinie: HPV-Infektion / präinvasive Läsionen des weiblichen Genitale: Prävention, Diagnostik und Therapie. https://leitlinien.net, 2008.
15. Wright TC Jr, Cox JT, Massad LS, Twiggs LB and Wilkinson EJ; ASCCP-Sponsored Consensus Conference: 2001 Consensus Guidelines for the management of women with cervical cytological abnormalities. J Am Med Assoc 287: 2120-2129, 2002.

16. Stier EA, Sebring MC, Mendez AE, Ba FS, Trimble DD and Chiao EY: Prevalence of anal human papillomavirus infection and anal HPV-related disorders in women: A systematic review. Am J Obstet Gynecol 213: 278-309, 2015.
17. Hessol NA, Holly EA, Efird JT, Minkoff H, Weber KM, Darragh TM, Burk RD, Strickler HD, Greenblatt RM and Palefsky JM: Concomitant anal and cervical human papillomavirus infections and intraepithelial neoplasia in HIV-infected and uninfected women. AIDS 27: 1743-1751, 2013.
18. Crawford R, Grignon AL, Kitson S, Winder DM, Ball SL, Vaughan K, Stanley MA, Sterling JC and Goon PK: High prevalence of HPV in non-cervical sites of women with abnormal cervical cytology. BMC Cancer 11: 473, 2011.
19. Islami F, Ferlay J, Lortet-Tieulent J, Bray F and Jemal A: International trends in anal cancer incidence rates. Int J Epidemiol: Oct 27, 2016 (Epub ahead of print).
20. Mesher D, Panwar K, Thomas SL, Beddows S and Soldan K: Continuing reductions in HPV 16/18 in a population with high coverage of bivalent HPV vaccination in England: An ongoing cross-sectional study. BMJ Open 6: e009915, 2016.
21. Bruni L, Diaz M, Barrionuevo-Rosas L, Herrero R, Bray F, Bosch FX, de Sanjosé S and Castellsagué X: Global estimates of human papillomavirus vaccination coverage by region and income level: A pooled analysis. Lancet Glob Health 4: e453-e463, 2016.
22. Stier EA, Chigurupati NL and Fung L: Prophylactic HPV vaccination and anal cancer. Hum Vaccin Immunother 12: 1348-1351, 2016.
23. Herbert A: Primary HPV testing: A proposal for co-testing in initial rounds of screening to optimise sensitivity of cervical cancer screening. Cytopathology: Mar 23, 2016 (Epub ahead of print).
24. Park IU, Ogilvie JW Jr, Anderson KE, Li ZZ, Darrah L, Madoff R and Downs L Jr: Anal human papillomavirus infection and abnormal anal cytology in women with genital neoplasia. Gynecol Oncol 114: 399-403, 2009.
25. Denny LA, Franceschi S, de Sanjosé S, Heard I, Moscicki AB and Palefsky J: Human papillomavirus, human immunodeficiency virus and immunosuppression. Vaccine 30 (Suppl 5): F168-F174, 2012.
26. Beachler DC, D'Souza G, Sugar EA, Xiao W and Gillison ML: Natural history of anal vs oral HPV infection in HIV-infected men and women. J Infect Dis 208: 330-339, 2013.
27. Goodman MT, Shvetsov YB, McDuffie K, Wilkens LR, Zhu X, Thompson PJ, Ning L, Killeen J, Kamemoto L and Hernandez BY: Sequential acquisition of human papillomavirus (HPV) infection oft he anus and the cervix: The Hawaii HPV Cohort Study. J Infect Dis 201: 1331-1339, 2010.
28. Sehnal B, Dusek L, Cibula D, Zima T, Halaska M, Driak D and Slama J: The relationship between the cervical and anal HPV infection in women with cervical intraepithelial neoplasia. J Clin Virol 59: 18-23, 2014.
29. Winer RL, Hughes JP, Feng Q, Xi LF, Cherne S, O'Reilly S, Kiviat NB and Koutsky LA: Detection of genital HPV types in fingertip samples from newly sexually active female univerity students. Cancer Epidemiol Biomarkers Prev 19: 1682-1685, 2010.
30. Widdice LE, Breland DJ, Jonte J, Farhat S, Ma Y, Leonard AC and Moscicki AB: Human papillomavirus concordance in heterosexual couples. J Adolesc Health 47: 151-159, 2010.
31. Brickman C and Palewsky JM: Human papillomavirus in the HIV-infected host: Epidemiology and pathogenesis in the antiretroviral era. Curr HIV/AIDS Rep 12: 6-15, 2015.
32. Cambou MC, Luz PM, Lake JE, Levi JE, Coutinho JR, de Andrade A, Heinke T, Derrico M, Veloso VG, Friedman RK and Grinsztejn B: Anal human papillomavirus (HPV) prevalences and factors associated with abnormal anal cytology in HIV-infected women in an urban cohort from Rio de Janeiro, Brazil. AIDS Patient Care STDS 29: 4-12, 2015.
33. Palefsky J: Human papillomavirus-related disease in people with HIV. Curr Opin HIV AIDS 4: 52-56, 2009.
34. Konopnicki D, Manigart Y, Gilles C, Barlow P, de Marchin J, Feoli F, Larsimont D, Delforge M, De Wit S and Clumeck N: Sustained viral suppression and higher CD4+ T-cell count reduces the risk of persistent cervical high-risk human papillomavirus infection in HIV-positive women. J Infect Dis 207: 1723-1729, 2013.
35. Zeier MD, Botha MH, Engelbrecht S, Machekano RN, Jacobs GB, Isaacs S, van Schalkwyk M, van der Merwe H, Mason D and Nachega JB: Combination antiretroviral therapy reduces the detection risk of cervical human papillomavirus infection in women living with HIV. AIDS 29: 59-66, 2015.
36. Moscicki AB, Ma Y, Farhat S, Jay J, Hanson E, Benningfield S, Jonte J, Godwin-Medina C, Wilson R and Shiboski S: Natural history of anal human papillomavirus infection in heterosexual women and risks associated with persistence. Clin Infect Dis 58: 804-811, 2014.

ANTICANCER RESEARCH *36*: 3195-3198 (2016)

Immunohistochemical Evaluation of E6/E7 HPV Oncoproteins Staining in Cervical Cancer

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Abstract. *Background/Aim: High-risk human papillomavirus (HPV) subtypes (i.e. 16 and 18) lead to uterine cervical cancer as well as HPV-positive oropharyngeal cancer (OSCC), a form of head and neck cancer. The induction of HPV-induced cancer is driven by virus-specific oncoproteins E6 and E7. E6 protein of HPV types 16 and 18 interacts with the E3 ubiquitin protein ligase, resulting in ubiquitination and proteolysis of tumor protein p53. E7 inactivates retinoblastoma protein (Rb) by phosphorylation followed by an increase of free eukaryotic transcription factor E2F (E2F) in the cell. This leads to an increase of cyclin-dependent kinase inhibitor p16, that is used as an immunohistochemical marker of HPV-associated OSCC. Unfortunately, p16 is not exclusively increased by E7 oncoprotein in carcinogenesis. Therefore, the aim of this study was to develop an immunohistochemical approach for the direct detection of E6/E7 oncoproteins in uterine cervical cancer as well as in OSCC. Material and Methods: Paraffin sections of uterine cervical cancer and 130 were analyzed. Immunohistochemical staining protocols were evaluated with tissue slides from patients with cervical dysplasia (CIN III) and squamous epithelial carcinoma tissue with HPV infection. Liver and placental tissues were used as negative controls. E6-Specific antibody (Biorbyt) was used as primary antibody. The polymer staining method and diaminobenzidine were applied for further development. Panels of E7-specific antibodies were tested. Again, the polymer staining method and diaminobenzidine were applied for further development. Results: E6-Specific antibody revealed specific and intense staining after pre-incubation of tissue slides with citrate buffer solution. Only the E7 antibody obtained from Chemicon showed intense and specific staining in patients with CIN III and squamous epithelial carcinoma tissue. Pre-incubation with proteinase K diminished non-specific reaction. Conclusion: Our results revealed a useful staining protocol for the immunohistochemical evaluation of E6/E7 oncoprotein expression in uterine cervical cancer, as well as in HPV-positive oropharyngeal cancer. Advantages of this method compared to mRNA in situ hybridization of E6/E7 are the much lower costs, as well as the broader applicability in pathological practice.*

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Key Words: E6, E7, HPV, cervical cancer.

Uterine cervical cancer is the second most common malignant tumor in women worldwide, which means that there are about 530,000 new cases and more than 270,000 such tumor-associated deaths every year (1). The main cause of uterine cervical cancer is persistent infection with high-risk human papillomavirus (HR-HPV) (2); specifically HPV subtypes 16 and 18 are responsible for about 70% of all cases (2, 3). Apart from uterine cervical cancer, HPVs are also responsible for 25 to 60% of oral squamous cell carcinomas (OSCCs), a form of head and neck cancer (4, 5). At the moment, over 170 HPV types are known (6) and it is highly likely that infection with 15 types (namely types 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 68, 73 and 82) leads to cancer. Therefore, these 15 types are called carcinogenic or high-risk types (7). Human papillomaviruses are small double-stranded DNA viruses, their genome contains barely 8,000 base pairs arranged as a circle, containing six 'early' genes (*E6, E7, E1, E2, E4, E5*) and two 'late' genes (L1, L2) (4). As HPVs do not contain genes coding for replication enzymes, they are dependent on host cells for their replication (4). The replication of the viral genes *E6* and *E7* leads to cellular expression of E6 and E7 oncoproteins which interferes with the cell cycle (8). E6 oncoprotein links to E6- associated protein (E6-AP) forming a complex which selectively binds to p53, leading to its ubiquitin-dependent

0250-7005/2016 $2.00+.40

proteolytic degradation (9). p53 acts as a tumor suppressor, inducing cell-cycle arrest or apoptosis in cases of DNA damage (10). As a result of the E6-dependent degradation of p53, it can no longer fulfil its tasks (11). p53 is not the only tumor suppressor affected by HPV oncproteins. The retinoblastoma protein (Rb) blocks cell-cycle progression from G_1 to S phase by binding and inactivating transcription factors of the free eukaryotic transcription factor E2F family (12, 13). E7 oncoprotein interacts with Rb and leads to its ubiquitin-dependent degradation (14, 15). Furthermore, E7 leads to increased phosphorylation of Rb, but only a small proportion of phosporylated Rb can form a complex with E2F, hence the E2F–Rb interaction is disturbed and E2F is no longer inactivated (16, 17). Consequently, the amount of cyclin-dependent kinase p16 is increased and this is what is used as an immunohistochemical marker for the presence of HPV infection concerning OSCC (18, 19).

In routine practice, the detection of E6/E7 is based on either *in situ* hybridization (20) or PCR (21) methodology due to unspecific staining results of antibodies used in former studies (22). Therefore, we tested a variety of different antibodies and different unmasking, antigen retrieval and staining protocols. The aim of this study was the establishment of an immunohistochemical staining procedure for a safe and simple detection of E6/E7 at the protein level in HPV-infected tumor tissue.

Materials and Methods

Specimens. For the evaluation of the immunohistochemical staining, paraffin-embedded tissue slides (4 μm thickness) from patients with normal control cervical tissue (10 cases), cervical intraepithelial neoplasia (CIN; 10 CIN III cases) and from patients with uterine cervical carcinoma (10 cases) were used. The CIN III cases were considered as being HPV-positive. Placenta (10 cases) and liver (10 cases) were obtained from the Department of Pathology and used as tissues for providing negative staining results.

Immunohistochemistry. The formalin-fixed paraffin-embedded sections (3 μm) were dewaxed in xylol, the endogenous peroxidase was inhibited by 3% methanol/H_2O_2 and rehydrated in a descending ethanol gradient. The slides were pretreated in Citrate Buffer (100˚C, pH: 6.0) to unmask the antigen. Afterwards, non-specific binding of the primary antibodies was blocked by using the appropriate blocking solution. Incubation with the primary antibodies followed (Table I). Incubation with the secondary antibody and following steps of the convenient detection system and color development were carried out according to the manufacturer protocol. Finally, the slides were counterstained by hemalaun (2 min), dehydrated in a rising ethanol gradient and covered.

Evaluation of staining. The immunoreactive score (IRS) used examines the intensity and distribution of antigen expression and is calculated by multiplying the percentage of positively stained cells (0: no staining; 1<10% of the cells; 2: 11-50%; 3: 51- 80%, 4>81%) with the cell's intensity of staining (0: none; 1: weak; 2: moderate; 3: strong). Two independent investigators examined the sections using a Leitz Diaplan microscope (Leitz, Wetzlar, Germany). The concordance was 98%. In case of different staining evaluation, reevaluation was made by both investigators until an agreement was reached (2% of all cases). Positive controls were carried out with tissues of CIN (10 CIN III cases). Negative controls were performed by replacement of the primary antibody and alternative incubation of the slides with IgG rabbit or mouse control antibodies (Biogenex, San Ramen, USA). In addition, specificity of the staining was evaluated by using control tissue that should not express the E6/E7 antigen (placental and liver tissue).

Table I. *Antibodies used for the study.*

Antigen	Antibody	Isotype	Concentration	Source
E6	orb.10837	Rabbit polyclonal	0.5 mg/ml	Biorbyt
E6	C1P5	Mouse IgG1	1.0 mg/ml	Abcam
E7	orb. 10839	Rabbit polyclonal	0.5 mg/ml	Biorbyt
E7	clone 8C9	Mouse IgG1	0.1 μg/ml	Invitrogen
E7	TGV701Y	Mouse IgG2a	1 mg/ml	Chemicon

Results

E6 immunohistochemistry. Concerning the detection of E6 oncoprotein, the best results were obtained by using the E6 antibody by Abcam (Table I). By adhering to this procedure, the expression of E6 oncoprotein was shown by intense staining of the cytoplasm of the tumor cells (Figure 1A). As can be seen in Figure 1A, only the cytoplasm of the tumor cells is stained and not the surrounding connective tissue, hence the staining is very selective. It was even possible for us to show that there is a graduation of the expression of the oncoprotein. Within this study, we identified cases of uterine cervical carcinoma with intense staining of E6 (Figure 1A) but also with lower expression of this oncoprotein (Figure 1B). Staining in carcinoma tissue was more intense compared to staining of CIN III (Figure 1C). No expression of the oncoprotein in a sample of non-dysplastic cervix and consequently no staining are shown in Figure 1D. For the establishment of the staining procedure, uterine cervical carcinoma tissue was used (Figure 1E). Liver tissue served for negative control staining (Figure 1F) and as isotype control, we used the same cervical carcinoma as used for staining establishment (Figure 1G).

E7 immunohistochemistry. The evaluation of the E7 antibodies revealed intense and specific expression with the antibody obtained from Chemicon (Table I). By following this instruction, in cases of expression of E7 oncoprotein, the cytoplasm of the tumor cells is stained. We identified uterine cervical carcinoma cases with intensively stained cytoplasm (Figure 2A) and, in addition, cases with lower intensity (Figure 2B). CIN III cases exhibited a less intense staining compared

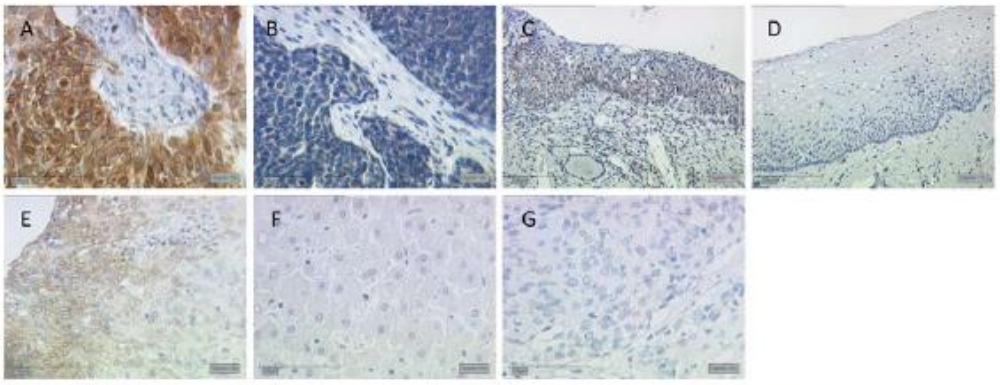

Figure 1. *Uterine cervical carcinoma showing intense staining of E6 by using the Abcam antibody (A) but also lower expression of this oncoprotein in another specimen (B). Staining in carcinoma tissue was more intense compared to staining of cervical dysplasia (CIN) grade III (C). No expression of the oncoprotein and consequently no staining were seen in all samples of non-dysplastic cervix (D). For the establishment of the staining procedure, uterine cervical carcinoma tissue was used (E). Liver tissue served for negative control staining (F), and as isotype control, the same cervical carcinoma, as used for staining establishment (G) was used. Magnification, ×25 in all cases.*

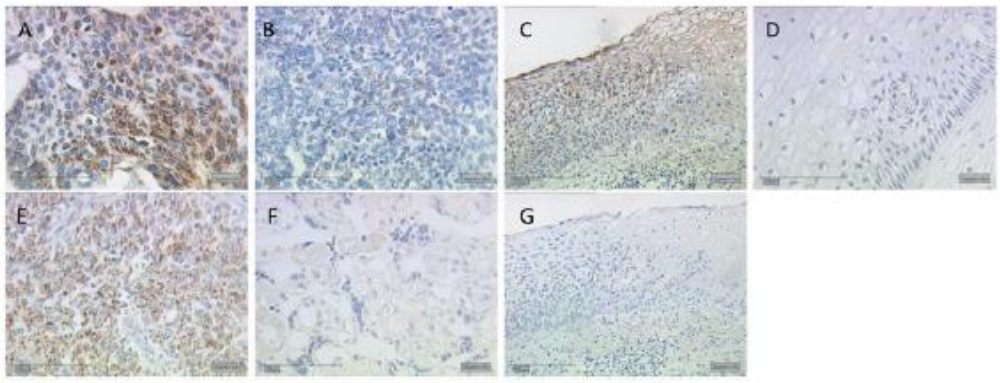

Figure 2. *E7 oncoprotein in uterine cervical carcinoma cases is reflected in intensively stained cytoplasm (A), but there were also cases with lower intensity (B). Cases of cervical dysplasia (CIN) grade III showed less intense staining compared to tumor cases (C). There was no expression of E7 oncoprotein in all samples of non-dysplastic cervix (D). As positive control tissue, tissue from conization of uterine cervix was used (E), as negative control tissue, placenta was obtained (F), and as isotype control, the same cervical carcinoma as used for staining establishment (G) was used. Magnification, ×25 in all cases.*

to tumor cases (Figure 2C). There was no expression of E7 oncoprotein found in a non-dysplastic cervix sample (Figure 2D). As positive control tissue tissue from conization of uterine cervix was used (Figure 2E), as negative control, placental tissue was used (Figure 2F), and as isotype control, we used the same cervical carcinoma as used for staining establishment (Figure 2G).

Discussion

In this study, we were able to establish a fast and simple method for the detection of the HPV-related oncoproteins E6/E7 in uterine cervical cancer tissue by using immunohistochemistry. As a result, a useful immunohistochemical evaluation protocol for the detection of the HPV oncoproteins

E6 and E7 was established. The best results were obtained with the E6 antibody from Abcam and the E7 antibody from Chemicon. The advantage of this procedure is the possibility of immunohistochemical evaluation of E6/E7 in routine pathology by following our staining protocols. An additional advantage of the immunohistochemical evaluation is that this method is easier to apply and less expensive in comparison to *in situ* mRNA hybridization. We are aware that *in situ* mRNA hybridization might be the best way to detect HPV, but on the other hand it is too complicated to be used for routine detection of HPV oncoproteins (23) compared to a simple immunohisto-chemistry protocol.

In summary, we showed that E6 and E7 oncoproteins expressed in uterine cervical carcinoma and CIN III carcinoma *in situ* tissues can be easily detected by immunohistochemistry using the E6 antibody from Abcam and the E7 antibody from Chemicon.

References

1 Tota JE, Chevarie-Davis M, Richardson LA, Devries M and Franco EL: Epidemiology and burden of HPV infection and related diseases: Implications for prevention strategies. Prev Med *53(Suppl 1)*: S12-21, 2011.

2 Munoz N, Bosch FX, Castellsague X, Diaz M, de Sanjose S, Hammouda D, Shah KV and Meijer CJ: Against which human papillomavirus types shall we vaccinate and screen? The international perspective. Int J Cancer *111(2)*: 278-285, 2004.

3 Schiffman M, Castle PE, Jeronimo J, Rodriguez AC and Wacholder S: Human papillomavirus and cervical cancer. Lancet *370(9590)*: 890-907, 2007.

4 Assmann G and Sotlar K: HPV-associated squamous cell carcinogenesis. Pathologe *32(5)*: 391-398, 2011.

5 Wittekindt C, Wagner S, Mayer CS and Klussmann JP: Basics of tumor development and importance of human papilloma virus (HPV) for head and neck cancer. GMS Curr Top Otorhinolaryngol Head Neck Surg *11*: Doc09, 2012.

6 Wittekindt C, Wagner S, Mayer CS and Klussmann JP: Basics of tumor development and importance of human papilloma virus (HPV) for head and neck cancer. Laryngorhinootologie *91(Suppl 1)*: S1-26, 2012.

7 Munoz N, Bosch FX, de Sanjose S, Herrero R, Castellsague X, Shah KV, Snijders PJ, Meijer CJ and International Agency for Research on Cancer Multicenter Cervical Cancer Study G: Epidemiologic classification of human papillomavirus types associated with cervical cancer. N Engl J Med *348(6)*: 518-527, 2003.

8 Gupta S, Takhar PP, Degenkolbe R, Koh CH, Zimmermann H, Yang CM, Guan Sim K, Hsu SI and Bernard HU: The human papillomavirus type 11 and 16 e6 proteins modulate the cell-cycle regulator and transcription cofactor TRIP-BR1. Virology *317(1)*: 155-164, 2003.

9 Scheffner M, Huibregtse JM, Vierstra RD and Howley PM: The HPV-16 E6 and E6-AP complex functions as a ubiquitin-protein ligase in the ubiquitination of p53. Cell *75(3)*: 495-505, 1993.

10 Tang D, Wu D, Hirao A, Lahti JM, Liu L, Mazza B, Kidd VJ, Mak TW and Ingram AJ: ERK activation mediates cell cycle arrest and apoptosis after DNA damage independently of p53. J Biol Chem *277(15)*: 12710-12717, 2002.

11 Fehrmann F and Laimins LA: Human papillomaviruses: Targeting differentiating epithelial cells for malignant transformation. Oncogene *22(33)*: 5201-5207, 2003.

12 Kim KY, Wang DH, Campbell M, Huerta SB, Shevchenko B, Izumiya C and Izumiya Y: PRMT4-mediated arginine methylation negatively regulates retinoblastoma tumor suppressor protein and promotes e2f-1 dissociation. Mol Cell Biol, 2014.

13 Lipinski MM and Jacks T: The retinoblastoma gene family in differentiation and development. Oncogene *18(55)*: 7873-7882, 1999.

14 Jones DL and Munger K: Interactions of the human papillomavirus E7 protein with cell cycle regulators. Semin Cancer Biol *7(6)*: 327-337, 1996.

15 Boyer SN, Wazer DE and Band V: E7 protein of human papilloma virus-16 induces degradation of retinoblastoma protein through the ubiquitin-proteasome pathway. Cancer Res *56(20)*: 4620-4624, 1996.

16 Yim EK and Park JS: The role of HPV E6 and E7 oncoproteins in HPV-associated cervical carcinogenesis. Cancer Res Treat *37(6)*: 319-324, 2005.

17 Chellappan S, Kraus VB, Kroger B, Munger K, Howley PM, Phelps WC and Nevins JR: Adenovirus E1A, simian virus 40 tumor antigen, and human papillomavirus E7 protein share the capacity to disrupt the interaction between transcription factor E2F and the retinoblastoma gene product. Proc Natl Acad Sci USA *89(10)*: 4549-4553, 1992.

18 Hoffmann M, Ihloff AS, Gorogh T, Weise JB, Fazel A, Krams M, Rittgen W, Schwarz E and Kahn T: P16(INK4A) overexpression predicts translational active human papillo-mavirus infection in tonsillar cancer. Int J Cancer *127(7)*: 1595-1602, 2010.

19 Preuss SF, Klussmann JP, Semrau R and Huebbers C: [update on HPV-induced oropharyngeal cancer]. HNO *59(10)*: 1031-1037, 2011.

20 Evans MF, Peng Z, Clark KM, Adamson CS, Ma XJ, Wu X, Wang H, Luo Y and Cooper K: HPV E6/E7 RNA *in situ* hybridization signal patterns as biomarkers of three-tier cervical intraepithelial neoplasia grade. PLoS One *9(3)*: e91142, 2014.

21 Hafner N, Gajda M, Altgassen C, Hertel H, Greinke C, Hillemanns P, Schneider A and Durst M: HPV16-e6 mrna is superior to cytokeratin 19 mRNA as a molecular marker for the detection of disseminated tumour cells in sentinel lymph nodes of patients with cervical cancer by quantitative reverse-transcription pcr. Int J Cancer *120(9)*: 1842-1846, 2007.

22 Hoffmann M, Tribius S, Quabius ES, Henry H, Pfannenschmidt S, Burkhardt C, Gorogh T, Halec G, Hoffmann AS, Kahn T, Rocken C, Haag J, Waterboer T and Schmitt M: HPV DNA, E6*i-mRNA expression and $p16^{INK4A}$ immunohistochemistry in head and neck cancer – how valid is $p16^{INK4A}$ as surrogate marker? Cancer Lett *323(1)*: 88-96, 2012.

23 Bishop JA, Ma XJ, Wang H, Luo Y, Illei PB, Begum S, Taube JM, Koch WM and Westra WH: Detection of transcriptionally active high-risk HPV in patients with head and neck squamous cell carcinoma as visualized by a novel E6/E7 mRNA *in situ* hybridization method. Am J Surg Pathol *36(12)*: 1874-1882, 2012.

Received March 15, 2016
Revised May 2, 2016
Accepted May 8, 2016

ONCOLOGY LETTERS 14: 4467-4476, 2017

The involvement of E6, p53, p16, MDM2 and Gal-3 in the clinical outcome of patients with cervical cancer

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Received December 7, 2016; Accepted February 3, 2017

DOI: 10.3892/ol.2017.6752

Abstract. High-risk human papilloma virus (HPV) is the leading cause of cervical cancer. HPV oncogenes are responsible for the development of malignancy, and the E6 oncoprotein that HPV expresses induces the degradation of tumour suppressor protein p53 (p53). This degradation leads to the upregulation of p16; however, unidentified proteins may also serve a role in the development and progression of cervical cancer. Therefore, the aim of the present study was to analyse the expression levels of E6, p53, p16, MDM2 proto-oncogene (MDM2) and galectin-3 (gal-3) in cervical cancer specimens. A total of 250 cervical cancer tissue slides were used. The expression of E6, p53, p16, MDM2 and gal-3 was analysed with immunohistochemical methods and a semi-quantitative scoring. SPSS software was used for the statistical evaluation of staining results and survival analysis of patients with cervical cancer. Cervical cancer specimens demonstrated significantly increased E6 staining with advanced T-status and increased International Federation of Gynecology and Obstetrics classification. E6, p53 and p16 demonstrated significantly different expression levels in squamous epithelial tissue compared with adenocarcinomas. MDM2 and gal-3 demonstrated positively correlated expression levels in cervical cancer. In addition, gal-3 expression was correlated with poor prognosis in p16-negative cases. A negative correlation between the expression of E6 and a mutated form of p53 was also identified in cervical cancer. p53 mutation was demonstrated to be common in cervical cancer, and gal-3 and MDM2 appeared to act in a combined manner in this type of tumour. As gal-3 is overexpressed in the cervical cancer tissue of patients with poor prognosis, the use of gal-3 inhibitors should be investigated in future studies.

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Key words: cervical cancer, E6 oncoprotein, p53, p16, MDM2, galectin-3

Introduction

Cervical cancer is the fourth most frequent cancer in women globally with ~530,000 new cases in 2012, accounting for 7.5% of all female cancer-associated mortalities (1). A major cause of cervical cancer is persistent infection with high-risk human papillomavirus (HR-HPV) (1). HPV subtypes 16 and 18 cause ~70% of all cases of HPV (1,2). At present, >170 HPV types have been identified (3). Infection with 15 subtypes of HPV (16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 68, 73 and 82) may lead to cancer, which is why these 15 types are known as carcinogenic or high-risk types (4). The genome of human papillomaviruses consists of ~8,000 base pairs and contains six early genes (E6, E7, E1, E2, E4 and E5) and two late genes (L1 and L2) (5). Upon replication of the viral gene E6, E6 oncoprotein is expressed, which alters the cell cycle (6). E6 oncoprotein and E6-associated protein (E6-AP) form a complex that binds to p53 and causes its proteolytic degradation (7).

The tumour suppressor protein p53 (p53) signalling pathways leads to cell cycle arrest or apoptosis in case of DNA damage (8). As E6 oncoprotein induces the degradation of p53, the function of this important cell cycle protein is disturbed following HPV infection (9). In addition, the cell cycle regulation protein p16 is expressed at high levels in HPV-infected epithelial cells, and thus acts as a marker for the diagnosis of HPV-associated carcinoma (9,10). In non-carcinoma tissues p53 is regulated by MDM2 proto-oncogene (MDM2) through a negative feedback mechanism. MDM2 promotes the ubiquitination and proteasome-dependent degradation of p53 (11). There is also an association between MDM2, p53 polymorphism and the progression of cervical carcinoma (12).

A protein previously demonstrated to be associated with cervical cancer is galectin-3 (gal-3) (5). Galectins are defined as lectins with a galactose-binding ability and a characteristic amino-acid sequence (13). Galectin is a name proposed by Hirabayashi and Kasai (14) for a family of animal lectins. Galectins are typically soluble and metal-independent in

their activity (15). They have similar features to cytoplasmic proteins, including no disulfide bridges, no sugar chains, no signal sequences and, in most cases, their N-terminal amino acids are acetylated (16). It is possible to classify galectins into the following three types on the basis of their structural architecture: Proto, chimera and tandem-repeat types. Gal-3 is a chimera-type galectin (17).

Gal-3 may increase the invasiveness of cervical cancer by activating vascular endothelial growth factor receptor-3 (5). Therefore, the aim of the present study was to systematically analyse the expression and interactions of E6, p53, p16, MDM2 and gal-3 in cervical cancer specimens.

Materials and methods

Ethical approval. The present study was approved by the local Ethics Committee of the Ludwig-Maximilians-University of Munich (approval no. 259-16; Munich, Germany), and was performed in compliance with the guidelines of the Helsinki Declaration. Patient data were fully anonymised.

Specimens. Archived formalin-fixed paraffin-embedded (FFPE) sections from 250 cases of cervical cancer were used in the present study; it was possible to analyse 248 cases as there was no tumour tissue present on two sections (Table I). Cervical dysplasia [cervical intra-epithelial neoplasia (CIN) stage III] (18) and non-dysplastic cervical tissue (3 sections of each) was used for the E6 immunohistochemical staining, and breast cancer tissue was used for the mutated p53 immunohistochemistry. Specimens were obtained from the Department of Obstetrics and Gynecology of Ludwig-Maximilians-University of Munich, and were obtained from patients undergoing surgery there between 1993 and 2002. Follow-up data were received from the Munich Cancer Registry (Munich Tumour Centre, Munich, Germany).

Table I. Clinical parameters of the patients included in the present study.

Clinical parameter	No./total no.	%
Age (years)		
≤50	143/248	58
>50	105/248	42
No. of metastasis positive lymph nodes		
0	149/248	60
1-4	97/248	39
NA	2/248	1
Tumour size (cm)		
<2	111/248	45
2-4	128/248	52
>4	9/248	3
Tumour grade		
I	20/248	8
II	141/248	57
III	78/248	31
NA	9/248	4
Tumour subtype		
Squamous	199/248	80
Adenocarcinoma	49/248	20
Progression (over 236 months)		
None	190/248	77
≥1	58/248	23
Survival (over 236 months)		
Right censured	210/248	85
Succumbed	38/248	15

NA, not applicable as data not available.

Immunohistochemistry. The FFPE sections (3-μm-thick) were dewaxed in xylol, endogenous peroxidase was inhibited with 3% methanol/H_2O_2 and sections were rehydrated in a descending ethanol gradient. To stain for mutated p53, wild-type p53, E6, gal-3 and MDM2, the slides were pre-treated in citrate buffer (100˚C; pH 6.0) for antigen retrieval. Following this, non-specific binding of the primary antibodies was blocked, and incubation with the primary antibodies followed (Tables II and III). Incubation with the secondary antibodies and the following steps of the detection system and colour development are illustrated in Tables II and III. For p16 detection, the specimens were automatically stained using the Ventana BenchMark XT Stainer (Ventana Medical Systems, Inc., Oro Valley, AZ, USA) and the CINtec Histology kit (cat. no. 9517; Roche Applied Science, Mannheim, Germany) according to the manufacturer's instructions, while all other antibodies were stained for manually. For wild-type p53, the slides were washed in PBS/0.05% Tween-20. All other slides were washed in PBS only. Finally, the slides were counterstained with hemalaun (Waldeck GmbH, Münster, Germany) for 2 min at room temperature, dehydrated in an ascending series of ethanol and stored.

Slides were examined with a Zeiss Axiophot light photomicroscope (Zeiss GmbH, Jena, Germany). Digital images were obtained with a digital-camera system (CF20DXC; KAPPA Messtechnik, Gleichen, Germany). All specimens were evaluated by a pathologist. The intensity and distribution patterns of the staining reaction was evaluated by two blinded, independent observers, including the gynecological pathologist, using the semi-quantitative immunoreactive (IRS)-score, as previously described (19), to asses steroid receptors (20) and cathepsin D (21) expression. The IRS score was calculated by multiplication of optical staining intensity (graded as 0, no staining; 1, weak staining; 2, moderate staining; and 3, strong staining) and the percentage of positive stained cells (0, no staining; 1, ≤10% of the cells, 2, 11-50% of the cells, 3=51-80% of the cells and 4, ≥81% of the cells) and without knowing the pathological evaluation, the diagnosis or the standard performed hematoxylin reaction for 2 min at room temperature for each specimen.

Statistical analysis. Data were analysed using SPSS software (version 19.0; IBM SPSS, Armonk, NY, USA) for Microsoft Windows and visualised using Microsoft Office 7 (Microsoft Corporation, Redmond, WA, USA). Spearman coefficients were calculated to assess correlations, while the

Table II. Procedures for gal-3 and MDM2 staining.

Protocol	Gal-3	MDM2
Blocking method	Horse serum[a], 20 min, RT	Goat serum[a], 20 min, RT
Primary antibody, dilution, incubation duration, incubation temperature, cat. no.	Anti-galectin-3, 1:1,000 in PBS, 16 h, 4˚C; NCL-GAL3[b]	Anti-MDM2, 1:100 in PBS, 16 h, 4˚C, NCL-MDM2[b]
Secondary antibody, dilution, incubation duration, incubation temperature, cat. no.	Biotynilated anti-mouse IgG[a], 30 min, RT; PK-6100	Biotinylated goat anti-mouse IgM, 30 min, RT ZMB2020[c]
Detection of secondary antibody	ABC-complex[a], 30 min	ABC-complex[a], 30 min
Chromogen	1 mg/ml DAB[d], 5 min	1 mg/ml DAB[d], 1 min

[a]Vectastain ABC kit; Vector Laboratories, Inc., Burlingame, CA, USA. [b]Novocastra; Leica Microsystems GmbH, Wetzlar, Germany. [c]Linaris GmbH, Dossenheim, Germany. [d]Dako, Glostrup, Denmark. Gal-3, galectin-3; MDM2, MDM2 proto-oncogene; Ig, immunoglobulin; DAB, 3,3'-diaminobenzidine; RT, room temperature.

Table III. Procedures for mutated p53, wild-type p53 and E6 staining.

Protocol	Mutated p53	p53 wild-type	E6
Blocking method	Reagent 1[a]; 5 min, RT	Reagent 1[a]; 5 min, RT	Reagent 1[a]; 5 min, RT
Primary antibody, dilution, incubation duration, incubation temperature, cat. no.	Anti-p53, 1:100 in PBS, 16 h, 4˚C, ab32049[b]	Anti-p53, 1:200 in PBS, 16 h, 4˚C, ab26[b]	Anti-E6, 1:150 in PBS, 1 h, RT, ab70[b]
Post blocking method	Reagent 2[a]; 20 min, RT	Reagent 2[a]; 20 min, RT	Reagent 2[a]; 20 min, RT
Secondary antibody, dilution, incubation duration, incubation temperature, cat. no.	HRP-Polymer Reagent 3[a], 30 min, RT POLHRP-100	HRP-Polymer Reagent 3[a], 30 min, RT POLHRP-100	HRP-Polymer Reagent 3[a]; 30 min, RT POLHRP-100
Chromogen	1 mg/ml DAB[c], 1 min	1 mg/ml DAB[c], 1 min	1 mg/ml DAB[c], 1 min

[a]From the ZytoChem-Plus HRP Polymer-kit; Zytomed Systems GmbH, Berlin, Germany. [b]Abcam, Cambridge, UK. [c]Dako, Glostrup, Denmark. RT, room temperature; HRP, horseradish peroxidase; DAB, 3,3'-diaminobenzidine.

Mann-Whitney U test was applied to examine differences between groups. Differences in survival were assessed using the log-rank test and survival curves were plotted in accordance with Kaplan-Meier estimator. $P<0.05$ was considered to indicate a statistically significant difference and data were expressed as the mean ± standard error. Cox regression analysis was used to compare the risk of mortality in patients with and without gal-3 expression when the effects of further factors were accounted for. Independent variables included in the Cox regression model were gal-3 expression, age at the time of surgery, histological subtype, tumour size, lymph node status (pN), metastasis, tumour grade, International Federation of Gynecology and Obstetrics (FIGO) stage (22,23), and E6, mutated p53 and MDM2 expression status.

Results

Evaluation of E6 oncoprotein immunohistochemistry and the detection of mutated p53 on control slides. CIN III tissue slides were used for the evaluation of E6 oncoprotein staining. Moderate expression levels of E6 were observed in the CIN III sections (Fig. 1A). There was no expression of the E6 oncoprotein, and therefore no staining observed, in the non-dysplastic cervical tissue (Fig. 1B). Breast cancer tissue was used to evaluate the staining of mutated p53 (Fig. 1C), which exhibited nuclear and cytoplasmic staining.

E6 oncoprotein staining. A total of 81% of all cervical cancer tissue examined expressed E6 oncoprotein (data not shown). Cervical cancer specimens demonstrated significantly increased staining with a higher T stage (according to the Tumor-Node-Metastasis classification system) (24). T1 stage carcinomas (Fig. 2A) demonstrated E6 staining with a median IRS of 2, while T2 (Fig. 2B) and T3 (Fig. 2C) stage carcinoma tissues had a significantly higher median E6 expression of IRS 3 ($P=0.017$; Fig. 2D).

FIGO 1 carcinoma tissues had a median E6 expression of IRS 2 (Fig. 2E). FIGO 2 (Fig. 2F) and FIGO 3 (Fig. 2G) carcinoma tissues had a median IRS of 4. FIGO 4-classified cervical cancer tissue had a median E6 IRS score of 6 (Fig. 2H). E6 demonstrated a significant positive correlation with the FIGO classification ($R=0.277$, $P<0.001$; Fig. 2I).

Figure 1. Representative images of the staining for E6 in dysplastic and non-dysplastic cervical tissue samples, and mutated tumour protein p53 in breast cancer samples. (A) E6 staining in cervical dysplasia. (B) No expression of E6 was detected in non-dysplastic cervix samples. (C) Breast cancer tissue demonstrated expression of mutated p53. Scale bar, 200 μm.

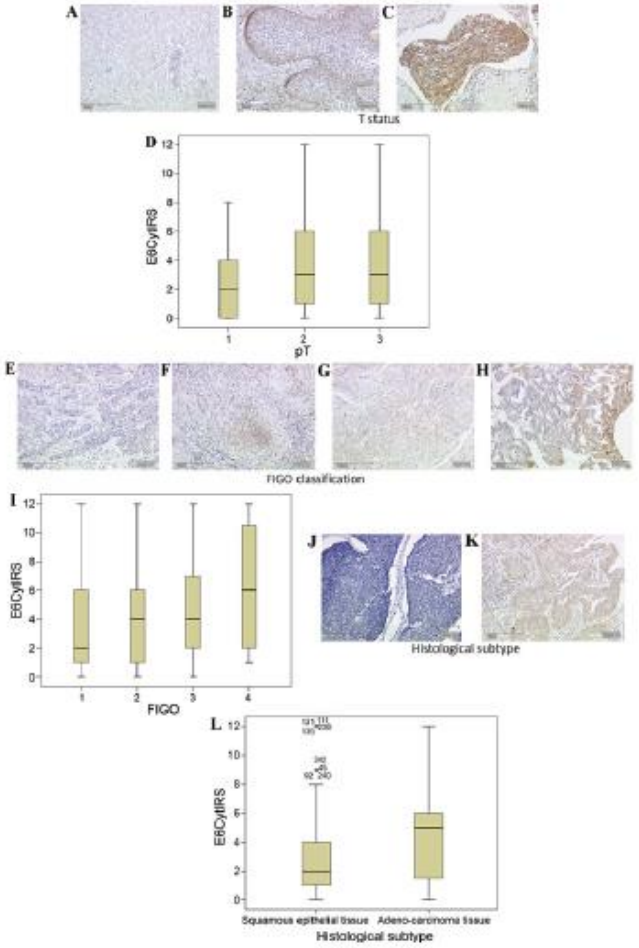

Figure 2. E6 expression is enhanced with cervical cancer tumour staging. (A) Low intensity E6 expression was observed in T1 tumours, whereas (B) T2 and (C) T3 staged tumours demonstrated increased expression of E6. (D) Box plot summary of the IRS for each tumour stage (P=0.017, FIGO 1 vs. 3). E6 expression was positively correlated with FIGO classification, with (E) FIGO 1 classified tissue demonstrating low expression of E6 while (F) FIGO 2 and (G) FIGO 3 classified tissue demonstrated increased expression levels, and (H) FIGO 4 tissue further increased expression levels. (I) Box plot summary of the IRS for each FIGO stage (P<0.001, FIGO 1 vs. 4). (J) Squamous epithelial tissue demonstrated lower levels of E6 staining than (K) adenocarcinoma tissue. (L) Box plot summary of the IRS for each histological subtype. Scale bar, 200 μm. FIGO, International Federation of Gynecology and Obstetrics; IRS, immunoreactive score; E6Cyt, E6 cytoplasmic; pT, pathological tumour stage.

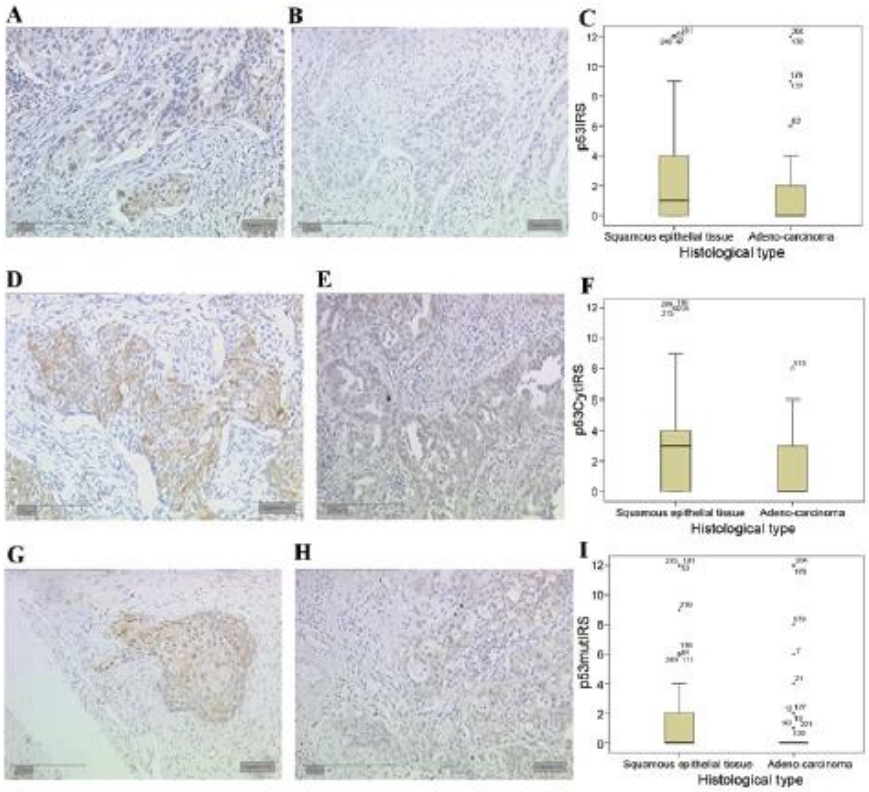

Figure 3. Tumour protein p53 expression in cervical cancer tissues. (A) Squamous epithelial tissue demonstrated a nuclear expression of wild-type p53, whereas (B) adenocarcinoma tissue had lower levels of p53 nuclear staining. (C) Box plot summary of the nuclear p53 IRS for each histological subtype. (D) Squamous epithelial tissue demonstrated higher expression of wild-type p53 in the cytoplasm compared with (E) adenocarcinoma tissue. (F) Box plot of the cytoplasmic p53 IRS for each histological subtype. (G) Squamous epithelial tissue demonstrated expression of mutated p53, while (H) adenocarcinoma tissue demonstrated almost no staining. (I) Box plot summary of the mutated p53 IRS for each histological subtype. Scale bar, 200 μm. IRS, immunoreactive score; p53Cyt, cytoplasmic wild-type p53; p53mut, mutant p53.

E6 demonstrated significantly different expression levels in cervical cancer tissue dependent on the histological subtype. Squamous epithelial carcinomas (Fig. 2J) had a median expression of IRS 2. Adenocarcinoma tissue (Fig. 2K) had significantly increased staining with a median of IRS 5 (P=0.015 vs. squamous epithelial carcinoma; Fig. 2L).

Wild-type and mutated p53 expression. Expression of wild-type p53 was observed in the nucleus and cytoplasm of 60 and 66% of all cervical cancer specimens, respectively. Significantly different expression levels in cervical cancer tissue in different histological subtypes were also observed for p53 expression. Wild-type p53 demonstrated a median nuclear expression (Fig. 3A) of IRS 1 in squamous epithelial tissue, whereas in adenocarcinoma tissue (Fig. 3B) the median nuclear expression was significantly decreased in comparison (IRS 0, P=0.024; Fig. 3C).

In addition to nuclear expression, wild-type cytosolic p53 expression also demonstrated significant differences associated with the histological subtype. In squamous epithelial tissue (Fig. 3D) a median expression of IRS 3 was observed, whereas in comparison the median cytosolic expression of p53 was significantly decreased in adenocarcinoma tissue (Fig. 3E) to IRS 0 (P<0.001; Fig. 3F).

The monoclonal antibody that recognises a previously described mutated form of p53, (25) also revealed significant staining differences associated with the histological subtype of cervical cancer. In addition, 42% of all cervical cancer tissue slides demonstrated nuclear expression of mutated p53, and 67% of all cases demonstrated mutated p53 expression in the cytoplasm. Although the median expression of mutated p53 in squamous epithelial tissue (Fig. 3G) and adenocarcinoma tissue (Fig. 3H) was 0, differences between the subtypes were significant (P=0.011; Fig. 3I).

Expression of p16 oncoprotein in cervical cancer tissue. p16 overexpression is routinely used in the Pathology Department of the Ludwig-Maximilians-University of Munich as a marker for HPV-associated head and neck squamous carcinoma (11). A total of 94% of cervical carcinoma cases tested

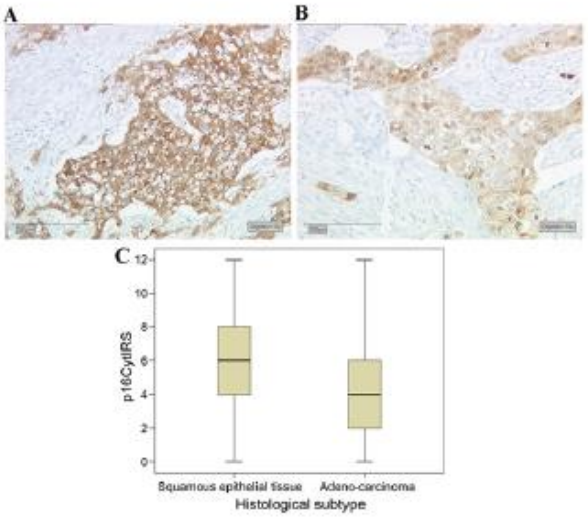

Figure 4. Expression of p16 in cervical cancer tissues (A) Squamous epithelial tissue demonstrated higher p16 expression levels compared with (B) adenocarcinoma tissue. (C) Box plot summary of the p16 IRS for each histological subtype. Scale bar, 200 μm. IRS, immunoreactive score; p16Cyt, cytoplasmic p16.

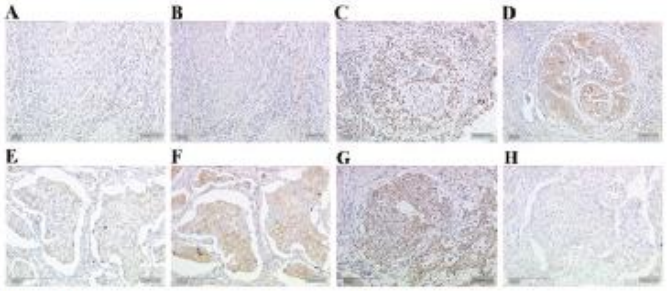

Figure 5. Expression of MDM2 and gal-3 in cervical cancer. Low expression of (A) MDM2 and (B) gal-3 was identified in the same area of cervical cancer. In another case, high expression of (C) MDM2 and (D) gal-3 were observed in the same are. Samples with (E) low E6 expression demonstrated high expression levels of (F) mutated p53. Another serial slide series with high expression of (G) E6 demonstrated low expression of (H) mutated p53. Scale bar, 200 μm. MDM2, MDM2 proto-oncogene; gal-3, galectin-3.

demonstrated p16 expression, and 61% of these cases were p16-overexpressing according to pathological evaluation. The cell cycle protein p16 demonstrated significant differences in expression between different histological subtypes of cervical cancer. Squamous epithelial tissue (Fig. 4A) had a median expression of IRS 6, while adenocarcinoma tissue (Fig. 4B) had a significantly lower expression in comparison, with an IRS of 4 ($P<0.001$; Fig. 4C). Notably, p16 demonstrated no significant correlation with E6 oncoprotein expression (data not shown).

Correlation analysis. A significant correlation was identified between MDM2 and gal-3 expression in cervical cancer tissue ($R=0.181$, $P=0.005$; data not shown). Cases of cervical cancer with low MDM2 expression (Fig. 5A) also demonstrated low gal-3 expression (Fig. 5B). Likewise, a case with high MDM2 expression (Fig. 5C) demonstrated high gal-3 expression (Fig. 5D). Cases of cervical cancer with low E6 oncoprotein expression (Fig. 5E) demonstrated enhanced staining of the mutated form of p53 in the same area of the tumour (Fig. 5F). However, cases with high expression of E6 (Fig. 5G) revealed low expression of mutated p53 (Fig. 5H). The statistical evaluation confirmed these results of serial section staining ($R=-0.140$, $P=0.028$; Table IV). A significant correlation was also identified between the expression of MDM2 and mutated p53 in cervical cancer tissue ($R=0.144$, $P=0.025$; Table IV). The correlation analyses and clinical parameters are summarised in Table IV.

Gal-3 is a negative prognosticator in p16-negative patients with cervical cancer. In patients with cervical cancer with no or very low p16 expression, gal-3 expression was correlated with a

Table IV. Correlation analyses of clinical parameters and immunohistochemical staining parameters.

Clinical parameter	Statistic	Age	Histology	pT	pN	pM	Grade	FIGO	E6	p53-mutated	MDM2	Galectin-3
Age (years)	Correlation coefficient	-.019	.004	-.115	-.065	-.140[a]	.354[b]	.133[a]	.169[b]	.107	.026	.050
	Sig. (2-tailed)	.766	.945	.070	.307	.028	.000	.037	.008	.095	.686	.438
	N	250	250	250	250	246	250	245	244	246	241	240
Histology	Correlation coefficient	.028	1.000	.000	-.073	-.108	-.084	.045	.156[a]	-.162[a]	.075	.029
	Sig. (2-tailed)	.664	.	.994	.249	.089	.185	.477	.015	.010	.242	.652
	N	245	249	249	249	249	249	249	245	249	244	243
pT	Correlation coefficient	.276[b]	.000	1.000	.359[b]	-.202[b]	.182[b]	.380[b]	.174[b]	.055	-.019	-.039
	Sig. (2-tailed)	.000	.994	.	.000	.001	.004	.000	.006	.384	.762	.540
	N	246	249	250	250	250	250	250	246	250	245	244
pN	Correlation coefficient	.037	-.073	.359[b]	1.000	-.172[b]	.212[b]	.240[b]	.033	-.050	-.058	-.033
	Sig. (2-tailed)	.559	.249	.000	.	.007	.001	.000	.610	.435	.370	.608
	N	246	249	250	250	250	250	250	246	250	245	244
pM	Correlation coefficient	-.081	-.108	-.202[b]	-.172[b]	1.000	-.146[a]	-.150[a]	-.037	.004	.032	-.074
	Sig. (2-tailed)	.206	.089	.001	.007	.	.021	.018	.560	.951	.619	.252
	N	246	249	250	250	250	250	250	246	250	245	244
Grade	Correlation coefficient	-.081	-.084	.182[b]	.212[b]	-.146[a]	1.000	.093	.084	-.065	-.175[b]	-.047
	Sig. (2-tailed)	.203	.185	.004	.001	.021	.	.142	.191	.302	.006	.461
	N	246	249	250	250	250	250	250	246	250	245	244
FIGO	Correlation coefficient	.076	.045	.380[b]	.240[b]	-.150[a]	.093	1.000	.227[b]	-.099	.021	.067
	Sig. (2-tailed)	.233	.477	.000	.000	.018	.142	.	.000	.120	.748	.296
	N	246	249	250	250	250	250	250	246	250	245	244
E6	Correlation coefficient	.088	.156[a]	.174[b]	.033	-.037	.084	.227[b]	1.000	.093	.001	.009
	Sig. (2-tailed)	.173	.015	.006	.610	.560	.191	.000	.	.147	.986	.893
	N	242	245	246	246	246	246	246	246	246	244	243
p53-mutated	Correlation coefficient	.092	-.290[b]	.016	-.019	.004	-.115	-.065	-.140[a]	1.000	.133[a]	.169[b]
	Sig. (2-tailed)	.148	.000	.796	.766	.945	.070	.307	.028	.	.037	.008
	N	246	249	250	250	250	250	250	246	250	245	244
MDM2	Correlation coefficient	.026	.075	-.019	-.058	.032	-.175[b]	.021	.001	-.042	1.000	.181[b]
	Sig. (2-tailed)	.686	.242	.762	.370	.619	.006	.748	.986	.511	.	.005
	N	241	244	245	245	245	245	245	244	245	245	243
Galectin-3	Correlation coefficient	.050	.029	-.039	-.033	-.074	-.047	.067	.009	-.020	.181[b]	1.000
	Sig. (2-tailed)	.438	.652	.540	.608	.252	.461	.296	.893	.760	.005	.
	N	240	243	244	244	244	244	244	243	244	243	244

[a]P<0.05; [b]P<0.01. pT, pathological tumour stage; pN, pathological node stage; pM, pathological metastasis stage; FIGO, International Federation of Gynecology and Obstetrics stage; MDM2, MDM2 proto-oncogene.

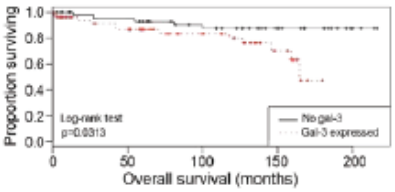

Figure 6. Kaplan-Meier overall survival analyses for patients with cervical cancer with gal-3 expression (red) compared with those with no gal-3 expression (black). Gal-3, galectin-3.

poor prognosis in overall survival analyses (P=0.0313; Fig. 6). Multivariate Cox regression analysis was performed to test which histopathological variables were independent prognosticators for survival rate in the tested breast cancer collective. It was demonstrated that the histological subtype (P=0.02), tumour size (P=0.011) and pN (P=0.045) were independent prognosticators for overall survival (Table V). No significant effect was demonstrated for the other histopathological variables.

Table V. Cox regression of overall survival on cervical cancer variables.

Parameters	P-value	Hazard ratio	95.0% CI Lower	95.0% CI Upper
Age (years)	0.071	1.029	0.997	1.062
Histology	0.002	3.576	1.586	8.063
pT	0.011	1.270	1.057	1.525
pN	0.045	2.113	1.016	4.395
pM	0.702	1.305	0.335	5.085
Tumour grade	0.065	1.717	0.968	3.048
FIGO	0.875	0.994	0.926	1.068
E6CytIRS	0.475	0.961	0.863	1.071
p16CytIRS	0.696	1.026	0.903	1.165
p53IRS	0.267	0.892	0.729	1.092
p53mutIRS	0.975	0.996	0.765	1.296
MDM2IRS	0.460	1.050	0.922	1.196
Gal-3 IRS score	0.452	0.937	0.792	1.109

CI, confidence interval; pT, pathological tumour stage; pN, pathological node stage; pM, pathological metastasis stage; FIGO, International Federation of Gynecology and Obstetrics stage; IRS, immunoreactive score; Cyt, cytoplasmic; mut, mutated; MDM2, MDM2 proto-oncogene; Gal-3, galectin-3.

Discussion

Within the present study, immunohistochemical evaluation of E6 oncoprotein expression was conducted. In addition, E6 expression levels were demonstrated to be associated with the histological subtype. The expression of wild-type p53 and a mutated form of p53 were identified in the cervical cancer specimens tested. Finally, correlation analyses revealed a combined positive expression pattern for galectin-3 and MDM2, and a negative correlation between E6 and mutated p53 expression.

Although the early era of HPV research identified that ≤99.5% of cervical cancer cases are HPV-associated (26), it remains controversial in the literature whether viral load and disease severity are positively correlated (12). Therefore, the present study investigated a number of markers that are associated with HPV-driven changes in cell cycle proteins. Using these markers permitted a comparative analysis of the influence of HPV on the progression of cervical cancer for >10 years following surgery.

The replication of the viral genes E6 and E7 results in the cellular expression of E6 and E7 oncoproteins, which interfere with the cell cycle (6). E6 oncoprotein binds to E6-AP, forming a complex that selectively binds to p53 and leads to its ubiquitin-dependent proteolytic degradation (7). The present study demonstrated that E6 immunohistochemistry was a fast and simple method for the detection of the HPV-associated oncoprotein E6 in cervical cancer tissues. As a routine practice, E6 and E7 are detected using either *in situ* hybridisation (11) or polymerase chain reaction (27) methodology due to the non-specific immunohistochemical staining results of antibodies used in former studies (28).

In the present study a well-tested antibody, and specific antigen retrieval and staining protocol was used, resulting in the establishment of a useful immunohistochemical evaluation protocol for the detection of the HPV E6 oncoprotein. The optimal results were obtained with the E6 antibody supplied by Abcam (Cambridge, UK). The advantage of immunohistochemical evaluation is that it is easier to apply and less expensive compared with mRNA *in situ* hybridisation. mRNA *in situ* hybridisation may be the optimal way to detect HPV; however, this method is more complicated for routine detection compared with immunohistochemistry (26). Evaluation of E6 immunohistochemical staining in cervical cancer tissue has previously revealed positive correlations with advanced T staging and FIGO classification (22). Although specific studies have indicated correlations between E6/E7 gene expression and the clinicopathological parameters of cervical cancer (26), such a correlation was not demonstrated in the present study.

An additional finding of the present study is the negative correlation between E6 and mutated p53 expression. Mutations of the gene encoding p53 (*TP53*) are the most frequent alterations in multiple human malignancies (29-31). In total, >50% of human tumours contain a mutation/deletion of *TP53*, ranging from 5-80% depending on the type, stage and etiology of the tumours (32). A number of previous studies have investigated a potential genetic link between these variations and cancer susceptibility, but the results have been controversial. A previous meta-analysis study from 49 pooled studies failed to demonstrate a link between a common *TP53* mutation (25) and cervical cancer susceptibility (33). Later on, the same mutation was revealed to be associated with higher pancreatic cancer risk among males; however, results also indicated that it may protect Arab women against the development of breast cancer (34,35). Multiple other mutations of *TP53* have since been described. Mutations that deactivate p53 in cancer are primarily located in the central DNA binding domain. These mutations typically ablate the ability of the protein to bind to

its target DNA sequences, prevent the transcriptional activation of p53 target genes. In total, ~80% of the most common p53 mutants demonstrate the capacity to exert dominant-negative effects over wild-type p53 and thus prevent the activation of transcription. In contrast, only 45% of the less frequent mutants studied have this capacity (36).

The mutation detected by the antibody used in the present study is an mutation at position 20 (serine to aspartic acid), which abolishes the phosphorylation site on p53. The phosphorylation of this serine when DNA damage is detected weakens the interaction between p53 and MDM2, thereby stabilising p53 (37-39). Thus, this mutation maintains increased protein levels of p53 following DNA damage. The analysis of the immunohistochemical detection of mutated p53 revealed that cervical cancer specimens derived from squamous epithelial tissue demonstrated significantly higher expression levels compared with adenocarcinoma tissue. In addition, a positive correlation between the expression of mutated p53 and MDM2, and a negative correlation between the expression of mutated p53 and E6, were identified. Therefore, it is possible to speculate that E6 also degrades the mutated form of p53. In a previously published study, this mutation was demonstrated to be associated with the improved survival of patients with cervical cancer (25).

Finally, a positive correlation between MDM2 and gal-3 expression was demonstrated in cervical cancer tissue. Little information concerning the involvement of galectins in cervical cancer exists at present. Research has primarily focused on gal-1 (40,41), gal-7 (42,43) and gal-9 (44). A previous publication described the influence of gal-3 on vascular endothelial growth factor C expression and its influence on the enhancement of cervical cancer cell invasiveness (5). The present study demonstrated that gal-3 was a negative independent prognosticator for the overall survival of patients with p16-negative cervical cancer. In this group of patients, gal-3 may be responsible for the aggressiveness of cervical cancer, whereas in p16-positive carcinomas different factors/signal transduction pathways may be responsible.

In the present study, a total of 250 cervical cancer cases were systematically analysed for the expression and interaction of E6, p53, p16, MDM2 and gal-3 in FFPE tumour tissue. Significantly increased levels of E6 staining were correlated with an advanced T stage and FIGO classification. Furthermore, MDM2 and gal-3 expression levels were positively correlated in cervical cancer. In addition, gal-3 expression levels were negatively correlated with prognosis in p16-negative cases. As gal-3 is overexpressed in the cervical cancer tissue of patients with a worse prognosis, the investigation of gal-3 inhibiting compounds is an additional task for the development of alternative treatments for this tumour type. In addition, a negative correlation between E6 and a mutated form of p53 in cervical cancer was identified. In conclusion, the results of the present study indicate that immunohistochemical staining may be a useful method for the detection the HPV E6 oncoprotein.

Acknowledgements

The present study was supported by the German Research Foundation. The authors would like to thank Professor Jutta Engel and Mr. Max Wiedemann (The Munich Cancer Registry, Munich Tumour Centre, Munich, Germany) for providing the follow-up data.

References

1. Munoz N, Bosch FX, Castellsagué X, Díaz M, de Sanjose S, Hammouda D, Shah KV and Meijer CJ: Against which human papillomavirus types shall we vaccinate and screen? The international perspective. Int J Cancer 111: 278-285, 2004.
2. Schiffman M, Castle PE, Jeronimo J, Rodriguez AC and Wacholder S: Human papillomavirus and cervical cancer. Lancet 370: 890-907, 2007.
3. Wittekindt C, Wagner S, Mayer CS and Klussmann JP: Basics of tumor development and importance of human papilloma virus (HPV) for head and neck cancer. Laryngorhinootologie 91 (Suppl 1): S1-S26, 2012 (In German).
4. Muñoz N, Bosch FX, de Sanjosé S, Herrero R, Castellsagué X, Shah KV, Snijders PJ and Meijer CJ; International Agency for Research on Cancer Multicenter Cervical Cancer Study Group: Epidemiologic classification of human papillomavirus types associated with cervical cancer. N Engl J Med 348: 518-527, 2003.
5. Zengel P, Assmann G, Mollenhauer M, Jung A, Sotlar K, Kirchner T and Ihrler S: Cancer of unknown primary originating from oropharyngeal carcinomas are strongly correlated to HPV positivity. Virchows Archiv 461: 283-290, 2012.
6. Gupta S, Takhar PP, Degenkolbe R, Koh CH, Zimmermann H, Yang CM, Guan Sim K, Hsu SI and Bernard HU: The human papillomavirus type 11 and 16 E6 proteins modulate the cell-cycle regulator and transcription cofactor TRIP-Br1. Virology 317: 155-164, 2003.
7. Scheffner M, Huibregtse JM, Vierstra RD and Howley PM: The HPV-16 E6 and E6-AP complex functions as a ubiquitin-protein ligase in the ubiquitination of p53. Cell 75: 495-505, 1993.
8. Tang D, Wu D, Hirao A, Lahti JM, Liu L, Mazza B, Kidd VJ, Mak TW and Ingram AJ: ERK activation mediates cell cycle arrest and apoptosis after DNA damage independently of p53. J Biol Chem 277: 12710-12717, 2002.
9. Mao C, Balasubramanian A, Yu M, Kiviat N, Ridder R, Reichert A, Herkert M, von Knebel Doeberitz M and Koutsky LA: Evaluation of a new p16(INK4A) ELISA test and a high-risk HPV DNA test for cervical cancer screening: Results from proof-of-concept study. Int J Cancer 120: 2435-2438, 2007.
10. Melkane AE, Mirghani H, Aupérin A, Saulnier P, Lacroix L, Vielh P, Casiraghi O, Griscelli F and Temam S: HPV-related oropharyngeal squamous cell carcinomas: A comparison between three diagnostic approaches. Am J Otolaryngol 35: 25-32, 2014.
11. Assmann G and Sotlar K: HPV-associated squamous cell carcinogenesis. Pathologe 32: 391-398, 2011 (In German).
12. Adams AK, Wise-Draper TM and Wells SI: Human papillomavirus induced transformation in cervical and head and neck cancers. Cancers (Basel) 6: 1793-1820, 2014.
13. Barondes SH, Castronovo V, Cooper DN, Cummings RD, Drickamer K, Feizi T, Gitt MA, Hirabayashi J, Hughes C, Kasai K, *et al*: Galectins: A family of animal beta-galactoside-binding lectins. Cell 76: 597-598, 1994.
14. Hirabayashi J and Kasai KI: Evolution of animal lectins. Prog Mol Subcell Biol 19: 45-88, 1998.
15. Barondes SH, Cooper DN, Gitt MA and Leffler H: Galectins. Structure and function of a large family of animal lectins. J Biol Chem 269: 20807-20810, 1994.
16. Kasai K and Hirabayashi J: Galectins: A family of animal lectins that decipher glycocodes. J Biochem 119: 1-8, 1996.
17. Hirabayashi J, Hashidate T, Arata Y, Nishi N, Nakamura T, Hirashima M, Urashima T, Oka T, Futai M, Muller WE, *et al*: Oligosaccharide specificity of galectins: A search by frontal affinity chromatography. Biochim Biophys Acta 1572: 232-254, 2002.
18. Boonlikit S and Srisantiroj N: Is there any clinical advantage in separating CIN 2 from CIN 3 in the current two-tiered cytological classification? Asian Pac J Cancer Prev 10: 115-118, 2009.
19. Remmele W, Hildebrand U, Hienz HA, Klein PJ, Vierbuchen M, Behnken LJ, Heicke B and Scheidt E: Comparative histological, histochemical, immunohistochemical and biochemical studies on oestrogen receptors, lectin receptors, and Barr bodies in human breast cancer. Virchows Arch A Pathol Anat Histopathol 409: 127-147, 1986.

20. Mylonas I, Speer R, Makovitzky J, Richter DU, Briese V, Jeschke U and Friese K: Immunohistochemical analysis of steroid receptors and glycodelin A (PP14) in isolated glandular epithelial cells of normal human endometrium. Histochem Cell Biol 114: 405-411, 2000.
21. Mylonas I, Makovitzky J, Richter DU, Jeschke U, Briese V and Friese K: Cathepsin D expression in normal, hyperplastic and malignant endometrial tissue: An immunohistochemical analysis. Acta Histochem 105: 245-252, 2003.
22. Kraljevic Z, Visković K, Ledinsky M, Zadravec D, Grbavac I, Bilandzija M, Soljacić-Vranes H, Kuna K, Klasnić K and Krolo I: Primary uterine cervical cancer: Correlation of preoperative magnetic resonance imaging and clinical staging (FIGO) with histopathology findings. Coll Antropol 37: 561-568, 2013.
23. Ozsarlak O, Tjalma W, Schepens E, Corthouts B, Op de Beeck B, Van Marck E, Parizel PM and De Schepper AM: The correlation of preoperative CT, MR imaging, and clinical staging (FIGO) with histopathology findings in primary cervical carcinoma. Eur Radiol 13: 2338-2345, 2003.
24. Horn LC, Schierle K, Schmidt D, Ulrich U, Liebmann A and Wittekind C: Current TNM/FIGO classification for cervical and endometrial cancer as well as malignant mixed mullerian tumors. Facts and background. Pathologe 32: 239-243, 2011 (In German).
25. Freier CP, Stiasny A, Kuhn C, Mayr D, Alexiou C, Janko C, Wiest I, Jeschke U and Kost B: Immunohistochemical evaluation of the role of p53 mutation in cervical cancer: Ser-20 p53-Mutant correlates with better prognosis. Anticancer Res 36: 3131-3137, 2016.
26. zur Hausen H: Papillomaviruses causing cancer: Evasion from host-cell control in early events in carcinogenesis. J Natl Cancer Inst 92: 690-698, 2000.
27. Hafner N, Gajda M, Altgassen C, Hertel H, Greinke C, Hillemanns P, Schneider A and Dürst M: HPV16-E6 mRNA is superior to cytokeratin 19 mRNA as a molecular marker for the detection of disseminated tumour cells in sentinel lymph nodes of patients with cervical cancer by quantitative reverse-transcription PCR. Int J Cancer 120: 1842-1846, 2007.
28. Hoffmann M, Tribius S, Quabius ES, Henry H, Pfannenschmidt S, Burkhardt C, Görögh T, Halec G, Hoffmann AS, Kahn T, *et al*: HPV DNA, E6*I-mRNA expression and p16INK4A immunohistochemistry in head and neck cancer-how valid is p16INK4A as surrogate marker? Cancer Lett 323: 88-96, 2012.
29. Nigro JM, Baker SJ, Preisinger AC, Jessup JM, Hostetter R, Cleary K, Bigner SH, Davidson N, Baylin S, Devilee P, *et al*: Mutations in the p53 gene occur in diverse human tumour types. Nature 342: 705-708, 1989.
30. Petitjean A, Achatz MI, Borresen-Dale AL, Hainaut P and Olivier M: TP53 mutations in human cancers: Functional selection and impact on cancer prognosis and outcomes. Oncogene 26: 2157-2165, 2007.
31. Vogelstein B, Lane D and Levine AJ: Surfing the p53 network. Nature 408: 307-310, 2000.
32. Hainaut P and Hollstein M: p53 and human cancer: The first ten thousand mutations. Adv Cancer Res 77: 81-137, 2000.
33. Klug SJ, Ressing M, Koenig J, Abba MC, Agorastos T, Brenna SM, Ciotti M, Das BR, Del Mistro A, Dybikowska A, *et al*: TP53 codon 72 polymorphism and cervical cancer: A pooled analysis of individual data from 49 studies. Lancet Oncol 10: 772-784, 2009.
34. Sonoyama T, Sakai A, Mita Y, Yasuda Y, Kawamoto H, Yagi T, Yoshioka M, Mimura T, Nakachi K, Ouchida M, *et al*: TP53 codon 72 polymorphism is associated with pancreatic cancer risk in males, smokers and drinkers. Mol Med Rep 4: 489-495, 2011.
35. Alawadi S, Ghabreau L, Alsaleh M, Abdulaziz Z, Rafeek M, Akil N and Alkhalaf M: P53 gene polymorphisms and breast cancer risk in Arab women. Med Oncol 28: 709-715, 2011.
36. Petitjean A, Mathe E, Kato S, Ishioka C, Tavtigian SV, Hainaut P and Olivier M: Impact of mutant p53 functional properties on TP53 mutation patterns and tumor phenotype: Lessons from recent developments in the IARC TP53 database. Hum Mutat 28: 622-629, 2007.
37. Bode AM and Dong Z: Post-translational modification of p53 in tumorigenesis. Nat Rev Cancer 4: 793-805, 2004.
38. Chehab NH, Malikzay A, Stavridi ES and Halazonetis TD: Phosphorylation of Ser-20 mediates stabilization of human p53 in response to DNA damage. Proc Natl Acad Sci USA 96: 13777-13782, 1999.
39. Wade M, Wong ET, Tang M, Stommel JM and Wahl GM: Hdmx modulates the outcome of p53 activation in human tumor cells. J Biol Chem 281: 33036-33044, 2006.
40. Kim HJ, Do IG, Jeon HK, Cho YJ, Park YA, Choi JJ, Sung CO, Lee YY, Choi CH, Kim TJ, *et al*: Galectin 1 expression is associated with tumor invasion and metastasis in stage IB to IIA cervical cancer. Hum Pathol 44: 62-68, 2013.
41. Huang EY, Chen YF, Chen YM, Lin IH, Wang CC, Su WH, Chuang PC and Yang KD: A novel radioresistant mechanism of galectin-1 mediated by H-Ras-dependent pathways in cervical cancer cells. Cell Death Dis 3: e251, 2012.
42. Matsui Y, Ueda S, Watanabe J, Kuwabara I, Ogawa O and Nishiyama H: Sensitizing effect of galectin-7 in urothelial cancer to cisplatin through the accumulation of intracellular reactive oxygen species. Cancer Res 67: 1212-1220, 2007.
43. Tsai CJ, Sulman EP, Eifel PJ, Jhingran A, Allen PK, Deavers MT and Klopp AH: Galectin-7 levels predict radiation response in squamous cell carcinoma of the cervix. Gynecol Oncol 131: 645-649, 2013.
44. Liang MY, Lu YM, Zhang Y and Zhang SL: Serum galectin-9 in cervical cancer. Zhonghua Yi Xue Za Zhi 88: 2783-2785, 2008 (In Chinese).

ANTICANCER RESEARCH *36*: 3131-3138 (2016)

Immunohistochemical Evaluation of the Role of *p53* Mutation in Cervical Cancer: Ser-20 *p53*-Mutant Correlates with Better Prognosis

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Abstract. *Background: Cervical cancer is driven by human papillomavirus virus-specific oncoprotein E6. E6 interacts with E3 ubiquitin-protein ligase, resulting in the proteolysis of p53 protein. The aim of this study was to analyze one TP53 mutation in patients with cervical cancer and to correlate it to prognosis. Materials and Methods: A total of 248 paraffin-embedded tumor samples were stained for mutated p53 protein. The distribution and intensity of staining both in the nucleus and cytoplasm were evaluated with a semi-quantitative immunohistochemical score. Results: A total of 66% of studied cervical carcinomas expressed the mutated p53 protein. The overall survival was better for patients expressing the mutated p53 protein in the nucleus. Conclusion: Interestingly, we found a very high mutation rate of TP53 in a cancer type where p53 is initially inactivated via E6 during the development of cervical cancer. An unexpected finding is the correlation of this mutation with better survival, possibly due to better response to therapy.*

Worldwide, cervical cancer is the fourth most common tumor diagnosed and cause of death in women, with an estimated 528,000 cases and 266,000 deaths in 2012 (1, 2). About 80% of cervical cancer cases occur in low-to-medium resource countries (3). Over the past decades, the introduction of tumor screening programs in many high-resource countries contributed to reduced incidence and mortality due to cervical cancer (4).

Oncogenic human papillomavirus (HPV)s, mainly HPV 16 and 18 genotypes, have been strongly associated with the risk of developing intraepithelial lesions and appear to be involved in the development of more than 90% of all squamous cell carcinomas and adenocarcinomas of the cervix (5). However, most individuals who have had HPV infections do not develop cervical cancer since the low-grade squamous epithelial lesions induced spontaneously regress in more than 90% of cases (1). Cervical cancer typically develops from pre-cancerous changes over a period of 10 to 20 years (6). HPV-induced cervical cancer is driven by the virus-specific oncoproteins E6 and E7. The E6 protein of HPV types 16 and 18 interacts with E3 ubiquitin-protein ligase, resulting in the proteolysis of p53 protein (7). In addition, E6 binds E1A binding protein p300 and CREB-binding proteins and reduces the ability to activate p53-responsive promotor elements (7). Variable levels of *E6* mRNA have been found in both cervical intraepithelial neoplasia and cervical cancer. However, the constitutive expression of early viral genes is not in itself sufficient to induce and maintain the transformation status (6, 8). Accumulation of genetic and epigenetic alterations over time may, therefore, be crucial for the ultimate progression to cancer.

Mutations of *TP53* gene are among the most common genetic alterations in many human malignancies (9-11). More than 50% of human tumors contain a mutation or deletion of the *TP53* gene, ranging from 5 to 80% depending on the type, stage, and etiology of tumors (12). Many studies have investigated a genetic link between these variation and cancer susceptibility. Results, however, have been controversial. In 2009, a meta-analysis study of 49 pooled studies failed to show a link between a very common mutation (namely substitution of an arginine for a proline at

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Key Words: Cervical cancer, *TP53* mutation, *MDM2*, immunohistochemistry, E6/E7, HPV.

0250-7005/2016 $2.00+.40

codon position 72) and cervical cancer susceptibility (13). In 2011, the same mutation was found to be associated with higher pancreatic cancer risk among males, but seemed to protect Arab women against breast cancer (14, 15). To date, many other mutations of *TP53* have been described. Mutations that deactivate p53 in cancer usually occur in the central DNA-binding domain. Most of these mutations destroy the ability of the protein to bind to its target DNA sequences, and thus prevent transcriptional activation of these genes. Furthermore, 80% of the most common mutants have the capacity to exert dominant-negative effect over wild-type p53 and thus prevent the activation of transcription. In comparison only 45% of the less frequent mutants studied have this capacity (16). Roles played by other mutations of the p53 protein are much less known. In this study, we focused on one mutation occurring in the *N*-terminal part of p53, which contains one of the two transcription activation domains of the protein involved in the regulation of several pro-apoptotic genes (17).

The aim of the study was to analyze the mutation of the *TP53* gene in patients with cervical cancer and to correlate mutation to clinical parameters and prognosis.

Materials and Methods

Patient data. Tissue samples from 248 formalin-fixed and paraffin-embedded cervical neoplasia biopsies from patients referred to the Gynecology Unit at the Department of Obstetrics and Gynecology, Ludwig Maximilian University of Munich, from 1993 to 2002, were included in this study. All cases had been previously characterized in terms of histology (grade, tumor size and lymph-node infiltration by tumor cells) at the Department of Pathology, Ludwig Maximilian University of Munich (Table I).

All material was sampled for diagnostic purposes and research was carried out in accordance with the legal requirements concerning confidential medical communication as well as the data protection act. Consequently, consulting the Ethics Committee of the Medical School, Ludwig Maximilian University of Munich, and written informed consent from the patients prior to participation in the study was not required.

Immunohistochemistry. Paraffin wax-embedded tissue sections of 3 μm from samples were deparaffinized in xylol for 20 min, washed in 100% ethanol and then incubated in methanol/H_2O_2 (3%) for 20 min. After rehydration in an alcohol gradient to distilled water, the slides were placed in a pressure cooker containing sodium citrate buffer (pH=6.0) and heated for 5 min. Slides were washed twice in phosphate buffer solution (PBS) and blocked using blocking solution (Zytomed, Berlin, Germany) for 5 min. Each slide was separately incubated with a specific antibody against mutated p53 protein (at position 20, serine to asparatic acid - ab32049; Abcam, Cambridge, UK) diluted 1/100 in PBS. Incubation of the sections with the primary antibodies lasted for 16 h at 4˚C. Afterwards, sections were washed twice in PBS before incubation with post-block reagent (Zytomed) for 20 min. Finally, slides were washed in PBS and then incubated with the horseradish peroxidase-polymer (3) (Zytomed) for 30 min. Staining was performed using 3,3-diaminobenzidine (DAB) substrate solution (Dako, Glostrup, Denmark) for 180 sec. Counterstaining was carried out with Mayer's hemalaun for 2 min. Finally, sections were washed in tap water for 5 minutes and afterwards dehydrated in an ascending alcohol series and washed in xylol. Slides were cover-slipped with Eukittquick-hardening mounting medium (Sigma-Aldrich, St. Louis, USA).

The intensity score (IRS) used examines the intensity and distribution of antigen expression and is calculated by multiplying the percentage of positively stained cells (0: no staining; 1 <10% of cells; 2: 11-50%; 3: 51- 80%, 4> 81%) with the intensity of cell staining (0: none; 1: weak; 2: moderate; 3: strong). Two independent investigators examined the sections using a Leitz Diaplan microscope (Leitz, Wetzlar, Germany). The concordance was of 95%. In cases of different staining evaluation, both investigators carried out re-evaluation until an agreement was reached (5% of all cases). Positive controls were carried out with human breast cancer sections. Negative controls were performed by replacement of the primary antibody and alternative incubation of the slides with IgG rabbit or mouse control antibodies (Biogenex, San Ramen, CA, USA).

Statistical analysis. Correlation of staining with grading, age, size, nodal status and survival analysis was evaluated with the statistical program R (Version 0.98.1028; RStudio, Inc., Boston, MA, USA). The Mann–Whitney *U*-test was used for evaluation of two independent groups. Values of $p<0.05$ were considered statistically significant.

Table I. *Clinical characteristics of the study population.*

	No./total.	%
Age, years		
≤50	143/248	58
>50	105/248	42
No. of positive nodes		
0	149/248	60
1-4	97/248	39
NA	2/248	1
Tumor size (cm)		
≤2	111/248	45
2-4	128/248	52
>4	9/248	3
Tumor grade		
I	20/248	8
II	141/248	57
III	78/248	31
NA	9/248	4
Tumor subtype		
Squamous	199/248	80
Adenocarcinoma	49/248	20
Tumor progression		
No	190/248	77
Yes	58/248	23
Survival*		
Alive	210/248	85
Dead	38/248	15

*At 236 months. NA: Not available.

Table II. *Variables taken into account for the proportional hazards regression analysis (Cox model) including the rule used to discriminate, the number of women attributed to each group and the number we could not attribute (NA).*

Variable	Rule	N	Yes	NA
Grade	At least grade 3	161	78	9
Age	Older than 47.5 years	124	124	0
Tumor size	Tumor bigger than 2 cm	111	136	1
Nodal status	Tumor nodal infiltration	149	97	2

Table III. *Proportional hazards regression analysis (COX model). Including regression coefficient (R. coef.), standard error (S. error), p-value and 95% confidence interval (CI) for the hazard ratio (lower and higher 0.95%).*

Variable	R. coef.	S. error	Hazard ratio	95% CI	*p*-Value
Mutant p53					
Cytoplasmic	0.3629	0.2774		0.7283-3.0205-	0.3942
Nuclear	−1.0188	0.4561		0.1477-0.8826	0.0255
Tumor grade	0.4900	0.3379		0.8146-3.2707	0.3546
Age	0.6928	0.3610		0.9826-4.0681	0.3624
Tumor size	1.3199	0.4761		1.4721-9.5171	0.0056
Nodal status	0.4599	0.3420		0.8103-3.0963	0.1786

Results

No difference in cytoplasmic expression of mutated p53 protein. A total of 66% of the studied cervical cancer cases expressed the mutated p53 protein (*i.e.* more than 10% of the cancer cells stained) in the cytoplasm. We found 81 patients with no visible expression for mutated p53 protein; 17 had a score of 1; 20 scored 2; 12 scored 3; 67 scored 4; 16 scored 6; 23 scored 8; five scored 9; four scored 12 and three were non quantifiable. Differential expression of mutated p53 in the cytoplasm of human cervical cancer assessed by immunoperoxidase staining is presented in Figure 1a-f. The expression was highly variable, from no expression at all to very strong and diffuse expression. However, the Kaplan–Meier curves showed no difference in the survival of the two groups (Figure 1g). The 5-year survival rate for the group with no cytoplasmic expression of mutated p53 was 87.3%, comparable to the 91.2% survival rate for the group with cytoplasmic expression of mutated p53. The 10-year survival rate for the group with no mutated p53 cytoplasmic expression was similarly 83.8%, not significantly different from that of the group with cytoplasmic expression of mutated p53 (85.0%). Overall these results show no significant advantage of cytoplasmic expression of mutated p53 protein (p=0.718).

Survival advantage associated with nuclear expression of mutated p53 protein. A total of 42% of studied cervical cancer cases expressed mutated p53 protein (*i.e.* more than 10% of the cancer cells stained) in the nucleus. We found 141 patients with no visible expression for mutated p53 protein; 19 scored 1; 56 scored 2; four scored 3; 10 scored 4; eight scored 6; one scored 8; one scored 9; five scored 12 and three were non quantifiable. Differential expression of mutated p53 in the nucleus of human cervical cancer assessed by immunoperoxidase staining is presented in Figure 2a-d. The expression was again, highly variable, from no expression at all to very strong and diffuse expression. However, the Kaplan–Meier curves showed there to be a difference in the survival of the group expressing mutated p53 in the nucleus, as shown in Figure 2e. The 5-year survival rate in the group with no nuclear expression of mutated p53 was 88.0%, significantly reduced compared with that of the group expressing nuclear mutated p53 (92.8%). The 10-year survival rate was similarly reduced (81.7% versus 90.4%, respectively). Overall, these results show a significant advantage of nuclear expression of mutated p53 protein (p=0.024).

Cox Proportional hazards regression analysis. To confirm these results, we performed a multivariable survival analyses based on the Cox model. We estimated the effect of the nuclear expression of mutated p53 after adjustment for other explanatory variables available at the time of the surgery: higher grade as graded after pathological characterization, age higher than the median (*i.e.* 47.5 years) at the onset of disease, larger size of the resected tumor (2 cm and greater) and presence of infiltrated tumor cells in the local lymph node. All these factors correlated with higher risk of death as presented in Table II. The proportional hazards regression analysis gives a more precise idea of the influence of these variables, as presented in Table III. The grading of the tumor and the presence of infiltrated tumor cells in local lymph nodes did not remain significant factors (p=0.167 and p=0.179, respectively). Moreover, the effect of age greater than the median at the onset of disease was reduced to a trend (p=0.059). However, resected tumor size and nuclear expression of mutated p53 protein were both significant (p=0.005 and p=0.025, respectively). The regression coefficient for the nuclear expression of mutated p53 protein was found to be negative, consequent with a given survival advantage. Overall these results confirm a significant advantage of nuclear expression of the mutated p53 protein in cervical cancer.

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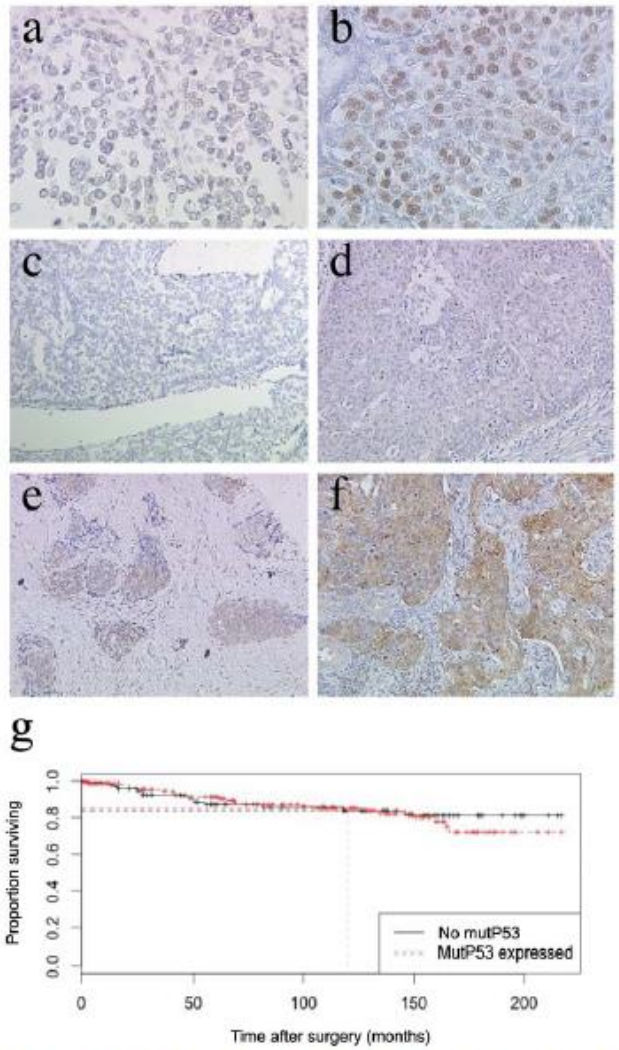

Figure 1. *Expression of mutated p53 in the cytoplasm of human cervical cancer tissue as assessed by immunoperoxidase staining: Negative control, IgG rabbit control antibody (breast cancer) (a); positive control (breast cancer); (b) no expression (c); low expression (d); intermediate expression (e); high expression (f). Magnification: a, b, ×200; c-f: ×80. g: Kaplan–Meier analysis of the overall survival of patients according to cytoplasmic expression of mutated p53.*

Discussion

The results of this study give a good indication as to where to focus the search for new tumor markers in cervical cancer. The high expression of the mutated version of p53 and the difference observed in survival of patients was not expected by analyzing the relevant literature. Despite its central role of p53 in the hallmarks of cancer, *TP53* mutation and

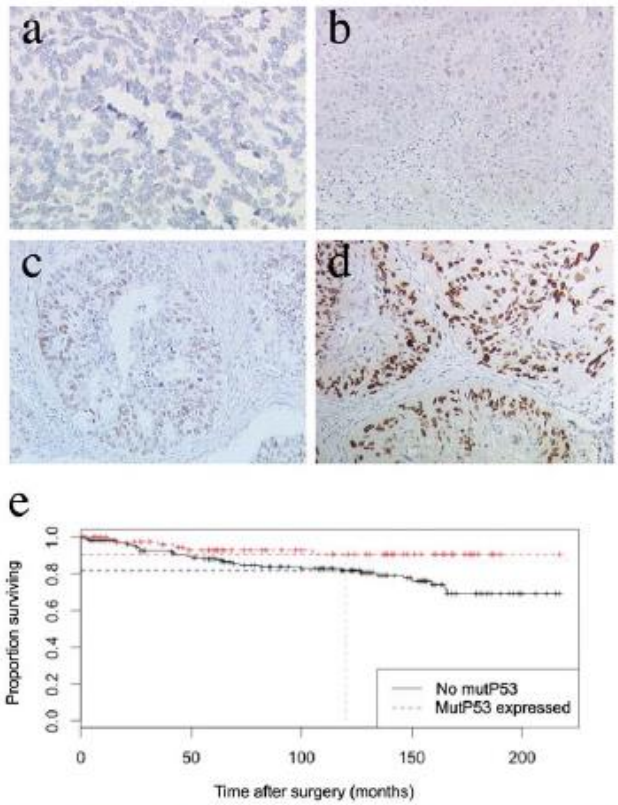

Figure 2. *Expression of mutated p53 in the nucleus of human cervical cancer tissue as assessed by immunoperoxidase staining: No expression (a); low expression (b); intermediate expression (c); high expression (d). Magnification: ×80. e: Kaplan–Meier analysis of the overall survival of patients according to nuclear expression of mutated p53. Survival advantage was shown for patients with tumor with nuclear expressed mutated p53 protein.*

expression status is not used for the prognosis of cervical cancer. Interestingly, we found a very high mutation rate of the *TP53* gene in a cancer type in which p53 is initially inactivated *via* the oncoprotein E6 during its development.

The *TP53* mutation is strongly associated with cervical cancer and was not detectable in normal tissues surrounding the tumor. The etiological role of infection with high-risk HPV in cervical cancer is well established (18). However, development of cancer, only occurs 10 or 20 years after the first infection and could be one explanation for the high rate of mutation associated with the pathology. Of all *TP53* mutations known, 90% are non-synonymous substitutions and concern single amino acid changes in the DNA-binding region (10). However, we herein analyzed one mutation occurring in the *N*-terminal part of p53, one of the two transcription activation domains of the protein involved in the regulation of several pro-apoptotic genes (17). In its normal functions, p53 senses DNA damage and can arrest growth by holding the cell cycle at the G_1/S regulation point on DNA damage recognition, or initiate apoptosis if DNA damage proves to be irreparable (11, 19-21).

The antibody we used detects a mutation at position 20 (serine into aspartic acid), which abolishes one phosphorylation site (Abcam personal communication). The phosphorylation of this serine on detection of DNA damage weakens the interaction of p53 with mouse double minute 2 (MDM2), also known as E3 ubiquitin protein ligase, thereby stabilizing p53 (22-24). Thus, the mutation abolishes the

normal increase in p53 protein level after DNA damage. Overall, the mutation was detected in the cytoplasm of 66% of cancer cases and in 42% in the nucleus. Since this mutation alters the cell's ability to stop the cell cycle on DNA damage recognition, it could work synergistically with E6 to reduce the ability of p53 to activate responsive promoter elements. Together, these two blockades could lead to mutation accumulation and, ultimately, to cervical cancer. Interestingly, MDM2 as an E3 ubiquitin protein ligase could also be directly targeted by E6 protein of HPV (7). MDM2 is also linked with increased risk of cancer and potential of treatment (25-27).

In accordance with its role as a transcription factor, we found mutated p53 had an influence on survival only if expressed in the nucleus. An unexpected finding is the correlation of *TP53* mutation alone with a better survival. *TP53* mutation is often observed in cancer. However, it is commonly associated with severely compromised tumor suppression due to an increased likelihood for uncontrolled cell division (28). One limitation of this study is the absence of information about other possible simultaneous *TP53* mutations, possibly destroying the ability of the protein to bind to its target DNA sequences, and thus preventing transcriptional activation of genes. However, we observed a significant effect of mutated p53 on patient survival, thus we assume that other mutations are less important. As a matter of fact, the capacity of p53 to bind to DNA promoters is of less importance if the protein stays bound to MDM2 and is therefore degraded. It remains to be assessed whether the milder prognosis associated with several mutants may be ascribed to specific functional properties and directly correlated to E6 protein expressed after HPV infection.

Patients included in this study were treated at our Institute and received radiotherapy targeting the DNA of tumor cells. The absence of an increase of p53 protein level after DNA damage due to mutation could be an advantage for tumorigenesis, since it would simplify the transmission of small nonsynonymous mutation; but it should also be a disadvantage in the case of great DNA damage such as that caused by radiotherapy, since the cell cycle would not be stopped and DNA would not be repaired, ultimately leading to more cell death. Indeed, phosphorylation of p53 at Ser-20 seems to be involved in cell radiosensitivity (29). That this kind of mutation often occurs in cervical cancer might be due to the constant E6-dependant p53 deactivation, making tumorigenesis less dependent on a dominant-negative mutation.

In summary, we found a very high mutation rate of the *TP53* gene in cervical cancer. According to this finding the overall survival was better in patients expressing the mutated p53 protein in the nucleus. Our results indicate that p53 mutation might serve as a useful biomarker to predict response to therapy.

Conflicts of Interest

The Authors declare no conflict of interest exists in regard to this study.

Acknowledgements

The work presented here is part of the Ph.D. thesis of CPF and was supported by a Ph.D. stipend of the DFG (Graduiertenkolleg 1202). This work was presented and awarded at the 16. HSTM, Hamburg 2014 under the name Immunohistochemical evaluation of the role of p53 mutation in cervical cancer.

References

1 Dunne EF and Park IU: HPV and HPV-associated diseases. Infect Dis Clin North Am *27*: 765-778, 2013.
2 DeSantis CE, Lin CC, Mariotto AB, Siegel RL, Stein KD, Kramer JL, Alteri R, Robbins AS and Jemal A: Cancer treatment and survivorship statistics, 2014. CA Cancer J Clin *64(4)*: 252-271, 2014.
3 Ferlay J, Shin H-R, Bray F, Forman D, Mathers C and Parkin DM: Estimates of worldwide burden of cancer in 2008: GLOBOCAN 2008. Int J Cancer *127*: 2893-2917, 2010.
4 Arbyn M, Castellsagué X, de Sanjosé S, Bruni L, Saraiya M, Bray F and Ferlay J: Worldwide burden of cervical cancer in 2008. Ann Oncol 22: 2675-2686, 2011.
5 Bouvard V, Baan R, Straif K, Grosse Y, Secretan B, El Ghissassi F, Benbrahim-Tallaa L, Guha N, Freeman C, Galichet L, Cogliano V and WHO International Agency for Research on Cancer Monograph Working Group: A review of human carcinogens–part B: biological agents. Lancet Oncol *10*: 321-322, 2009.
6 Steenbergen RDM, Snijders PJF, Heideman DAM and Meijer CJLM: Clinical implications of (epi)genetic changes in HPV-induced cervical precancerous lesions. Nat Rev Cancer *14*: 395-405, 2014.
7 Moody CA and Laimins LA: Human papillomavirus oncoproteins: pathways to transformation. Nat Rev Cancer *10*: 550-560, 2010.
8 Vinokurova S, Wentzensen N, Kraus I, Klaes R, Driesch C, Melsheimer P, Kisseljov F, Dürst M, Schneider A and von Knebel Doeberitz M: Type-dependent integration frequency of human papillomavirus genomes in cervical lesions. Cancer Res 68: 307-313, 2008.
9 Nigro JM, Baker SJ, Preisinger AC, Jessup JM, Hostetter R, Cleary K, Bigner SH, Davidson N, Baylin S and Devilee P: Mutations in the p53 gene occur in diverse human tumour types. Nature 342: 705-708, 1989.
10 Petitjean A, Achatz MIW, Borresen-Dale AL, Hainaut P and Olivier M: TP53 mutations in human cancers: functional selection and impact on cancer prognosis and outcomes. Oncogene 26: 2157-2165, 2007.
11 Vogelstein B, Lane D and Levine AJ: Surfing the p53 network. Nature 408: 307-310, 2000.
12 Hainaut P and Hollstein M: p53 and human cancer: the first ten thousand mutations. Adv Cancer Res 77: 81-137, 2000.
13 Klug SJ, Ressing M, Koenig J, Abba MC, Agorastos T, Brenna SMF, Ciotti M, Das BR, Del Mistro A, Dybikowska A, Giuliano

AR, Gudleviciene Z, Gyllensten U, Haws ALF, Helland A, Herrington CS, Hildesheim A, Humbey O, Jee SH, Kim JW, Madeleine MM, Menczer J, Ngan HYS, Nishikawa A, Niwa Y, Pegoraro R, Pillai MR, Ranzani G, Rezza G, Rosenthal AN, Roychoudhury S, Saranath D, Schmitt VM, Sengupta S, Settheetham-Ishida W, Shirasawa H, Snijders PJF, Stoler MH, Suárez-Rincón AE, Szarka K, Tachezy R, Ueda M, van der Zee AGJ, von Knebel Doeberitz M, Wu M-T, Yamashita T, Zehbe I and Blettner M: TP53 codon 72 polymorphism and cervical cancer: a pooled analysis of individual data from 49 studies. Lancet Oncol *10*: 772-784, 2009.

14 Sonoyama T, Sakai A, Mita Y, Yasuda Y, Kawamoto H, Yagi T, Yoshioka M, Mimura T, Nakachi K, Ouchida M, Yamamoto K and Shimizu K: TP53 codon 72 polymorphism is associated with pancreatic cancer risk in males, smokers and drinkers. Mol Med Rep *4(3)*: 489-495, 2011.

15 Alawadi S, Ghabreau L, Alsaleh M, Abdulaziz Z, Rafeek M, Akil N and Alkhalaf M: P53 gene polymorphisms and breast cancer risk in Arab women. Med Oncol *28*: 709-715, 2011.

16 Petitjean A, Mathe E, Kato S, Ishioka C, Tavtigian SV, Hainaut P and Olivier M: Impact of mutant p53 functional properties on TP53 mutation pat-terns and tumor phenotype: lessons from recent developments in the IARC TP53 database. Hum Mutat *28*: 622-669, 2007.

17 Venot C, Maratrat M, Dureuil C, Conseiller E, Bracco L and Debussche L: The requirement for the p53 proline-rich functional domain for mediation of apoptosis is correlated with specific PIG3 gene transactivation and with transcriptional repression. EMBO J *17*: 4668-4679, 1998.

18 zur Hausen H: Papillomaviruses in the causation of human cancers – a brief historical account. Virology *384*: 260-265, 2009.

19 Vousden KH and Lane DP: p53 in health and disease. Nat Rev Mol Cell Biol *8*: 275-283, 2007.

20 Lavin MF and Gueven N: The complexity of p53 stabilization and activation. Cell Death Differ *13*: 941-950, 2006.

21 Haupt Y, Maya R, Kazaz A and Oren M: Mdm2 promotes the rapid degradation of p53. Nature *387*: 296-299, 1997.

22 Bode AM and Dong Z: Post-translational modification of p53 in tumorigenesis. Nat Rev Cancer *4*: 793-805, 2004.

23 Chehab NH, Malikzay A, Stavridi ES and Halazonetis TD: Phosphorylation of Ser-20 mediates stabilization of human p53 in response to DNA damage. Proc Natl Acad Sci USA *96*: 13777-13782, 1999.

24 Wade M, Wong ET, Tang M, Stommel JM and Wahl GM: Hdmx modulates the outcome of p53 activation in human tumor cells. J Biol Chem *281*: 33036-33044, 2006.

25 Knappskog S and Lønning PE: Mdm2 SNP309 and risk of endometrial cancer. Tumour Biol *35*: 7285-7286, 2014.

26 Zhao Y, Yang X, Hao X, Pan X, Zhao B, Ma J, Fang J and Zhao M: Common variant on Mdm2 contributes to endometrial cancer susceptibility: evidence based on 7 studies. Tumour Biol *35*: 7555-7560, 2014.

27 Wang W, Qin J-J, Voruganti S, Srivenugopal KS, Nag S, Patil S, Sharma H, Wang M-H, Wang H, Buolamwini JK and Zhang R: The pyrido[b]indole Mdm2 inhibitor SP-141 exerts potent therapeutic effects in breast cancer models. Nat Commun *5*: 5086, 2014.

28 Hollstein M, Sidransky D, Vogelstein B and Harris CC: p53 mutations in human cancers. Science *253*: 49-53, 1991.

29 Okaichi K, Nose K, Kotake T, Izumi N and Kudo T: Phosphorylation of p53 modifies sensitivity to ionizing radiation. Anticancer Res *31*: 2255-2258, 2011.

Received March 17, 2016
Revised May 12, 2016
Accepted May 16, 2016

International Journal of
Molecular Sciences

MDPI

Article

Histone H3 Acetyl K9 and Histone H3 Tri Methyl K4 as Prognostic Markers for Patients with Cervical Cancer

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Academic Editor: Nicoletta Sacchi
Received: 13 December 2016; Accepted: 15 February 2017; Published: 23 February 2017

Abstract: Chromatin remodeling alters gene expression in carcinoma tissue. Although cervical cancer is the fourth most common cancer in women worldwide, a systematic study about the prognostic value of specific changes in the chromatin structure, such as histone acetylation or histone methylation, is missing. In this study, the expression of histone H3 acetyl K9, which is known to denote active regions at enhancers and promoters, and histone H3 tri methyl K4, which preferentially identifies active gene promoters, were examined as both show high metastatic potential. A panel of patients with cervical cancer was selected and the importance of the histone modifications concerning survival-time (overall survival and relapse-free survival) was analyzed in 250 cases. Histone H3 acetyl K9 staining was correlated with low grading, low FIGO (TNM classification and the International Federation of Gynecology and Obstetrics) status, negative N-status and low T-status in cervical cancer, showing a higher expression in adenocarcinoma than in squamous cell carcinoma. Cytoplasmic expression of histone H3 tri methyl K4 in a cervical cancer specimen was correlated with advanced T-status and poor prognosis. While cytoplasmic H3K4me3 expression seemed to be a marker of relapse-free survival, nuclear expression showed a correlation to poor prognosis in overall survival. Within this study, we analyzed the chemical modification of two histone proteins that are connected to active gene expression. Histone H3 acetyl K9 was found to be an independent marker of overall survival. Histone H3 tri methyl K4 was correlated with poor prognosis and it was found to be an independent marker of relapse-free survival. Therefore, we could show that chromatin remodeling plays an important role in cervical cancer biology.

Keywords: cervical cancer; histone H3 acetyl K9; histone H3 tri methyl K4; epigenetics; chromatin modification; histone proteins; prognosis

1. Introduction

Cervical cancer is the fourth most frequent cancer in women worldwide (about 530,000 new cases in 2012, 7.5% of all female cancer deaths). The leading cause of cervical cancer is a persistent infection with high-risk human papillomavirus (HR-HPV) [1]. Specifically, the HPV subtypes 16 and 18 cause about 70% of all cancer cases [1,2]. A total of 170 HPV-types have been described currently [3]. The infection with 15 types of HPV most likely leads to cancer, which is why these 15 types are called

carcinogenic or high-risk types [4]. The genome of human papillomaviruses consists of approximately 8000 base pairs and contains six "early genes" (E6, E7, E1, E2, E4, E5) and two "late genes" (L1, L2) [5]. In case of replication of the viral gene E6, the E6 oncoprotein is expressed, which disturbs the cell cycle [6]. E6 oncoprotein and E6-associated protein (E6-AP) form a complex which binds to p53 and causes its proteolytic degradation [7].

During the different stages of cervical cancer development, there is an accumulation of epigenetic alterations that leads to changes in gene expression [8]. Altered mechanisms of epigenetic regulation in cervical cancer include DNA methylation and post-translational modifications of histone proteins [8]. It has been reported that histone modifying enzymes such as histone deacetylase (HDAC)-1 and HDAC2 are over-expressed in cervical dysplasia and invasive carcinoma [9]. These results suggest that the dysregulation of enzymes that modify histones in cervical cancer are of importance for the biology of this tumor entity.

HR-HPVs establish persistent infection by maintaining their genomes as extrachromosomal elements—the so-called episome—that replicate, together with host DNA, in infected cells [10]. By associating with the host chromatin, HR-HPV redirects the normal cellular control of chromatin to create a cellular environment that is beneficial for both the HR-HPV multiplication and malignant progression of the infected cell. Therefore, the investigation of HPV–host chromatin interaction will offer new insights into the importance of HPV-driven chromatin regulation in cervical cancer tissue [10].

The state of histone modifications that are connected to the early and late HPV viral promoters—modification by acetylation and methylation—were examined in a previous study in cell culture systems using chromatin immunoprecipitation assays: in undifferentiated cells, di-methylated forms of histone H3K4 as well as acetylated histone H3 and H4 were found [11]. Together with differentiation, the levels of di-methylated H3K4 and acetylated H3 are increased, while the acetylated H4 is also increased, which suggests that nucleosomes are activated through histone modifications to coordinate the HPV transcription during cell differentiation [11].

The already-mentioned studies and several other studies showed that histone protein modifications play a fundamental role in HPV driven oncogenesis. Because a systematic investigation of posttranslational changes in histone proteins, for their prognostic relevance in cervical cancer tissue, was lacking, the aim of this study was an expression analyses of histone H3 acetyl K9 (H3K9ac) and histone H3 tri methyl K4 (H3K4me3) in cervical cancer, examined in 250 cases by immunohistochemical methods and assessed by a semi-quantitative score.

2. Results

2.1. H3K9ac Staining in Cervical Carcinoma

To control the quality of our H3K9ac staining, we used normal (non-pathological) colon tissue, which showed strong nuclear expression in >80% of epithelial cells without a cytoplasmic expression (Figure 1A).

A total of 92.8% of all cervical cancer specimens showed only a nuclear expression of H3K9ac with a median Immune Reactive Score (IRS) of 4 (36%), while 7.2% of all samples did not express H3K9ac at all. Compared to 50.8% with low expression (IRS = 1–5), an enhanced staining (IRS $\geq$ 6) was detected in 42.0% of samples.

In the following analyses, we examined the correlation between H3K9ac and several clinic pathological parameters such as histological subtype, grading, T-status, N-status and FIGO-classification by noticing the distribution of these parameters in our study group (Table 1).

Table 1. Clinic pathological variables of the patients included in this study.

Item	No./Total No.	%
Age, years		
<49	139/250	55.6
>49	111/250	44.4
Number of Positive Nodes		
0	151/250	60.4
≥1	97/250	38.8
Not available (NA's)	2/250	0.8
Tumor Size, pT		
pT1	110250	44
pT2/3/4	137/250	54.8
Not available (NA's)	3/250	1.2
FIGO		
I	64/250	25.6
II/III/IV	92/250	36.8
Not available (NA's)	94/250	37.6
Tumor Grade		
I	21/250	8.4
II	143/250	57.2
III	78/250	31.2
Not available (NA's)	8/250	3.2
Tumor Subtype		
Squamous	202/250	80.8
Adenocarcinoma	48/250	19.2
Progression (over 235 months)		
None	210/250	84
At least one	21/250	11.6
Not available (NA's)	11/250	4.4
Survival (over 235 months)		
Right censured	190/250	76
Died	49/250	19.6
Not available (NA's)	11/250	4.4

Examining the histological subtype, squamous epithelial carcinomas (Figure 1B) with a median IRS of 4 showed a lower H3K9ac expression than adenocarcinoma tissue (Figure 1C) with a median IRS of 8, differing significantly from each other ($p = 0.013$; Figure 1D; Table 2).

Regarding the grading, low graded (G1) specimens did not show the general median IRS of 4 in the H3K9ac staining. They presented a median IRS of 8 in 31% of samples (Figure 1E), while the median IRS of 4 in intermediate graded (G2) and high graded (G3, Figure 1F) samples was represented by 35.0% and 41.0%, respectively. Thus, enhanced staining was highly significantly correlated with low grading ($p = 0.004$; Rho = −0.209 with $p = 0.001$; Figure 1G and Table 2).

Analysing the N-Status (involved lymph nodes), 86.1% of all patients without lymph-node metastasis (N−; Figure 1H) had an IRS of ≥4 compared to 66.0% of all patients with lymph-node positive status (N+; Figure 1I), while both presented the same median IRS of 4 (Figure 1K). An enhanced expression of H3K9ac was accompanied by lymph node-negative status, while low expression was accompanied by lymph node-positive status ($p = 0.001$; Rho = −0.236 with $p < 0.001$; Table 2).

All tumor sizes (T-stages) showed an equal IRS of 4 (Figure 1L), being represented in 34/110 cases (31.0%) in T1-stage patients, and 56/137 cases (40.9%) in T2/3/4-stage patients. Data showed a significant difference ($p = 0.035$) with an inversed correlation meaning that enhanced H3K9ac staining correlated with low T-Status (Rho = −0.149 with $p = 0.019$; Table 2). Although the correlation was highly significant, it was not detectable in the boxplot.

Regarding the FIGO status, patients with FIGO I had a median IRS of 8 in 17 patients in this subgroup (17/64; 26.6%), compared to patients with a FIGO status of II or more with a median IRS of 4 (32/92; 34.8%). We could show a significant correlation between FIGO status and H3acet expression ($p = 0.016$) with a negative spearman's-rank correlation (Rho = −0.192; $p = 0.016$), meaning that strong H3K9ac staining correlated with low FIGO status (Figure 1M).

In summary, we detected associations of H3K9ac regarding histological subtype ($p = 0.013$), grading ($p = 0.004$), N-status ($p = 0.001$), T-status ($p = 0.035$) and FIGO status ($p = 0.016$) by using non-parametric tests (Table 2). In particular, the negative correlation between H3acet staining on the one hand and FIGO, T- and N-status on the other hand seem to go well together, as FIGO status is defined by T and N-status.

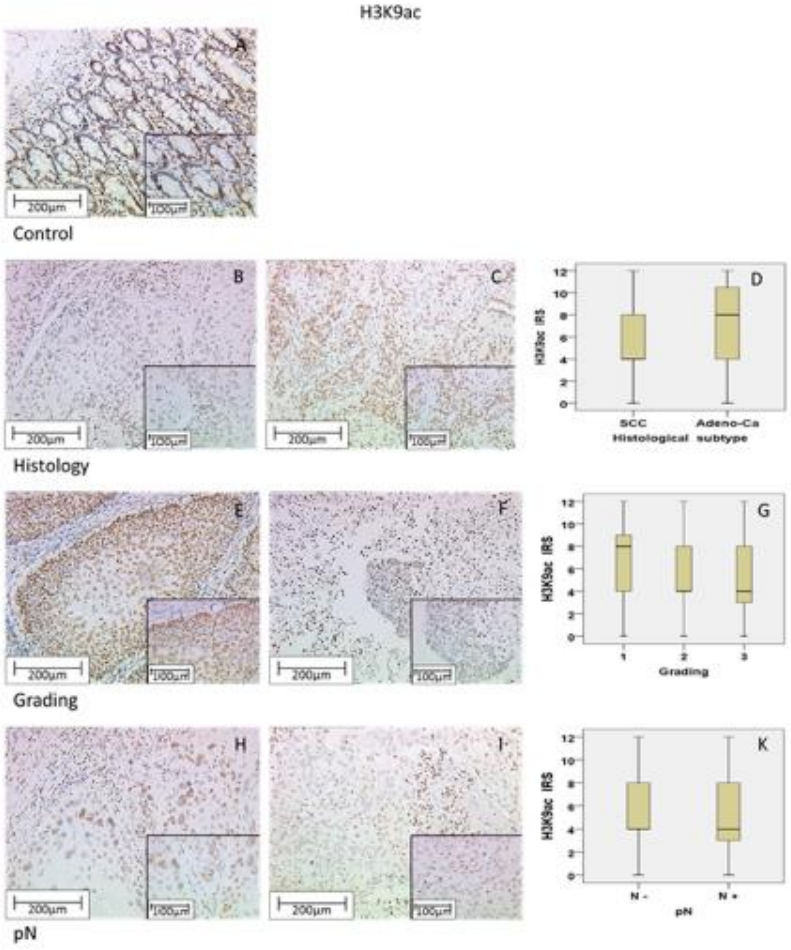


Figure 1. *Cont.*

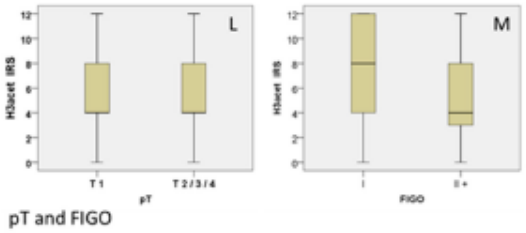


Figure 1. Positive control of H3K9ac staining in colon tissue with strong nuclear cytoplasmic expression and without cytoplasmic expression in epithelial cells (**A**). Squamous epithelial tissue (**B**) showed a median expression of H3K9ac, while adenocarcinoma tissue (**C**) showed significantly more intense H3K9ac staining; the summary regarding histological subtype is shown as a box plot (**D**). Grading: G1-stage tumors showed enhanced H3K9ac expression (**E**), G3-stage tumors (**F**) showed weak staining; the summary regarding grading is shown as a box plot (**G**). N-status: Negative N-status with high H3K9ac expression (**H**), positive N-status with low H3K9ac expression (**I**); the summary regarding N-status is shown as a box plot (**K**). T-status: The median Immune Reactive Score (IRS) is 4 for every T-status (**L**), although there is a strong correlation. TNM classification and the International Federation of Gynecology and Obstetrics (FIGO): Boxplot shows a different median IRS for FIGO-states (**M**). Scale bar 200 µm, small pictures 100 µm.

2.2. *H3K4me3 Staining in Cervical Cancer*

To evaluate the H3K4me3 staining, we used placenta tissue where a very strong expression in trophoblastic cells, in the nucleus as well as in the cytoplasm, was found (Figure 2A).

Of all the cervical cancer specimens, a total of 96.8% showed H3K4me3 expression, while 3.2% did not show any expression at all. H3K4me3 was found in the cytoplasm as well as in the nucleus, correlating significantly with each other (Rho = 0.290 with $p < 0.001$). All positive tested samples presented a nuclear expression with a median IRS of 8 (31%) compared to a median IRS of 0 (56.4%) in samples with cytoplasmic expression (Figure 2B). All in all, nuclear expression of H3K4me3 was detectable in 96.8% (negative: 3.2%) of patients, while cytoplasmic expression was only positive in 43.6% of all patients negative: 56.4%). Nuclear H3K4me3 expression was enhanced (IRS = 4–12) in 88.4% of all cases compared to a low expression (IRS = 0–3) in 11.6%. Regarding cytoplasmic expression, 36.8% slides showed a high expression (IRS = 4–12) and 63.2% slides presented a weak expression (IRS = 0–3).

Examining the T-stage, T1-stage carcinoma tissues showed the general median of 0 in 30.4% of all cases in the cytoplasmic H3K4me3 staining (Figure 2C). In contrast, T2/3/4-stage samples showed an enhanced median IRS of 2 (Figure 2D) in 3.6% of all cases. Performing nonparametric-tests and Spearman's rank correlation, enhanced cytoplasmic expression of H3K4me3 was correlated with advanced T-Status ($p = 0.002$; Rho = 0.191 with $p = 0.003$; Table 2). This means that an advanced cytoplasmic H3K4me3 expression correlated with higher T-status (Figure 2E).

In summary, we found associations of cytoplasmic H3K4me3 expression regarding T-status ($p = 0.002$) by using non-parametric tests (Table 2). No significant difference was found for cytoplasmic expression among N-Status, FIGO or grading and there were no correlations detected between nuclear H3K4me3 expression and the described pathological parameters.

Table 2. Staining results and correlation analysis.

	H3K9ac				H3K4me3 (Cytoplasmic)				H3K4me3 (Nuclear)			
	Median IRS (+/−SD)	%	p (NPAR)	ρ	Median IRS (+/−SD)	%	p (NPAR)	ρ	Median IRS (+/−SD)	%	p (NPAR)	ρ
Histology												
SCC	4 (+/−3.45)	40.10%	0.013	-	0 (+/−2.67)	57.40%	0.296	-	8 (+/−3.56)	32.20%	0.603	-
Adeno-Ca	8 (+/−3.78)	31.30%			0 (+/−3.35)	52.10%			8 (+/−3.58)	27.10%		
Grade												
G1	8 (+/−3.51)	31.00%	0.004	−0.209 ($p = 0.001$)	0 (+/−2.94)	57.10%	0.197	0.082 ($p > 0.05$)	8 (+/−3.66)	28.60%	0.917	−0.017 ($p > 0.05$)
G2	4 (+/−3.57)	35.00%			0 (+/−3.08)	52.40%			8 (+/−3.59)	31.50%		
G3	4 (+/−3.26)	41.00%			0 (+/−2.24)	62.80%			8 (+/−3.55)	33.30%		
pN												
N−	4 (+/−3.55)	86.10%	0.001	−0.236 ($p = 0.000$)	0 (+/−3.02)	57.00%	0.981	−0.001 ($p > 0.05$)	8 (+/−3.50)	32.50%	0.695	0.025 ($p > 0.05$)
N+	4 (+/−3.33)	66.00%			0 (+/−2.43)	55.70%			8 (+/−3.69)	28.90%		
pT												
T1	4 (+/−3.52)	30.90%	0.035	−0.149 ($p = 0.019$)	0 (+/−2.34)	69.10%	0.002	0.191 ($p = 0.003$)	8 (+/−3.49)	30.00%	0.171	0.081 ($p > 0.05$)
T2/3/4	4 (+/−3.49)	40.90%			2 (+/−2.93)	3.60%			8 (+/−3.60)	32.10%		
FIGO												
I	8 (+/−3.91)	26.60%	0.016	−0.192 ($p = 0.016$)	0 (+/−2.45)	64.10%	0.324	0.070 ($p = 0.384$)	8 (+/−3.37)	32.80%	0.862	−0.005 ($p = 0.948$)
II+	4 (+/−3.44)	23.90%			2 (+/−2.66)	5.40%			8 (+/−3.67)	30.40%		
p16	-	-	-	0.047 ($p > 0.05$)	-	-	-	0.009 ($p > 0.05$)	-	-	-	0.144 ($p = 0.027$)

SD = standard deviation; % = percentage of the subgroup with median IRS; NPAR = non-parametric test; p = p-value; ρ = correlation coefficient; SCC = squamous cell carcinoma, pT = tumor size, FIGO = TNM classification and the International Federation of Gynecology and Obstetrics.

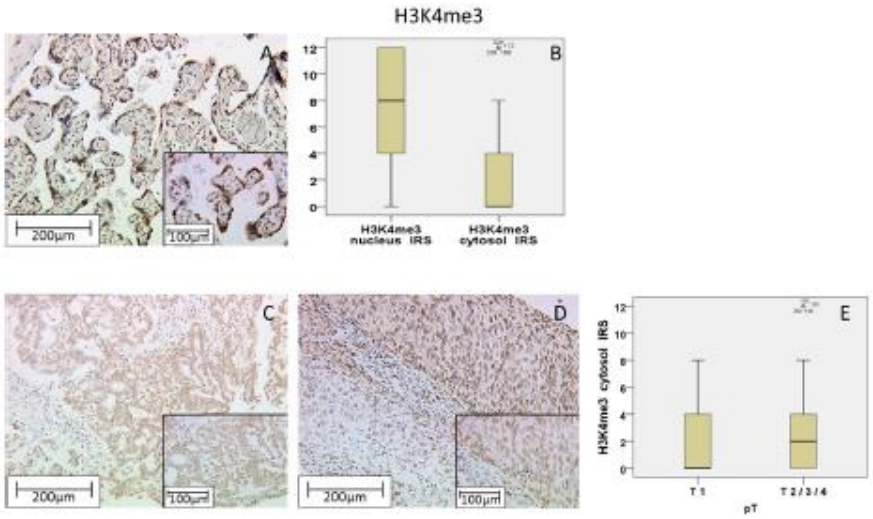


Figure 2. Positive control of H3K4me3 staining in placenta tissue with strong nuclear cytoplasmic expression and weak cytoplasmic expression in trophoblastic cells (**A**); H3K4me3 showed a higher expression in the nucleus than in the cytoplasm (**B**); T1-stage tumors (**C**) with significantly lower expression than T2/3/4-stage tumors (**D**); The summary regarding T-status is shown as a box plot (**E**). Scale bar 200 µm, small pictures 100 µm.

2.3. Correlation Analysis between H3K4me3 and p16 Oncoprotein

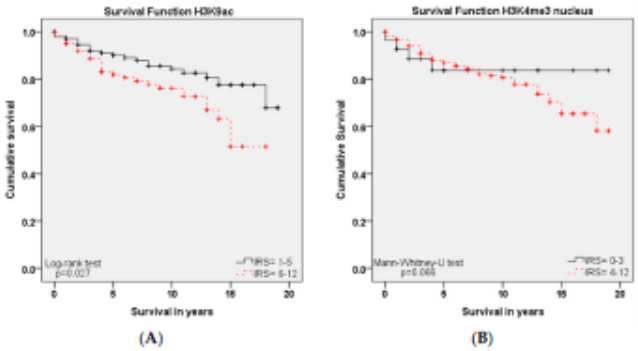
It is well known that the expression of p16 oncoprotein in cancer is not only associated with DNA methylation but also with histone modification [12]. By using recently published data by our institute [13,14], we looked for a similar association in cervical cancer. Analyses showed that a nuclear H3K4me3 expression was positively correlated with p16 expression (Rho = 0.144; $p = 0.027$; Table 2). No correlation was found regarding the cytoplasmic expression of H3K4me3 (Rho = 0.009; $p > 0.05$) or nuclear H3K9ac expression (Rho = 0.047; $p > 0.05$).

2.4. Role of H3K9ac and H3K4me3 for Overall Survival

Enhanced H3K9ac expression (IRS $\geq$ 6) was—as well as H3K4me3 expression (IRS $\geq$ 4)—associated with survival-time after diagnosis.

As shown in the Kaplan–Meier curve (Figure 3A), high expression of H3K9ac (IRS $\geq$ 6) in cervical cancer patients was correlated with poor prognosis in overall survival rates ($p = 0.027$). This was in contrast to the correlation between H3K9ac staining and the described clinic pathological parameters (T-status, N-status, Grading and FIGO), where high H3K9ac expression was correlated with the low stage of these parameters.

In addition to H3K9ac, we examined the role of H3K4me3 for survival, where we found a similar correlation: advanced nuclear H3K4me3 expression was also correlated with poor prognosis concerning overall survival (Figure 3B, $p = 0.066$). For cytoplasmic H3K4me3 expression, there was no significance concerning overall survival.



(A) (B)

Figure 3. Kaplan–Meier analyses for overall survival: H3K9ac ($p = 0.027$; **A**) with high expression (IRS $\geq$ 6; red) compared to low expression (IRS $\leq$ 5; black); High nuclear H3K4me3 expression (IRS $\geq$ 4; red) compared to low expression (IRS $\leq$ 3; black) regarding overall survival ($p = 0.066$; **B**).

2.5. Role of H3K9ac and H3K4me3 for Progress-Free Survival

Although H3K9ac and nuclear H3K4me3 expressions showed significant differences regarding overall survival, their expressions showed no significant correlation for relapse-free survival ($p = 0.763$ and $p = 0.08$).

In contrast, cytoplasmic H3K4me3 expression, which was not a marker of overall survival, was significantly correlated with progress-free survival: high cytoplasmic expression of H3K4me3 (IRS $\geq$ 4) meant short relapse-free survival (Figure 4, $p = 0.025$), matching the correlation between high expression and advanced T-Status.

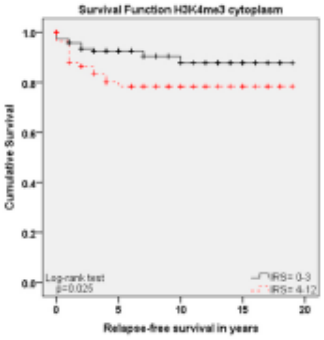


Figure 4. Kaplan–Meier analyses for relapse-free survival: high cytoplasmic H3K4me3 expression (IRS $\geq$ 4; red) compared to low expression (IRS $\leq$ 3; black) regarding relapse-free survival ($p = 0.025$).

2.6. Cox Regression of H3K9ac and H3K4me3 and Clinic Pathological Variables

The additionally performed multivariate cox-regression tested which histopathological parameters were independent prognosticators for survival in our study-group.

For overall survival, the histological subtype ($p = 0.040$), pN-status ($p = 0.003$), FIGO classification ($p = 0.012$), age at surgery ($p < 0.001$) and the expression of H3K9ac ($p = 0.027$) were independent prognosticators, but not the H3MK4me3 expression or other tested clinic pathological parameters (Table 3).

Regarding relapse-free survival, only FIGO status (p = 0.044) and cytoplasmic H3K4me3 expression (p = 0.030) turned out to be independent markers in multivariate cox-analysis (Table 4), but not H3K9ac expression, nuclear H3K4me3 expression or other described parameters.

Table 3. Cox regression of clinic pathological variables regarding overall survival.

Variable	Significance	Hazard Ratio of Exp(B)	Lower 95% CI of Exp(B)	Upper 95% CI of Exp(B)
Histology	0.040	1.893	1.030	3.479
pT	0.751	0.910	0.509	1.626
pN	0.003	2.447	1.367	4.380
FIGO	0.012	3.181	1.287	7.863
Grading	0.198	1.360	0.852	2.170
Age at surgery	0.000	1.049	1.026	1.072
H3K9ac	0.027	1.900	1.076	3.356
H3K4me3 nucleus	0.708	1.216	0.436	3.389
H3K4me3 cytoplasm	0.159	1.503	0.853	2.651

Table 4. Co12x regression of clinic pathological variables regarding progress-free survival.

Variable	Significance	Hazard Ratio of Exp(B)	Lower 95% CI of Exp(B)	Upper 95% CI of Exp(B)
Histology	0.753	1.156	0.469	2.851
pT	0.760	0.890	0.423	1.875
pN	0.843	1.082	0.495	2.368
FIGO	0.044	3.085	1.031	9.235
Grading	0.521	1.228	0.656	2.299
Age at surgery	0.157	1.021	0.992	1.052
H3K9ac	0.763	0.890	0.417	1.900
H3K4me3 nucleus	0.476	0.681	0.236	1.963
H3K4me3 cytoplasm	0.030	2.278	1.084	4.790

3. Discussion

Within this study, we showed that the immunohistochemical evaluation of histone H3K9ac staining was correlated with low grading, low FIGO-classification, low T-status and negative N-status in cervical cancer. We could also find a higher expression of histone H3K9ac in adenocarcinoma compared to squamous cell carcinoma. Due to its correlation between expression and poor prognosis (overall survival), it could be used as an independent marker of prognosis. Cytoplasmic expression of histone H3K4me3 in a cervical cancer specimen was correlated with advanced T-status and poor prognosis. It seems to be a marker of relapse-free survival, while nuclear expression showed a correlation to poor prognosis without being an independent marker regarding cervical cancer.

Histone proteins give the genome the ability to pack very large amounts of DNA in a very small space, but at the same time they leave their N-terminal tails flexible [15]. The N-terminal tail of the histone proteins can undergo post-translational modification by enzymes, adding chemical modifications such as acetylation, methylation, phosphorylation and deamination that alter the structure of the DNA package and allow or prevent gene transcription [16]. It is already known that histone modifications at histone 3 lysine 9 acetylation (H3K9ac) denote active regions at enhancers as well as promoters, whereas the tri-methyl form, H3K4me3, preferentially identifies the gene promoters that are active [17,18]. In addition, it has been shown that epigenetic modulations of the genome involve histone modifications that alter the gene chromatin configuration. A decondensed ("open") configuration allows transcription factors access to binding sites, whereas a condensed ("closed") configuration blocks transcription binding sites, thereby regulating gene transcription [19,20]. Based on these findings, it has been shown that high metastatic potential had greater acetylation of histone H3 lysine 9 (H3K9ac) and tri-methylation of histone H3 lysine 4 (H3K4me3) [19]. Therefore, these two modifications that are correlated to enhanced gene activity and in addition show high metastatic potential were used in the present study as markers for the identification of the prognostic relevance of those histone modifications for cervical cancer survival.

It is already known that E6 oncoprotein and E6-associated protein (E6-AP) form a complex which binds to p53 and causes its proteolytic degradation [7]. P53 is a tumor suppressor, as it leads to cell cycle arrest or apoptosis in the case of DNA damage [21]. As E6 oncoprotein induces the degradation of p53, the function of this important cell cycle protein is disturbed [12] after HPV infection. The cell cycle regulation protein p16 is expressed at high levels in HPV-infected epithelial cells, which is why it acts as a marker for the diagnosis of a HPV associated carcinoma [22,23]. On the other hand, studies have shown that p16 expression is induced by an oncogene senescence-related mechanism that involves histone H3K27 demethylation by histone lysine demethylase, and that p16 expression is necessary for the survival of HPV-infected cells expressing E7 viral oncoprotein [24,25].

Unfortunately, p16 is not exclusively increased by E7 oncoprotein in carcinogenesis. Therefore, in a recent study, we established and published an immunohistochemical approach for the direct detection of E6 oncoprotein in uterine cervical cancer [14]. In addition, we found a very high mutation rate of TP53 in this cancer type where p53 is initially inactivated via E6 during the development of cervical cancer. An unexpected finding is the correlation of this mutation with better survival, possibly due to better response to therapy [13].

Because both H3K9ac and H3K4me3 are negative prognosticators for cervical cancer patients, the use of epigenetic drugs or the search for epigenetic targets could be a useful goal for cervical cancer treatment.

Recently, two main classes of epigenetic drugs—methylation inhibitors and HDAC inhibitors—are in clinical trials for the treatment of cervical cancer [26]. One of these potential new drugs could be valproic acid (VPA). VPA was found to be an effective inhibitor of histone deacetylases and has been shown to induce anti-tumor effects by modulating cellular pathways, including cell cycle arrest, apoptosis, angiogenesis, metastasis, differentiation, and senescence [27]. The antitumor effect of VPA in cervical cancer can be explained by either the hyper-acetylation of p53 protein, protecting it from degradation by E6 and increasing p53 activity; or via the inhibition of Akt1 and Akt2 expression, which results in apoptotic cell death [28,29]. Acetylation of p53 is a process that occurs in response to DNA damage and stress and is necessary for p53 transcriptional activity. Therefore, p53 was one of the first non-histone proteins that could be acetylated by histone acetyl transferases [30].

In addition, HDAC inhibitors also interfere with cervical cancer via non-histone targets. The HDAC inhibitor suberoylanilide hydroxamic acid (SAHA) induces apoptosis in HeLa cervical cancer cells in vitro with bortezomib by activating caspase-3 and increasing the ratio of bax/bcl-2 expression [31]. Epigenetic aberrations, such as histone protein modification have the ability to regulate the expression of oncogenes or repression of tumor suppressor genes. Therefore, these modified histone proteins are powerful candidates for the investigation of cancer pathogenesis and progression. For cervical cancer, for instance, Feng et al. [26] highlighted a number of genes that underwent epigenetic alteration at the level of DNA methylation, histone modification, or noncoding RNA action in this type of cancer.

Further investigation of these alterations and information about them could lead to new and reliable screening methods for women at high risk of cervical cancer and can help to establish new candidates for a better treatment of this disease.

4. Materials and Methods

4.1. Patients and Specimens

We used 250 paraffin-embedded cervical cancer samples obtained from patients having undergone surgery for cervical cancer in the Department of Obstetrics and Gynecology of the Ludwig-Maximilians-University of Munich between 1993 and 2002. The median age of the patients was 47.0 years (range 20–83 years), and overall median survival was 100.0 months. For distribution of clinic pathological variables see Table 1. Only patients with adenocarcinoma or squamous cell carcinoma (SCC) of the cervix were included in our study, other histological subtypes were excluded

due to low number. As positive controls for immunohistochemically staining, we utilized colon tissue for histone H3 acetyl K9 and placenta tissue for histone H3 tri methyl K4; both received from the Department of Obstetrics and Gynecology of the Ludwig-Maximilians-University of Munich. Clinical and follow-up data for statistical analyses were provided by the Munich cancer registry and retrieved from medical records.

4.2. Ethics Approval

All cervical cancer specimens had originally been collected for histopathological diagnostics and were no longer used for clinical tests, when they were recruited for this survey. Patient data were totally anonymized and the authors were blinded for clinical information—including survival-time during experimental analyses. The study was conducted conforming to the Declaration of Helsinki and was approved by the local ethics committee of the Ludwig-Maximilians University of Munich (reference number 259-16, 2016).

4.3. Immunohistochemistry

The paraffin-embedded and formalin-fixed samples (3 µm) had been stored at room temperature and were first dewaxed in xylol. After rinsing the tissue in 100% ethanol and blocking the endogenous peroxidase with 3% methanol/H_2O_2, the samples were rehydrated in a descending alcohol series. To avoid heat-associated protein-agglomeration and to unmask the antigen, the slides were warmed up to 100 °C in a pressure cooker for 5 min, adding a trisodium citrate buffer solution with pH = 6. After having prepared the slides by washing them in distilled water and PBS-buffer, we added the suitable blocking solution to avoid unspecific (hydrophobic) bindings between immunoglobulins on the one side and cell membranes or fatty tissue on the other side by saturation of electrostatic charges. Afterwards, the samples were incubated at a temperature of +4 °C with the primary antibodies (Table 5).

After increasing the staining by the post-block-reagent and applying the HRP-polymer, the substrate-staining with DAB was performed and the counterstaining by haemalm (2 min) was carried out subsequently. More details concerning the suitable detection system and the following steps were defined exactly in Table 5. Finally, the samples were dehydrogenated in a rising alcohol series and covered. Colon tissue and placenta tissue were used for each staining as positive and negative controls for H3K9ac and H3K4me3.

The intensity of the expression was evaluated by the immunoreactive score (IRS). Well-established and applied in numerous other studies, this semi-quantitative score multiplies the intensity of the staining (0 = not stained; 1 = low intensity; 2 = moderate intensity; 3 = high intensity) and the percentage of stained cells (0 = 0%; 1 = 1%–10%; 2 = 11%–50%; 3 = 51%–80%; 4 ≥ 80%). Finally, we distinguished between 0 = no expression and 12 = very high expression of histones.

Table 5. Antibodies and chemicals used for the immunohistochemistry.

Histone H3 Acetyl K9 [1]	**Histone H3 Tri Methyl K4** [2]
Blocking solution [3]: 5 min	Blocking solution [3]: 5 min
primary antibody [1]: 1:200 in PBS [5], incubation: 16 h, 4 °C	primary antibody [2]: 1:500 in PBS [5], incubation: 16 h, 4 °C
PostBlock [3]: 20 min	PostBlock [3]: 20 min
HRP Polymer [3]: 30 min	HRP Polymer [3]: 30 min
Chromogen: DAB [4] (0.5 min)	Chromogen: DAB [4] (1 min)

[1] Anti Histone H3 acetyl K9, clone Y28 (rabbit IgG), concentration: 0.059 mg/mL, company: Abcam (Cambridge, UK), order number: ab32129; [2] Anti Histone H3 tri methyl K4, rabbit IgG polyclonal, concentration: 1 mg/mL, company: Abcam, order number: ab8580; [3] ZytoChem Plus HRP Polymer Kit (Mouse/Rabbit) 3 × 100; company: Zytomed Systems (Berlin, Germany) Nr. POLHRP-100; [4] Liquid DAB + Substrate Chromogen System 1 mg/mL, DAKO; [5] Dulbecco's Phosphate Buffered Saline.

4.4. Statistics

For statistical analyses, IBM SPSS Statistics version 23 (Armonk, NY, USA) was used. Bivariate correlations were calculated by Spearman's-rank-correlation coefficient and non-parametric tests (NPAR: Mann–Whitney U test, Kruskal–Wallis test) were employed to compare independent groups. To visualize differences concerning survival rates, Kaplan–Meier curves were created and afterwards compared by a log-rank test and—if necessary—Mann–Whitney-U test. Survival times are shown in years, but for more exact results they were calculated in months. To show statistical difference, p had to be <0.05.

5. Conclusions

The expression of histone H3 acetyl K9 and histone H3 tri methyl K4 was examined in 250 cases of cervical cancer. Both histone protein modifications turned out to be independent negative prognosticators for the overall survival or the relapse-free survival of cervical cancer patients. For cervical cancer, it is the first study that showed a direct link between histone protein modification and survival in a large cohort of patients.

Acknowledgments: The study was supported by the "Heuer Stiftung" for Bernd P. Kost.

Author Contributions: Susanne Beyer, Bernd P. Kost and Christian Dannecker conceived and designed the experiments; Susanne Beyer, Christina Kuhn, Sandra Schulze and Simone Hofmann performed the experiments; Susanne Beyer, Junyan Zhu and Doris Mayr analyzed the data; Doris Mayr contributed analysis tools as gynecological pathologist; Susanne Beyer and Udo Jeschke wrote the paper. Bernd P. Kost finally approved the manuscript.

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

Abbreviations

H3K9ac	histone H3 acetyl K9
H3K4me3	histone H3 tri methyl K4
SCC	squamous cell carcinoma

References

1. Chatterjee, S.; Chattopadhyay, A.; Samanta, L.; Panigrahi, P. HPV and cervical cancer epidemiology—Current status of HPV vaccination in India. *Asian Pac. J. Cancer Prev.* **2016**, *17*, 3663–3673. [PubMed]
2. Schiffman, M.; Castle, P.E.; Jeronimo, J.; Rodriguez, A.C.; Wacholder, S. Human papillomavirus and cervical cancer. *Lancet* **2007**, *370*, 890–907. [CrossRef]
3. Wittekindt, C.; Wagner, S.; Mayer, C.S.; Klussmann, J.P. Basics of tumor development and importance of human papilloma virus (HPV) for head and neck cancer. *Laryngorhinootologie* **2012**, *91* (Suppl. 1), S1–S26. [PubMed]
4. Liu, X.M.; Pan, C.W.; Wang, G.D.; Cai, X.H.; Chen, L.; Meng, C.F.; Huang, J.C. Finite element analysis of the stability of combined plate internal fixation in posterior wall fractures of acetabulum. *Int. J. Clin. Exp. Med.* **2015**, *8*, 13393–13397. [PubMed]
5. Liu, J.; Cheng, Y.; He, M.; Yao, S. Vascular endothelial growth factor C enhances cervical cancer cell invasiveness via upregulation of galectin-3 protein. *Gynecol. Endocrinol.* **2014**, *30*, 461–465. [CrossRef] [PubMed]
6. Gupta, S.; Takhar, P.P.; Degenkolbe, R.; Koh, C.H.; Zimmermann, H.; Yang, C.M.; Guan Sim, K.; Hsu, S.I.; Bernard, H.U. The human papillomavirus type 11 and 16 E6 proteins modulate the cell-cycle regulator and transcription cofactor TRIP-Br1. *Virology* **2003**, *317*, 155–164. [CrossRef] [PubMed]
7. Meng, C.F.; Su, B.; Li, W. DNA demethylation is superior to histone acetylation for reactivating cancer-associated genes in ovarian cancer cells. *Mol. Med. Rep.* **2011**, *4*, 1273–1278. [CrossRef] [PubMed]
8. Sandoval-Basilio, J.; Serafin-Higuera, N.; Reyes-Hernandez, O.D.; Serafin-Higuera, I.; Leija-Montoya, G.; Blanco-Morales, M.; Sierra-Martinez, M.; Ramos-Mondragon, R.; Garcia, S.; Lopez-Hernandez, L.B.; et al. Low proteolytic clipping of histone H3 in cervical cancer. *J. Cancer* **2016**, *7*, 1856–1860. [CrossRef] [PubMed]

9. Huang, B.H.; Laban, M.; Leung, C.H.; Lee, L.; Lee, C.K.; Salto-Tellez, M.; Raju, G.C.; Hooi, S.C. Inhibition of histone deacetylase 2 increases apoptosis and p21Cip1/WAF1 expression, independent of histone deacetylase 1. *Cell Death Differ.* **2005**, *12*, 395–404. [CrossRef] [PubMed]
10. You, J. Papillomavirus interaction with cellular chromatin. *Biochim. Biophys. Acta* **2010**, *1799*, 192–199. [CrossRef] [PubMed]
11. Wooldridge, T.R.; Laimins, L.A. Regulation of human papillomavirus type 31 gene expression during the differentiation-dependent life cycle through histone modifications and transcription factor binding. *Virology* **2008**, *374*, 371–380. [CrossRef] [PubMed]
12. Meng, C.F.; Zhu, X.J.; Peng, G.; Dai, D.Q. Promoter histone H3 lysine 9 di-methylation is associated with DNA methylation and aberrant expression of p16 in gastric cancer cells. *Oncol. Rep.* **2009**, *22*, 1221–1227. [PubMed]
13. Freier, C.P.; Stiasny, A.; Kuhn, C.; Mayr, D.; Alexiou, C.; Janko, C.; Wiest, I.; Jeschke, U.; Kost, B. Immunohistochemical evaluation of the role of p53 mutation in cervical cancer: Ser-20 p53-mutant correlates with better prognosis. *Anticancer Res.* **2016**, *36*, 3131–3137. [PubMed]
14. Stiasny, A.; Kuhn, C.; Mayr, D.; Alexiou, C.; Janko, C.; Wiest, I.; Jeschke, U.; Kost, B. Immunohistochemical evaluation of E6/E7 HPV oncoproteins staining in cervical cancer. *Anticancer Res.* **2016**, *36*, 3195–3198. [PubMed]
15. Iwasaki, W.; Miya, Y.; Horikoshi, A.; Taguchi, H.; Tachiwana, H.; Shibata, T.; Kagawa, W.; Kurumizaka, H. Contribution of histone N-terminals to the structure and stability of nucleosomes. *FEBS Open Bio* **2013**, *3*, 363–369. [CrossRef] [PubMed]
16. Taverna, S.D.; Li, H.; Ruthenburg, A.J.; Allis, C.D.; Patel, D.J. How chromatin-binding modules interpret histone modifications: Lessons from professional pocket pickers. *Nat. Struct. Mol. Biol.* **2007**, *14*, 1025–1040. [CrossRef] [PubMed]
17. Yavartanoo, M.; Choi, J.K. Encode: A sourcebook of epigenomes and chromatin language. *Genom. Inform.* **2013**, *11*, 2–6. [CrossRef] [PubMed]
18. Wang, Z.; Zang, C.; Rosenfeld, J.A.; Schones, D.E.; Barski, A.; Cuddapah, S.; Cui, K.; Roh, T.Y.; Peng, W.; Zhang, M.Q.; et al. Combinatorial patterns of histone acetylations and methylations in the human genome. *Nat. Genet.* **2008**, *40*, 897–903. [CrossRef] [PubMed]
19. Yu, Y.; Zeng, P.; Xiong, J.; Liu, Z.; Berger, S.L.; Merlino, G. Epigenetic drugs can stimulate metastasis through enhanced expression of the pro-metastatic ezrin gene. *PLoS ONE* **2010**, *5*, e12710. [CrossRef] [PubMed]
20. Jaenisch, R.; Bird, A. Epigenetic regulation of gene expression: How the genome integrates intrinsic and environmental signals. *Nat. Genet.* **2003**, *33*, 245–254. [CrossRef] [PubMed]
21. Tang, D.; Wu, D.; Hirao, A.; Lahti, J.M.; Liu, L.; Mazza, B.; Kidd, V.J.; Mak, T.W.; Ingram, A.J. ERK activation mediates cell cycle arrest and apoptosis after DNA damage independently of p53. *J. Biol. Chem.* **2002**, *277*, 12710–12717. [CrossRef] [PubMed]
22. Mao, C.; Balasubramanian, A.; Yu, M.; Kiviat, N.; Ridder, R.; Reichert, A.; Herkert, M.; von Knebel Doeberitz, M.; Koutsky, L.A. Evaluation of a new p16(INK4a) ELISA test and a high-risk HPV DNA test for cervical cancer screening: Results from proof-of-concept study. *Int. J. Cancer* **2007**, *120*, 2435–2438. [CrossRef] [PubMed]
23. Melkane, A.E.; Mirghani, H.; Auperin, A.; Saulnier, P.; Lacroix, L.; Vielh, P.; Casiraghi, O.; Griscelli, F.; Temam, S. HPV-related oropharyngeal squamous cell carcinomas: A comparison between three diagnostic approaches. *Am. J. Otolaryngol.* **2014**, *35*, 25–32. [CrossRef] [PubMed]
24. McLaughlin-Drubin, M.E.; Crum, C.P.; Munger, K. Human papillomavirus E7 oncoprotein induces KDM6A and KDM6B histone demethylase expression and causes epigenetic reprogramming. *Proc. Natl. Acad. Sci. USA* **2011**, *108*, 2130–2135. [CrossRef] [PubMed]
25. McLaughlin-Drubin, M.E.; Park, D.; Munger, K. Tumor suppressor p16INK4A is necessary for survival of cervical carcinoma cell lines. *Proc. Natl. Acad. Sci. USA* **2013**, *110*, 16175–16180. [CrossRef] [PubMed]
26. Fang, J.; Zhang, H.; Jin, S. Epigenetics and cervical cancer: From pathogenesis to therapy. *Tumour Biol.* **2014**, *35*, 5083–5093. [CrossRef] [PubMed]
27. Duenas-Gonzalez, A.; Lizano, M.; Candelaria, M.; Cetina, L.; Arce, C.; Cervera, E. Epigenetics of cervical cancer. An overview and therapeutic perspectives. *Mol. Cancer* **2005**, *4*. [CrossRef] [PubMed]

28. De la Cruz-Hernandez, E.; Perez-Cardenas, E.; Contreras-Paredes, A.; Cantu, D.; Mohar, A.; Lizano, M.; Duenas-Gonzalez, A. The effects of DNA methylation and histone deacetylase inhibitors on human papillomavirus early gene expression in cervical cancer, an in vitro and clinical study. *Virol. J.* **2007**, *4*. [CrossRef] [PubMed]
29. Chen, J.; Ghazawi, F.M.; Bakkar, W.; Li, Q. Valproic acid and butyrate induce apoptosis in human cancer cells through inhibition of gene expression of Akt/protein kinase B. *Mol. Cancer* **2006**, *5*. [CrossRef] [PubMed]
30. Brooks, C.L.; Gu, W. The impact of acetylation and deacetylation on the p53 pathway. *Protein Cell* **2011**, *2*, 456–462. [CrossRef] [PubMed]
31. Jiang, Y.; Wang, Y.; Su, Z.; Yang, L.; Guo, W.; Liu, W.; Zuo, J. Synergistic induction of apoptosis in HeLa cells by the proteasome inhibitor bortezomib and histone deacetylase inhibitor SAHA. *Mol. Med. Rep.* **2010**, *3*, 613–619. [PubMed]

Archives of Gynecology and Obstetrics
https://doi.org/10.1007/s00404-018-4928-9

GYNECOLOGIC ONCOLOGY

Glucocorticoid receptor in cervical cancer: an immunhistochemical analysis

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Received: 18 June 2018 / Accepted: 1 October 2018

Abstract

Purpose Cervical cancer is one of the most frequent cancers in women worldwide. In most of all cases, a persistent HPV infection is the leading cause. HPV-specific sequences are able to bind glucocorticoid receptor (GR). Dexamethasone can increase the activity of early promoters in HPV16 and HPV18 interfering in transcription control of viral oncogenes. The aim of our study was to evaluate glucocorticoid receptor as transcriptional factor in its active form in the nucleus of in cervical cancer cells and to correlate the results with clinical patient specific parameters.

Methods A total of 250 paraffin-embedded cervical cancer samples obtained from patients having undergone surgery for cervical cancer were used for the study. The expression of GR was immunhistochemical examined and evaluated by a semi-quantitative scoring. SPSS software was used for the statistical evaluation of staining results and survival analysis of patients with cervical cancer.

Results GR is frequently expressed in cervical carcinoma tissue in favor of squamous cell carcinoma (SCC). An enhanced expression is correlated with rather small clinical stages. The expression of the GR is correlated with better overall survival and progression-free survival.

Conclusions The glucocorticoid receptor is frequently expressed in cervical carcinoma tissue in favor of squamous cell carcinoma. An enhanced expression is correlated with rather small clinical stages. The expression of the analyzed receptor is correlated with better overall survival. Further studies are needed to determine useful treatment targets for glucocorticoid receptor manipulation.

Keywords Cervical cancer · Glucocorticoid receptor · Survival

Introduction

Cervical cancer is one of the most frequent cancers in women worldwide. Regarding women's outcome, major prognostic factors are known as International Federation of Gynecology and Obstetrics (FIGO) stage, histological type or grade, tumor size, lymph node metastasis or rather lymphatics invasion. According to international guidelines, patients are treated with surgery or radiotherapy depending on staging and individual risk assessment [1–3].

In most of all cases, a persistent infection with high-risk human papillomavirus (HR-HPV) is the reason for cervical cancer [1, 4]. A total of 170 HPV types are known [5, 6]. In the genome of human papillomaviruses, there are approximately 8000 base pairs and six "early genes" (E6, E7, E1, E2, E4, E5), two "late genes" (L1, L2) and noncoding regions [7]. Integration of HPV is a vector for cervical carcinogenesis resulting in a loss of a suppressive function on E6 and E7. In consequence, disturbance of cell cycle, uncontrolled cell proliferation and possible carcinogenesis occur [8–10]. Although viral-specific pathogenesis of cervical cancer is well known, additional mechanisms as co-factors are assumed to induce HPV-related carcinogenesis. The role of steroid hormones in the pathogenesis of HPV-related cervical cancer is under investigation [8, 11–14].

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Published online: 10 October 2018

HPV-specific sequences (LCR, long control regions) are able to bind receptors like glucocorticoid receptor (GR) [8, 15]. Dexamethasone can result in an increased activity of early promoters in HPV16 and HPV18 interfering in transcription control of viral oncogenes E6 and E7 [8, 13, 16, 17].

Materials and methods

Specimens

We used 250 paraffin-embedded cervical cancer samples. They were obtained from patients with a median age of 47.0 years (range 20–83 years), and an overall median survival of 100.0 months. Patients had undergone surgery for cervical cancer in the Department of Obstetrics and Gynecology of the LMU Munich between 1993 and 2002. Clinic pathological variables are described in Table 1. Only patients with the most frequent histological subtype (adenocarcinoma or squamous cell carcinoma) of the cervix were included in our study; due to low number, other histological subtypes were excluded. For positive and negative controls, placenta tissue received from the Department of Obstetrics and Gynecology of the LMU Munich was used. Clinical and follow-up data for statistical analyses were provided by The Munich Cancer Registry and recruited from medical records. We used, therefore, the original data. This means that we used the FIGO classification which was available for 2002—as patients underwent surgery between 1993 and 2002. In this classification, patients were also staged in FIGO IIIC, which means that they were FIGO III with positive lymph nodes.

Table 1 Clinic pathological parameters of the patients included in this study

	No./Total no.	%
Age, years		
< 49	139/250	55.6
> 49	111/250	44.4
No. of positive nodes		
0	151/250	60.4
≥ 1	097/250	38.8
NA's	002/250	00.8
pT		
pT1	111/250	44.4
pT2	129/250	51.6
pT3/4	009/250	03.6
NA's	001/250	0.04
FIGO		
I	64/250	25.6
II	49/250	19.6
III	37/250	14.8
IV	07/250	02.8
NA's	93/250	37.2
Tumor grade, G		
I	021/250	08.4
II	143/250	57.2
III	078/250	31.2
NA's	008/250	03.2
Tumor subtype		
Squamous	202/250	80.8
Adenocarcinoma	048/250	19.2
Progression(over 235 months)		
None	210/250	84.0
At least one	29/250	11.6
NA's	011/250	04.4
Survival (over 235 months)		
Right censured	190/250	76.0
Died	049/250	19.6
NA's	011/250	04.4

Immunohistochemical staining

Specimens were formalin fixed and paraffin embedded, while stored at room temperature. Slides (3 µm) were dewaxed in xylol and afterwards washed in 100% alcohol. After blocking the endogenous peroxidase by 3% methanol/H_2O_2, rehydration of the tissue took place in a descending alcohol series. Sections were cooked at 100 °C in a sodium citrate buffer solution with pH = 6 to unmask the antigen and to prevent heat-associated protein agglomeration. After washing the slides (aqua dist./PBS buffer), the following antibody was added on the tissue and incubation at +4 °C for 16 h was performed (Table 2): Anti-GR (mouse IgG2a; clone 4H2; Novocastra, Wetzlar, Germany). Post-block reagent and HRP polymer were added to increase the staining. Finally, the tissue was dehydrated in a rising series of alcohol and finally covered. The power of the staining was evaluated by an optical microscope with the immunoreactivity score (IRS), where intensity (0 = not stained; 1 = low intensity; 2 = moderate intensity; 3 = high intensity) and percentage of stained cells (0 = 0%; 1 = 1–10%; 2 = 11–50%; 3 = 51–80%; 4 ≥ 80%) were multiplied. The higher the result, the more powerful the expression (0 = no expression, 12 = very high expression). The slides were examined by two independent persons.

In our study, we examined the function of the glucocorticoid receptor as transcription factor, which is the case if it is present in the nucleus as active form. In the cytoplasm, the receptor is present but not active as a transcription factor; so, it was not detected.

Table 2 Antibody and chemicals used for the immunohistochemistry

Glucocorticoid receptor (GR)[a]
Blocking solution[b]: 5 min
Primary antibody[a]: 1:30 in PBS[d] incubation: 16 h, 4 °C
PostBlock[b]: 20 min
HRP Polymer[2]: 30 min
Chromogen: DAB[c] (1 min)

[a]Anti-GR, clone 4H2 (mouse IgG2a), company: Novocastra (Wetzlar, Germany), Order number: NCL-GCR

[b]ZytoChem Plus HRP Polymer Kit (Mouse/Rabbit) 3*100; company: Zytomed Systems (Germany) Nr. POLHRP-100

[c]Liquid DAB + Substrate Chromogen System 1 mg/ml, DAKO

[d]Dulbecco's phosphate-buffered saline

Ethics approval

The study was approved by the ethics committee of the Ludwig-Maximilians University Munich (reference number 259-16) and considered the Declaration of Helsinki. Patient data were anonymized. During experimental and statistical analyses, the authors were blinded for clinic pathological parameters and information regarding survival. All used cancer tissue was no longer needed for clinical tests as it had initially been collected for histopathological diagnostics after surgery.

Statistics

SPSS Statistics data version 23 (IBM, Armonk, USA) was taken to perform statistical analyses. Non-parametric tests (Mann–Whitney *U* test and Kruskal–Wallis test) were used to compare independent groups and bivariate correlations were showed by Spearman's rho correlation coefficient. Survival analyses were plotted in Kaplan–Meier curves and boxplots; for significant differences regarding survival, log-rank test was used, or additionally Mann–Whitney *U* test. Cox analysis was performed to find independent markers for survival. If p was <0.05, we considered the result to be statistically significant.

Results

GR staining in cervical carcinoma

To control the GR staining, we used normal (non-pathological) placenta tissue, which showed strong nuclear expression in > 80% of epithelial cells (Fig. 1a) without any cytoplasmic reaction.

A total of 92.4% of all cervical cancer specimens showed an expression of GR receptor with a median IRS of 4, represented in 41.6% of all cases. In contrast, 7.6% did not show any expression at all. A low GR expression (IRS ≤ 3) was shown in 35.6% compared to an enhanced expression (IRS ≥ 4) in 64.4%.

GR staining in correlation with clinical parameters

Analyzing the histological subtype (Fig. 1b), squamous epithelial carcinomas showed a median IRS of 4 being represented in 45.0% of all cases (Fig. 1c), compared to a median IRS of 3 in 10.4% in adenocarcinoma tissue (Fig. 1d). The expression of the staining was significantly different between these two histological subtypes ($p=0.000$; Table 3).

Correlating the GR findings with FIGO classification, the median IRS varied between 0 and 8 (Fig. 1e). FIGO I showed a median IRS of 4 (Fig. 1f), compared to a median IRS of 8 in FIGO IIA (Fig. 1g) and 0 in FIGO IIIC (Fig. 1h). GR staining was significantly correlated with FIGO stage ($p=0.002$), whereas an enhanced staining was accompanied by a low FIGO stage (Rho = −0.174, $p=0.030$; Table 3).

No significant difference between GR staining and grading, T- and N-status was found

GR staining and survival

Kaplan–Meier analysis showed a significant correlation between GR expression (IRS ≥ 4) and overall survival ($p=0.045$): an advanced GR expression went along with significant better overall survival compared to low GR expression (IRS ≤ 3) in cervical cancer (Fig. 2a). Regarding release-free survival, an increased GR expression was also correlated with longer release-free survival ($p=0.009$; Fig. 2b). This fitted to the correlation between GR and the FIGO status, where high GR expression was correlated with a low FIGO state.

Cox regression was performed to find independent prognosticators concerning survival. Regarding overall survival, clinical parameters like histological subtype ($p=0.038$), N-status ($p=0.002$), FIGO classification ($p=0.003$) and age at surgery ($p<0.001$) were independent prognosticators, as well as T-stage ($p=0.003$) but not grading. Expression of GR turned out to be an independent marker for overall survival being correlated with better overall survival (Table 4).

Regarding relapse-free survival, neither analyzed clinic pathological markers (histological subtype, T-status, N-stage, FIGO stage, grading or age at surgery) nor GR expression turned out to be significant.

Discussion

Glucocorticoids are well-known substances in cancer treatment. They are used as co-medication to reduce side effects of cancer therapy or by effecting cell-cycle progression and

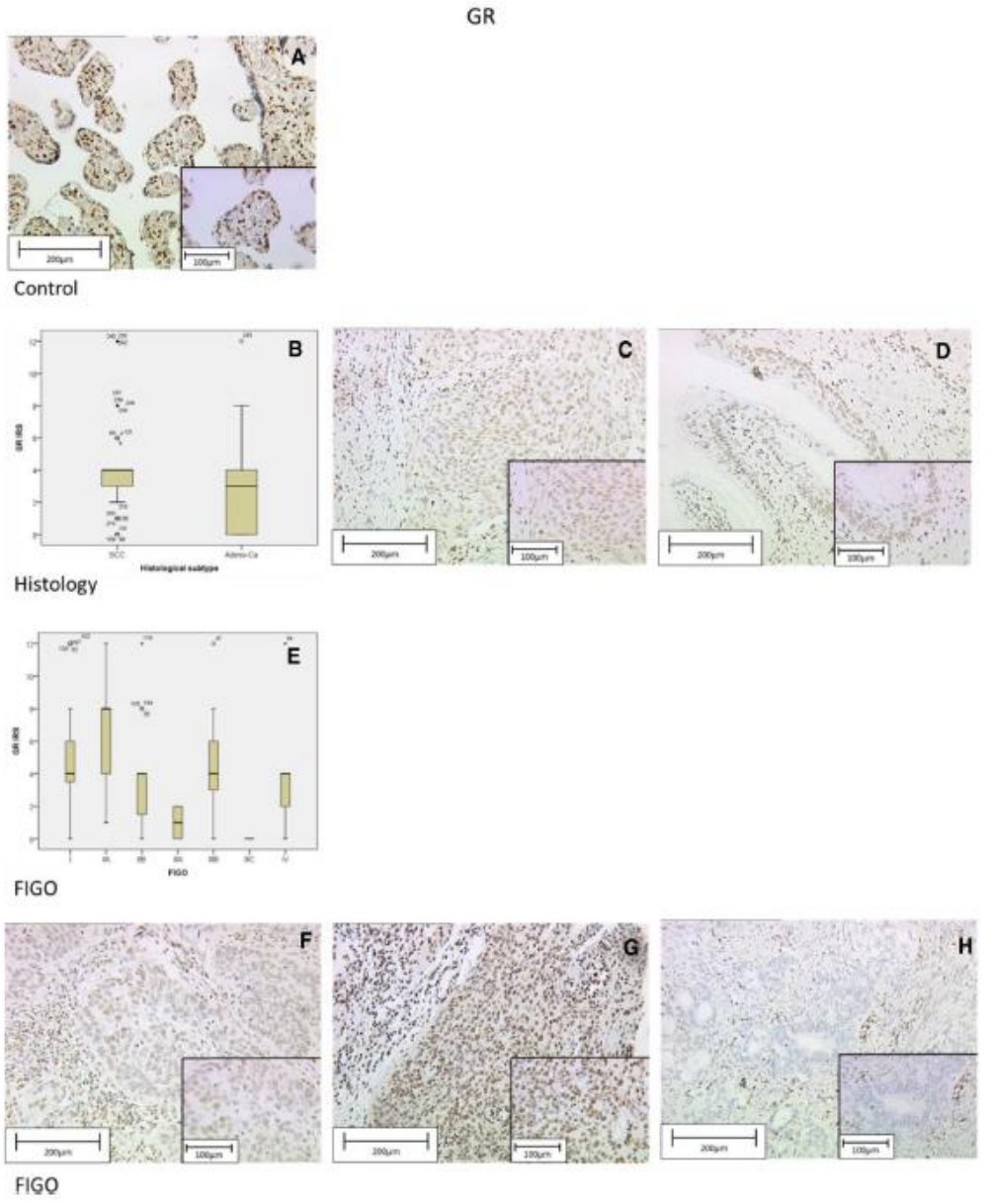

Fig. 1 Positive control of GR staining in placenta tissue with strong nuclear expression (**a**). Correlation between GR expression and histological subtypes: (**b**). Median IRS of SCC was 4 (**c**), compared to the median IRS of Adeno-Ca of 3 (**d**). GR expression correlated with FIGO status, as summarized in the boxplot (**e**). FIGO I staged patient showed an IRS of 4 (**f**), FIGO II staged patient, a median IRS of 8 (**g**) and FIGO III staged patient, an IRS of 0 (**h**). Scale bar 200 µm, small pictures 100 µm

Table 3 Staining results and correlation analysis for GR expression

	GR			
	Median IRS (±SD)	%	*p* (NPAR)	*ρ*
Histology				
SCC	4 (±3.0)	45.0	0.000	–
Adeno-Ca	3 (±2.7)	10.4		
FIGO				
I	4 (±3.2)	48.4	0.002	−0.174 ($p=0.030$)
IIA	8 (±3.4)	50.0		
IIB	4 (±2.5)	45.0		
IIIA	1 (±1.4)	00.0		
IIIB	4 (±2.7)	33.3		
IIIC	0 (±0.0)	100		
IV	4 (±3.8)	42.9		
pT				
T1	4 (±3.1)	41.8	0.492	−0.068 ($p>0.05$)
T2	4 (±2.9)	41.4		
T3/4	4 (±3.0)	44.4		

SD standard deviation, % percentage of the subgroup with median IRS, *NPAR* non-parametric test, *p* p value, *ρ* correlation coefficient

apoptosis to treat malignancy itself [18]. The effect of glucocorticoids and their corresponding receptor or the interaction with other pathogens like HPV on cervical carcinoma is not clear yet.

Altogether, there are limited data about GR expression in cervical carcinoma. In a study by Block et al., glucocorticoid receptor expression in 20 solid tumor types was analyzed. 82% of the analyzed cervical cancer tissue in a small sample size was tested positive in their analysis [8]. Regarding our study, > 92% of the analyzed samples showed detectable GR expression in the nucleus with a high staining in > 64% of all cases. The study by Block et al. [19] indicated that GR expression varies by tumor subtype comparing different histological lung cancer types. Due to the low number of cervical carcinoma samples in their study, a sufficient analysis of histological subtypes was not done.

In favor of squamous epithelial carcinomas, a significant difference between squamous epithelial carcinoma and adenocarcinoma concerning GR expression was measured in our study. Differential expression of prognosis marker proteins in both carcinoma entities was described recently by our group. In cervical cancer, Histone H3 acetyl K9 staining was associated with low grading, low FIGO status, negative N-status and low T-status and showed a higher expression in adenocarcinoma compared to squamous cell carcinoma [23]. In addition, we found a positive correlation of the nuclear GR staining with p16 ($\rho=0.301$, $p<0.001$) and p53 ($\rho=0.237$, $p<0.001$), which were obtained from a former study [20]. The glucocorticoid receptor showed also a positive correlation with the G protein-coupled estrogen receptor (GPER, $\rho=0.233$, $p<0.001$), RIP140 ($\rho=0.171$, $p=0.008$) and Histone H3 Tri Methyl K4 ($\rho=0.143$, $p=0.023$) [21–23].

According to international guidelines, cervical cancer patients are treated with surgery or radiotherapy depending on individual staging and risk assessment. Risk assessment includes tumor size, stage, depths of tumor invasion, lymph node status, lympho-vascular space invasion and histological subtype. Regarding prognosis and risk assessment, there might be other biological markers in cervical cancer to assess individual therapy policy or prognosis [24–28].

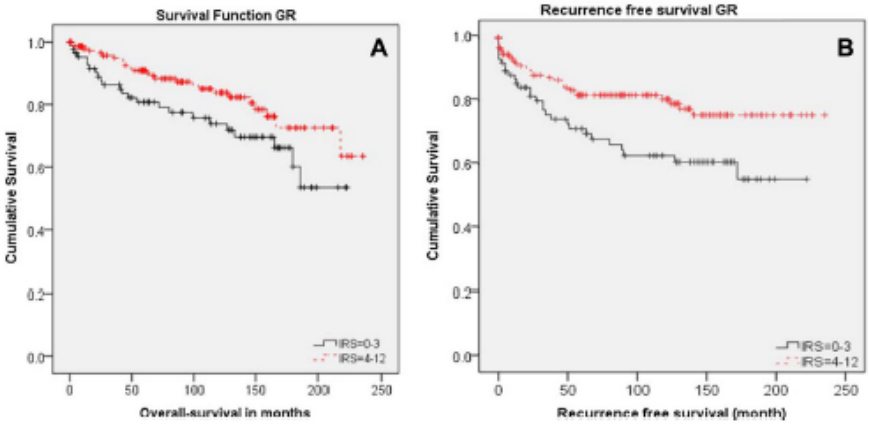

Fig. 2 Kaplan–Meier analysis regarding overall survival in cervical cancer: low GR expression (IRS ≤ 3) compared to high expression (IRS ≥ 4) regarding overall survival (**a**; $p=0.045$) and recurrence-free survival (**b**; $p=0.009$)

Table 4 Cox regression of clinic pathological variables regarding overall survival in cervical cancer

	Significance	Hazard ratio [Exp(B)]	Lower 95% CI of Exp(B)	Upper 95% CI of Exp(B)
Histology	**0.038**	**1.905**	**1.036**	**3.500**
pT	0.624	0.882	0.534	1.457
pN	**0.002**	**2.465**	**1.378**	**4.412**
FIGO	**0.003**	**1.258**	**1.080**	**1.465**
Grading	0.193	1.365	0.855	2.181
Age at surgery	**0.000**	**1.049**	**1.026**	**1.072**
GR	**0.054**	**0.575**	**0.328**	**1.009**

Significant results are shown in bold

In the present study, expression of GR was significantly correlated with survival: an advanced GR expression went along with significant better overall- and release-free survival compared to low GR expression in our analyzed cancer cells. In a study by Vanderbilt et al. [29], the effect of glucocorticoid growth arrest in lymphoid cell lines was proportional to GR content. Gehring et al. [30] showed a correlation between low-level GR expression with a poor treatment response or patient prognosis in ALL. Nevertheless, consistent with data in hematological malignancies, our data indicate a better prognosis of cervical cancer patients correlating with GR expression, fitting to the correlation between FIGO and GR expression. More data exist regarding mRNA expression of GR in cervical cancer. We think that it is not allowed to transfer these data one–one to our study design as we examined the active form of the glucocorticoid receptor with its expression in the nucleus. Interestingly, in a recent study, we could show that RIP140 as co-regulator of the glucocorticoid receptor is also an independent prognosticator for cervical cancer patients [22].

As therapeutic agents, glucocorticoids are effective in inducing apoptosis in many hematological malignancies. Besides positive effects of glucocorticoids in leukemia, different cancer cells seem to respond with increased resistance towards glucocorticoid induced apoptosis [31]. Limited data concerning the apoptotic effect of glucocorticoids in solid tumor cells exist from osteosarcoma or small-cell lung cancers. Their might be negative effects of glucocorticoids in solid tumors by causing faster growth or metastasis by providing a selection pressure [18].

Other studies identified the induction of glucocorticoid receptor as feature of drug resistance leading to worse survival rates, if GR is expressed [32]. These data seem to be in contrast to our results but they refer to special cases and mechanisms, for example, in prostate cancer. It is not clear if a one–one transfer to cervical cancer is possible.

Altogether, the wide range of mechanisms by which glucocorticoids are able to develop in different cell lines are not fully understood [31]. An ongoing clinical trial (NCT02762981) will provide initial data, if the glucocorticoid receptor can be targeted by the selective glucocorticoid receptor modulator CORT125134 in combination with nab-paclitaxel in different solid tumors [33, 34]. If the glucocorticoid effect on cervical carcinoma tissue is dependent on GR itself or other mechanisms has to be investigated.

Author contributions BPK: project development, data collection. SB: experiments, manuscript writing. LS: data collection, manuscript editing. JZ: data analyses. DM: supervision, data analyses. CK: experiments, methodology. SS: experiments, methodology. SH: experiments, methodology. SM: data analyses, supervision, funding. UJ: supervision. HH: manuscript edition, data analyses

Funding The study was supported by the "Heuer Stiftung" for Bernd P. Kost. The authors would like to thank Prof. Dr. med. Jutta Engel, M.P.H. and Max Wiedemann (The Munich Cancer Registry of the Tumorzentrum München [TZM—Munich Tumor Center]) for the follow-up data.

Compliance with ethical standards

Conflict of interest All authors declare that they have no conflict of interest.

Ethical approval All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards. This article does not contain any studies with animals performed by any of the authors.

Informed consent The study was approved by the ethics committee of the Ludwig-Maximilians University Munich (reference number 259-16). Patient data were anonymized.

References

1. Munoz N, Bosch FX, Castellsague X, Diaz M, de Sanjose S, Hammouda D, Shah KV, Meijer CJ (2004) Against which human papillomavirus types shall we vaccinate and screen? The international perspective. Int J Cancer 111:278–285
2. Serrano-Olvera A, Cetina L, Coronel J, Duenas-Gonzalez A (2014) Follow-up consultations for cervical cancer patients in a mexican cancer center. Comparison with NCCN guidelines. Asian Pac J Cancer Prev 15:8749–8752
3. Horn LC, Beckmann MW, Follmann M, Koch MC, Mallmann P, Marnitz S, Schmidt D, German Cancer S (2015) s3 guidelines on diagnostics and treatment of cervical cancer: demands on pathology. Pathologe 36:585–593
4. Schiffman M, Castle PE, Jeronimo J, Rodriguez AC, Wacholder S (2007) Human papillomavirus and cervical cancer. Lancet 370:890–907
5. Wittekindt C, Wagner S, Mayer CS, Klussmann JP (2012) basics of tumor development and importance of human papilloma virus (HPV) for head and neck cancer. Laryngorhinootologie 91(Suppl 1):S1–S26

6. Munoz N, Bosch FX, de Sanjose S, Herrero R, Castellsague X, Shah KV, Snijders PJ, Meijer CJ, International Agency for Research on Cancer Multicenter Cervical Cancer Study G (2003) Epidemiologic classification of human papillomavirus types associated with cervical cancer. N Engl J Med 348:518–527
7. Liu J, Cheng Y, He M, Yao S (2014) Vascular endothelial growth factor c enhances cervical cancer cell invasiveness via upregulation of galectin-3 protein. Gynecol Endocrinol 30:461–465
8. Meng CF, Su B, Li W (2011) DNA demethylation is superior to histone acetylation for reactivating cancer-associated genes in ovarian cancer cells. Mol Med Rep 4:1273–1278
9. Gupta S, Takhar PP, Degenkolbe R, Koh CH, Zimmermann H, Yang CM, Guan Sim K, Hsu SI, Bernard HU (2003) The human papillomavirus type 11 and 16 e6 proteins modulate the cell-cycle regulator and transcription cofactor trip-br1. Virology 317:155–164
10. Scheffner M, Huibregtse JM, Vierstra RD, Howley PM (1993) The hpv-16 e6 and e6-ap complex functions as a ubiquitin-protein ligase in the ubiquitination of p53. Cell 75:495–505
11. Pittayakhajonwut D, Angeletti PC (2010) Viral trans-factor independent replication of human papillomavirus genomes. Virol J 7:123
12. Bromberg-White JL, Meyers C (2002) The upstream regulatory region of human papillomavirus type 31 is insensitive to glucocorticoid induction. J Virol 76:9702–9715
13. Chan WK, Klock G, Bernard HU (1989) Progesterone and glucocorticoid response elements occur in the long control regions of several human papillomaviruses involved in anogenital neoplasia. J Virol 63:3261–3269
14. Webster K, Taylor A, Gaston K (2001) Oestrogen and progesterone increase the levels of apoptosis induced by the human papillomavirus type 16 e2 and e7 proteins. J Gen Virol 82:201–213
15. Kwasniewska A, Postawski K, Gozdzicka-Jozefiak A, Kwasniewski W, Grywalska E, Zdunek M, Korobowicz E (2011) Estrogen and progesterone receptor expression in HPV-positive and HPV-negative cervical carcinomas. Oncol Rep 26:153–160
16. Fonseca-Moutinho JA, Cruz E, Carvalho L, Prazeres HJ, de Lacerda MM, da Silva DP, Mota F, de Oliveira CF (2004) Estrogen receptor, progesterone receptor, and BCL-2 are markers with prognostic significance in cin iii. Int J Gynecol Cancer 14:911–920
17. Chen YH, Huang LH, Chen TM (1996) Differential effects of progestins and estrogens on long control regions of human papillomavirus types 16 and 18. Biochem Biophys Res Commun 224:651–659
18. Schlossmacher G, Stevens A, White A (2011) Glucocorticoid receptor-mediated apoptosis: mechanisms of resistance in cancer cells. J Endocrinol 211:17–25
19. Block TS, Murphy TI, Munster PN, Nguyen DP, Lynch FJ (2017) Glucocorticoid receptor expression in 20 solid tumor types using immunohistochemistry assay. Cancer Manag Res 9:65–72. https://doi.org/10.2147/CMAR.S124475
20. Stiasny A, Freier CP, Kuhn C, Schulze S, Mayr D, Alexiou C, Janko C, Wiest I, Dannecker C, Jeschke U et al (2017) The involvement of e6, p53, p16, mdm2 and gal-3 in the clinical outcome of patients with cervical cancer. Oncol Lett 14:4467–4476
21. Friese K, Kost B, Vattai A, Marme F, Kuhn C, Mahner S, Dannecker C, Jeschke U, Heublein S (2018) The g protein-coupled estrogen receptor (gper/gpr30) may serve as a prognostic marker in early-stage cervical cancer. J Cancer Res Clin Oncol 144:13–19
22. Vattai A, Cavailles V, Sixou S, Beyer S, Kuhn C, Peryanova M, Heidegger H, Hermelink K, Mayr D, Mahner S et al (2017) Investigation of rip140 and lcor as independent markers for poor prognosis in cervical cancer. Oncotarget 8:105356–105371
23. Beyer S, Zhu J, Mayr D, Kuhn C, Schulze S, Hofmann S, Dannecker C, Jeschke U, Kost BP (2017) Histone h3 acetyl k9 and histone h3 tri methyl k4 as prognostic markers for patients with cervical cancer. Int J Mol Sci. https://doi.org/10.3390/ijms18030477
24. Marth C, Landoni F, Mahner S, McCormack M, Gonzalez-Martin A, Colombo N, Committee E G (2017) Cervical cancer: ESMO clinical practice guidelines for diagnosis, treatment and follow-up. Ann Oncol 28:iv72–iv83
25. Reich O, Regauer S, Marth C, Schmidt D, Horn LC, Dannecker C, Menton M, Beckmann MW (2015) Precancerous lesions of the cervix, vulva and vagina according to the 2014 who classification of tumors of the female genital tract. Geburtshilfe Frauenheilkd 75:1018–1020
26. Freier CP, Stiasny A, Kuhn C, Mayr D, Alexiou C, Janko C, Wiest I, Jeschke U, Kost B (2016) Immunohistochemical evaluation of the role of p53 mutation in cervical cancer: Ser-20 p53-mutant correlates with better prognosis. Anticancer Res 36:3131–3137
27. Stiasny A, Kuhn C, Mayr D, Alexiou C, Janko C, Wiest I, Jeschke U, Kost B (2016) Immunohistochemical evaluation of e6/e7 hpv oncoproteins staining in cervical cancer. Anticancer Res 36:3195–3198
28. Kolben TM, Kraft F, Kolben T, Goess C, Semmlinger A, Dannecker C, Schmoeckel E, Mayr D, Sommer NN, Mahner S et al (2017) Expression of sialyl lewis a, sialyl lewis x, lewis y, gal-3, gal-7, stmn1 and p16 in cervical dysplasia. Future Oncol 13:145–157
29. Vanderbilt JN, Miesfeld R, Maler BA, Yamamoto KR (1987) Intracellular receptor concentration limits glucocorticoid-dependent enhancer activity. Mol Endocrinol 1:68–74
30. Gehring U, Mugele K, Ulrich J (1984) Cellular receptor levels and glucocorticoid responsiveness of lymphoma cells. Mol Cell Endocrinol 36:107–113
31. Herr I, Buchler MW, Mattern J (2009) Glucocorticoid-mediated apoptosis resistance of solid tumors. Results Probl Cell Differ 49:191–218
32. Arora Vivek K, Schenkein Emily, Murali Rajmohan et al (2013) Glucocorticoid receptor confers resistance to anti-androgens by bypassing androgen receptor blockade. Cell 155(6):1309–1322
33. Hunt H, Donaldson K, Strem M, Zann V, Leung P, Sweet S, Connor A, Combs D, Belanoff J (2017) Assessment of safety, tolerability, pharmacokinetics, and pharmacological effect of orally administered cort125134: An adaptive, double-blind, randomized, placebo-controlled phase 1 clinical study. Clin Pharmacol Drug Dev 7(4):408–421. https://doi.org/10.1002/cpdd.389
34. Hunt HJ, Belanoff JK, Walters I, Gourdet B, Thomas J, Barton N, Unitt J, Phillips T, Swift D, Eaton E (2017) Identification of the clinical candidate (r)-(1-(4-fluorophenyl)-6-((1-methyl-1 h-pyrazol-4-yl)sulfonyl)-4,4a,5,6,7,8-hexah ydro-1 h-pyrazolo[3,4-g] isoquinolin-4a-yl)(4-(trifluoromethyl)pyridin-2-yl)methano ne (cort125134): a selective glucocorticoid receptor (gr) antagonist. J Med Chem 60:3405–3421

www.impactjournals.com/oncotarget/ Oncotarget, 2017, Vol. 8, (No. 62), pp: 105356-105371

Research Paper

Investigation of RIP140 and LCoR as independent markers for poor prognosis in cervical cancer

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Keywords: *cervical carcinoma; squamous cell carcinoma; adenocarcinoma; RIP140/NRIP1; LCoR*

Received: *May 18, 2017* ***Accepted:*** *July 25, 2017* ***Published:*** *October 31, 2017*

ABSTRACT

Introduction: RIP140 (Receptor Interacting Protein) is involved in the regulation of oncogenic signaling pathways and in the development of breast and colon cancers. The aim of the study was to analyze the expression of RIP140 and its partner LCoR in cervical cancers, to decipher their relationship with histone protein modifications and to identify a potential link with patient survival.

Methods: Immunohistochemical analyses were carried out to quantify RIP140 and LCoR expression in formalin-fixed paraffin-embedded tissue sections cervical cancer samples. Correlations of RIP140 and LCoR expression with histopathological variables were determined by correlation analyses. Survival rates of patients expressing low or high levels of RIP140 and LCoR were compared by Kaplan-Meier curves.

Results: RIP140 overexpression was associated with a significantly shorter overall survival of cervical cancer patients. This effect was significant in the squamous cell carcinoma subtype but not in adenocarcinomas. RIP140 is no longer a significant negative prognosticator for cervical cancer when LCoR expression is low.

Discussion: RIP140 is an independent predictor of poor survival of patients with cervical cancer. Patients with tumors expressing low levels of both RIP140 and LCoR showed a better survival compared to patients expressing high levels of RIP140. Modulation of RIP140 and LCoR may represent a novel targeting strategy for cervical cancer prevention and therapy.

INTRODUCTION

Cervical cancer is the second most frequent female cancer and the third leading cause for cancer death in female patients worldwide [1]. The two main malignant epithelial cervical cancer types are the squamous cell carcinoma and the adenocarcinoma (about 70% and 10-25% of all cervix carcinomas, respectively) [2]. A persistent infection with high-risk human papillomavirus (HR-HPV) is the major leading cause of cervical cancer [3]. When HPV replicates, the viral E6 oncoprotein is expressed and disturbs the cell cycle [4]. The E6 oncoprotein and the E6-associated protein (E6-AP) form a complex which binds to the tumor suppressor protein p53 (an inducer of cell-cycle arrest or apoptosis [5]) and causes its proteolytic degradation [6].

The epigenetic regulation in cervical cancers can be modified through altered mechanisms such as DNA methylation and post-translational modifications of histone

proteins [7]. In a recent study, we showed that histone H3 acetyl K9 (H3K9ac) and histone H3 trimethyl K4 (H3K4me3) were independent markers for poor prognosis and short overall survival (OS) in cervical cancer patients [8].

Steroid hormones act as cofactors of HPVs in the etiology of cervical cancer [9]. For instance, the regulatory region of the HR-HPV-16 contains three glucocorticoid hormone receptor response elements, which bind the glucocorticoid receptor and thereby allow viral transcription by glucocorticoids [9].

RIP140 (Receptor Interacting Protein of 140 kDa), also known as NRIP1 (Nuclear Receptor Interacting Protein 1), is a transcription coregulator of various nuclear receptors and transcription factors [10-12]. It was first identified as an ERα (estrogen receptor α) interacting protein which binds in a ligand-dependent manner to nuclear receptors and thereby limits their transactivation [13, 14]. Indeed, by means of four inhibitory domains that recruit C-terminal binding proteins and histone deacetylase, RIP140 mainly acts as a transcriptional repressor [15, 16]. More recently, an interaction of RIP140 with ERβ has also been described in ovarian cancer cells [17].

RIP140 is involved in the progression and development of cancer [18-20]. RIP140 directly interacts with E2F transcription factors, suppresses their transcriptional activity and thereby could inhibit cell proliferation [12]. However, more recently, Aziz *et al.* (2015) reported that inhibition of RIP140 expression by siRNA in breast cancer cell lines can significantly induce apoptosis and reduce cell growth [18]. In colon cancer, RIP140 has an opposing effect in comparison to breast cancer tissue as it can inhibit Wnt target gene expression and thereby decreases the ability of human colon cancer cells to proliferate [21].

Apart from RIP140, the ligand dependent corepressor (LCoR) is another transcriptional corepressor of agonist-bound nuclear receptors and other transcription factors, which also acts by recruiting histone deacetylases and C-terminal binding proteins [22-24]. Like RIP140, overexpression of LCoR represses estrogen-dependent gene expression and decreased breast cancer cell proliferation [22, 23]. Very recently, Jalaguier et al. demonstrated an interaction between RIP140 and LCoR and a strong regulation of LCoR expression by RIP140 in human breast cancer cells [22]. Interestingly, loss of RIP140 expression switches the effect of LCoR from inhibition to promotion of cell proliferation [22]. Finally, correlation of gene expression levels with clinical outcome indicated that low LCoR and RIP140 levels were associated with shorter OS in patients with breast cancer [22].

The goal of the present study was the analysis of RIP140 and LCoR expression in cervical carcinoma tissue and the correlation of their expression with patient OS. Since neither RIP140 nor LCoR has been studied in cervical cancer, this investigation represents the first analysis of these transcription factors in this pathology.

RESULTS

Expression of RIP140 in cervical carcinoma and correlation with histopathological variables

A total of 172 (71.7%) of the cervical cancer tissue samples showed positive RIP140 staining in the nucleus with a median IRS of 3 while 68 (28.3 %) did not express nuclear RIP140 (IRS=0 or 1). 10 cases could not be assessed for technical reasons. Cytoplasmic RIP140 staining was detected in 207 cases (86.3%) and 33 cases (13.7%) showed no cytoplasmic expression. Median IRS for cytoplasmic RIP140 expression was 4. The levels of nuclear RIP140 expression were assessed in the two main histological subtypes of cervical cancers. The median IRS of nuclear RIP140 expression (IRS=3) was equivalent in squamous cell carcinoma and adenocarcinoma of the cervix (Figure 1).

The Spearman test was applied for the correlation analysis of RIP140 with LCoR expression and various histopathological parameters. For positive nuclear RIP140 expression in cervical cancer tissues (with an IRS>1), a significant correlation with cytoplasmic RIP140 ($p<0.001$), nuclear LCoR ($p=0.034$), H3K9ac ($p=<0.001$) and tumor grading ($p=0.037$) were detected (Table 1). Tumor grading was negatively correlated with RIP140 expression (Spearman's rho: -0.135). Although a significant negative correlation exists between tumor grading and nuclear RIP140 expression, there is no significant difference between the different tumor grading subgroups according to the Kruskal-Wallis-test (G1 and G3: $p=0.13$; G1 and G2: $p=0.5$; G2 and G3: $p=0.089$) (Figure 2).

For cytoplasmic RIP140 expression, significant positive correlation with cytoplasmic LCoR expression ($p=0.001$), E6 ($p=0.006$) and H3K9ac ($p=0.013$) could be shown (Table 1). Further, a positive correlation between cytoplasmic RIP140 and mutated p53 in the nucleus was detected ($p=0.034$, Spearman's rho: 0.137) (Table 1). Low and high expression of mutated nuclear p53 staining and cytoplasmic RIP140 in cervical cancer are shown in Figure 3.

Correlation analysis of adjuvant radiotherapy and nuclear RIP140 expression showed no significant correlation between the two factors ($p=0.894$).

LCoR staining in cervical carcinoma and correlation analysis with histopathological variables

LCoR staining in the nucleus of cervical carcinoma tissue of the collective was expressed in 47 cases (18.8%)

with a median IRS of 2 and in 203 cases (81.2%) no LCoR staining could be detected. Similarly, most of the cases did not express LCoR in the cytoplasm (n=213, 85.2%) and 37 cases were positive (14.8%), with a median IRS of 4.

Correlation analysis of LCoR with various histopathological parameters (Table 2) showed that nuclear LCoR expression is significantly positively correlated with cytoplasmic LCoR ($p<0.001$), nuclear RIP140 expression ($p=0.034$, Spearman's rho: 0.137) and H3K9ac ($p=0.025$, Spearman's rho: 0.142) and negatively correlated with tumor size ($p=0.039$; Spearman's rho: -0.131). A smaller tumor (pT1a-b) is associated with a higher LCoR IRS score and a larger tumor is associated with a lower IRS score (Figure 4). For cytoplasmic LCoR expression, a significant positive correlation with cytoplasmic RIP140 expression ($p=0.001$), E6 ($p=0.022$) and H3K4me3 ($p=0.031$) could be detected (Table 2). The correlation between cytoplasmic LCoR and E6 expression is also shown in Figure 5.

Correlation of RIP140 and LCoR expression with OS and relapse-free survival of cervical cancer patients

Cervical cancer patients with positive nuclear RIP140 expression (n=171) were compared with patients without nuclear RIP140 expression (n=68), demonstrating that high nuclear RIP140 expression was associated with a less favorable OS in comparison to patients with low RIP140 expression ($p=0.015$). The significant difference is shown in the Kaplan-Meier curve in Figure 6. The OS is defined as the time period from primary surgical treatment to the time point of death in the follow up period. A receiver operating characteristic curve (ROC-curve) was used to determine the best cut-off level for

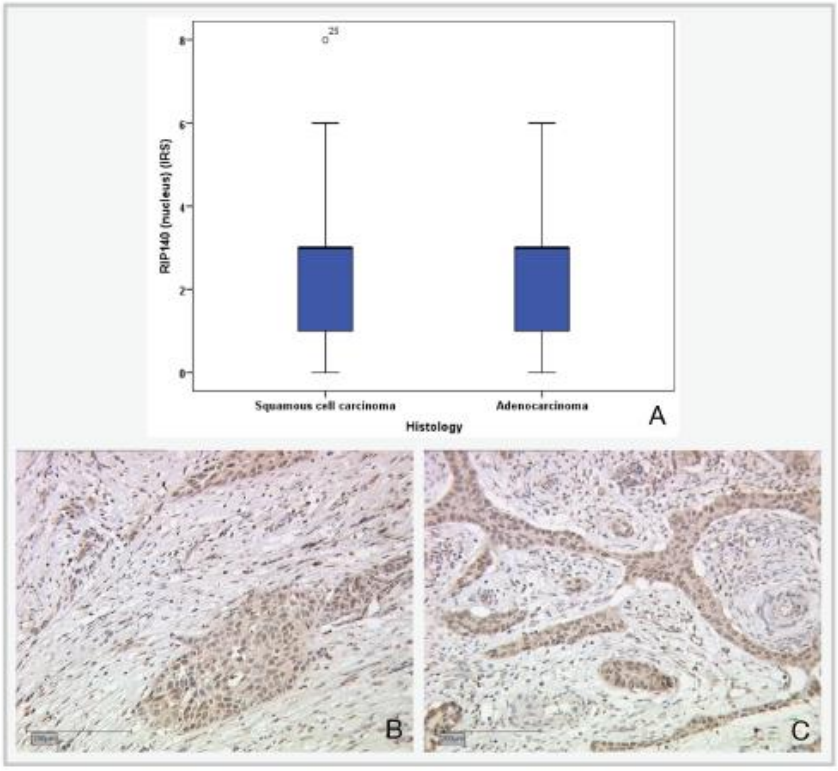

Figure 1: Expression of nuclear RIP140 in cervical epithelial tumor subtypes. (A) Boxplot of RIP140 expression and histological subtype. (B) Squamous cell carcinoma, n=192; median RIP140 expression: IRS 3; magnification x10. (C) Adenocarcinoma, n=47; median RIP140 expression: IRS 3; magnification x10.

Table 1: Correlation analysis of RIP140 and histopathological variables

	RIP140 (nucleus) IRS>1		RIP140 (cytoplasm) IRS>1	
	Significance	Correlation coefficient	Significance	Correlation coefficient
RIP140 (nucleus)	-	-	**<.001*** **	.552
RIP140 (cytoplasm)	**<.001*** **	.552	-	-
LCoR (nucleus)	**.034***	.137	.213	.081
LCoR (cytoplasm)	.370	.058	**.001****	.213
E6 (cytoplasm)	.199	.083	**.006****	.177
P53 (cytoplasm)	.892	.009	.256	.074
Mutated p53 (cytoplasm)	.588	.035	**.034***	.137
H3K9ac	**.001****	.205	**.013***	.160
H3K4me3	.733	.022	.233	.077
Tumor grading	**.037***	*-.135*	.060	*-.122*
Histology	.959	.003	.512	*-.043*
pT	.717	*-.024*	.321	.064
pN	.128	*-.098*	.723	*-.023*
FIGO	.986	.001	.094	.108

Significant correlations are marked with asterisks (*=p≤0.05, **=p≤0.01, ***=p≤0.001). Correlation coefficients of negative correlations are marked in italics.

high and low RIP140 expression based on the maximum difference between sensitivity and specificity. There was no significant correlation between the expression level of RIP140 in the cytoplasm (RIP140 low.: n=106; RIP140 high.: n=133) and patient OS (p=0.615). RIP140 expression, specifically in the nucleus, therefore appears to be a negative prognosticator for the OS of cervical cancer patients. The subsequent survival analysis of the two main histological subtypes showed that a significant inverse correlation of nuclear RIP140 expression with OS was observed in squamous cell carcinoma (p=0.015) (Figure 7) but not in cervix adenocarcinoma (p=0.828) (Figure 8).

LCoR expression in cervical cancer tissue was not associated with patient OS (p=0.329). This was also the case when squamous cell carcinoma and cervix adenocarcinoma were separately analyzed. As for RIP140, a ROC-curve was used to determine the cut-off level for low and high nuclear LCoR expression. Very interestingly, RIP140 is a negative prognosticator for OS of cervical cancer patients when LCoR expression is high (IRS>2) (p=0.021 - Figure 9) but not when nuclear LCoR expression is low (Figure 10). The longest OS outcome could be observed in patients with high RIP140 expression and LCoR expression being low (n=28) (Figure 10).

There is no significant difference in progression regarding LCoR/RIP140 expression. There was a trend for a shorter relapse-free survival in patients with nuclear LCoR IRS>0.5 expression in the primary cervical tumor (p=0.081) (Supplementary Figure 1).

Multivariate cox regression analysis

Multivariate Cox regression analysis was performed to test which histopathological variables including age, FIGO-classification, histology, tumor size (pT), nodal status (pN), tumor differentiation grade, RIP140 and LCoR status were independent prognosticators for OS

in our cohort of patients with cervical cancers. RIP140 expression with an IRS>1 (p=0.014), histology (p=0.002), tumor size (p=0.005) and lymph node status (p=0.020) were independent prognosticators for patient OS (Table 3). No significant effect could be seen for the other histopathological variables.

DISCUSSION

Patients with breast cancer where RIP140 is expressed at high and LCoR at low levels, show a better survival rate. Our data show that, in cervical cancer biopsies, expression of RIP140 is associated with poor prognosis. In line with these observations, a previous study reported a 5.84-times enhanced expression of the *NRIP1* gene at the mRNA level in cervical cancer compared to normal tissue [25]. Moreover, integration of viral DNA into the host genome can lead to the disruption of invaded genes and can consequently play a role in the process of HPV carcinogenesis in HPV-associated cervical cancer. Recently, Olthof and colleagues (2015) identified an integration site of HPV16 within the *NRIP1* gene [26]. They showed that viral E2, E6 and E7 gene expression proved to be independent of the number of integration sites and viral load, hence integration might not affect viral gene expression [26].

RIP140 targets different pathways that are relevant for the development of cervical cancer such as estrogen receptor (ER) signaling [19]. Indeed, increased estrogen levels (through the usage of oral contraceptives or repeated parity) lead to an increased risk of cervical cancer in HPV-infected women [27]. Steroid hormones are able to increase the transcription of HPV oncogenes leading to an increased viral persistence [28] and to the degradation of p53 which might favor tumorigenesis through disruption of the normal cell cycle [29].

In addition to its influence on ER, RIP140 inhibits the transactivation potential of E2Fs transcription factors

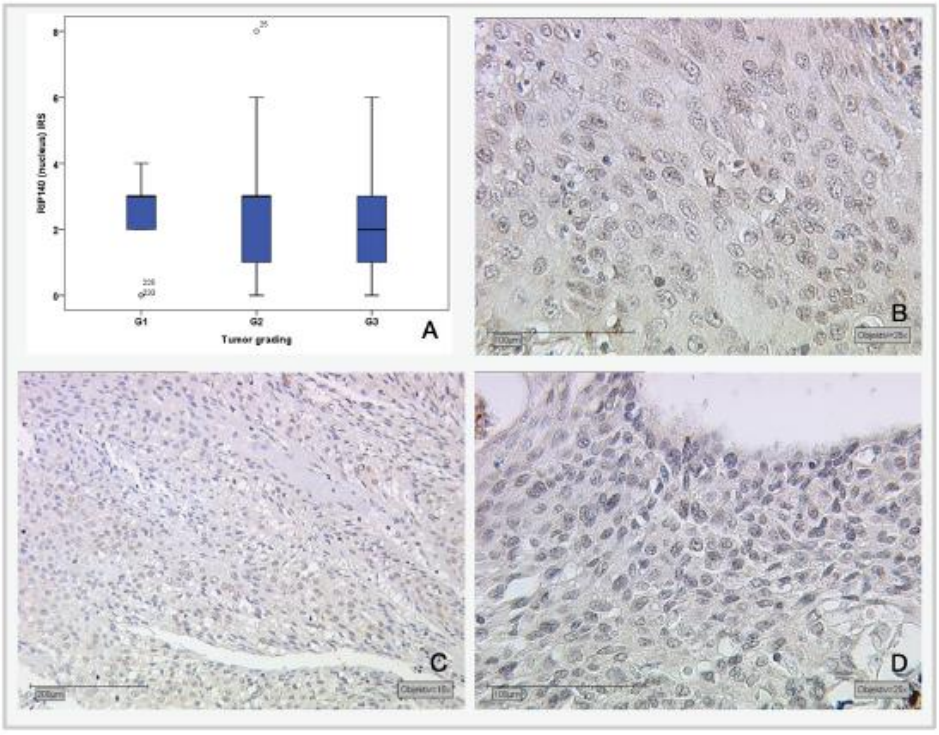

Figure 2: Correlation of nuclear RIP140 expression (IRS) and tumor grading. **(A)** Boxplot of RIP140 expression and tumor grading. **(B)** G1-stage tumors (n=19) with median RIP140 IRS score of 3; magnification x25. **(C)** G2-stage tumors (n=137) with median RIP140 expression of 3; magnification x10. **(D)** G3-stage tumors (n=75) with median RIP140 expression of 2; magnification x25.

[12]. E2Fs are regulators of genes required for cell cycle progression, DNA replication, apoptosis and cell differentiation [30]. Srivastava et al. (2014) identified E2F as a potential biomarker that is assumed to regulate the transcription of a group of genes associated with cervical cancer and might thereby act as a potential molecular target for the treatment of this malignancy [31]. The inactivation of E2F repressor-Rb by HPV E7 leads to a deregulation of E2F activity and consequently to increased levels of proteins whereby transcriptional regulation is controlled [32, 33]. Genes that are involved in invasive cervical carcinoma are therefore presumably under E2F regulation [32].

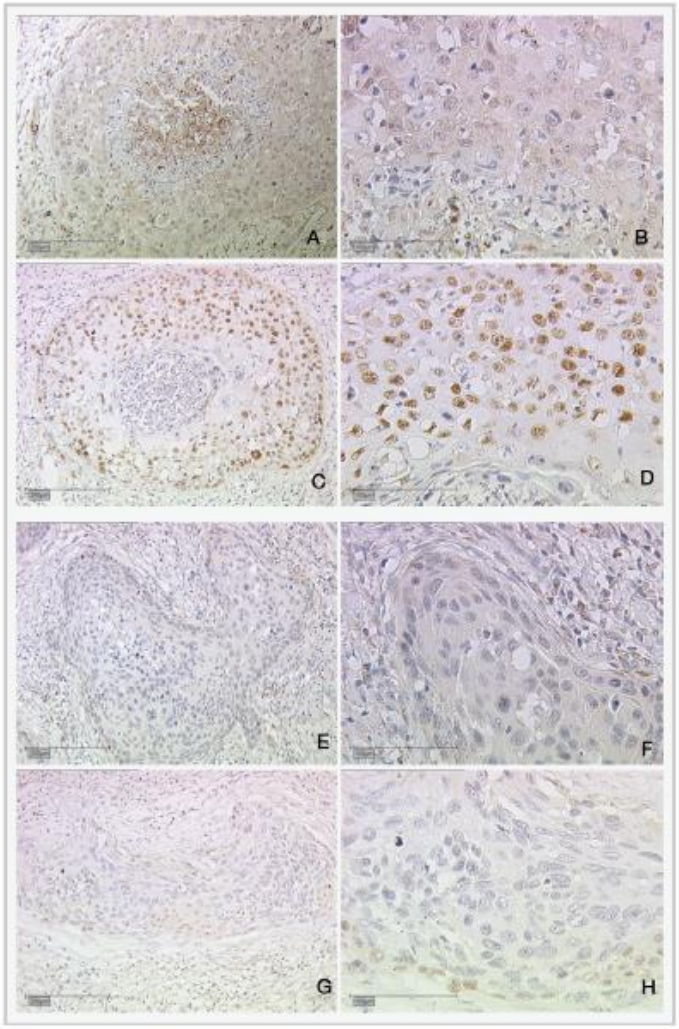

Figure 3: Expression of cytoplasmic RIP140 and nuclear mutated p53 in cervical cancer. (A and B) High RIP140 expression (cytoplasm), SCC; (C and D) high mutated p53 (nucleus); (E and F) low RIP140 expression (cytoplasm), SCC; (G and H) low mutated p53 (nucleus). Magnifications are x10 for (A), (C), (E) and (G) and x25 for (B), (D), (F) and (H).

Table 2: Correlation analysis of LCoR expression (IRS>2) and various histopathological variables

	LCoR (nucleus)		LCoR (cytoplasm)	
	Significance	Correlation coefficient	Significance	Correlation coefficient
LCoR (nucleus)	-	-	**<0.001*****	
LCoR (cytoplasm)	<0.001***	.295	-	-
RIP140 (nucleus)	**.034***	.137	.370	.058
RIP140 (cytoplasm)	.213	.081	**.001*****	.213
E6 (cytoplasm)	.604	.033	**.022***	.146
P53 (cytoplasm)	.987	*-.001*	.560	*-.037*
Mutated p53 (cytoplasm)	.413	.052	.698	.025
H3K9ac	**.025***	.142	.896	*-.008*
H3K4me3	.448	*-.048*	**.031***	.136
Grading	.164	*-.088*	.456	*-.047*
Histology	.948	.004	.851	.012
pT	**.039***	*-.131*	.623	.031
pN	.140	*-.094*	.329	.062
FIGO	.338	*-.061*	.603	.033

Significant correlations are marked with asterisks (*=p≤0.05, **=p≤0.01, ***=p≤0.001). Correlation coefficients of negative correlations are marked in italics.

Furthermore, RIP140 is involved in Wnt-signaling [34]. In colon cancer, RIP140 has a negative impact on Wnt/β-catenin target genes and thereby inhibits epithelial cell progression, cell proliferation and tumor growth [19, 21]. This contradictory effect of RIP140 in colon cancer compared to its effect on breast or cervical cancers indicates the complex roles of RIP140 on cell growth and tumor development in different tissues [18]. Depending on the tissue and the physiological or pathophysiological condition, are the corepressors RIP140 and LCoR able to function differently [24]. A participation of the Wnt/β-catenin signaling pathway in HPV-related cancers and the possible mechanisms by which the oncoproteins E6 and E7 activate this pathway has been demonstrated [35]. The Wnt/β-catenin pathway can regulate cell proliferation and apoptosis in cervical carcinomas and seems to be important for cervical oncogenesis [36].

In our correlation analysis, we showed that cytoplasmic RIP140 expression was positively correlated with cytoplasmic virus-specific oncoprotein E6 (p=0.006) and mutated p53 in the cytoplasm (p=0.034). Cytoplasmic LCoR expression also correlated significantly with E6 expression (p=0.022), but no significant correlation could be identified between LCoR and p53. The oncoproteins E6 and E7 are involved in the development of HPV-induced cervical cancer [37]. E6 protein interacts with the E3 ubiquitin-protein ligase, resulting in the proteolysis of p53 protein [38]. Mutations of p53 are genetic alterations in different human malignancies where the ability of the protein to bind to its target DNA sequence is destroyed and transcriptional activation is reduced [39, 40]. In a previous study, we could show a significant advantage of nuclear p53 protein expression (p=0.024) on the OS of cervical cancer patients [37].

In our current study, we observed a positive correlation between mutated p53 in the cytoplasm and cytoplasmic RIP140 which is associated with a worse prognosis in cervical cancer patients. As p53 can only function in the nucleus [37], we may speculate that p53 is kept in the cytoplasm and therefore correlated with

the negative prognosticator for cervical cancer, RIP140, when they interact with each other. Interestingly, only the mutated form of p53 showed a negative correlation with E6 in the cytoplasm (p=0.028, Spearman's rho: -0.140) (Stiasny et al. (July 2017) in print at Oncology Letters "The role of E6 oncoprotein, p53, p16, MDM2 and Galectin-3 for the clinical outcome of cervical cancer patients"). In our case, E6 has an influence on the expression of mutated p53 in the cytoplasm and RIP140 correlates with mutated p53 in the cytoplasm. Therefore, E6 may be involved in the RIP140/mutated p53 correlation.

Finally, post-translational modifications like acetylation play major roles in controlling the repressive activity of RIP140 [41]. The transcriptional contortion in cancer induced through expression changes of co-repressors is altered by the actions of histone modifying enzymes [42]. In a recent study, we could show that the histone protein H3K4me3 was correlated with poor prognosis in cervical cancer patients and is an independent marker for relapse-free survival [8]. Moreover, the histone protein H3K9ac was found to be an independent marker of OS in cervical cancer patients [8]. In the present work, we demonstrated a significant correlation of H3K9ac levels with nuclear (p<0.001) and cytoplasmic (p=0.013) RIP140 expression as well as with nuclear LCoR expression (p=0.025). In addition, cytoplasmic LCoR levels were correlated with H3K4me3 (p=0.031). The positive correlation of RIP140 with H3K9ac levels is in line with our findings as we show that RIP140 is associated with a less favorable OS in cervical cancer patients just as the histone protein modification. It is already known that RIP140 directly interacts with HDACs [15, 16]. It has been proposed that this interaction might sequester HDACs out of their target sites and could therefore explain part of the positive effects that RIP140 exerts on

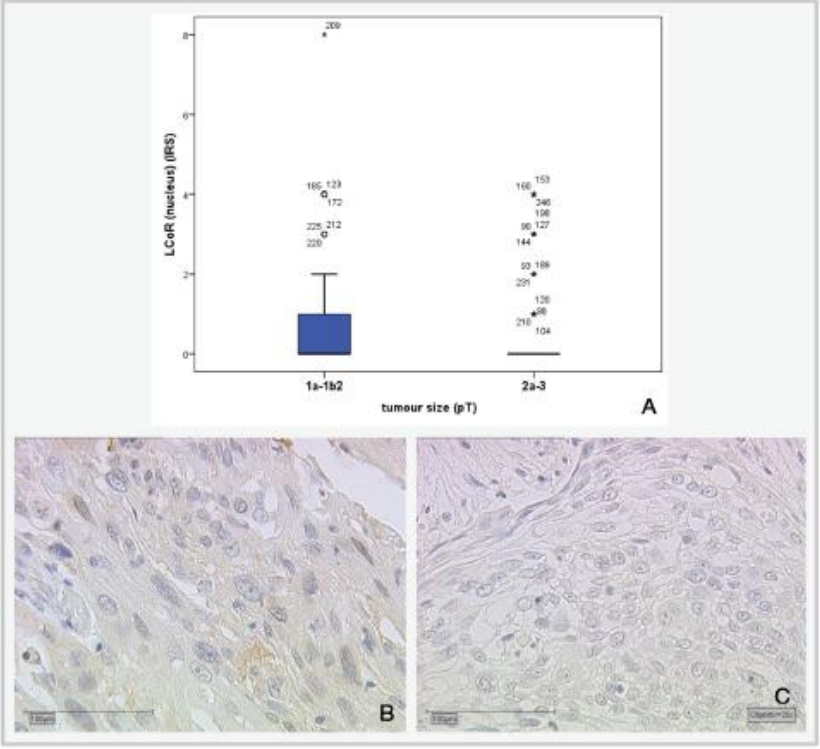

Figure 4: Correlation of cervical tumor size with LCoR IRS staining. (A) Boxplot of LCoR expression and tumor size showing a significant negative correlation (p=0.039; Spearman's rho: -0.131); (B) median LCoR IRS in pT1a-b2 tumors = 1., magnification x25 (C) Median LCoR IRS in pT2a-3 cervical tumors = 0; magnification x25.

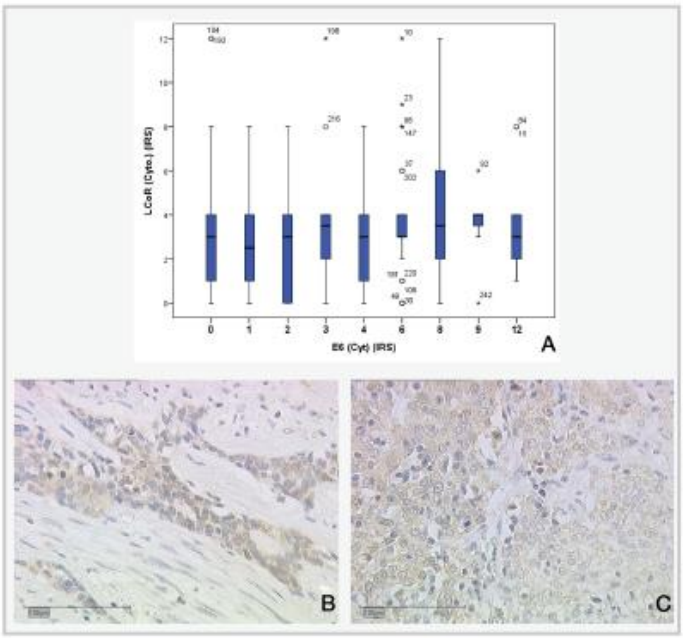

Figure 5: Correlation of LCoR expression with E6 expression. **(A)** Boxplot showing positive correlation between LCoR and E6 expression (IRS) (p=0.022; Spearman's rho: -0.146). **(B)** LCoR expression in SCC cervical cancer, x25. **(C)** LCoR expression in SCC cervical cancer, x25.

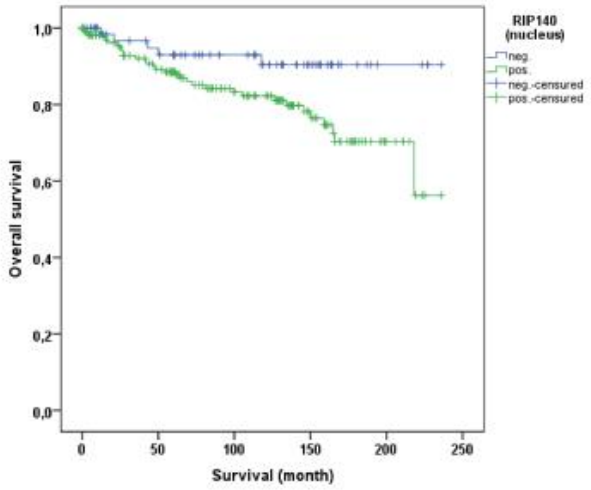

Figure 6: OS of patients with cervix carcinoma with a high (n=171) and a low RIP140 expression (n=68). Low RIP140 expression is associated with a longer OS in cervical cancer patients (p=0.015).

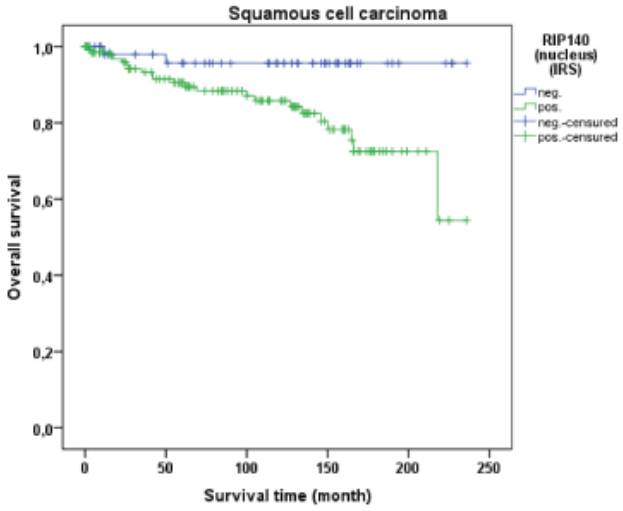

Figure 7: OS of patients with squamous cell carcinoma with low and high RIP140 expression. There is a significant longer OS in patients with squamous cell carcinoma expressing low RIP140 levels (n=54) in comparison to tumors with high RIP140 expression (n=137) (p=0.034).

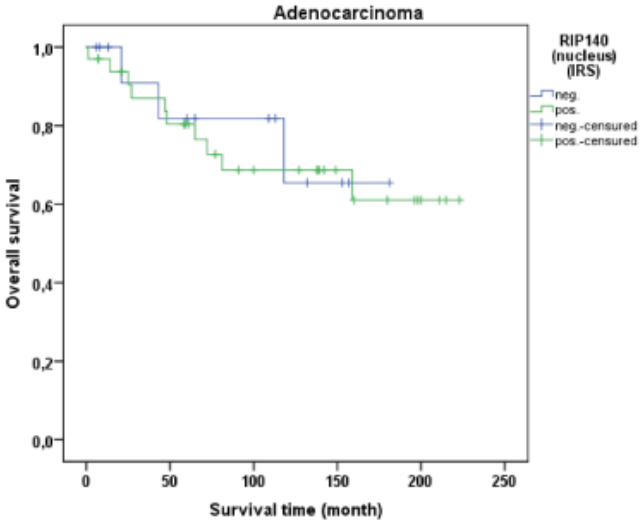

Figure 8: OS of patients with cervix adenocarcinomas with low and high RIP140 expression. There is no significant difference in OS of patients with RIP140 negative adenocarcinoma (n=14) in comparison to RIP140 positive adenocarcinoma (n=33) (p=0.828).

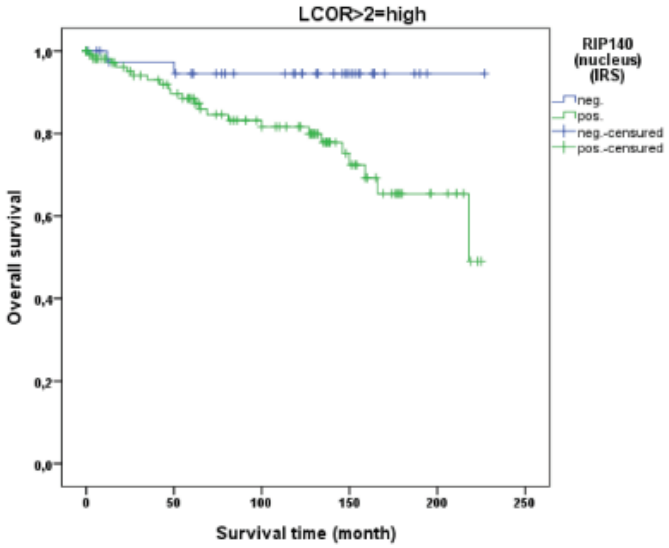

Figure 9: OS in patients with cervical cancer and high LCoR expression (IRS>2) classified by positive (n=113) and negative (n=40) RIP140 status. RIP140 is a negative prognosticator for OS in cervical cancer patients (p=0.021) when LCoR expression is positive (IRS>2) (n=153 patients).

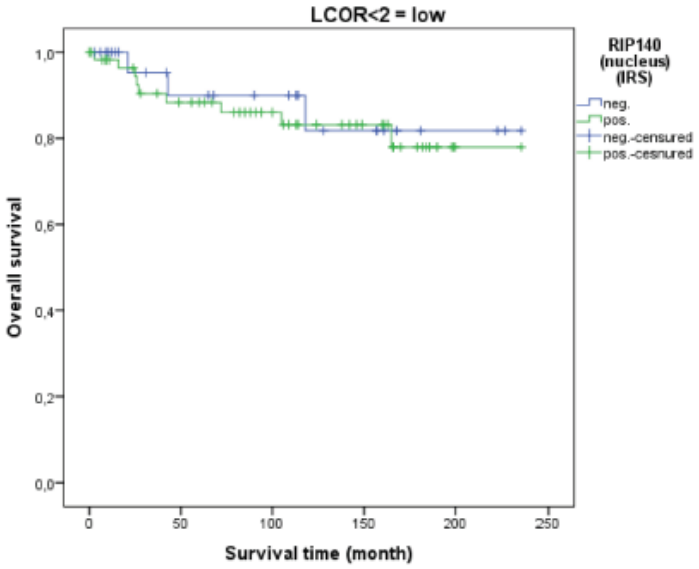

Figure 10: OS in patients with cervical cancer and low LCoR expression classified by positive (n=58) and negative (n=28) RIP140 status. RIP140 expression is not associated with OS (p=0.710) when LCoR expression is low (IRS<2) (n=86).

Table 3: Cox-regression of significant histopathological variables regarding OS in cervical cancers

	Significance	Hazard ratio Exp (B)	Lower 95% CI of Exp(B)	Upper 95% CI of Exp(B)
Tumor size (pT)	.005**	1.264	1.072	1.492
Histology	.002**	3.076	1.521	6.220
RIP140 (nucleus) (IRS>1)	.014*	3.385	1.285	8.918
Nodal status (pN)	.020*	2.417	1.151	5.076

Significant correlations are marked with asterisks (*=p≤0.05, **=p≤0.01, ***=p≤0.001).

Table 4: Description of the cohort clinical pathological variables of the patients

	Number (total number of patients: n=250)	%
Age, years		
< 49	139	55.6
≥49	111	44.4
Tumor subtype		
Squamous	202	80.8
Adenocarcinoma	48	19.2
Tumor size, pT		
pT1	110	44.0
pT2	128	51.2
pT3/4	9	3.6
NA	3	1.2
Tumor grade		
I	21	8.4
II	143	57.2
III	78	31.2
NA	8	3.2
FIGO		
I	64	25.6
II	48	19.2
III	37	14.8
IV	7	2.8
NA	94	37.6
Number of positive nodes		
0	151	60.4
≥ 1	97	38.8
NA	2	0.8

(Continued)

	Number (total number of patients: n=250)	%
Progression (over 235 months)		
None	210	84.0
At least one	21	11.6
NA	19	7.6
Survival (over 235 months)		
Censured	190	76.0
Dead	49	19.6
NA	11	4.4

gene expression such as when the transcription is driven by the transcription factor Sp1 [15]. Such a sequestration of HDACs might lead to an increase in H3K9 acetylation. Alternatively, H3K9ac marks on the genome (which are essentially related to transcriptional activation) could be linked to a global increase in gene expression including that of the *RIP140* and *LCOR* genes, thus explaining the correlations observed in the present work.

In this study, we also demonstrated that RIP140 expression (IRS≥1) is associated with poor OS of patients with cervical cancer. The inverse correlation of RIP140 with OS of patients is significant in squamous cell carcinoma of the cervix (p=0.015) and not in adenocarcinoma samples but this could be due to the smaller number of cases. A positive correlation between nuclear RIP140 and LCoR expression was demonstrated. Differentiated Kaplan-Meier analysis of RIP140 showed that RIP140 was no longer a negative prognosticator in cervical carcinoma if LCoR nuclear expression was low. Hence, joint expression of both transcription factors RIP140 and LCoR in cervical tissue is associated with a worse prognosis. Of importance, nuclear RIP140 levels (together with histological subtype, tumor size and nodal status) is an additional independent parameter which prognosticated survival in the tested cervical cancer cohort.

One limitation of this study is that the study is a retrospective which analyses the data of the patients who had undergone surgery for cervical cancer from 1993 until 2002. The advantage of a retrospective study is that this enables a long follow-up period, however, therapy options have been modified in the meantime which can further have an influence on the follow-up period. Additionally, the whole patient cohort originates from a single hospital and, for a more detailed analysis, a multi-centre study should be carried out.

In conclusion, RIP140 and LCoR transcription factors may lead to the progression of cervical cancer, and possibly represent novel therapeutic targets for the treatment of this malignancy. Further studies are required to analyze their roles in the biology of cervical cancer and, more precisely, their interaction with p53, E6 and histone proteins. Additionally, the mechanisms of how RIP140 and LCoR interact with other pathways in order to influence the development of cervical cancer have to be studied. Genome-wide profiling of RIP140 and LCoR binding sites in cervical cancer cells will be needed to examine these different cross-talks. In addition, because there is a direct correlation between RIP140 and LCoR with the histone protein modifications H3K4me3 and H3K9ac, analysis of their involvement in the maintenance of the epigenome should be investigated in cervical cancer.

MATERIALS AND METHODS

Characteristics of patients and biopsies

Formalin fixed paraffin embedded samples of all assessable cervical cancer cases (250 patients, all without distant metastasis (pM0) at the time point of primary surgery) who had undergone surgery at the Department of Gynecology and Obstetrics, Ludwig-Maximilians-University Munich, Germany, from 1993 until 2002 were included in the study. All patients who had undergone surgery for the treatment of cervical cancer and where the paraffin-embedded tumor was available were included in the study. There was no pre-selection of the patients. Histopathological tumor subtypes were assigned according to the WHO criteria by a gynecological pathologist. Squamous cell carcinoma (SCC) (202 cases) and cervix adenocarcinoma (48 cases) were included in the cohort (Table 4). Other histological subtypes were excluded from the study as there were only few cases. Clinical and follow-up data regarding patient age, OS, tumor size, lymph node status, FIGO classification, tumor grade and tumor subtype were retrieved from the Munich Cancer Registry (Table 4). Median age of patients was 47.0 years (range 20-83 years). Tumor grade included grade I (well differentiated), grade II (moderately differentiated) and grade III (poorly differentiated). In total, five patients received an adjuvant chemotherapy.

Ethical approval and informed consent

All procedures involving human participants were in accordance with the ethical standards of the

institutional and/or national research committee and with the Helsinki declaration of 1964 and its later amendments or comparable ethical standards. The study was approved by the local ethics committee of the Ludwig-Maximilians University of Munich (reference number 259-16, 2016).

Immunohistochemistry

Expression of RIP140 and LCoR was immunohistochemically quantified from the embedded cervical cancer samples. Tissue samples were fixed in neutral-buffered formalin (3.7%) straight after resection and then underwent standardized paraffin embedding. For immunohistochemistry, formalin-fixed paraffin-embedded tissue sections (3µm) were first deparaffinised in xylol, rehydrated in a descending ethanol gradient and then prepared for epitope retrieval in a pressure cooker using sodium citrate buffer (pH 6.0). Next, sections were blocked with 3% H_2O_2 in methanol at room temperature for 20 min in order to inactivate the endogenous peroxidase. Blocking solution was applied for blocking of the non-specific binding of the primary antibodies. Sections were then consecutively incubated with the following primary antibodies: anti-RIP140(polyclonal rabbit IgG, Sigma Aldrich, St. Louis, USA) and anti-LCoR (polyclonal rabbit IgG, Novus Biologicals, Littleton, USA). Antibody reactivity was analysed using the ZytoChemPlus HRP Polymer System (mouse/rabbit) (Zytomed Systems, Berlin, Germany) according to the manufacturer's protocol. Next, substrate and chromogen (3,3'-diaminobenzidine DAB; Dako, Glostrup, Denmark) were added to the slides, which were then counterstained with Mayer's acidic haematoxylin and cover slipped. Appropriate positive controls (placenta samples) and negative controls (negative control serum added on the placenta: Negative Control for Super Sensitive Rabbit Antibodies, Rabbit IgG, Biogenics, Fremont, USA) were included in each experiment. Nuclear as well as cytoplasmic RIP140 and LCoR staining was then correlated with cytoplasmic staining of E6, nuclear p53 (wild type and mutated on Ser20), H3K9ac and H3K4me3, which has been carried out for former publications [37] [8]. Most of the mutations of p53 destroy the ability of the protein to bind to its target DNA and thereby prevent transcriptional gene activation. In a recent study, we detected a high mutation rate of TP53 in a cervical cancer type where p53 is initially inactivated when cervical cancer develops [37]. The mutation could be correlated with a better OS, presumably due to a better response to therapy.

Signal quantification

Cervical cancer sections were examined by two independent observers using a Leitz Diaplan microscope (Leitz, Wetzlar, Germany). For each slide, staining was quantified by applying the semiquantitative immunoreactive score (IRS) which is used for optical assessment of the intensity and distribution pattern of antigen expression [43]. The IRS was calculated by multiplying the number of positively stained cells (in %) (0: no staining; 1: ≤10% of the cells; 2: 11% to 50%; 3: 51% to 80%, 4: >80%) with staining intensity (0: none; 1: weak; 2: moderate; 3: strong). We used a scale from 0-1 (no expression) to 12 (very high expression). A receiver operating characteristic curve (ROC-curve) was used to determine the cut-off level between RIP140 and LCoR overexpression and reduced RIP140 respectively LCoR expression. For identification of the cut-off level for RIP140 and LCoR, the maximum difference between sensitivity and specificity was used. Images were taken with a CCD color camera (JVC, Victor Company of Japan, Japan).

Statistics

IBM SPSS Statistics for Windows, Version 22.0. Armonk, NY: IBM Corp. was used for data analysis. P-values lower than $p<0.05$ were considered statistically significant. Survival times were compared by Kaplan-Meier analysis, and differences in the OS times of patients were tested for significance by the Cox-Mantel log-rank test. Group comparisons of independent groups regarding ordinal analysis variables were tested with the Mann-Whitney U test or the Kruskal-Wallis test as appropriate. All histopathological variables which had been documented, have been tested in the multivariable analysis. Correlations between ordinal variables were tested using Spearman's rank correlation coefficient. Cox regression analysis was used to compare the risk of death in patients with and without RIP140 and LCoR expression when the effects of further factors were accounted for. Independent variables included in the Cox regression model were RIP140 and LCoR expression, age, tumor size (pT) (T1-T4), histological subtype (Squamous cell carcinoma and Adenocarcinoma), tumor grade (G1, G2, G3), FIGO classification (Stage I-IVB) and lymph node status (pN) (pN0=no regional lymph node metastasis, pN1=regional lymph node metastasis). We used neither forward nor backward variable selection because all stepwise procedures have strongly be criticized [44, 45]. Variables were therefore selected based on theoretical considerations and forced into the model.

Abbreviations

ERα	Estrogen receptor α
H3K4me3	Histone H3 trimethyl K4
H3K9ac	Histone H3 acetyl K9 H3K9ac
LCoR	Ligand dependent corepressor
NRIP1	Nuclear Receptor Interacting Protein 1
pN	Nodal status
pT	Tumor size
OS	Overall survival
RIP140	Receptor Interacting Protein of 140 kDa
SCC	Squamous cell carcinoma

Author contributions

AV, BK, UJ, VC, SS, MP and HH and conceived and designed the experiments. CK, SB and AV performed the experiments. AV, BK, KH, VC, SS, DM and UJ analysed the data. AV, BK, VC, SS, KH and UJ wrote the research article. All authors read and approved the manuscript. SM, CD, VC, SS, KH, DM and UJ did the final revision of the manuscript.

ACKNOWLEDGMENTS

We thank Simone Hofmann and Sandra Schulze for their excellent technical assistance.

CONFLICTS OF INTEREST

We, the authors, declare that we have no conflicts of interest.

FUNDING

This work was supported by the Bayerische Gleichstellungsförderung (BGF) scholarship of Bavaria. The funder had no role in study design, data collection and analysis, decision to publish or preparation of the manuscript.

REFERENCES

1. den Boon JA, Pyeon D, Wang SS, Horswill M, Schiffman M, Sherman M, Zuna RE, Wang Z, Hewitt SM, Pearson R, Schott M, Chung L, He Q, et al. Molecular transitions from papillomavirus infection to cervical precancer and cancer: role of stromal estrogen receptor signaling. Proc Natl Acad Sci U S A. 2015; 112: E3255-64.
2. Young RH, Clement PB. Endocervical adenocarcinoma and its variants: their morphology and differential diagnosis. Histopathology. 2002; 41: 185-207.
3. Schiffman M, Wentzensen N, Wacholder S, Kinney W, Gage JC, Castle PE. Human papillomavirus testing in the prevention of cervical cancer. J Natl Cancer Inst. 2011; 103: 368-83.
4. Gupta S, Takhar PP, Degenkolbe R, Koh CH, Zimmermann H, Yang CM, Guan Sim K, Hsu SI, Bernard HU. The human papillomavirus type 11 and 16 E6 proteins modulate the cell-cycle regulator and transcription cofactor TRIP-Br1. Virology. 2003; 317: 155-64.
5. Tang D, Wu D, Hirao A, Lahti JM, Liu L, Mazza B, Kidd VJ, Mak TW, Ingram AJ. ERK activation mediates cell cycle arrest and apoptosis after DNA damage independently of p53. J Biol Chem. 2002; 277: 12710-7.
6. Scheffner M, Werness BA, Huibregtse JM, Levine AJ, Howley PM. The E6 oncoprotein encoded by human papillomavirus types 16 and 18 promotes the degradation of p53. Cell. 1990; 63: 1129-36.
7. Sandoval-Basilio J, Serafín-Higuera N, Reyes-Hernandez OD, Serafín-Higuera I, Leija-Montoya G, Blanco-Morales M, Sierra-Martínez M, Ramos-Mondragon R, García S, López-Hernández LB, Yocupicio-Monroy M, Alcaraz-Estrada SL. Low proteolytic clipping of histone H3 in cervical cancer. J Cancer. 2016; 7: 1856-60.
8. Beyer S, Zhu J, Mayr D, Kuhn C, Schulze S, Hofmann S, Dannecker C, Jeschke U, Kost BP. Histone H3 acetyl K9 and histone H3 tri methyl K4 as prognostic markers for patients with cervical cancer. Int J Mol Sci. 2017.
9. Khare S, Pater MM, Tang SC, Pater A. Effect of glucocorticoid hormones on viral gene expression, growth, and dysplastic differentiation in HPV16-immortalized ectocervical cells. Exp Cell Res. 1997; 232: 353-60.
10. Carascossa S, Gobinet J, Georget V, Lucas A, Badia E, Castet A, White R, Nicolas JC, Cavaillès V, Jalaguier S. Receptor-interacting protein 140 is a repressor of the androgen receptor activity. Mol Endocrinol. 2006; 20: 1506-18.
11. Docquier A, Augereau P, Lapierre M, Harmand PO, Badia E, Annicotte JS, Fajas L, Cavaillès V. The RIP140 gene is a transcriptional target of E2F1. PLoS One. 2012; 7: e35839.
12. Docquier A, Harmand PO, Fritsch S, Chanrion M, Darbon JM, Cavaillès V. The transcriptional coregulator RIP140 represses E2F1 activity and discriminates breast cancer subtypes. Clin Cancer Res. 2010; 16: 2959-70.
13. Augereau P, Badia E, Carascossa S, Castet A, Fritsch S, Harmand PO, Jalaguier S, Cavaillès V. The nuclear receptor transcriptional coregulator RIP140. Nucl Recept Signal. 2006; 4: e024.
14. Cavailles V, Dauvois S, L'Horset F, Lopez G, Hoare S, Kushner PJ, Parker MG. Nuclear factor RIP140 modulates transcriptional activation by the estrogen receptor. EMBO J. 1995; 14: 3741-51.
15. Castet A, Boulahtouf A, Versini G, Bonnet S, Augereau P, Vignon F, Khochbin S, Jalaguier S, Cavaillès V. Multiple domains of the Receptor-Interacting Protein 140 contribute to transcription inhibition. Nucleic Acids Res. 2004; 32: 1957-66.
16. Christian M, Tullet JM, Parker MG. Characterization of four autonomous repression domains in the corepressor receptor interacting protein 140. J Biol Chem. 2004; 279: 15645-51.
17. Docquier A, Garcia A, Savatier J, Boulahtouf A, Bonnet S, Bellet V, Busson M, Margeat E, Jalaguier S, Royer C, Balaguer P, Cavaillès V. Negative regulation of estrogen signaling by ERbeta and RIP140 in ovarian cancer cells. Mol Endocrinol. 2013; 27: 1429-41.
18. Aziz MH, Chen X, Zhang Q, DeFrain C, Osland J, Luo Y, Shi X, Yuan R. Suppressing NRIP1 inhibits growth of breast cancer cells *in vitro* and *in vivo*. Oncotarget. 2015; 6: 39714-24. https://doi.org/10.18632/oncotarget.5356.
19. Lapierre M, Docquier A, Castet-Nicolas A, Gitenay D, Jalaguier S, Teyssier C, Cavaillès V. The emerging role

of the transcriptional coregulator RIP140 in solid tumors. Biochim Biophys Acta. 2015; 1856: 144-50.

20. Ghoussaini M, Fletcher O, Michailidou K, Turnbull C, Schmidt MK, Dicks E, Dennis J, Wang Q, Humphreys MK, Luccarini C, Baynes C, Conroy D, Maranian M, et al. Genome-wide association analysis identifies three new breast cancer susceptibility loci. Nat Genet. 2012; 44: 312-8.
21. Lapierre M, Bonnet S, Bascoul-Mollevi C, Ait-Arsa I, Jalaguier S, Del Rio M, Plateroti M, Roepman P, Ychou M, Pannequin J, Hollande F, Parker M, Cavailles V. RIP140 increases APC expression and controls intestinal homeostasis and tumorigenesis. J Clin Invest. 2014; 124: 1899-913.
22. Jalaguier S, Teyssier C, Nait Achour T, Lucas A, Bonnet S, Rodriguez C, Elarouci N, Lapierre M, Cavaillès V. Complex regulation of LCoR signaling in breast cancer cells. Oncogene. 2017; 36: 4790-801.
23. White JH, Fernandes I, Mader S, Yang XJ. Corepressor recruitment by agonist-bound nuclear receptors. Vitam Horm. 2004; 68: 123-43.
24. Calderon MR, Verway M, An BS, DiFeo A, Bismar TA, Ann DK, Martignetti JA, Shalom-Barak T, White JH. Ligand-dependent corepressor (LCoR) recruitment by Kruppel-like factor 6 (KLF6) regulates expression of the cyclin-dependent kinase inhibitor CDKN1A gene. J Biol Chem. 2012; 287: 8662-74.
25. Pyeon D, Newton MA, Lambert PF, den Boon JA, Sengupta S, Marsit CJ, Woodworth CD, Connor JP, Haugen TH, Smith EM, Kelsey KT, Turek LP, Ahlquist P. Fundamental differences in cell cycle deregulation in human papillomavirus-positive and human papillomavirus-negative head/neck and cervical cancers. Cancer Res. 2007; 67: 4605-19.
26. Olthof NC, Huebbers CU, Kolligs J, Henfling M, Ramaekers FC, Cornet I, van Lent-Albrechts JA, Stegmann AP, Silling S, Wieland U, Carey TE, Walline HM, Gollin SM, et al. Viral load, gene expression and mapping of viral integration sites in HPV16-associated HNSCC cell lines. Int J Cancer. 2015; 136: E207-18.
27. Ramachandran B. Functional association of oestrogen receptors with HPV infection in cervical carcinogenesis. Endocr Relat Cancer. 2017; 24: R99-108.
28. Mitrani-Rosenbaum S, Tsvieli R, Tur-Kaspa R. Oestrogen stimulates differential transcription of human papillomavirus type 16 in SiHa cervical carcinoma cells. J Gen Virol. 1989; 70: 2227-32.
29. Moodley M, Moodley J, Chetty R, Herrington CS. The role of steroid contraceptive hormones in the pathogenesis of invasive cervical cancer: a review. Int J Gynecol Cancer. 2003; 13: 103-10.
30. Chen HZ, Tsai SY, Leone G. Emerging roles of E2Fs in cancer: an exit from cell cycle control. Nat Rev Cancer. 2009; 9: 785-97.
31. Srivastava P, Mangal M, Agarwal SM. Understanding the transcriptional regulation of cervix cancer using microarray gene expression data and promoter sequence analysis of a curated gene set. Gene. 2014; 535: 233-8.
32. Rosty C, Sheffer M, Tsafrir D, Stransky N, Tsafrir I, Peter M, de Crémoux P, de La Rochefordière A, Salmon R, Dorval T, Thiery JP, Couturier J, Radvanyi F, et al. Identification of a proliferation gene cluster associated with HPV E6/E7 expression level and viral DNA load in invasive cervical carcinoma. Oncogene. 2005; 24: 7094-104.
33. van der Watt PJ, Ngarande E, Leaner VD. Overexpression of Kpnβ1 and Kpnα2 importin proteins in cancer derives from deregulated E2F activity. PLoS One. 2011; 6: e27723.
34. Zhang D, Wang Y, Dai Y, Wang J, Suo T, Pan H, Liu H, Shen S, Liu H. Downregulation of RIP140 in hepatocellular carcinoma promoted the growth and migration of the cancer cells. Tumour Biol. 2015; 36: 2077-85.
35. Bello JO, Nieva LO, Paredes AC, Gonzalez AM, Zavaleta LR, Lizano M. Regulation of the Wnt/β-catenin signaling pathway by human papillomavirus E6 and E7 oncoproteins. Viruses. 2015; 7: 4734-55.
36. Rodriguez-Sastre MA, González-Maya L, Delgado R, Lizano M, Tsubaki G, Mohar A, García-Carrancá A. Abnormal distribution of E-cadherin and beta-catenin in different histologic types of cancer of the uterine cervix. Gynecol Oncol. 2005; 97: 330-6.
37. Freier CP, Stiasny A, Kuhn C, Mayr D, Alexiou C, Janko C, Wiest I, Jeschke U, Kost B. Immunohistochemical evaluation of the role of p53 mutation in cervical cancer: Ser-20 p53-mutant correlates with better prognosis. Anticancer Res. 2016; 36: 3131-7.
38. Moody CA, Laimins LA. Human papillomavirus oncoproteins: pathways to transformation. Nat Rev Cancer. 2010; 10: 550-60.
39. Petitjean A, Achatz MI, Borresen-Dale AL, Hainaut P, Olivier M. TP53 mutations in human cancers: functional selection and impact on cancer prognosis and outcomes. Oncogene. 2007; 26: 2157-65.
40. Vogelstein B, Lane D, Levine AJ. Surfing the p53 network. Nature. 2000; 408: 307-10.
41. Yang XJ, Seto E. Lysine acetylation: codified crosstalk with other posttranslational modifications. Mol Cell. 2008; 31: 449-61.
42. Battaglia S, Maguire O, Campbell MJ. Transcription factor co-repressors in cancer biology: roles and targeting. Int J Cancer. 2010; 126: 2511-9.
43. Remmele W, Stegner HE. [Recommendation for uniform definition of an immunoreactive score (IRS) for immunohistochemical estrogen receptor detection (ER-ICA) in breast cancer tissue]. [Article in German]. Pathologe. 1987; 8: 138-40.
44. Millis S. Statistical practices: the seven deadly sins. Child Neuropsychol. 2003; 9: 221-33.
45. Mundry R, Nunn CL. Stepwise model fitting and statistical inference: turning noise into signal pollution. Am Nat. 2009; 173: 119-23.

International Journal of *Molecular Sciences*

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Article

The Prostaglandin EP3 Receptor Is an Independent Negative Prognostic Factor for Cervical Cancer Patients

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Received: 26 April 2017; Accepted: 13 July 2017; Published: 19 July 2017

Abstract: We know that one of the main risk factors for cervical cancer is an infection with high-risk human papillomavirus (HR-HPV). Prostaglandins and their receptors are very important for the tumour growth and tumour-associated angiogenesis. Little is known about the expression of the Prostaglandin E receptor type 3 (EP3) or the Prostaglandin (PG)E_2-EP3 signalling in cervical cancer, so the aim of the study was to analyse the expression of the EP3 receptor in cervical cancer and find prognostic factors in relation to survival; EP3 immunohistological staining of 250 cervical cancer slides was performed and analysed with a semi-quantitative score. The statistical evaluation was performed with Statistical Package for the Social Sciences (SPSS) to evaluate the staining results and the survival analyses of the cervical cancer cases. A significant difference was observed in EP3 expression in Fédération Internationale de Gynécologie et d'Obstétrique (FIGO) stadium I versus FIGO stadium II–IV cases. High expression of EP3 (IRS $\geq$ 1.5) in cervical cancer patients was correlated with poor prognosis in overall survival rates. Survival in adenocarcinoma (AC) of the cervix was lower than in squamous cell carcinoma (SCC). Cox regression analysis shows that EP3 is an independent prognosticator. In this study we could show that the membrane-bound prostaglandin receptor EP3 is an independent prognosticator for cervical cancer patient survival. Targeting the EP3 receptor seems to be an interesting candidate for endocrine therapy. Therefore, more research is needed on the influence of the receptor system and its influence on cervical cancer growth.

Keywords: cervical cancer; squamous cell carcinoma; adenocarcinoma; EP3 receptor; overall survival; prognostic factor; cox regression

1. Introduction

Approximately half a million women are diagnosed annually with invasive cervical cancer worldwide. In the year 2012 we had about 530,000 new cases, which is about 8% of all female cancer deaths [1]. The infection with genital human papillomavirus (HPV) is one of the most common sexually-transmitted infections worldwide [2]. We know that one of the main risk factors for cervical cancer is an infection with high-risk human papillomavirus (HR-HPV). Especially HPV-16 and HPV-18

subtypes cause nearly 70% of all cases of cervical cancer [3]. The most common HPV subtypes in woman with normal cytological findings are HPV-16, HPV-18, HPV-52, HPV-31, and HPV-58 [2].

Prostanoids are metabolites of arachidonic acid synthesized by cyclooxygenase-1 (COX-1) and cyclooxygenase-2 (COX-2) [4]. The prostaglandin (PG) D_2, PGE_2, $PGF_{2\alpha}$, PGI_2, and the thromboxane A_2 are found in most tissues and organs. They are produced by almost all nucleated cells and act as autocrine and paracrine lipid mediators [5]. Each prostaglandin has, as a ligand, its own receptor. The receptor for the PGE_2, named EP receptor, has four subtypes (EP1, EP2, EP3, EP4). The receptors are G protein-coupled receptors with seven transmembrane domains [6]. The prostaglandins play an important role in the induction of fever, pain, infection, immunity, and the stimulation of the hypothalamic-pituitary-adrenal axis [7]. Some of the prostaglandins are implicated in many aspects of reproductive functions. In addition the PG play an important role in vascular homeostasis, like inducing hypertension, thrombosis, and haemostasis [6].

The prostaglandins and their receptors are very important for tumour growth and tumour-associated angiogenesis. However, the identity of the responsible prostaglandins and the prostaglandin receptors is at the moment unknown [8]. Amano et al. characterized the role of PG-signalling in tumour-associated angiogenesis and tumour progression in a mouse model and declare that the PGE_2-EP3 signalling is critical for tumour-associated angiogenesis and tumour growth [8]. Recent studies suggest that many tumours are regulated by COX enzyme products [9]. COX-2 is upregulated in numerous cancers like pancreas, lung, bladder, colon, and prostate [10].

The EP3 receptor subtype is very special among the EP receptors, because in that there are multiple isoforms generated through mRNA splicing. So various splicing variants have been identified [11–13]. The different isoforms differ in the C-terminus and through different signal transduction pathways [13]. Regarding the EP3 isoforms and their effects, little is known and their different physiological roles remain unknown [12].

Little is known about the expression of the EP3 receptor or the PGE_2-EP3 signalling in cervical cancer. A few studies suggest the overexpression of COX-2 in cervical cancer [14]. However, the mechanism of the upregulation of COX-2 in cervical cancer remains unknown [15].

The aim of this study was a systematic analysis of the expression of the EP3 receptor in human squamous cell carcinomas and adenocarcinomas of the cervix. In addition, we want to investigate if there exists some prognostic factors in relation to survival. A selective EP3 antagonist may exhibit a chemoprotective effect and, in the future, it could become a new important tool for cancer therapy [8].

2. Results

2.1. Positive Control of EP3 Staining

Paraffin-embedded sections of ovarian carcinoma metastasis in the colon were used to control the quality of the EP3 staining (Figure 1A,B). The anti-PTGER3 antibody binding site, the first cytoplasmatic domain with the amino acid sequence: RRESKRKKSFLLC position 79—91 is present in all EP3 isoforms 1–12 and was picked after researching the Human Protein Atlas.

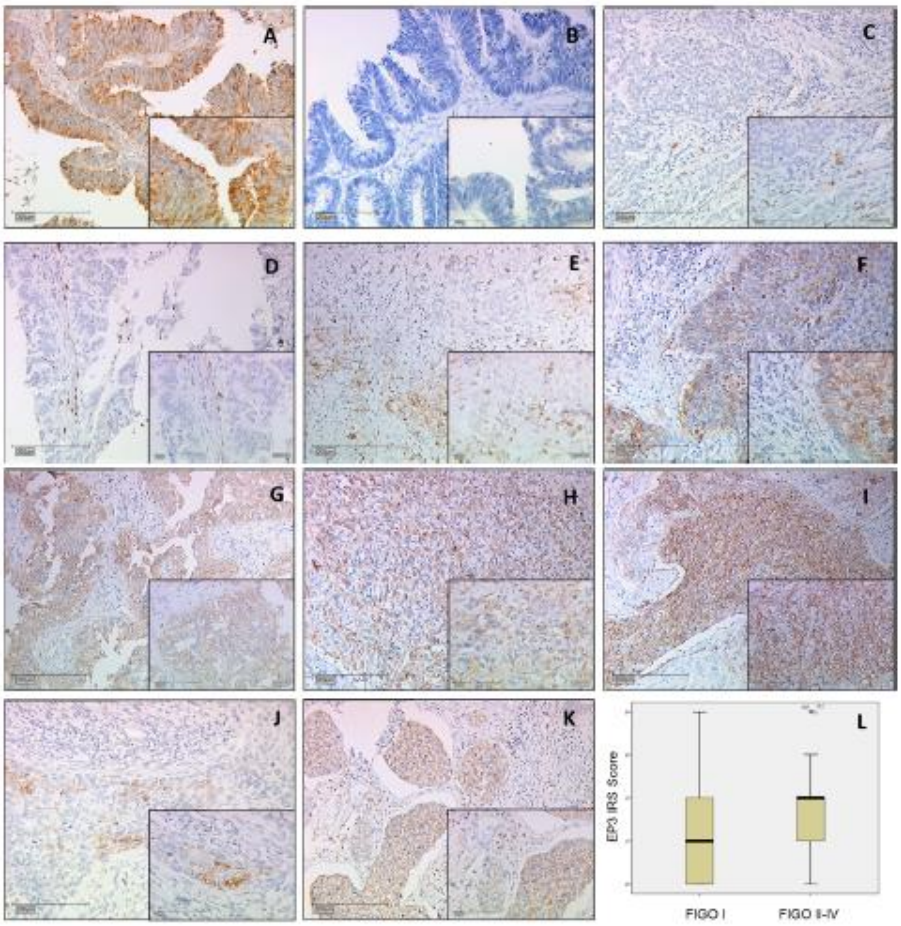

Figure 1. All images are at 10× magnification with an insert at 25× magnification. (**A**) Positive control of ovarian cancer metastasis in the colon shows cystoplasmatic and membrane-associated staining; (**B**) Negative control of ovarian cancer metastasis in the colon; (**C**) Squamous cell carcinoma Immunoreactive score (IRS) 1; (**D**) Adenocarcinoma IRS 1; (**E**) Squamous cell carcinoma IRS 4; (**F**) Adenocarcinoma IRS 4; (**G**) Adenocarcinoma carcinoma IRS 8; (**H**) Squamous cell carcinoma IRS 8, (**I**) Squamous cell carcinoma IRS 9; (**J**) Prostaglandin E receptor type 3 (EP3) staining of an Fédération Internationale de Gynécologie et d'Obstétrique (FIGO) Ib diagnosed IRS 2 stained squamous cell carcinoma; (**K**) EP3 staining of an FIGO 4 diagnosed IRS 4 stained squamous cell carcinoma; (**L**) Boxplot of FIGO I and FIGO II–IV cases with median IRS.

2.2. *EP3 Staining in Cervical Carcinoma*

The intensity of the expression was evaluated by the immunoreactive score (IRS) using a Leitz (Wetzlar, Germany) microscope, and is well-established and applied in numerous other studies. In brief, this semi-quantitative score multiplies the intensity of the staining (0 = not stained; 1 = low intensity; 2 = moderate intensity; 3 = high intensity) and the percentage of stained cells (0 = 0%; 1 = 1–10%;

2 = 11–50%; 3 = 51–80%; 4 ≥ 80%). Finally, we distinguished between 0 = no expression and 12 = very high expression of EP3 [16]. Two independent observers were blinded and evaluated the intensity and distribution pattern of the immunochemical staining reaction. The two observers differed in eight cases (n = 3.2%) of the evaluation. These cases were re-evaluated together and both observers came to the same result. The concordance before the re-evaluation was 96.8%.

A total of 77.2% of all cervical cancer specimens showed cytosolic expression of EP3. The IRS was 2.75 in 76% of the samples, compared to cases that did not express EP3 (18.0%) at all. Compared to 21.1% with low expression (IRS < 1.5), an enhanced staining (IRS ≥ 1.5) was detected in 78.9% of the samples. The cut off of IRS 1.5 was obtained through receiver operator curve (ROC) analysis. We found significant positive correlation using Spearman's test between EP3 IRS staining and tumor size (pT) (p = 0.018; Rho = 0.154) and FIGO stadium (p = 0.040; Rho = 0.133).

We separated two groups regarding invasiveness: FIGO stadium patients with the diagnosis of FIGO I, IA, IB (Figure 1J) which have a limited tumour in the cervical part of the uterus and the second group with FIGO II, III, IV (Figure 1K) stadium. The result was that the first group of 57 cases had a median EP3 IRS score of 2 and the second group of 91 cases showed a median EP3 IRS score of 4 with a significance of p = 0.012 (Figure 1L).

2.3. Correlation Analysis between Prostaglandin E Receptor Type 3 (EP3) and Fédération Internationale de Gynécologie et d'Obstétrique (FIGO) Classification

We examined the correlation between EP3 and several clinic pathological parameters, such as grading, histology, size of the primary tumour (T-status), nearby lymph nodes (N-status), and FIGO-classification by noticing the distribution of these parameters in our study group. In addition, a significant difference was observed in EP3 expression in FIGO stadium I cases versus FIGO stadium II–IV cases (Table 1).

Table 1. EP3 Immunoreactive score (IRS) staining results and correlation analysis, pN = lymph node stage, pT = tumour stage, FIGO = Fédération Internationale de Gynécologie et d'Obstétrique.

Variables	p (NPAR)	Correlation Coefficient
Histology	0.700	(−0.025)
pN	0.229	0.078
pT	0.018	0.154
FIGO	0.040	0.133

2.4. Role of EP3 for Overall Survival

Enhanced EP3 expression (IRS ≥ 1.5, obtained by ROC-analysis) was associated with shorter survival time after diagnosis. As shown in the Kaplan-Meier curve (Figure 2A), high expression of EP3 (IRS ≥ 1.5) in cervical cancer patients was correlated with poor prognosis in overall survival rates (p = 0.012).

2.5. Survival Function of Squamous Cell Carcinoma Versus Adenocarcinoma

Additionally, we compared the cumulative survival of all EP3-positive (IRS ≥ 1.5) squamous cell carcinomas versus adenocarcinomas. The Kaplan-Meier curve (Figure 2B) shows, as expected, that adenocarcinoma patients have a poor survival time after diagnosis p = 0.009 [17].

2.6. EP3 Staining of Squamous Cell Carcinoma Versus Adenocarcinoma

In addition, we performed a Kaplan-Meier test for EP3 positive (IRS ≥ 1.5) squamous cell carcinoma and adenocarcinomas versus their EP3 negative ones and were able to show that EP3 expression in squamous carcinoma patients is significant with poor survival (p = 0.003; Figure 2C). Overall survival in cervical adenocarcinomas indicates that none of the EP3 negative patients in our collective died (Figure 2D).

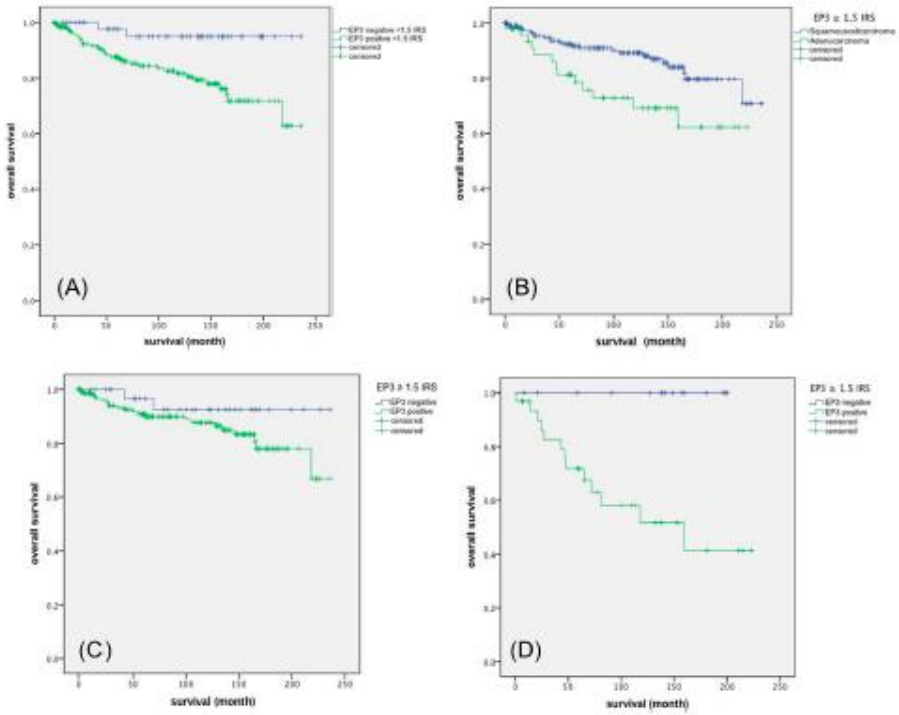

Figure 2. Kaplan-Meier curves: (**A**) EP3 survival function of all cervical cancer carcinoma $p = 0.012$; (**B**) EP3 survival function of all cervical squamous-versus adenocarcinoma $p = 0.009$; (**C**) EP3 survival function of cervical squamous cell carcinoma diagnosed patients $p = 0.003$; (**D**) EP3 survival function of cervical adenocarcinomas patients $p = 0.003$.

2.7. Cox Regression of EP3 Immunoreactive Score (IRS) with Clinic Pathological Variables

The additionally performed multivariate cox-regression tested which histopathological parameter were independent prognosticators for survival in our study group.

For overall survival the histological subtype ($p = 0.002$), lymph node metastasis (pN)-status ($p = 0.025$) and tumor size (pT)-status ($p = 0.001$) were independent prognosticators (Table 2).

Table 2. Cox regression of clinic pathological variables regarding overall survival, pM = distant metastasis stage, IRS = Immunoreactive score, CI = confidence interval, Exp (B) = hazard ratio.

Variable	Significance	Hazard Ratio of Exp (B)	Lower 95% CI of Exp (B)	Upper 95% CI of Exp (B)	B
EP3 IRS	0.007	1.264	1.066	1.498	0.234
Histology	0.002	3.118	1.538	6.322	1.137
pT	0.001	1.32	1.115	1.562	0.277
pN	0.025	2.208	1.103	4.42	0.792
FIGO	0.398	0.971	0.905	1.04	−0.030
Grading	0.242	1.381	0.804	2.372	0.323
Age	0.136	1.021	0.993	1.05	0.021
pM	0.261	2.214	0.554	8.857	2.214

3. Discussion

In recent years, attention has been focused on understanding the role of inflammation in tumour biology. It is known that COX-2 plays an important role for the induction of inflammation either individually or through sustained production of PGE_2 [18]. The overexpression of COX-2 is reported in numerous human malignancies including colon, breast, lung, and prostate [19]. It is even reported that COX-2 is overexpressed in HPV-related diseases, like cervical cancer [20].

In our investigation we examined the expression of EP3 receptor in cervical cancer (adenocarcinoma and squamous cell carcinoma), the EP3 receptor as an independent marker and tried to find prognostic factors in relation to survival.

Within this study, we showed that the immunohistochemical evaluation of EP3 receptor staining was correlated with high FIGO-classification in cervical cancer. This is in line with prognostic implications of higher EP3 receptor expression and higher FIGO-classification, which are both associated with poorer survival. We demonstrated that an increased EP3 receptor expression correlates with a negative outcome of overall survival of cervical carcinoma patients.

Further studies suggest that tumour histology has an important impact on survival for women with cervical cancer and, additionally, a poorer survival in patients with cervical adenocarcinoma [21]. We find the same results but, additionally, we could demonstrate that patients with adenocarcinoma and an IRS less than 1.5 had a very good overall survival rate. It is useful to distinguish between patients with AC and patients with AC and a high expression of EP3 receptor too, because the latter had a significantly worse outcome regarding survival. Thus, targeting the EP3 receptor, diagnostically, generally seems possible. On the other hand a new study from 2017 suggest that there was no significant difference in survival when patients were compared by cell type, so the prognosis of adenocarcinoma is controversially discussed in the literature and further studies are required [22].

The frequency of cervical adenocarcinoma is variable, but a prevalence between 15% and 25% is reported in the current literature [23]. Although the AC is less frequent than the SCC, we think that an immunohistochemical evaluation of the EP3 receptor could be an interesting tool for the clinical routine in the future.

In our study next to the EP3 receptor we found the T-status, the histology and the N-status to be an independent marker of overall survival. To our knowledge, this is the first time that associations of EP3 receptor with other biological characteristics of cervical cancer and the effect of EP3 on survival of cervical cancer patients have been analysed. We could not find another report describing EP3 as an independent prognosticator for long time survival in cervical cancer patients. Other independent markers for overall survival in patients with cervical cancer have also been investigated by Beyer et al. [16]. They found the histone H3 acetyl K9 to be an independent marker of overall survival. Chen et al. supposed that cervical carcinoma high-expressed long non coding RNA 1 (lncRNA-CCHE1) is an independent poor prognostic biomarker [24].

The role of EP3 and cancer in other studies show various effects. Some studies demonstrate an indirect pro-tumorigenic effect of EP3 receptor expression in various kinds of cancer, which was similar to our data. Miyata et al. have shown that the density of EP3 receptor positive stromal cells is associated with cancer cell progression and malignant potential, including angiogenesis and lymphangiogenesis [25]. The EP3 receptor has been shown to contribute to malignant aggressiveness, carcinogenesis and poor prognosis in several cancer types like lung adenocarcinoma and breast carcinoma [26]. On the contrary, other studies suggest an anti-tumorigenic effect of EP3 receptor expression. Shoji et al. show a colon tumour development in EP3 receptor knockout mice and suggest an important role of EP3 in suppression of cell growth [27]. Another study shows that an upregulation of EP3 expression in prostate cancer cells has preventive and anticancer effects [28,29].

Important to respect is the fact that we have different isoforms of the EP3 receptor. Many details of the EP3 receptor and its isoforms are uncovered and the data have a number of discrepancies, especially with regard to its effects [12]. The isoforms of the EP3 receptor may have different effects and physiological roles based on the tissue, in which they are expressed [12]. Thus, further studies are

required to investigate the PGE_2/EP3 isoforms for a better understanding of the physiological and pathophysiological effects.

4. Materials and Methods

4.1. Patients and Specimens

In this study, cervical cancer tissue samples of 250 patients who underwent surgery for cervical cancer from 1993 to 2002 at the Department of Gynecology and Obstetrics, Ludwig-Maximilians-University of Munich, Germany were used. The patient's median age was 47 years (range 20–83 years), and overall median survival was 100 months. The distribution of clinic-pathological variables can be seen in Table 3. In our study patients with squamous cell carcinoma or adenocarcinoma of the cervix were included, other histological subtypes were excluded due to the low number. No pre-selection besides that took place. As a positive control for immunohistochemical staining, we utilized ovarian carcinoma metastasis of the colon tissue for EP3 which was received from the Department of Obstetrics and Gynecology of the Ludwig-Maximilians-University of Munich. The Munich Cancer Registry (MCR) provided clinical and follow-up data for statistical analyses and retrieved from medical records. All of this is supported by the Bavarian Cancer Registry act and results in a loss of 4.4% follow-up patients.

Table 3. Patient characteristics.

Item	Numbers/Total Numbers	Percentage
Age		
<49	139/250	55.6%
>49	111/250	44.4%
Number of positive lymph nodes		
0	151/250	60.4%
>1	97/250	38.8%
Not available	2/250	0.8%
pT, Tumour size		
pT1	110/250	44,0%
pT2/3/4	137/250	54.8%
Not available	3/250	1.2%
FIGO		
I	64/250	25.6%
II/III/IV	92/250	36.8%
Not available	94/250	37.6%
Tumour grade		
I	21/250	8.4%
II	143/250	57.2%
III	78/250	31.2%
Not available	8/250	3.2%
Tumour subtype		
Squamous	202/250	80.8%
Adenocarcinoma	48/250	19.2%
Progression (over 235 months)		
None	210/250	84,0%
At least one event	21/250	11.6%
Not available	11/250	4.4%
Survival (over 235 months)		
Right censured	190/250	76,0%
Died	49/250	19.6%
Not available	11/250	4.4%

4.2. Ethics Approval

The initially collected cervical cancer specimens for histopathological diagnostics were no longer used for clinical tests. We recruited all patients for this survey out of this histopathological collective.

The data of the patients were totally anonymised. The authors were blinded for clinical information during statistical analyses, including survival time. The ethics committee of the Ludwig-Maximilians University approbated the ethical vote of this study. The Helsinki Declaration guidelines were respected (reference number 259-16, 13 June 2016).

4.3. Immunohistochemistry

The paraffin-embedded and formalin-fixed samples were cut (3 µm) from all specimens and mounted on positively charged glass slides. Stored at +20 °C before dewaxing for 20 min in xylol was performed. After washing the tissue in 100% ethanol, the endogenous peroxidase was blocked with 3% methanol/H_2O_2 for 20 min. The tumour slides were rehydrated in a descending alcohol series. To unmask the antigen after formalin-fixation-associated protein-agglomeration, the slides were warmed up in an airtight pot for 5 min at +100 °C, adding a trisodium citrate buffer solution (Merck 244 and Merck 6448) with pH = 6. After preparing the slides by washing them in distilled water and PBS-buffer the first step of the Polymer kit (ZytoChem Plus HRP Polymer System, Berlin, Germany) was applied for 5 min to avoid unspecific (hydrophobic) bindings. Incubation of the samples at +4 °C for 16 h with the EP3 primary antibody (anti-PTGER3 antibody polyclonal rabbit IgG; ABCAM ab189131) followed. After steps 2 and 3 of the polymer kit (Reagents 2 and 3), the substrate-staining with DAB (chromogen substrate kit, Dako, Munich, Germany) was performed for two and a half minutes, followed by the counterstaining by hemalaun colouring (2 min). The samples were finally dehydrogenated in an ascending alcohol series and covered.

5. Conclusions

In this study we showed that the immunohistochemical evaluation of the EP3 receptor expression is correlated to the FIGO classification, so we could demonstrate that an increased EP3 receptor expression correlates with a negative outcome of overall survival of cervical carcinoma patients.

In addition we found a different expression of EP3 in correlation to the histological subtype. Patients with AC and a high expression of EP3 receptor had a significant worse outcome regarding survival. Targeting the EP3 receptor diagnostically seems generally possible.

Acknowledgments: The study was supported by the "Heuer Stiftung" for Bernd P. Kost.

Author Contributions: Helene Heidegger, Aurelia Vattai, Caroline Aberl, Udo Jeschke, and Bernd Kost conceived and designed the experiments; Sebastian Dietlmeier, Christina Kuhn, and Yao Ye performed the experiments; Helene Heidegger, Sebastian Dietlmeier, and Udo Jeschke analysed the data; Helene Heidegger, Sebastian Dietlmeier, and Udo Jeschke wrote the paper. Sven Mahner and all other authors read and approved the manuscript.

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

References

1. Ferlay, J.; Soerjomataram, I.; Dikshit, R.; Eser, S.; Mathers, C.; Rebelo, M.; Parkin, D.M.; Forman, D.; Bray, F. Cancer incidence and mortality worldwide: Sources, methods and major patterns in globocan 2012. *Int. J. Cancer* **2015**, *136*, E359–E386. [CrossRef] [PubMed]
2. Bruni, L.; Diaz, M.; Castellsague, X.; Ferrer, E.; Bosch, F.X.; de Sanjose, S. Cervical human papillomavirus prevalence in 5 continents: Meta-analysis of 1 million women with normal cytological findings. *J. Infect. Dis.* **2010**, *202*, 1789–1799. [CrossRef] [PubMed]
3. Munoz, N.; Bosch, F.X.; Castellsague, X.; Diaz, M.; de Sanjose, S.; Hammouda, D.; Shah, K.V.; Meijer, C.J. Against which human papillomavirus types shall we vaccinate and screen? The international perspective. *Int. J. Cancer* **2004**, *111*, 278–285. [CrossRef] [PubMed]

4. Knabl, J.; Huttenbrenner, R.; Hutter, S.; Gunthner-Biller, M.; Vrekoussis, T.; Karl, K.; Friese, K.; Kainer, F.; Jeschke, U. Peroxisome proliferator-activated receptor-γ (PPARγ) is down regulated in trophoblast cells of gestational diabetes mellitus (GDM) and in trophoblast tumour cells bewo in vitro after stimulation with ppargamma agonists. *J. Perinat. Med.* **2014**, *42*, 179–187. [CrossRef] [PubMed]
5. Karnezis, T.; Shayan, R.; Fox, S.; Achen, M.G.; Stacker, S.A. The connection between lymphangiogenic signalling and prostaglandin biology: A missing link in the metastatic pathway. *Oncotarget* **2012**, *3*, 893–906. [CrossRef] [PubMed]
6. Narumiya, S.; Sugimoto, Y.; Ushikubi, F. Prostanoid receptors: Structures, properties, and functions. *Physiol. Rev.* **1999**, *79*, 1193–1226. [CrossRef] [PubMed]
7. Sugita, R.; Kuwabara, H.; Kubota, K.; Sugimoto, K.; Kiho, T.; Tengeiji, A.; Kawakami, K.; Shimada, K. Simultaneous inhibition of PGE_2 and PGI_2 signals is necessary to suppress hyperalgesia in rat inflammatory pain models. *Mediat. Inflamm.* **2016**, *2016*, 9847840. [CrossRef] [PubMed]
8. Amano, H.; Hayashi, I.; Endo, H.; Kitasato, H.; Yamashina, S.; Maruyama, T.; Kobayashi, M.; Satoh, K.; Narita, M.; Sugimoto, Y.; et al. Host prostaglandin E_2-EP3 signaling regulates tumor-associated angiogenesis and tumor growth. *J. Exp. Med.* **2003**, *197*, 221–232. [CrossRef] [PubMed]
9. Sales, K.J.; Katz, A.A.; Davis, M.; Hinz, S.; Soeters, R.P.; Hofmeyr, M.D.; Millar, R.P.; Jabbour, H.N. Cyclooxygenase-2 expression and prostaglandin E_2 synthesis are up-regulated in carcinomas of the cervix: A possible autocrine/paracrine regulation of neoplastic cell function via EP_2/EP_4 receptors. *J. Clin. Endocrinol. Metab.* **2001**, *86*, 2243–2249. [CrossRef] [PubMed]
10. Tsujii, M.; Kawano, S.; DuBois, R.N. Cyclooxygenase-2 expression in human colon cancer cells increases metastatic potential. *Proc. Natl. Acad. Sci. USA* **1997**, *94*, 3336–3340. [CrossRef] [PubMed]
11. Regan, J.W.; Bailey, T.J.; Donello, J.E.; Pierce, K.L.; Pepperl, D.J.; Zhang, D.; Kedzie, K.M.; Fairbairn, C.E.; Bogardus, A.M.; Woodward, D.F.; et al. Molecular cloning and expression of human EP3 receptors: Evidence of three variants with differing carboxyl termini. *Br. J. Pharmacol.* **1994**, *112*, 377–385. [CrossRef] [PubMed]
12. Israel, D.D.; Regan, J.W. EP3 prostanoid receptor isoforms utilize distinct mechanisms to regulate ERK 1/2 activation. *Biochim. Biophys. Acta* **2009**, *1791*, 238–245. [CrossRef] [PubMed]
13. Kotani, M.; Tanaka, I.; Ogawa, Y.; Usui, T.; Mori, K.; Ichikawa, A.; Narumiya, S.; Yoshimi, T.; Nakao, K. Molecular cloning and expression of multiple isoforms of human prostaglandin e receptor EP3 subtype generated by alternative messenger RNA splicing: Multiple second messenger systems and tissue-specific distributions. *Mol. Pharmacol.* **1995**, *48*, 869–879. [PubMed]
14. Herfs, M.; Herman, L.; Hubert, P.; Minner, F.; Arafa, M.; Roncarati, P.; Henrotin, Y.; Boniver, J.; Delvenne, P. High expression of PGE_2 enzymatic pathways in cervical (pre)neoplastic lesions and functional consequences for antigen-presenting cells. *Cancer Immunol. Immunother.* **2009**, *58*, 603–614. [CrossRef] [PubMed]
15. Kulkarni, S.; Rader, J.S.; Zhang, F.; Liapis, H.; Koki, A.T.; Masferrer, J.L.; Subbaramaiah, K.; Dannenberg, A.J. Cyclooxygenase-2 is overexpressed in human cervical cancer. *Clin. Cancer Res.* **2001**, *7*, 429–434. [PubMed]
16. Beyer, S.; Zhu, J.; Mayr, D.; Kuhn, C.; Schulze, S.; Hofmann, S.; Dannecker, C.; Jeschke, U.; Kost, B.P. Histone H3 acetyl K9 and histone H3 tri methyl K4 as prognostic markers for patients with cervical cancer. *Int. J. Mol. Sci.* **2017**, *18*, 477. [CrossRef] [PubMed]
17. Lai, C.H.; Hsueh, S.; Hong, J.H.; Chang, T.C.; Tseng, C.J.; Chou, H.H.; Huang, K.G.; Lin, J.D. Are adenocarcinomas and adenosquamous carcinomas different from squamous carcinomas in stage IB and II cervical cancer patients undergoing primary radical surgery? *Int. J. Gynecol. Cancer* **1999**, *9*, 28–36. [CrossRef] [PubMed]
18. Jawanjal, P.; Salhan, S.; Dhawan, I.; Das, N.; Aggarwal, R.; Tripathi, R.; Rath, G. Augmented activity of cyclooxygenase-2 in tissue and serum of patients with cervical cancer. *J. Clin. Lab. Anal.* **2016**, *30*, 1198–1207. [CrossRef] [PubMed]
19. Harris, R.E. Cyclooxygenase-2 (cox-2) blockade in the chemoprevention of cancers of the colon, breast, prostate, and lung. *Inflammopharmacology* **2009**, *17*, 55–67. [CrossRef] [PubMed]
20. Ryu, H.S.; Chang, K.H.; Yang, H.W.; Kim, M.S.; Kwon, H.C.; Oh, K.S. High cyclooxygenase-2 expression in stage IB cervical cancer with lymph node metastasis or parametrial invasion. *Gynecol. Oncol.* **2000**, *76*, 320–325. [CrossRef] [PubMed]
21. Galic, V.; Herzog, T.J.; Lewin, S.N.; Neugut, A.I.; Burke, W.M.; Lu, Y.S.; Hershman, D.L.; Wright, J.D. Prognostic significance of adenocarcinoma histology in women with cervical cancer. *Gynecol. Oncol.* **2012**, *125*, 287–291. [CrossRef] [PubMed]

22. Bean, L.M.; Ward, K.K.; Plaxe, S.C.; McHale, M.T. Survival of women with microinvasive adenocarcinoma of the cervix is not improved by radical surgery. *Am. J. Obstet. Gynecol.* **2017**. [CrossRef] [PubMed]
23. Young, R.H.; Clement, P.B. Endocervical adenocarcinoma and its variants: Their morphology and differential diagnosis. *Histopathology* **2002**, *41*, 185–207. [CrossRef] [PubMed]
24. Chen, Y.; Wang, C.X.; Sun, X.X.; Wang, C.; Liu, T.F.; Wang, D.J. Long non-coding RNA CCHE1 overexpression predicts a poor prognosis for cervical cancer. *Eur. Rev. Med. Pharmacol. Sci.* **2017**, *21*, 479–483. [PubMed]
25. Miyata, Y.; Ohba, K.; Matsuo, T.; Watanabe, S.; Hayashi, T.; Sakai, H.; Kanetake, H. Tumor-associated stromal cells expressing E-prostanoid 2 or 3 receptors in prostate cancer: Correlation with tumor aggressiveness and outcome by angiogenesis and lymphangiogenesis. *Urology* **2013**, *81*, 136–142. [CrossRef] [PubMed]
26. Yano, T.; Zissel, G.; Muller-Qernheim, J.; Jae Shin, S.; Satoh, H.; Ichikawa, T. Prostaglandin E_2 reinforces the activation of ras signal pathway in lung adenocarcinoma cells via EP3. *FEBS Lett.* **2002**, *518*, 154–158. [CrossRef]
27. Shoji, Y.; Takahashi, M.; Kitamura, T.; Watanabe, K.; Kawamori, T.; Maruyama, T.; Sugimoto, Y.; Negishi, M.; Narumiya, S.; Sugimura, T.; et al. Downregulation of prostaglandin E receptor subtype EP3 during colon cancer development. *Gut* **2004**, *53*, 1151–1158. [CrossRef] [PubMed]
28. Kashiwagi, E.; Shiota, M.; Yokomizo, A.; Itsumi, M.; Inokuchi, J.; Uchiumi, T.; Naito, S. Prostaglandin receptor EP3 mediates growth inhibitory effect of aspirin through androgen receptor and contributes to castration resistance in prostate cancer cells. *Endocr. Relat. Cancer* **2013**, *20*, 431–441. [CrossRef] [PubMed]
29. Huang, H.F.; Shu, P.; Murphy, T.F.; Aisner, S.; Fitzhugh, V.A.; Jordan, M.L. Significance of divergent expression of prostaglandin EP_4 and EP3 receptors in human prostate cancer. *Mol. Cancer Res.* **2013**, *11*, 427–439. [CrossRef] [PubMed]

3 Zusammenfassung

Die Inzidenz des Zervixkarzinoms ist in Deutschland in den letzten Jahren rückläufig. Noch in den 70er Jahren des vergangenen Jahrhunderts war das Zervixkarzinom das häufigste Karzinom der Frau. Durch Früherkennungsuntersuchungen konnte die Anzahl an invasiven Karzinomen deutlich reduziert werden. Insgesamt ist auch der Anteil höhergradiger Tumorstadien und die Mortalität rückläufig, allerdings stagniert der Rückgang in den letzten Jahren. Zur Risikoabschätzung erkrankter Patientinnen mit Zervixkarzinom sind wichtige klinische Prognosefaktoren bekannt. Nach durchgeführter Primärtherapie kommt es im weiteren Verlauf bei etwa einem Drittel der Patientinnen zu einem Rezidiv innerhalb eines Zeitraums von 2 Jahren. In Studien kam es bei bis zu 60% der Patientinnen zu einer Fernmetastasierung. Diese Patientinnen sind in der Regel, bei insgesamt schlechtem Ansprechen, nur noch einer palliativen Therapie zugänglich. Als klinische Risikofaktoren konnten in Studien das initiale Tumorstadium und der Befall an Lymphknoten identifiziert werden (S3 Leitlinie Zervixkarzinom 2014).
Neben klinischen Faktoren sind für diverse andere Tumorentitäten molekulare Prognosefaktoren untersucht, aus denen neben einer Risikoklassifizierung auch weiterführende Therapieoptionen abgeleitet werden können. Seit mehreren Jahren beschäftigt sich die Forschungsgruppe der Klinik und Poliklinik für Frauenheilkunde der LMU München mit Fragen zur molekularen Tumorbiologie bei verschiedenen Tumorentitäten aus dem Fachgebiet der Frauenheilkunde. Hierbei sind für einige gynäkologische Karzinome auch prognostisch relevante klinische und molekulare Faktoren untersucht worden. Das Zervixkarzinom ist diesbezüglich sowohl durch die Forschungsgruppe als auch international noch unzureichend erforscht.

Eine Infektion mit dem Humanen Papillomavirus (HPV) über sexuelle Kontakte ist sehr wahrscheinlich und weit verbreitet. Hierbei scheint es vor allem in Risikopopulationen zu einer ubiquitären anogenitalen Infektion zu kommen. Bei auffälliger Zervix-Zytologie ist eine

gleichzeitige anale Infektion mit dem vergleichbaren HPV-Subtyp sehr wahrscheinlich. Ebenso verhält es sich bei Patientinnen mit HIV-Infektion, unabhängig von ihrem individuellen Zervixbefund.

Verschiedene molekulare Faktoren und Mechanismen, welche in der Karzinogenese des Zervixkarzinoms eine wichtige Rolle spielen, sind bekannt. Zur Detektion molekularer Faktoren werden unterschiedliche, naturwissenschaftliche Analysevefahren verwendet. Zur Verbesserung der Analysemethode und zur Etablierung einer einfachen, sicheren und kostengünstigen Detektion von molekularen Faktoren, wie Onkoproteinen, Rezeptoren, Regulatoren und Repressoren oder Histonen wurde in einer molekularen Arbeit unter Testung einer Vielzahl an Antikörpern und Färbeprotokollen ein immunhistochemisches Färbe- und Analyseverfahren etabliert.

Diverse molekulare Faktoren, die mit HPV-getriggerten onkogenen Veränderungen des Zellzyklus assoziiert sind, wurden innerhalb dieser Arbeit analysiert. Die molekularen Daten wurden mit klinischen Daten der jeweiligen Patientinnen korreliert. Die Analyse ermöglichte eine Aussage auf die Progression des Zervixkarzinoms über 10 Jahre nach stattgehabter Operation und damit Probengewinnung. Mittels immunhistochemischer Analyse wurden die molekularen Faktoren E6 und E7, der Tumorsuppressor p53, Ser-20 p53 Mutant, p16, das Proto-Onkogen MDM2, Gal-3, die Histone H3 Acetyl K9 und H3 Tri Methyl K4, der Prostaglandinrezeptor EP3, die Ko-Regulatoren RIP 140 und LCoR sowie der Glucocorticoidrezeptor untersucht.

Für die meisten analysierten Marker konnte eine hohe Expression in den jeweiligen Proben nachgewiesen werden. Hierbei konnte für einige dieser Faktoren eine Korrelation mit klinischen Parametern wie histologischem Typ, Größe des Karzinoms mittels T-Stadium, FIGO-Klassifikation oder Nodalstatus hergestellt werden. Durch Korrelation der molekularen Daten mit klinischen Parametern konnten Daten zur Frage der Prognose der Patientinnen in unserer Untersuchung gewonnen werden. Eine interessante Korrelation zwischen Überleben der Patientinnen und molekularem Marker konnte für die immunhistochemische Expression der

Faktoren Galektin-3 (gal-3), Glukokortikoidrezeptor (GR), Ko-Regulator RIP 140 (Receptor Interacting Protein), Histon H3acetylK9Histon H3trimethylK4 und dem Prostaglandinrezeptor EP3 gezeigt werden. Aus der Literatur ist bekannt, dass gal-3 in Tumorzellen epigenetisch reguliert wird. Durch die vorliegenden Korrelationsanalysen konnte gezeigt werden, dass auch modifizierte Histonproteine beteiligt sein können. Auch der Glukokortikoidrezeptor wirkt epigenetisch. Hierfür ist die Aktivierung von Ko-Regulatorproteinen wie RIP 140 oder auch LCoR nötig.

Wie bereits bei anderen Tumorentitäten etabliert, soll, basierend auf einer potentiellen molekularen Risikostratifizierung, der Anreiz für die Forschung an weiterführenden Therapieoptionen beim Zervixkarzinom gelegt werden. Diese Faktoren könnten neue innovative Ansätze einer zielgerichteten Strategie in der Prävention, Prognoseeinschätzung und Therapie des Zervixkarzinoms darstellen.

4 Literatur

1. Adams AK, Wise-Draper TM, Wells SI. Human papillomavirus induced transformation in cervical and head and neck cancers. Cancers (Basel). 2014 Sep 15;6(3):1793-820. doi: 10.3390/cancers6031793. Review.

2. Amano, H.; Hayashi, I.; Endo, H.; Kitasato, H.; Yamashina, S.; Maruyama, T.; Kobayashi, M.; Satoh, K.;Narita, M.; Sugimoto, Y.; et al. Host prostaglandin E2-EP3 signaling regulates tumor-associated angiogenesis and tumor growth. J. Exp. Med. 2003, 197, 221–232.

3. Arbyn M, Castellsagué X, de Sanjosé S, Bruni L, Saraiya M, Bray F, Ferlay J.
Worldwide burden of cervical cancer in 2008. Ann Oncol. 2011 Dec;22(12):2675-86. doi: 10.1093/annonc/mdr015. Epub 2011 Apr 6.

4. Assmann G, Sotlar K.HPV-associated squamous cell carcinogenesis.
Pathologe. 2011 Sep;32(5):391-8. doi: 10.1007/s00292-011-1442-2. Review. German.

5. Beck, I.M.E., et al., Crosstalk in Inflammation: The Interplay of Glucocorticoid Receptor-Based Mechanisms and Kinases and Phosphatases. Endocrine Reviews, 2009. 30(7): p. 830-882.

6. Bodily J, Laimins LA: Persistence of human papillomavirus infection: keys to malignant progression. Trends Microbiol 2011;19(1):33 – 9. doi: 10.1016/j.tim.2010.10.002

7. Boyer SN, Wazer DE, Band V. E7 protein of human papilloma virus-16 induces degradation of retinoblastoma protein through the ubiquitin-proteasome pathway.
Cancer Res. 1996 Oct 15;56(20):4620-4.

8. Bromberg-White et al. Bromberg-White JL, Meyers C (2002) The upstream regulatory region of human papillomavirus type 31 is insensitive to glucocorticoid induction. J Virol 76:9702–9715

9. Carascossa S, Gobinet J, Georget V, Lucas A, Badia E, Castet A, White R, Nicolas JC, Cavaillès V, Jalaguier S. Receptor-interacting protein 140 is a repressor of the androgen receptor activity. Mol Endocrinol. 2006; 20: 1506-18.

10. Catalan Institute of Oncology (ICO), International Agency for Research on Cancer (IARC): HPV Information Centre 2018 [Available from: www.hpvcentre.net accessed 27 November 2017

11. Chan WK, Klock G, Bernard HU (1989) Progesterone and glucocorticoid response elements occur in the long control regions of several human papillomaviruses involved in anogenital neoplasia. J Virol 63:3261–3269

12. Chen YH, Huang LH, Chen TM (1996) Differential effects of progestins and estrogens on long control regions of human papillomavirus types 16 and 18. Biochem Biophys Res Commun 224:651–659

13. Clifford G, Gallus S, Herrero R, Munoz N, Snijders P, Vaccarella S, et al. Worldwide distribution of human papillomavirus types in cytologically normal women in the International Agency for Research on Cancer HPV prevalence surveys: a pooled analysis. The Lancet. 2005;366(9490):991-8

14. Dellinger TH, Smith DD, Ouyang C, Warden CD, Williams JC, Han ES. L1CAM is an independent predictor of poor survival in endometrial cancer - An analysis of The Cancer

Genome Atlas (TCGA). Gynecol Oncol. 2016 May;141(2):336-340. doi: 10.1016/j.ygyno.2016.02.003. Epub 2016 Feb 6.

15. Docquier A, Harmand PO, Fritsch S, Chanrion M, Darbon JM, Cavaillès V. The transcriptional coregulator RIP140 represses E2F1 activity and discriminates breast cancer subtypes. Clin Cancer Res. 2010; 16: 2959-70.

16. Doorbar J. The papillomavirus life cycle. Journal of clinical virology. 2005;32:7-15.
Dyson N. The regulation of E2F by pRB-family proteins. Genes & development. 1998;12(15):2245-62.

17. Doorbar J, Quint W, Banks L, et al. The biology and life-cycle of human papillomaviruses. Vaccine 2012;30 Suppl 5:F55-70. doi: 10.1016/j.vaccine. 2012.06.083

18. Evander M, Frazer IH, Payne E, Qi YM, Hengst K, McMillan N. Identification of the alpha6 integrin as a candidate receptor for papillomaviruses. Journal of virology. 1997;71(3):2449-56.

19. Fonseca-Moutinho JA, Cruz E, Carvalho L, Prazeres HJ, de Lacerda MM, da Silva DP, Mota F, de Oliveira CF (2004) Estrogen receptor, progesterone receptor, and BCL-2 are markers with prognostic significance in cin iii. Int J Gynecol Cancer 14:911–920

20. Franco EL, Villa LL, Sobrinho JP, Prado JM, Rousseau M-C, Désy M, et al. Epidemiology of acquisition and clearance of cervical human papillomavirus infection in women from a high-risk area for cervical cancer. Journal of Infectious Diseases. 1999;180(5):1415-23.

21. Gupta S, Takhar PP, Degenkolbe R, Koh CH, Zimmermann H, Yang CM, Guan Sim K, Hsu SI, Bernard HU. The human papillomavirus type 11 and 16 E6 proteins modulate the cell-cycle regulator and transcription cofactor TRIP-Br1. Virology. 2003 Dec 5;317(1):155-64.

22. Horn LC, Beckmann MW, Follmann M, Koch MC, Mallmann P, Marnitz S, Schmidt D, German Cancer S (2015) s3 guidelines on diagnostics and treatment of cervical cancer: demands on pathology. Pathologe 36:585–593

23. https://www.krebsdaten.de/Krebs/DE/Content/Krebsarten/Gebaermutterhalskrebs/gebaermutterhalskrebs_node.html

24. Huang BH, Laban M, Leung CH, Lee L, Lee CK, Salto-Tellez M, Raju GC, Hooi SC. Inhibition of histone deacetylase 2 increases apoptosis and p21Cip1/WAF1 expression, independent of histone deacetylase 1. Cell Death Differ. 2005 Apr;12(4):395-404.

25. Jalaguier S, Teyssier C, Nait Achour T, Lucas A, Bonnet S, Rodriguez C, Elarouci N, Lapierre M, Cavaillès V. Complex regulation of LCoR signaling in breast cancer cells. Oncogene. 2017; 36: 4790-801.

26. Jiang, Y., et al., Synergistic induction of apoptosis in HeLa cells by the proteasome inhibitor bortezomib and histone deacetylase inhibitor SAHA. Mol Med Rep, 2010. 3(4): p. 613-9.

27. Khleif SN, DeGregori J, Yee CL, Otterson GA, Kaye FJ, Nevins JR, et al. Inhibition of cyclin D-CDK4/CDK6 activity is associated with an E2F-mediated induction of cyclin kinase inhibitor activity. Proceedings of the National Academy of Sciences. 1996;93(9):4350-4.

28. Kim YT, Choi EK, Cho NH, Ko JH, Yang WI, Kim JW, et al. Expression of cyclin E and p27 KIP1 in cervical carcinoma. Cancer letters. 2000;153(1):41-50.

29. Klussmann PDJ, Preuss S, Speel E. Humane Papillomviren und Oropharynxkarzinome. Hno. 2009;57(2):113-22.

30. Kuwabara I, Liu F-T. Galectin-3 promotes adhesion of human neutrophils to laminin. The Journal of Immunology. 1996;156(10):3939-44.

31. Lapierre M, Docquier A, Castet-Nicolas A, Gitenay D, Jalaguier S, Teyssier C, Cavaillès V. The emerging role of the transcriptional coregulator RIP140 in solid tumors. Biochim Biophys Acta. 2015; 1856: 144-50.

32. Liu F-T, Rabinovich GA. Galectins as modulators of tumour progression. Nature Reviews Cancer. 2005;5(1):29-41.

33. Lu, Y.-S., et al., Effects of glucocorticoids on the growth and chemosensitivity of carcinoma cells are heterogeneous and require high concentration of functional glucocorticoid receptors. World Journal of Gastroenterology : WJG, 2005. 11(40): p. 6373-6380.

34. Marth, C, Landoni F, Mahner S, McCormack M, Gonzalez-Martin A, Colombo N. ESMO Guidlines Committee. Cervical Cancer: ESMO Clinical Practice Guidlines for diagnosis, treatment and follow-up. Ann Oncol. 2017 Jul 1;28(suppl_4):iv72-iv83. doi: 10.1093/annonc/mdx220.

35. Matarrese P, Fusco O, Tinari N, Natoli C, Liu FT, Semeraro ML, et al. Galectin-3 overexpression protects from apoptosis by improving cell adhesion properties. International journal of cancer. 2000;85(4):545-54.

36. Mao C, Balasubramanian A, Yu M, Kiviat N, Ridder R, Reichert A, Herkert M, von Knebel Doeberitz M, Koutsky LA. Evaluation of a new p16(INK4A) ELISA test and a high-risk HPV DNA test for cervical cancer screening: results from proof-of-concept study. Int J Cancer. 2007 Jun 1;120(11):2435-8.

37. McKenna, N. J. et al. Nuclear receptor coactivators: multiple enzymes, multiple complexes, multiple functions. J. Steroid Biochem. Mol. Biol. 69, 3-12 (1999).

38. Melkane AE, Mirghani H, Aupérin A, Saulnier P, Lacroix L, Vielh P, Casiraghi O, Griscelli F, Temam S. HPV-related oropharyngeal squamous cell carcinomas: a comparison between three diagnostic approaches. Am J Otolaryngol. 2014 Jan-Feb;35(1):25-32. doi: 10.1016/j.amjoto.2013.08.007. Epub 2013 Oct 7.

39. Meng CF, Su B, Li W (2011) DNA demethylation is superior to histone acetylation for reactivating cancer-associated genes in ovarian cancer cells. Mol Med Rep 4:1273–1278

40. Moody CA, Laimins LA. Human papillomavirus oncoproteins: pathways to transformation. Nature Reviews Cancer. 2010;10(8):550-60.

41. Münger K, Baldwin A, Edwards KM, Hayakawa H, Nguyen CL, Owens M, et al. Mechanisms of human papillomavirus-induced oncogenesis. Journal of virology. 2004;78(21):11451-60.

42. Muñoz N, Bosch FX, Castellsagué X, Díaz M, de Sanjose S, Hammouda D, Shah KV, Meijer CJ. Against which human papillomavirus types shall we vaccinate and screen? The international perspective. Int J Cancer. 2004 Aug 20;111(2):278-85.

43. Muñoz N, Bosch FX, de Sanjosé S, Herrero R, Castellsagué X, Shah KV, Snijders PJ,

Meijer CJ; Epidemiologic classification of human papillomavirus types associated with cervical cancer. International Agency for Research on Cancer Multicenter Cervical Cancer Study Group. N Engl J Med. 2003 Feb 6;348(6):518-27.

44. Munoz N, Bosch FX, Castellsague X, Diaz M, de Sanjose S, Hammouda D, Shah KV, Meijer CJ (2004) Against which human papillomavirus types shall we vaccinate and screen? The international perspective. Int J Cancer 111:278–285

45. Nangia-Makker P, Honjo Y, Sarvis R, Akahani S, Hogan V, Pienta KJ, et al. Galectin-3 induces endothelial cell morphogenesis and angiogenesis. The American journal of pathology. 2000;156(3):899-909.

46. Narisawa-Saito M, Kiyono T. Basic mechanisms of high-risk human papillomavirus-induced carcinogenesis: Roles of E6 and E7 proteins. Cancer science. 2007;98(10):1505-11

47. Narumiya, S.; Sugimoto, Y.; Ushikubi, F. Prostanoid receptors: Structures, properties, and functions. Physiol. Rev. 1999, 79, 1193–1226.

48. Ochieng J, Leite-Browning ML, Warfield P. Regulation of cellular adhesion to extracellular matrix proteins by galectin-3. Biochemical and biophysical research communications. 1998;246(3):788-91.

49. Pauletti G, Dandekar S, Rong H, Ramos L, Peng H, Seshadri R, Slamon DJ. Assessment of methods for tissue-based detection of the HER-2/neu alteration in human breast cancer: a direct comparison of fluorescence in situ hybridization and immunohistochemistry. J Clin Oncol. 2000 Nov 1;18(21):3651-64.

50. Petitjean A, Achatz MI, Borresen-Dale AL, Hainaut P, Olivier M. TP53 mutations in human cancers: functional selection and impact on cancer prognosis and outcomes. Oncogene. 2007 Apr 2;26(15):2157-65. Review.

51. Perissi, V. & Rosenfeld, M. G. Controlling nuclear receptors: the circular logic of cofactor cycles. Nat. Rev. Mol. Cell Biol. 6, 542-554 (2005).

52. Pittayakhajonwut D, Angeletti PC (2010) Viral trans-factor independent replication of human papillomavirus genomes. Virol J 7:123

53. Ryu, H.S.; Chang, K.H.; Yang, H.W.; Kim, M.S.; Kwon, H.C.; Oh, K.S. High cyclooxygenase-2 expression in stage IB cervical cancer with lymph node metastasis or parametrial invasion. Gynecol. Oncol. 2000, 76,320–325.

54. S3-Leitlinie Diagnostik, Therapie und Nachsorge der Patientin mit Zervixkarzinom 2014 https://www.leitlinienprogramm-onkologie.de/leitlinien/zervixkarzinom/

55. S3-Leitlinie Früherkennung, Diagnostik, Therapie und Nachsorge des Mammakarzinoms (Version 4.3) 2020 https://www.leitlinienprogramm-onkologie.de/leitlinien/mammakarzinom/

56. Sano H, Hsu DK, Yu L, Apgar JR, Kuwabara I, Yamanaka T, et al. Human galectin-3 is a novel chemoattractant for monocytes and macrophages. The Journal of Immunology. 2000;165(4):2156-64.

57. Schiffman M, Castle PE, Jeronimo J, Rodriguez AC, Wacholder S. Human papillomavirus and cervical cancer. Lancet. 2007 Sep 8;370(9590):890-907. Review.

58. Serrano-Olvera A, Cetina L, Coronel J, Duenas-Gonzalez A (2014) Follow-up consultations for cervical cancer patients in a mexican cancer center. Comparison with NCCN guidelines. Asian Pac J Cancer Prev 15:8749–8752

59. Stubenrauch F, Laimins LA, editors. Human papillomavirus life cycle: active and latent phases. Seminars in cancer biology; 1999: Elsevier.

60. Sugita, R.; Kuwabara, H.; Kubota, K.; Sugimoto, K.; Kiho, T.; Tengeiji, A.; Kawakami, K.; Shimada, K. Simultaneous inhibition of PGE2 and PGI2 signals is necessary to suppress hyperalgesia in rat inflammatory pain models. Mediat. Inflamm. 2016, 2016, 9847840.

61. Tang D, Wu D, Hirao A, Lahti JM, Liu L, Mazza B, Kidd VJ, Mak TW, Ingram AJ.
ERK activation mediates cell cycle arrest and apoptosis after DNA damage independently of p53. J Biol Chem. 2002 Apr 12;277(15):12710-7. Epub 2002 Jan 30.

62. Vogelstein B, Lane D, Levine AJ. Surfing the p53 network. Nature. 2000;408(6810):307-10.

63. WHO, International Agency for Research on Cancer. GLOBOCAN 2018. Fact Sheet: Cervix uteri. 08.05.2019. 2018.

64. WHO, International Agency for Research on Cancer. GLOBOCAN 2018. Estimated number of incident cases Germany, females, all ages. http://gco.iarc.fr. 08.05.2019. 2019.

65. WHO, International Agency for Research on Cancer. GLOBOCAN 2018. Estimated number of incident cases from 2018 to 2040, cervix uteri, females, all ages. https://gco.iarc.fr. 08.05.2019. 2019.

66. Wooldridge TR, Laimins LA. Regulation of human papillomavirus type 31 gene expression during the differentiation-dependent life cycle through histone modifications and transcription factor binding. Virology. 2008 May 10;374(2):371-80. doi: 10.1016/j.virol.2007.12.011. Epub 2008 Jan 31.

67. Zengel P, Assmann G, Mollenhauer M, Jung A, Sotlar K, Kirchner T, Ihrler S. Cancer of unknown primary originating from oropharyngeal carcinomas are strongly correlated to HPV positivity. Virchows Arch. 2012 Sep;461(3):283-90. doi: 10.1007/s00428-012-1290-3. Epub 2012 Aug 2.

5 Abkürzungen

HPV = Humanes Papillomavirus

WHO = World Health Organization

UICC = Union internationale contre le cancer

TNM = T: Tumor, N: Nodus, M: Metastase

DNA = Desoxyribonukleinsäure

LCR = Long Control Region

LR = Low-Risk

HR = High-Risk

p16 = CDK-Inhibitor 2A Protein

E6-AP = E6-assoziiertes Protein

MDM2 = MDM2 Proto-Onkogen

Rb = Retinoblastom-Protein

Gal-3 = Galektin-3

NR = Nukleäre Rezeptoren

RIP140 = Receptor Interacting Protein

NRIP1 = Nuclear Receptor Interacting Protein 1)

LCoR = Ligand dependent Corepressor

COX = Cyclooxegenase

FIGO = International Federation of Gynecology and Obstetrics

Her-2 = human epidermal growth factor receptor 2

HIV = Humanes Immundefizienz-Virus

G = Gruppe

PCR = Polymerase Kettenreaktion

CIN = Cervikale Intraepitheliale Neoplasie

LK = Lymphknoten

EP 3 = Prostaglandin E Rezeptor Typ3

LMU = Ludwig-Maximilians-Universität

6 Publikationen

Originalarbeiten als Erst- oder Letztautor

Glucocorticoid receptor in cervical cancer: an immunhistochemical analysis.

Kost BP, Beyer S, Schröder L, Zhou J, Mayr D, Kuhn C, Schulze S, Hofmann S, Mahner S, Jeschke U, Heidegger H.

Arch Gynecol Obstet. 2019 Jan;299(1):203-209. doi: 10.1007/s00404-018-4928-9. Epub 2018 Oct 10.

The involvement of E6, p53, p16, MDM2 and Gal-3 in the clinical outcome of patients with cervical cancer.

Stiasny A, Freier CP, Kuhn C, Schulze S, Mayr D, Alexiou C, Janko C, Wiest I, Dannecker C, Jeschke U, **Kost BP**.

Oncol Lett. 2017 Oct;14(4):4467-4476. doi: 10.3892/ol.2017.6752. Epub 2017 Aug 14.

Prevalence of human papillomavirus infection of the anal canal in women: A prospective analysis of high-risk populations.

Kost BP, Hofmann J, Stoellnberger S, Bergauer F, Blankenstein T, Alba-Alejandre I, Stein A, Stuckart C, Weizsäcker K, Mylonas I, Mahner S, Gingelmaier A.

Oncol Lett. 2017 Apr;13(4):2495-2501. doi: 10.3892/ol.2017.5714. Epub 2017 Feb 10.

Investigation of RIP140 and LCoR as independent markers for poor prognosis in cervical cancer.

Vattai A, Cavailles V, Sixou S, Beyer S, Kuhn C, Peryanova M, Heidegger H, Hermelink K, Mayr D, Mahner S, Dannecker C, Jeschke U, **Kost B**.

Oncotarget. 2017 Oct 31;8(62):105356-105371. doi: 10.18632/oncotarget.22187. eCollection 2017 Dec 1.

The Prostaglandin EP3 Receptor Is an Independent Negative Prognostic Factor for Cervical Cancer Patients.

Heidegger H, Dietlmeier S, Ye Y, Kuhn C, Vattai A, Aberl C, Jeschke U, Mahner S, **Kost B**.

Int J Mol Sci. 2017 Jul 19;18(7). pii: E1571. doi: 10.3390/ijms18071571.

Histone H3 Acetyl K9 and Histone H3 Tri Methyl K4 as Prognostic Markers for Patients with Cervical Cancer.

Beyer S, Zhu J, Mayr D, Kuhn C, Schulze S, Hofmann S, Dannecker C, Jeschke U, **Kost BP**.

Int J Mol Sci. 2017 Feb 23;18(3). pii: E477. doi: 10.3390/ijms18030477.

Immunohistochemical Evaluation of the Role of p53 Mutation in Cervical Cancer: Ser-20 p53-Mutant Correlates with Better Prognosis.

Freier CP, Stiasny A, Kuhn C, Mayr D, Alexiou C, Janko C, Wiest I, Jeschke U, **Kost B**.

Anticancer Res. 2016 Jun;36(6):3131-7.

Immunohistochemical Evaluation of E6/E7 HPV Oncoproteins Staining in Cervical Cancer.

Stiasny A, Kuhn C, Mayr D, Alexiou C, Janko C, Wiest I, Jeschke U, **Kost B**.

Anticancer Res. 2016 Jun;36(6):3195-8.

HIV testing in pregnancy: are we testing enough?

Kost BP, Gingelmaier A, Kainer F, Friese K, Mylonas I.

Arch Gynecol Obstet. 2011 Aug;284(2):357-60. doi: 10.1007/s00404-010-1639-2. Epub 2010 Aug 18.

Platelets, a typical source of error in real-time PCR quantification of mitochondrial DNA content in human peripheral blood cells.

Banas B, **Kost BP**, Goebel FD. Geteilte Erstautorenschaft

Eur J Med Res. 2004 Aug 31;9(8):371-7.

Originalarbeiten als Koautor:

The prostaglandin receptor EP2 determines prognosis in EP3-negative and galectin-3-high cervical cancer cases.

Dietlmeier S, Ye Y, Kuhn C, Vattai A, Vilsmaier T, Schröder L, **Kost BP**, Gallwas J, Jeschke U, Mahner S, Heidegger HH.

Sci Rep. 2020 Jan 24;10(1):1154. doi: 10.1038/s41598-020-58095-3.

Higher CCL22+ Cell Infiltration is Associated with Poor Prognosis in Cervical Cancer Patients.

Wang Q, Schmoeckel E, **Kost BP**, Kuhn C, Vattai A, Vilsmaier T, Mahner S, Mayr D, Jeschke U, Heidegger HH.

Cancers (Basel). 2019 Dec 12;11(12). pii: E2004. doi: 10.3390/cancers11122004.

Differential prognostic relevance of patho-anatomical factors among different tumor-biological subsets of breast cancer: Results from the adjuvant SUCCESS A study.

Deniz M, DeGregorio A, DeGregorio N, Bekes I, Widschwendter P, Schochter F, Ernst K, Scholz C, Bauer EC, Aivazova-Fuchs V, Weissenbacher T, **Kost B**, Jueckstock J, Andergassen U, Steidl J, Trapp E, Fasching PA, Häberle L, Beckmann MW, Schneeweiss A, Schrader I, Janni W, Rack B, Friedl TW.

Breast. 2019 Apr;44:81-89. doi: 10.1016/j.breast.2018.12.008. Epub 2018 Dec 20.

TA-MUC1 as detected by the fully humanized, therapeutic antibody Gatipotzumab predicts poor prognosis in cervical cancer.

Heublein S, Friese K, **Kost B**, Marmé F, Kuhn C, Mahner S, Dannecker C, Mayr D, Jeschke U, Vattai A.

J Cancer Res Clin Oncol. 2018 Oct;144(10):1899-1907. doi: 10.1007/s00432-018-2706-5. Epub 2018 Jul 30.

The G protein-coupled estrogen receptor (GPER/GPR30) may serve as a prognostic marker in early-stage cervical cancer.

Friese K, **Kost B**, Vattai A, Marmé F, Kuhn C, Mahner S, Dannecker C, Jeschke U, Heublein S.

J Cancer Res Clin Oncol. 2018 Jan;144(1):13-19. doi: 10.1007/s00432-017-2510-7. Epub 2017 Sep 18.

Lowered Rilpivirine Exposure During the Third Trimester of Pregnancy in Human Immunodeficiency Virus Type 1-Infected Women.

Schalkwijk S, Colbers A, Konopnicki D, Gingelmaier A, Lambert J, van der Ende M, Moltó J, Burger D; Pharmacokinetics of newly developed antiretroviral agents in HIV-infected pregnant women (PANNA) Network.

Clin Infect Dis. 2017 Oct 15;65(8):1335-1341. doi: 10.1093/cid/cix534.

The Effects of Petroselinum Crispum on Estrogen Receptor-positive Benign and Malignant Mammary Cells (MCF12A/MCF7).

Schröder L, Koch J, Mahner S, **Kost BP**, Hofmann S, Jeschke U, Haumann J, Schmedt J, Richter DU.

Anticancer Res. 2017 Jan;37(1):95-102.

Induction of DNA damage and apoptosis in human leukemia cells by efavirenz.

Brüning A, Jückstock J, **Kost B**, Tsikouras P, Weissenbacher T, Mahner S, Mylonas I.

Oncol Rep. 2017 Jan;37(1):617-621. doi: 10.3892/or.2016.5243. Epub 2016 Nov 15.

Comparison of HER2 Expression in Primary Tumor and Disseminated Tumor Cells in the Bone Marrow of Breast Cancer Patients.

Rack B, Zombirt E, Trapp E, Jückstock J, Andergassen U, Neugebauer J, **Kost B**, Weissenbacher T, Jeschke U, Schindlbeck C, Janni W, Alunni-Fabbroni M.

Oncology. 2016;90(4):232-8. doi: 10.1159/000442986. Epub 2016 Mar 4.

Efavirenz Causes Oxidative Stress, Endoplasmic Reticulum Stress, and Autophagy in Endothelial Cells.

Weiß M, **Kost B**, Renner-Müller I, Wolf E, Mylonas I, Brüning A.

Cardiovasc Toxicol. 2016 Jan;16(1):90-9. doi: 10.1007/s12012-015-9314-2.

The expression of thyroid hormone receptors (THR) is regulated by the progesterone receptor system in first trimester placental tissue and in BeWo cells in vitro.

Vattai A, Ziegelmüller B, **Kost B**, Kuhn C, Hofmann S, Bayer B, Anslinger K, Jeschke U, Ditsch N.

Eur J Obstet Gynecol Reprod Biol. 2015 Dec;195:31-9. doi: 10.1016/j.ejogrb.2015.09.003. Epub 2015 Sep 30.

The influence of obesity on survival in early, high-risk breast cancer: results from the randomized SUCCESS A trial.

Widschwendter P, Friedl TW, Schwentner L, DeGregorio N, Jaeger B, Schramm A, Bekes I, Deniz M, Lato K, Weissenbacher T, **Kost B**, Andergassen U, Jueckstock J, Neugebauer J, Trapp E, Fasching PA, Beckmann MW, Schneeweiss A, Schrader I, Rack B, Janni W, Scholz C.

Breast Cancer Res. 2015 Sep 18;17:129. doi: 10.1186/s13058-015-0639-3.

Expression of Thyroid Hormone Receptors in Villous Trophoblasts and Decidual Tissue at Protein and mRNA Levels Is Downregulated in Spontaneous and Recurrent Miscarriages.

Ziegelmüller B, Vattai A, **Kost B**, Kuhn C, Hofmann S, Bayer B, Toth B, Jeschke U, Ditsch N.

J Histochem Cytochem. 2015 Jul;63(7):511-23. doi: 10.1369/0022155415582052. Epub 2015 Mar 26.

Analysis of endoplasmic reticulum stress in placentas of HIV-infected women treated with protease inhibitors.

Brüning A, Kimmich T, Brem GJ, Buchholtz ML, Mylonas I, **Kost B**, Weizsäcker K, Gingelmaier A.

Reprod Toxicol. 2014 Dec;50:122-8. doi: 10.1016/j.reprotox.2014.10.012. Epub 2014 Oct 25.

Pooled analysis of the prognostic relevance of progesterone receptor status in five German cohort studies.

Salmen J, Neugebauer J, Fasching PA, Haeberle L, Huober J, Wöckel A, Rauh C, Schuetz F, Weissenbacher T, **Kost B**, Stickeler E, Klar M, Orlowska-Volk M, Windfuhr-Blum M, Heil J, Rom J, Sohn C, Fehm T, Mohrmann S, Loehberg CR, Hein A, Schulz-Wendtland R, Hartkopf AD, Brucker SY, Wallwiener D, Friese K, Hartmann A, Beckmann MW, Janni W, Rack B.

Breast Cancer Res Treat. 2014 Nov;148(1):143-51. doi: 10.1007/s10549-014-3130-4. Epub 2014 Sep 25.

High-fidelity simulation increases obstetric self-assurance and skills in undergraduate medical students.

Scholz C, Mann C, Kopp V, **Kost B**, Kainer F, Fischer MR.

J Perinat Med. 2012 Nov;40(6):607-13. doi: 10.1515/jpm-2012-0052.

Anal cytology as a screening tool for early detection of anal dysplasia in HIV-infected women.

Gingelmaier A, Weissenbacher T, **Kost B**, Kaestner R, Sovric M, Mylonas I, Friese K, Bergauer F.

Anticancer Res. 2010 May;30(5):1719-23.

Protease inhibitor-based antiretroviral prophylaxis during pregnancy and the development of drug resistance.

Gingelmaier A, Eberle J, **Kost BP**, Bogner JR, Hofmann J, Weissenbacher T, Kästner R, Friese K, Weizsaecker K.

Clin Infect Dis. 2010 Mar 15;50(6):890-4. doi: 10.1086/650747.

Mitochondrial toxicity in HIV type-1-exposed pregnancies in the era of highly active antiretroviral therapy.

Gingelmaier A, Grubert TA, **Kost BP**, Setzer B, Lebrecht D, Mylonas I, Mueller-Hoecker J, Jeschke U, Hiedl S, Friese K, Walker UA.

Antivir Ther. 2009;14(3):331-8.

The influence of HIV infection and antiretroviral therapy on the mitochondrial membrane potential of peripheral mononuclear cells.

Sternfeld T, Schmid M, Tischleder A, Mudra S, Schlamp A, **Kost BP**, Gruber R, Youle M, Bogner JR, Goebel FD.

Antivir Ther. 2007;12(5):769-78.

Kasuistiken/Casereports:

Congenital cytomegalovirus infection in pregnancy: a case report of fetal death in a CMV-infected woman.

Kost BP, Mylonas I, Kästner R, Rack B, Gingelmaier A, Friese K.

Arch Gynecol Obstet. 2007 Sep;276(3):265-8. Epub 2007 Feb 28.

Sonstige Veröffentlichungen:

Endometriosis -Update of Therapeutic Management.

L. Hertlein, A. Burges, F. Trillsch, **B. Kost**, S. Mahner, N. Rogenhofer

No. 23 Digital Edition / eBook 2_2018 www.german-medical-journal.eu

Lehre im Fach Frauenheilkunde und Geburtshilfe – Zwischen digitaler Euphorie und analoger Resignation.

C. Scholz, F. Kainer, **B. Kost**, B. Toth, W. Janni.

Frauenarzt 53 (2012), 630-633.

Effect of antiretroviral drugs on mitochondrial DNA in HIV-infected pregnant women and their newborns.

Kost BP, Sovric M, Notheis G, Kästner R, Mylonas I, Kainer F, Friese K, Gingelmaier A.

Arch Gynecol Obstet 2010 Oct;Volume 282, supplement1 Abstract 03.54:250.

Mitochondrial DNA depletion and changes in lactate metabolism as a marker of nucleoside toxicity in HIV-infected patients.

Bauer AM, **Kost B**, Hillebrand S, Bogner JR, Banas B.

Eur J Med Res. 2003; 8 Suppl. 1: 46

Lactic acidosis in HAART treated patients is not a result of mitochondrial depletion.

Kost B, Hillebrand S, Goebel FD, Bogner JR, Banas B.

Antiviral Therapy. 2003; 8 Suppl. 1: 391

7 Lebenslauf

Dienstanschrift

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Förderungen:

Zweifach Friedrich-Bauer-Stiftung
Heuer-Stiftung
Else-Kröner-Freseniusstiftung – EKFS

Mitgliedschaften:

Deutsche Gesellschaft für Gynäkologie und Geburtshilfe - DGGG
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8 Danksagung

An erster Stelle möchte ich Herrn Prof. Udo Jeschke und meinem „neuen“ Chef Herrn Prof. Sven Mahner, sowie meinem „alten“ Chef Herrn Prof. Klaus Friese danken.

Prof. Udo Jeschke hat mich wissenschaftlich und menschlich mit all seinem Können beim Entstehen und bei der Durchführung dieser Arbeit, mit unermüdlichem Optimismus, unterstützt. Dafür möchte ich ihm und dem gesamten Team des Forschungslabors in der Maistrasse von Herzen danken. Zu nennen sind hier Christina, Simone, Irmi, Laurent und Stephan, die stets fleißigen Doktoranden sowie hervorgehoben Christine Friedl. Ihr möchte ich besonders danken, denn ohne Ihren unermüdlichen Antrieb und Glauben an mich, würde es diese Arbeit nicht geben.

Danken möchte ich meinem jetzigen Chef Herrn Prof. Sven Mahner, daß er mich tatkräftig bei der Umsetzung dieser Arbeit und meinem Weg an der Klinik mit Vertrauen unterstützt und meine Begeisterung für die universitären Aufgaben teilt. Auch möchte ich mich bei meinem „alten“ Chef Herrn Prof. Klaus Friese bedanken. Er hat den Grundstein meiner klinischen und universitären Arbeit und meine Begeisterung für das Fach Frauenheilkunde und Geburtshilfe gelegt und hat mich im universitären sowie klinischen Alltag stets gefördert und gefordert.

Ein besonderer Dank gilt Konrad und meiner gesamten Familie, die durch meine Aufgaben große Entbehrungen hinnehmen müssen und dennoch stets an mich glauben und mich unermüdlich unterstützen.

Danke an Euch alle, dass ihr die Entstehung dieser Arbeit möglich gemacht habt!