Untersuchung der Mediatorkomplex-Untereinheiten MED12 und MED15 in verschiedenen Tumorentitäten

Inaugural-Dissertation

zur Erlangung des Doktorgrades

der Hohen Medizinischen Fakultät

der Rheinischen Friedrich-Wilhelms-Universität

Bonn

Anne Kathrin Offermann

aus Simmerath

2016

Angefertigt mit der Genehmigung
der Medizinischen Fakultät der Universität Bonn

1. Gutachter: Prof. Dr. med. Sven Perner
2. Gutachter: Prof. Dr. med. Dominik Wolf

Tag der Mündlichen Prüfung: 21.12.2015

Aus dem Institut für Pathologie
Direktor: Prof. Dr. med. Glen Kristiansen

Für meine Familie

Inhaltsverzeichnis

1. Zusammenfassung in deutscher Sprache

1.1 Einleitung

Der Mediatorkomplex ist ein evolutionär konservierter, aus 30 Untereinheiten bestehender Multiproteinkomplex, der strukturell und funktionell in die vier Module Kopf-, Mittel-, Schwanz- und Kinase-Teil gegliedert wird (Lewis und Reinberg, 2003). Abhängig von Einflüssen auf und Anforderungen an die Zelle binden spezifische Untereinheiten des Mediators an verschiedene Ko-Aktivatoren von Signalwegen, wodurch der Mediatorkomplex seine Formation ändert und schließlich als „Kernkomplex“ im Nukleus vorliegt. Hier interagiert er direkt mit der RNA-Polymerase II und ermöglicht somit als Bindeglied zwischen Signalwegen und der basalen Transktiptionsmachinerie eine adaptierte Genexpression (Malik und Roeder, 2010). Eine veränderte Expression oder Funktion des Mediators kann zu dysregulierter Transkriptionsregulation führen und wurde bereits in Zusammenhang mit verschiedenen Erkrankungen gebracht (Napoli et al., 2012).

Die Untereinheit MED15 ist Bestandteil des Schwanz-Moduls und spielt eine wichtige Rolle in der Vermittlung des TGFβ-, Nodal- und SREBP-Signalweges (Kato et al., 2002; Yang et al., 2006). Genetische Veränderungen von MED15 wurden im Mikrodeletionssyndrom DiGeorge beschrieben (Berti et al., 2001) und Ergebnisse aus Mammakarzinomzellen weisen darauf hin, dass eine verstärkte MED15-Expression mit einem aggressiveren Verhalten der Tumorzellen assoziiert ist (Zhao et al., 2013).

MED12 wird dem Kinase-Modul des Mediators zugeordnet, und ist in verschiedene Signalwege während der Embryogenese und Zelldifferenzierung involviert (Gillmor et al. 2010; Rocha et al. 2010; Tutter et al. 2010). Die Beobachtung von MED12-Mutationen in Patienten mit FGS1- oder Lujansyndrom (Risheg et al., 2007; Schwartz et al., 2007), beides genetisch bedingte Erkrankungen mit typischen Entwicklungsstörungen, weisen übereinstimmend mit experimentellen Studien auf eine essentielle biologische Relevanz von MED12 bei der Regulation embryologischer Zellprozesse hin. In 2011 wurden MED12-Mutationen erstmals im Tumorgewebe identifiziert, wobei 70 % untersuchter Leiomyome eine Mutation in Exon 2 von MED12 aufwiesen (Mäkinen et al., 2011). Bei der Exom-Sequenzierung von Prostatakarzinomen konnten in einer weiteren Studie in 5,4 % primärer Tumore rekurrente MED12-Mutationen nachgewiesen werden (Barbieri et al., 2012).

Weltweit stellen Tumorerkrankungen eine führende Ursache für Morbidität und Mortalität

dar, und führten im Jahre 2012 zu 8,2 Millionen Todesfällen weltweit (World Health Organization, 2015). Insbesondere das Prostatakarzinom, die häufigste Krebserkrankung des Mannes, ist gekennzeichnet durch einen sehr variablen Krankheitsverlauf (Jemal et al., 2011). Die meisten Patienten weisen einen indolenten, langsam wachsenden Tumor auf, dessen Progredienz lediglich im Verlauf kontrolliert wird oder der mittels radikaler Prostatektomie oder lokaler Bestrahlung geheilt werden kann (National Institute for Health and Care Excellence, 2014). Die Identifikation aggressiver Tumore, die der Kontrolle exogener Wachstumshormone entgehen und damit kastrations-resistent sind, stellt dagegen die größte Herausforderung bei der Behandlung von Prostatakarzinompatienten dar (Karantanos et al., 2016).

Tumore des Kopf- und Halsbereiches sind die sechsthäufigste Krebserkrankung in westlichen Nationen und werden histopathologisch in über 90 % der Fälle den Plattenepithelkarzinomen zugeordnet (Jemal et al., 2011). Ursachen für ein mittleres Überleben von unter 50 % umfassen unter anderem späte Diagnosestellung, frühe Metastasierung und multifokale Tumorrekurrenz trotz durchgeführter Primärtherapie, der chirurgischen Tumorresektion (Warnakulasuriya, 2009).

Neben der Erforschung der Ätiologie und Initiation von Tumoren nimmt die Entschlüsselung von Faktoren, die zur Tumorprogression, -rekurrenz und -resistenz gegenüber Therapien in der onkologischen Forschung einen wichtigen Stellenwert ein. Ziel ist es, die molekularen Grundlagen maligner Prozesse in Tumorzellen zu verstehen, um Angriffspunkte für neue Therapien zu finden.

1.2 Fragestellung

Da Veränderungen molekularer Prozesse in Tumorzellen ein spezifisches Transkriptionsmuster zugrunde liegt und der Mediatorkomplex einen entscheidenden Regulator spezifischer Genexpression darstellt (Malik und Roeder, 2010), war Ziel dieser Arbeit zu untersuchen, ob den Mediator-Untereinheiten MED12 und MED15 eine Bedeutung in der Tumorentwicklung und –progression zukommt. Zunächst sollten Gewebsanalysen zeigen, ob MED12 und MED15 ein unterschiedliches Expressionsmuster im benignen und Tumorgewebe verschiedener Stadien aufweisen. Die Korrelation der Expression zu Patientendaten soll eine Aussage über die Relevanz der MED12- und MED15-Expression zur Prognoseabschätzung sowie einen Zusammen-

hang zu klinisch-pathologischen Parametern ermöglichen.
Anschließend wurde die Bedeutung von MED12 und MED15 für die Proliferation, Apoptose und Migration von Tumorzellen mittels Zelllinien-Experimente untersucht. Die Ergebnisse dieser in-vitro Versuche sollten erste Anhaltspunkte dafür geben, ob MED12 und MED15 potenzielle Angriffspunkte zur Reduktion maligner Eigenschaften darstellen könnten.
Vorherige Studien anderer Arbeitsgruppen geben bereits Hinweise darauf, dass MED12 und MED15 in die Vermittlung potenziell onkogener Signaltransduktionswege involviert sind. Basierend darauf zielten weitere Experimente darauf ab zu untersuchen, ob die MED12- und MED15-Expression mit der Aktivität onkogener Signalwege assoziiert ist und ob eine Herunterregulation von MED12 und MED15 diese Aktivität reduzieren kann.
Zusammenfassend sollte mit dieser Studie untersucht werden, 1) ob sich die MED12- und MED15-Expression im benignen und malignem Gewebe einerseits und in verschiedenen Tumorstadien andererseits signifikant unterscheidet, 2) ob MED12 und MED15 eine Bedeutung für maligne Eigenschaften von Tumorzellen aufweisen und 3) in welche Signalwege, deren Aktivierung die molekulare Grundlage für ein aggressives Tumorzell-Verhalten darstellen, MED12 und MED15 involviert sind. Basierend auf diesen Ergebnissen soll anschließend eine Aussage darüber gemacht werden können, inwiefern MED12 und MED15 als prognostischer Marker und therapeutisches Angriffziel in Krebszellen dienen könnte.

1.3 Material und Methoden

Für die Untersuchung der MED15-Expression im Prostatakarzinom wurde das Gewebe von 718 Patienten in Form von Tissue microarrays (TMAs) analysiert. Die untersuchte Progressionskohorte umfasst 40 benigne Prostatagewebe, 110 nicht-metastasierte Primärtumore, 89 lokal fortgeschrittene Primärtumore, 92 lymphknoten-metastasierte Tumore, 160 kastrations-resistente Prostatakarzinome sowie eine Validierungskohorte von 290 Primärtumoren.
Die MED12-Expression wurde auf 656 Gewebeproben ausgewertet, darunter 40 benigne Prostatagewebe, 405 nicht-metastasierte Primärtumore, 91 lymphknoten-metastasierte Tumore und 160 kastrations-resistente Prostatakarzinome.
Um die MED15-Expression während der Progression von Kopf- und Halstumoren zu

untersuchen, wurden 113 Primärtumore, 85 Lymphknotenmetastasen, 30 Rezidivtumore sowie 20 Proben von benignem Plattenepithel analysiert.

Immunohistochemische Färbungen wurden auf in Paraffin eingebettetem Gewebe unter Einsatz des Ventana XT Immunostainer (Vantana, Tuscon) durchgeführt. Folgende Antikörper wurden verwendet: anti-MED15 rabbit polyclonal (1:50, clone 11566-1-AP, Proteintech), anti-MED12 rabbit polyclonal (1:50, Bethyl) ready-to-use anti-Ki-67 rabbit monoclonal (clone 30-9, Ventana), anti-phospho-SMAD3 rabbit monoclonal (1:50, EP823Y, Abcam), anti-phospho-AKT(Ser473) rabbit monoclonal (1:50, 736E11, Cell Signaling).

Die nukleäre MED12- und MED15-Expression des Prostatagewebes wurde mithilfe des “Immunoreaktiven Scores” (IRS) quantifiziert, indem der Prozentsatz immunopositiver Zellen (0, 0%; 1, <10%; 2, 10-50%; 3, 51-80%; 4 >80%) mit der Färbeintensität (0, keine Expression; 1, schwache Expression; 2, mittelstarke Expression; 3, starke Expression) multipliziert wurde. Ein immunoreaktiver Score ab 3 (MED12) bzw. 4 (MED15) wurde vor Durchführung der Analyse als “Überexpression” definiert.

Für die computerunterstützte, bildanalytische Quantifizierung der nukleären MED15-Expression im Gewebe der Kopf- und Halstumoren verwendeten wir eine kommerzielle Software (Tissue Studio, Definiens Developer XD 2.0). Hierbei wurde ebenfalls ein Expressions-Score gebildet, der sowohl die Färbeintensität als auch den Index immunopositiver Zellen berücksichtigt und zur Definierung folgender Gruppen diente: keine Expression (Score < 0.07), schwache Expression (Score ≥ 0.07 < 0.2) oder Überexpression (Score ≥ 0.2).

Zur Expressionsanalyse des Proliferationsmarkers Ki67 diente der Prozentsatz immunopositiver und somit proliferierender Zellen. Um die TGFβ- und PI3K(PI3 Kinase)-Aktivität im Gewebe zu quantifizieren, wurde das phospho-SMAD3- bzw. phospho-AKT-Level bestimmt, der Median des Expressionscores der jeweiligen Kohorte als Schwellenwert definiert und die Gruppen 0 (negativ) und 1 (positiv) gebildet.

Die Prostatazelllinie BPH1 sowie die metastatischen Prostatakarzinomzelllinien LNCaP, DU145 und PC3 wurden beim „American Type Culture Collection“ (ATCC), die HNSCC-Zellen SCC25 bei der „German Collection of Microorganisms and Cell Cultures“ (DSMZ) und HSC3 bei der „Japanese Collection of Research Bioresources Cell Bank“ (JCRB) erworben. BPH1- und PC-3-Zellen wurden in RPMI 1640-Medium mit 10 % Fetal Calf

Serum (FCS), 1 % Penicillin/Streptomycin und 1% Glutamin, LNCaP- und DU145-Zellen zusätzlich mit 25nM HEPES-Puffer und 1 % NEAA, SCC25- und HSC3-Zellen in DMEM-Medium mit 10 % Fetal Calf Serum (FCS) und 1 % Penicillin/Streptomycin kultiviert.
Um einen transienten MED12- bzw. MED15-Knockdown zu erzielen, wurden Zellen ausplattiert und 24 Stunden später mit scrambled oder genspezifischer siRNA mittels Lipofektion transfiziert. Nach weiteren 72 Stunden wurden Proteinlysate transfizierter Zellen hergestellt, um mittels Western blot den Erfolg der Herunterregulation von MED12 bzw. MED15 zu kontrollieren. Gleichzeitig transfizierte Zellen dienten der Viabilitätsmessung sowie der Bestimmung der TGFβ-Aktivität, indem nach verschiedenen Zeitpunkten nach siRNA-Transfektion ein MTT-Assay sowie eine pSMAD3- und Vimentin-Quantifizierung durchgeführt wurde.
Um den Einfluss des TGFβ-Signalweges auf das Expressionslevel und die subzelluläre Lokalisation von MED12 und MED15 zu untersuchen, wurden PC3-, SCC25- und HSC3-Zellen zuvor 24 Stunden lang in Serum-reduziertem Medium mit 2 % FCS kultiviert, anschließend über verschiedene Zeiträume mit rekombinantem TGFβ1 oder TGFβ3 behandelt und schließlich über Western blot-Analysen und Immunozytochemie analysiert.
Um die Viabilität zwischen MED12- bzw. MED15-Knockdown-Zellen und Kontrollzellen zu vergleichen, wurden die Zellen mit 3-(4,5-Dimethylthiazol-2-yl)-2,5 diphenyltetrazoliumbromid (MTT) gefärbt und die Absorbtion bei 595 nm gemessen.
Um eine Zellzyklusanalyse durchzuführen, wurden MED12- bzw. MED15-Knockdown-Zellen und Kontrollzellen in 200 ml Bindepuffer mit 7,5 mg/ml Propidium Iodide (PI) und Ribonuclease (1:1000) resuspendiert. Nach 30 Minuten Inkubation bei Raumtemperatur wurde die PI-Färbung mittels Fluoreszenz-aktivierte Durchflusszytometrie (FACS) quantifiziert, was einen Rückschluss auf den Prozentsatz der Zellen in einer bestimmten Phase des Zellzyklus erlaubt.
Um den Prozentsatz apoptotischer Zellen zwischen MED12- bzw. MED15-Knockdown-Zellen und Kontrollzellen zu vergleichen, wurden die Zellen mit Bindepuffer gewaschen und mit Annexin V-FITC und PI gefärbt. Nach 15 Minuten Inkubationszeit wurden apoptotische Zellen mittels FACS-Analyse quantifiziert, wobei Annexin V-positiv/PI-negativ als früh-apoptotisch und Annexin V-positiv/PI-positiv als spät-apoptotisch definiert wurde.

Zur Expressionsanalyse verschiedener Proteine wurden Zellen auf Objektträgern ausplattiert und nach verschiedenen Zeitpunkten oder Zellbehandlungen unter Einsatz des Ventana XT Immunostainer mit Protein-spezifischen Antikörpern wie oben beschrieben gefärbt. Die Auswertung erfolgte mikroskopisch oder mithilfe der oben beschriebenen Software.
Alle statistischen Berechnungen wurden mithilfe SPSS 2.0. sowie einer Beratung und Kooperation mit Markus Reischl und Ralf Mikut (Institut für Angewandte Informatik, Karlsruher Institut für Technologie) durchgeführt.

1.4 Ergebnisse

Die Analyse der immunohistochemischen Färbung zeigt im Vergleich zum Normalgewebe eine verstärkte nukleäre MED12- und MED15-Expression in primären Prostatakarzinomen und Lymphknotenmetastasen. In kastrations-resistenten Tumoren (CRPC) sind MED12 und MED15 am stärksten exprimiert, wobei wir eine Überexpressionsrate von 21 % (MED12) bzw. 70 % (MED15) im lokalisierten CRPC und 40 % (MED12) und 76 % (MED15) im fernmetastasierten CRPC feststellten. Der Vergleich der MED12- und MED15-Expression zwischen kastrations-resistenten Karzinomen vor und nach Androgenentzugs-Behandlung zeigt, dass MED15 nach Therapie eine signifikant stärkere Expression aufweist. Dies konnten wir an gepaartem Tumormaterial von 29 individuellen Patienten sowie an einer Validierungskohorte bestehend aus Gewebe von Patienten mit kastrations-resistenten Prostatakarzinomen nach Therapie (n=145) oder mit hormon-naiven Tumoren ohne Androgenentzugs-Therapie (n=112) feststellen.
Während unsere Analysen keine Assoziation zwischen der MED12-Expression und der Überlebensrate der Patienten ergaben, weisen Patienten mit MED15-Überexpression im CRPC ein reduziertes Überleben auf.
Da wir eine verstärkte MED15-Expression im Verlauf der Erkrankung beobachten konnten, untersuchten wir anschließend plattenepitheliale Schleimhaut aus dem Kopf-Halsbereich. Im Vergleich zu benignem Plattenepithel weisen primäre Plattenepithelkarzinome, Lymphknotenmetastasen und Rezidivtumore ein stärkeres nukleäres MED15-Expressionslevel auf. Insgesamt ist MED15 in 35% der primären Plattenepithelkarzinome, 30 % der Lymphknotenmetastasen und 70 % der Rezidivtumore überexprimiert. Um einen Hinweis darauf zu bekommen, ob die MED15-Überexpression ein

klonales Ereignis darstellen könnte, untersuchten wir MED15 in Primärtumoren und gepaarten Lymphknotenmetastasen derselben Patienten. Hierbei stellten wir fest, dass der MED15-Überexpressionsstatus im Primärtumor dem in korrespondierenden Lymphknotenmetastasen in 74 % der Fälle gleicht. In Übereinstimmung damit korreliert die MED15-Expression in Primärtumoren signifikant mit der Expression in Lymphknotenmetastasen.

Unsere Korrelationsanalysen ergaben, dass die MED15-Überexpression mit einem starkem Alkoholkonsum der Patienten assoziiert ist. Dagegen konnten wir keine Assoziation zu weiteren klinisch-pathologischen Faktoren wie Alter, Geschlecht, T-Status, HPV-Status und dem Vorhandensein von Lymphknoten- oder Fernmetastasen feststellen.

Das Vorhandensein einer MED15-Überexpression in Tumoren von Patienten, die einen Rezidivtumor entwickelten, geht mit einer höheren Mortalitätsrate einher. Außerdem weisen Tumore aus der Mundhöhle und dem Oropharynx eine signifikant stärkere MED15-Expression auf als Tumore aus dem Hypopharynx oder Larynx, und sind mit einer höheren Mortalitätsrate assoziiert.

Um das MED12- und MED15-Expressionslevel in Tumor-Zelllinien zu untersuchen, führten wir eine immunozytochemische Färbung mit spezifischen Antikörpern durch. Hierbei beobachteten wir eine nukleäre Expression von MED12 und MED15 in benignen Prostatahyperplasiezellen (BPH1) sowie in den androgen-sensitiven Prostatakarzinomzellen LNCaP und VCaP und den kastrations-resistenten Zellen PC3 und DU145. Die Plattenepithelkarzinomzelllinien HSC3 und SCC25 zeigen ebenfalls eine nukleäre MED15-Expression. Basierend auf diesen Ergebnissen ermöglichen alle MED12- und MED15-positiven Zelllinien folgende Knockdown-Experimente.

Im Gewebe primärer Prostatakarzinome und Metastasen konnten wir mittels IHC feststellen, dass die nukleäre MED12-Expression mit der Anzahl der für die Proliferationsmarker Ki67 und pHH3 positiv gefärbter Kerne signifikant korreliert. Um diese Beobachtung an Zelllinien-Experimenten zu verifizieren, führten wir an DU145-und LNCaP-Zellen einen siRNA-vermittelten MED12-Knockdown durch und bestätigten mittels Western Blot und qRT-PCR die Herunterregulation von MED12 auf Protein- und mRNA-Ebene. Im MTT-Assay zeigten MED12-Knockdown-Zellen eine reduzierte Viabili-

tät. Der anschließend durchgeführte Annexin-V- und Zellzyklus-Assay diente der Untersuchung, ob die reduzierte Viabilität von MED12-Knockdown-Zellen auf einer induzierten Apoptose oder einem veränderten Zellzyklus beruht. Aus der Beobachtung, dass kein Unterschied der Annexin-V-Intensität zwischen Kontroll- und MED12-Knockdown-Zellen vorliegt, folgerten wir, dass MED12 keinen Einfluss auf die Apoptoserate der Zellen hat. Im Gegensatz dazu zeigten Zellen mit MED12-Knockdown einen veränderten Zellzyklus. In DU145-Zellen führte der MED12-Knockdown zu einer Akkumulation der Zellen in der G0/G1-Phase sowie zu einer reduzierten Rate an Zellen in der G2/M-Phase. LNCaP-Zellen mit MED12-Knockdown wiesen eine reduzierte Rate an Zellen in der S-Phase sowie eine gesteigerte Rate an Zellen in der G2/M-Phase auf. In Übereinstimmung damit beobachteten wir eine gesteigerte Expression des Zellzyklus-Inhibitors p27 in MED12-Knockdown-Zellen, die wir mittels ICC analysierten. Dagegen hatte MED12 keinen Einfluss auf den Zellzyklus-Inhibitor p21.

Sowohl im Prostatakarzinom als auch in Plattenepithelkarzinomen des Kopf- und Halsbereiches analysierten wir mittels IHC eine Korrelation zwischen der MED15-Expression und der Rate an Ki67-positiven Zellen. Dies veranlasste uns dazu, den Effekt eines MED15-Knockdowns auf die Zellproliferation in-vitro zu untersuchen. In den Plattenepithelkarzinomzelllinien HSC3 und SCC25 führte ein MED15-Knockdown zu signifikant reduzierter Zellviabilität im MTT-Assay sowie einer verringerten Expression des Proliferationsmarkers Ki67.
Unsere IHC-Analyse im Prostatakarzinomgewebe ergab eine verstärkte MED15-Expression im CRPC im Vergleich zum primären Prostatakarzinom sowie eine Zunahme von MED15 nach Androgenentzug-Therapie. Da diese Beobachtung auf eine Rolle von MED15 in der Progression zur Kastrationsresistenz hindeutet, zielten weiterführende Experimente darauf ab, dies mittels in-vitro Versuche zu verifizieren. Dazu verglichen wir die Zellviabilität von LNCaP-Zellen mit und ohne MED15-Knockdown, während die Zellen in Medium ohne Androgene kultiviert wurden. Hierbei zeigten MED15-Knockdown-Zellen eine um 50 % reduzierte Viabilität sowie eine Apoptoserate von 13 % im Vergleich zu 1 % in Kontrollzellen.

In anschließenden Untersuchungen sollte herausgearbeitet werden, in welche Signal-

wege MED12 und MED15 involviert sind und ob wir im Tumorgewebe sowie in Zelllinien eine Assoziation zwischen der Aktivierung potenziell onkogener Signalwege und der Expression von MED12 und MED15 feststellen können.
Vorherige Studien zeigten, dass MED12 in der Regulierung der TGFβ-Rezeptor-Aktivität involviert ist (Huang et al., 2012). Gleichzeitig konnte in verschiedenen Studien gezeigt werden, dass der TGFβ-Signalweg im CRPC aktiviert ist und zur Aggressivität von Tumorzellen beiträgt (Barrack et al., 1997). Diese Beobachtungen veranlassten uns dazu zu untersuchen, ob MED12 eine Bedeutung im TGFβ-Signalweg in CRPC-Zellen zukommt. Im Prostatakarzinomgewebe stellten wir fest, dass die MED12-Überexpression mit einer stärkeren Expression des TGFβ-Aktivitätsmarkers phospho-SMAD3 assoziiert ist. In Übereinstimmung damit führt eine TGFβ-Stimulation von PC3-Zellen zu einer stärkeren nukleären MED12-Expression. Um den Einfluss von MED12 auf den TGFβ-Signalweg zu untersuchen, behandelten wir PC3-Kontroll- und MED12-Knockdown-Zellen mit rekombinantem TGFβ und analysierten das Expressionslevel des TGFβ-Zielgens Vimentin. Wir beobachteten in der immunozytochemischen Färbung und im Western blot eine verminderte Vimentin-Expression in Zellen mit MED12-Knockdown.

Sowohl im CRPC-Gewebe als auch in androgen-sensitiven LNCaP-Zellen konnten wir zeigen, dass MED15 nach Androgenentzug verstärkt exprimiert wird. Verschiedene molekularbiologische Mechanismen tragen zur Kastrations-Resistenz von Prostatakarzinomzellen bei, unter anderem ermöglicht die Aktivierung des PI3K/AKT/mTOR-Signalweges, dass Prostatazellen ohne den Wachstumsstimulator Testosteron überleben können (Biting et al., 2013). Der TGFβ-Signalweg wiederum aktiviert Zielgene, die über eine gesteigerte Invasion der Tumorzellen zur Metastasenbildung beitragen (Danielpour, 2005). MED15 ist ein wichtiger Ko-Aktivator des TGFβ-Signalweges, der mit dem PI3K/AKT/mTOR-Signalweg interagiert (Vo et al., 2013). Diese Beobachtungen veranlassten uns herauszufinden, ob ein aktivierter PI3K/AKT/mTOR- und TGFβ-Signalweg zu der von uns beobachteten MED15-Überexpression unter Androgenentzug führt. Zunächst führten wir eine IHC für den TGFβ-Aktivitätsmarker phospho-SMAD3 sowie den PI3K-Aktivitätsmarker phospho-AKT am CRPC-Gewebe durch und stellten eine signifikante Korrelation zwischen der MED15-Expression und der Aktivität beider Signalwege fest. In Übereinstimmung damit führte eine Behandlung von PC3-Zellen mit

rekombinantem TGFβ sowie von LNCaP-Zellen mit dem PI3K-Aktivator Epidermal growth factor (EGF) zur verstärkten MED15-Expression, die wir mittels Western blot verifizierten. Umgekehrt zeigten Zellen, die unter Androgenentzug mit dem TGFβ-Rezeptor-Inhibitor SB431542 oder dem PI3K-Inhibitor LY294002 behandelten wurden, eine reduzierte MED15-Expression.

Um anschließend zu untersuchen, ob MED15 den TGFβ-Signalweg beeinflusst, verglichen wir die TGFβ-Aktivität in Kontroll- und MED15-Knockdown-Zellen, nachdem diese mit rekombinantem TGFβ behandelt wurden. Hierbei zeigte sich eine reduzierte phospho-SMAD3-Expression sowie eine verringerte TGFβ-stimulierte Proliferation in Zellen mit MED15-Knockdown.

In Plattenepithelkarzinomzellen des Kopf- und Halsbereiches untersuchten wir ebenfalls die Korrelation zwischen der MED15-Expression und der TGFβ-Aktivität. Hierbei zeigte sich wie in Prostatakarzinomzellen, dass MED15 mit stärkerer phospho-SMAD3-Expression assoziiert ist und das ein MED15-Knockdown zu verringerter TGFβ-Aktivität führt.

1.5 Diskussion

Die in dieser Studie beschriebene gesteigerte MED12- und MED15-Expression während der Progression verschiedener Tumorentitäten weist auf eine Involvierung dieser Mediator-Untereinheiten in molekulare Mechanismen aggressiver Tumorzellen hin. Die Ergebnisse unserer funktionellen Experimente zeigen einen Einfluss von MED12 und MED15 auf unterschiedliche Eigenschaften und Signalkaskaden von Tumorzellen, die zu gesteigerter Proliferation, Metastasenbildung und Überlebensfähigkeit beitragen. Basierend auf diesen Beobachtungen schlagen wir vor, dass MED12 und MED15 als therapeutische Angriffsziele in fortgeschrittenen Tumorstadien dienen könnten. Da Studien bisher auf eine ausschließlich intrazelluläre Lokalisation von Mediatorkomplex-Untereinheiten hinweisen, könnten kleinmolekulare Inhibitoren eine Möglichkeit zur Reduktion der MED12- und MED15-Aktivität darstellen. Zukünftige in-vitro und in-vivo-Experimente sind notwendig, den Effekt einer MED12- und MED15-Inhibition auf das Malignitätspotenzial von Tumorzellen zu untersuchen. Darüber hinaus muss eine MED12- und MED15-Inhibition bezüglich der Toxizität auf benigne Zellen und hinsichtlich ihrer Spezifität und Sensitivität bei der Tumorzellschädigung untersucht werden.

In Übereinstimmung mit unseren Ergebnissen der Zelllinien-Experimente, wonach die Herunterregulation von MED12 und MED15 die TGFβ-Aktivität reduziert, korreliert die nukleäre MED12- und MED15-Expression signifikant mit dem TGFβ-Aktivitätsmarker phospho-SMAD3 im Tumorgewebe. Obwohl in einer vorherigen Studie eine Interaktion von MED12 mit dem TGFβ-Rezeptor sowie von MED15 mit phospho-SMAD3 gezeigt wurde, lassen unsere Ergebnisse bisher keine Aussage darüber zu, ob MED12 und MED15 den TGFβ-Signalweg als Teil des nukleären Mediatorkomplexes oder als individuelle Ko-Aktivatoren beeinflussen. Weitergehende Experimente werden notwendig sein, um diese molekularen Mechanismen zu untersuchen.

Die Androgenenzug-Therapie stellt die Erstlinientherapie fortgeschrittener und metastasierter Prostatakarzinome dar (Amaral et al. 2012). Nach initialer Regression kommt es durch verschiedene molekulare Mechanismen zur Kastrationsresistenz, worauf eine progrediente Tumorerkrankung folgt (Amaral et al. 2012). Unsere Untersuchungen an Geweben und Zelllinien zeigen eine verstärkte MED15-Expression im CRPC im Vergleich zu Primärtumoren sowie nach Androgenentzug und ein MED15-Knockdown unter diesen Bedingungen führt zu geringerer Proliferation und vermehrter Apoptose. Diese Beobachtungen weisen darauf hin, dass MED15 Teil der Resistenzmechnismen unter Androgentzug darstellt und eine MED15-Inhibition die Entwicklung einer Kastrationsresistenz unter bestimmten Bedingungen verlangsamen könnte.

Die von uns ermittelte Korrelation zwischen der MED15-Expression und der Aktivität des TGFβ- und PI3K-Signalweges im Gewebe geben Hinweis darauf, dass MED15 in diese alternativen Überlebenssignalwege involviert ist. In Zelllinien beobachteten wir bereits eine geringere TGFβ-Aktivität in MED15-Knockdown-Zellen. In zukünftigen Studien werden wir analysieren, ob und auf welcher molekularen Grundlage MED15 die PI3K-Signaltransduktion beeinflusst und ob dies die molekulare Grundlage der von uns beobachteten Effekte des MED12- und MED15-Knockdowns darstellt.

1.6 Zusammenfassung und Ausblick

Zusammenfassend weisen unsere Ergebnisse von Untersuchungen an Prostatakarzinomen und Plattenepithelkarzinomen darauf hin, dass MED12 und MED15 an Regulationsmechanismen beteiligt sind, die das Malignitätspotenzial von Tumorzellen steigern. Im Tumorgewebe konnten wir zeigen, dass die MED12- und MED15-

Expression signifikant mit der Expression von Proliferationsmarkern sowie Aktivitätsmarkern potenziell onkogener Signalwege korreliert. Übereinstimmende Ergebnisse konnten wir mittels Experimente an Tumorzelllinien verifizieren. Gleichzeitig beobachteten wir ein vermindertes Wachstum, eine gesteigerte Apoptose und reduzierte Migration von Tumorzellen nach Herunterregulation von MED12 oder MED15. Basierend auf diesen Ergebnissen vermuten wir, dass Patienten in fortgeschrittenen Tumorstadien von einer zielgerichteten Therapie profitieren, die eine Reduktion der Aktivität von MED12 und MED15 beinhaltet. Weitere Untersuchungen werden zeigen, ob ein reduziertes Tumorwachstum oder eine verminderte Metastasenbildung in-vivo durch eine MED12- oder MED15-Herunterregulation erreicht werden kann. In weiteren Schritten müssen Mechanismen entwickelt werden, um MED12 und MED15 zielgerichtet in Zellen zu inhibieren. Da beide Proteine zytoplasmatisch und nukleär lokalisiert sind, stellen kleinmolekulare Inhibitoren, die passiv durch die Zellmembran diffundieren können, eine Möglichkeit dar.

In unseren Untersuchungen stellten wir darüber hinaus fest, dass MED15 mit der Aktivität des PI3K-Signalweges in Prostatakarzinomzellen assoziiert ist. Verschiedene Inhibitoren, die in diesen Signalwegen eingreifen, werden derzeit in zahlreichen Experimenten und bereits in klinischen Studien auf ihre Wirksamkeit geprüft. Im Rahmen zukünftiger Studien sollte untersucht werden, ob eine MED15-Überexpression im Tumorgewebe den Erfolg einer PI3K-Inhibition bei der Tumorreduktion vorhersagen kann, und MED15 somit als prädiktiver Marker für diese Therapiemöglichkeit dienen könnte. Außerdem könnte eine simultane Inhibition der PI3Kinase und MED15 den Effekt auf die Tumorprogression verstärken.

Schlussfolgernd weisen unsere Untersuchungen auf eine Bedeutung von MED12 und MED15 für die Tumorprogression hin und liefern Ausgangspunkte für weitere Studien, um die praktische Relevanz dieser Ergebnisse zu ermitteln.

Die von mir generierten und oben beschriebenen Ergebnisse sind in die der Dissertation beigefügten Publikationen eingeflossen.

1.7 Literaturverzeichnis der deutschen Zusammenfassung

Amaral TM, Macedo D, Fernandes I, Costa L. Castration-resistant prostate cancer: mechanisms, targets, and treatment. Prostate Cancer 2012; 2012: 327253

Barbieri CE, Baca SC, Lawrence MS, Demichelis F, Blattner M, Theurillat JP, White TA, Stojanov P, Van Allen E, Stransky N, Nickerson E, Chae SS, Boysen G, Auclair D, Onofrio RC, Park K, Kitabayashi N, MacDonald TY, Sheikh K, Vuong T, Guiducci C, Cibulskis K, Sivachenko A, Carter SL, Saksena G, Voet D, Hussain WM, Ramos AH, Winckler W, Redman MC, Ardlie K, Tewari AK, Mosquera JM, Rupp N, Wild PJ, Moch H, Morrissey C, Nelson PS, Kantoff PW, Gabriel SB, Golub TR, Meyerson M, Lander ES, Getz G, Rubin MA, Garraway LA. Exome sequencing identifies recurrent SPOP, FOXA1 and MED12 mutations in prostate cancer. Nature Genetics 2012; 44: 685-689

Barrack ER. TGF beta in prostate cancer: a growth inhibitor that can enhance tumorigenicity. Prostate 1997; 31: 61-70

Berti L, Mittler G, Przemeck GK, Stelzer G, Gunzler B, Amati F, Conti E, Dallapiccola B, Hrabe de Angelis M, Novelli G, Meisterernst M. Isolation and characterization of a novel gene from the DiGeorge chromosomal region that encodes for a mediator subunit. Genomics 2001; 74: 320-332

Bitting RL, Armstrong AJ. Targeting the PI3K/Akt/mTOR pathway in castration-resistant prostate cancer. Endocr Relat Cancer 2013; 20: R83-99

Danielpour D. Functions and regulation of transforming growth factor-β (TGF-β) in the prostate. Eur J Cancer 2005; 41: 846–857

Gillmor CS1, Park MY, Smith MR, Pepitone R, Kerstetter RA, Poethig RS.
The MED12-MED13 module of Mediator regulates the timing of embryo patterning in Arabidopsis. Development 2010;137: 113-122

Huang S, Hölzel M, Knijnenburg T, Schlicker A, Roepman P, McDermott U, Garnett M, Grernrum W, Sun C, Prahallad A, Groenendijk FH, Mittempergher L, Nijkamp W, Neefjes J, Salazar R, Ten Dijke P, Uramoto H, Tanaka F, Beijersbergen RL, Wessels LF, Bernards R. MED12 controls the response to multiple cancer drugs through regulation of TGF-β receptor signaling. Cell 2012; 151: 937-950

Jemal A, Bray F, Center MM, Ferlay J, Ward E, Forman D. Global cancer statistics. CA Cancer J Clin 2011; 61: 69-90

Karantanos T, Corn PG, Thompson TC. Prostate cancer progression after androgen deprivation therapy: mechanisms of castrate resistance and novel therapeutic approaches. Oncogene 2013; 32: 5501-5511

Kato Y, Habas R, Katsuyama Y, Naar AM, He X. A component of the ARC/Mediator complex required for TGF beta/Nodal signalling. Nature 2002; 418: 641-646

Lewis BA, Reinberg D. The mediator coactivator complex: functional and physical roles in transcriptional regulation. J Cell Sci 2003; 116: 3667-3675

Mäkinen N, Mehine M, Tolvanen J, Kaasinen E, Li Y, Lehtonen HJ, Gentile M, Yan J, Enge M, Taipale M, Aavikko M, Katainen R, Virolainen E, Böhling T, Koski TA, Launonen V, Sjöberg J, Taipale J, Vahteristo P, Aaltonen LA. MED12, the mediator complex subunit 12 gene, is mutated at high frequency in uterine leiomyomas. Science 2011; 334: 252–255

Malik S, Roeder RG: The metazoan Mediator co-activator complex as an integrative hub for transcriptional regulation. Nat Rev Genet 2010; 11: 761-772

Napoli C, Sessa M, Infante T, Casamassimi A. Unraveling framework of the ancestral Mediator complex in human diseases. Biochemie 2012; 94: 579-587

National Institute for Health and Care Excellence, 2014: Prostate cancer: diagnosis and treatment, NICE guidelines [CG175]. https://www.nice.org.uk/guidance/cg175

Risheg H, Graham JM Jr, Clark RD, Rogers RC, Opitz JM, Moeschler JB, Peiffer AP, May M, Joseph SM, Jones JR, Stevenson RE, Schwartz CE, Friez MJ. A recurrent mutation in MED12 leading to R961W causes Opitz-Kaveggia syndrome. Nat Genet 2007; 39: 451-453

Rocha PP1, Scholze M, Bleiss W, Schrewe H. Med12 is essential for early mouse development and for canonical Wnt and Wnt/PCP signaling. Development 2010; 137: 2723-2731

Schwartz CE, Tarpey PS, Lubs HA, Verloes A, May MM, Risheg H, Friez MJ, Futreal PA, Edkins S, Teague J, Briault S, Skinner C, Bauer-Carlin A, Simensen RJ, Joseph SM, Jones JR, Gecz J, Stratton MR, Raymond FL, Stevenson RE. The original Lujan syndrome family has a novel missense mutation (p.N1007S) in the MED12 gene. J Med Genet. 2007; 44: 472-477

Tutter AV1, Kowalski MP, Baltus GA, Iourgenko V, Labow M, Li E, Kadam S. Role for Med12 in regulation of Nanog and Nanog target genes. J Biol Chem 2009; 284: 3709-3718

Vo BT, Morton D Jr, Komaragiri S, Millena AC, Leath C, Khan SA. TGF-β effects on prostate cancer cell migration and invasion are mediated by PGE2 through activation of PI3K/AKT/mTOR pathway. Endocrinology 2013; 154: 1768-1779

Warnakulasuriya S. Global epidemiology of oral and oropharyngeal cancer. Oral Oncol 2009; 45: 309-316

World Health Organization, 2015: Fact sheet N°297. http://www.who.int/mediacentre/factsheets/fs297/en/

Yang F, Vought BW, Satterlee JS, Walker AK, Jim Sun ZY, Watts JL, DeBeaumont R, Saito RM, Hyberts SG, Yang S, Macol C, Iyer L, Tjian R, van den Heuvel S, Hart AC, Wagner G, Naar AM. An ARC/Mediator subunit required for SREBP control of cholesterol and lipid homeostasis. Nature 2006; 442: 700-704

Zhao M, Yang X, Fu Y, Wang H, Ning Y, Yan J, Chen YG, Wang G. Mediator MED15 modulates transforming growth factor beta (TGFbeta)/Smad signaling and breast cancer cell metastasis. J Mol Cell Biol 2013; 5: 57-60

Int J Cancer. 2014 Jul 1;135(1):19-26. doi: 10.1002/ijc.28647. Epub 2013 Dec 9.

MED15, encoding a subunit of the mediator complex, is overexpressed at high frequency in castration-resistant prostate cancer.

Shaikhibrahim Z[1], Menon R, Braun M, Offermann A, Queisser A, Boehm D, Vogel W, Rüenauver K, Ruiz C, Zellweger T, Svensson M, Andren O, Kristiansen G, Wernert N, Bubendorf L, Kirfel J, Biskup S, Perner S.

[1]Department of Prostate Cancer Research, University Hospital of Bonn, Bonn, Germany; Institute of Pathology, University Hospital of Bonn, Bonn, Germany.

Abstract

The mediator complex is an evolutionary conserved key regulator of transcription of protein-coding genes and an integrative hub for diverse signaling pathways. In this study, we investigated whether the mediator subunit MED15 is implicated in castration-resistant prostate cancer (CRPC). MED15 expression and copy number/rearrangement status were assessed by immunohistochemistry (IHC) and fluorescence in situ hybridization (FISH), respectively on 718 prostate cancer (PCa) specimens and sequenced by Sanger on a subset. Furthermore, SMAD3 phosphorylation, androgen receptor (AR) and proliferation markers were evaluated by IHC. In PCa cells, siRNA/shRNA knockdown of MED15 was followed by proliferation assays with/without dihydrotestosterone (DHT), and treatments with recombinant TGF-β3. Our results show that MED15 is overexpressed in 76% of distant metastatic CRPC (CRPC(MET)) and 70% of local-recurrent CRPC (CRPC(LOC)), in contrast to low frequencies in androgen-sensitive PCa, and no expression in benign prostatic tissue. Furthermore, MED15 overexpression correlates with worse clinical outcome thus defining a highly lethal phenotype. Moreover, TGF-β signaling activation associates with MED15 overexpression in PCa tissues, and leads to increased expression of MED15 in PCa cells. MED15 knockdown effects phosphorylation and shuttling of p-SMAD3 to the nucleus as well as TGF-β-enhanced proliferation. In PCa tissues, MED15 overexpression associates with AR overexpression/amplification and correlates with high proliferative activity. MED15 knockdown decreases both androgen-dependent and -independent proliferation in PCa cells. Taken together, these findings implicate MED15 in CRPC, and as MED15 is evolutionary conserved, it is likely to emerge as a lethal

phenotype in other therapeutic-resistant diseases, and not restricted to our disease model.

Introduction

Since the groundbreaking discovery of the multi-subunit complex mediator by Kornberg et al. in yeast,[1] and subsequently its identification in humans,[2] mediator emerged as a key regulator of protein coding-genes, and an integrative hub for diverse signaling pathways.[3] Mediator is comprised of distinct modules designated as “head,” “middle,” and “tail,” which form a relatively stable “core” structure, and a “kinase” module, which associates reversibly with the complex.[3] Generally, the head and middle modules are involved in interactions with the core RNA polymerase II (pol II)-associated basal transcription machinery, whereas the tail and kinase modules interact with activators and serve as a major locus for signal transduction and signaling pathways, respectively.[3] Even though the link between various mediator subunits and human diseases, ranging from neurological, cardiovascular, metabolic disorders to cancer is emerging, yet, we are still in the early stages of unraveling all of the activities and interactions of which the mediator complex is potentially capable.[3]

In an effort to unravel new activities of the mediator complex, we aimed in this study at investigating whether the mediator complex through its tail module subunit MED15 which is essential for transforming growth factor-β (TGF-β)/activin/nodal/Smad2/3 signal transduction,[4] is implicated in castration-resistant prostate cancer (CRPC) as a prototype of therapeutic-resistant diseases.[5] CRPC is invariably the lethal form of prostate cancer (PCa) with minimal treatment options and an average survival rate of few months to a couple of years thus remains a significant therapeutic challenge.

Material and Methods

This study was approved by the Internal Review Board of the University Hospital of Bonn in accordance with the Declaration of Helsinki.

Cohorts

Seven hundred and eighteen patients including 110 nonmetastasized primary PCa, 89 locally advanced primary PCa, 92 lymph node metastasized primary PCa with

corresponding lymph node metastases, and 160 CRPC (90 local recurrent CRPC (out of these, 28 with corresponding locally advanced primary PCa), 70 samples distant metastatic CRPC, 123 prostatectomy specimens from the University Hospital of Basel in Switzerland, and 172 from the University Hospital of Örebro in Sweden. Available patient clinicopathological data are also provided (Supporting Information Table 2).

Tissue microarrays (TMA)

TMAs were constructed as described earlier.[6]

Fluorescence in situ hybridization (FISH)

For AR amplification assay, we used a target probe (RP11–479J1, Invitrogen, Paisley, UK) and a centromeric probe for chromosome X (MetaSystems, Altlussheim, Germany); for MED14 copy number assay, a target probe (CTD-2375G6, Invitrogen) and a centromeric probe for chromosome X (MetaSystems), for MED15 copy number assay, a target probe (CTD-2544022, Invitrogen) and a centromeric probe for chromosome 22 (Abbott, Wiesbaden, Germany); for the MED15 rearrangement assay, two probes spanning the locus of MED15: a centromeric probe (CTD-2319K10, Invitrogen) and a centromeric probe (CTD-2576J23, Invitrogen). ERG break-apart FISH and FISH evaluation were performed as described earlier[7].

Immunohistochemistry (IHC)

IHC was conducted using the following primary antibodies (dilutions, clones/catalogue number, manufacture): anti-MED15 mouse monoclonal (1:50, 11566-1-AP, Proteintech, Chicago, IL), anti-MED14 antibody Rabbit Polyclonal (1:100, NBP1–67781, Novus Bioloicals, Germany), anti-PHH3 rabbit polyclonal (1:100, CMC36911010; Cell Marque, Rocklin, CA), ready-to-use anti-Ki-67 rabbit monoclonal (clone 30-9, Ventana, AZ), anti-AR mouse monoclonal (1:100, AR441, Dako, Glostrup, Denmark), anti-p-SMAD3 rabbit monoclonal (1:50, D12E11, Cell Signalling, Danvers, MA), and anti-ERG rabbit monoclonal (1:100, EPR3864, Abcam, Cambridge, MA). Positive and negative controls for antibodies against MED15 and p-SMAD3 in tissues was also performed (Supporting Information Fig. 4). Quantification of protein expression was evaluated according to the Immunoreactivity Score (IRS).[8] Briefly, IRS considers both the amount of immunoreactive cells as well as the intensity of the staining. IRS is calculated as

“percentage of immunreactive tumor cells” (0□=□0%; 1□≤□10%; 2□=□10–50%; 3□=□51–80%; 4□≥□80%) × “staining intensity” (from 0□=□no expression to 3□=□strong expression). An IRS of 0 was interpreted as none or a very weak expression. An IRS from 1 to 3 was interpreted as a weak to medium expression. An IRS of 4 and above was interpreted as a high expression (overexpression). We chose these categories prior to the analyses to avoid the risk of “data fitting.” We decided on a score of 4 or higher, as it reflects cases which either visibly express MED15 in virtually all cancer cells, or harbor at least a marked or strong expression in 11–50% (or more) of cancer cells. Of note, the vast majority of statistical correlations, e.g., with androgen receptor (AR) amplification or proliferation was performed with the full-range MED15 IRS, and not the dichotomized categories (i.e., overexpression vs. weak to medium expression). Therefore, the distinction between overexpressing and non-overexpressing cases had no effect on these analyses.

Cell lines

DU145, PC3, LNCaP, VCaP and BPH1 cells were cultured according to ATCC guidelines.

Immunofluorescence (IF)

IF was performed using an anti-MED15 mouse monoclonal (1:50, 11566-1-AP, Proteintech, Chicago, IL) on the DU145, PC3, LNCaP, VCaP and BPH1 cells according the published protocol for cultured cell lines by Cell Signaling Technology (Danvers, MA).

SiRNA knockdown

Sequences for siRNAs pools (Dharmacon, Lafayette, CO): MED15: 5′CCAAGACCCGGGACGAAUA, GGGUGUUGUUAGAGCGUCU3′, 5′GGUCAGUCAAAUCGAGGAU3′, 5′CCGGACAAGCACUCGGUCA3′. PC3 and DU145 cells were transfected with 200 nmol L^{-1} of siRNA using Lipofectamine RNAiMAX (Invitrogen, Karlsruhe, Germany) for 48 hr. LNCaP cells were grown in charcoal-stripped serum, in phenol red free medium for 3 days followed by 200 nmol L^{-1} siRNA transfection for 48 hr. Two days post-transfection, medium with or without 100 nmol L^{-1}

DHT (Sigma Aldrich, Steinheim, Germany) was added for an additional 24 hr.

ShRNA stable knockdown

Lentivirus generation, harvesting and infection were performed according to the manufacturer's instructions (Open Biosystems and Thermo Scientific). GFP-containing pGIPZ vector constructs expressing shRNAmir against MED15 or scrambled shRNAmir were used as well as the Trans-Lentiviral Packaging System.

Western blot

Cell lysates and nuclear extracts of PC3 (sc-2152, Santa Cruz, CA) and DU145 (sc-24960, Santa Cruz, CA) were analyzed by immunobloting using MED15 rabbit polyclonal antibody (Proteintech, Chicago, IL). P-SMAD3 expression in cell lysates was analyzed using anti-p-SMAD3 rabbit monoclonal (1:1000, EP823Y, Abcam, Cambridge, UK).

QRT-PCR

RNA was isolated using RNeasy Mini Kit (Qiagen, Germany) and reverse transcribed using an iScript cDNA synthesis kit, according to manufacturer's instructions (Biorad, Germany). PCR reactions with a Power SYBR Green kit were performed according to the manufacturer's instructions (Applied Biosystems, Darmstadt, Germany). Reactions were performed using Light-Cycler 480 II (Roche, Mannheim, Germany). For each sample in a given experiment, duplicate reactions were performed, as well as duplicate reactions with β-actin as reference. Fold changes were calculated using the formula 2^ddCT. Primer sets used were PAI-1-F 5′-attcaagcagctatgggattcaa-3′; PAI-1-R 5′-ctggacgaagatcgcgtctg-3′. Primer sets used for MED15-F 5′-caaggcttccgtgatcatct-3′ and MED15-R 5′-agcagacagcagtacagacagc-3′.

TGF-β treatments

For investigating the effect of TGF-β signaling upon MED15 overexpression, PC3 cells were serum starved in a medium containing 2% fetal calf serum (FCS) for 48 hr, then treated with recombinant TGF-β3 (Immunotools, Friesoythe, Germany) at concentrations of 5, 10 and 40 ng mL^{-1}. Cells were harvested after 24 hr for RNA extraction, and after

72 hr for protein analysis. For the effect of MED15 knock-down upon TGF-ß signaling by Western blot, PC3 cells with or without MED15 knock-down were treated with recombinant TGF-ß3 at a concentration of 10 ng mL^{-1} for 1 hr, and protein lysates were prepared thereafter.

On the other hand, TGF-β treatments for the purpose of IHC evaluations were performed as follows. PC3 cells were seeded on slides in medium containing 10% FCS. When cells were attached, cells were serum starved in a medium containing 2% FCS for 48 hr, and then treated with starved medium with or without a 10 ng mL^{-1} TGF-β3 for 72 hr. Slides were then washed with Phosphate buffered saline (PBS) and cells were fixed in paraformaldehyde overnight. IHC using a primary antibody against MED15 was then performed. MED15 cytoplasmic and nuclear expression was analyzed by two independent pathologists. For the effect of MED15 knock-down upon TGF-β signaling by IHC, PC3 cells were seeded in six-well plates and MED15 knockdown was performed using siRNA specific for MED15 or scrambled siRNA as control. After 48 hr, cells were harvested, plated on slides and serum starved for 48 hr. Cells were then treated with starved medium with and without a 10 ng mL^{-1} TGF-β3 for 2 hr, washed with PBS and fixed in paraformaldehyde overnight. IHC was performed using a primary antibody against p-SMAD3. P-SMAD3 expression in the cytoplasm and the nucleus was analyzed by two independent pathologists.

Cell proliferation assay

Following siRNA transfection/stable knockdown, cells were analyzed for proliferation using the MTT assay (Roche, Mannheim, Germany). Proliferation was measured after 24, 48 and 72 hr. Each experiment was repeated in triplicates and 3× independently. Following siRNA transfection and TGF-β3 treatments, cells were analyzed as above after 48 hr.

Sanger sequencing (PCR amplification and sequence analysis of MED15)

Sanger sequencing of MED15 was performed on seven castration resistant prostate cancer samples. Mutational screen of genomic DNA of all coding exons and flanking intron sequences of MED15 using PCR (AmpliTaq Gold® Fast PCR Master Mix, Invitrogen, Darmstadt, Germany) was performed according to manufacturer's

instructions. Sequencing PCR reactions were performed using the BigDye Version 3.1 cycle sequencing kit according to manufacturer's instructions. Amplified fragments were sequenced on the ABI 3730xl automated sequencer using the BigDye Version 3.1 cycle sequencing kit (Applied Biosystems, Darmstadt, Germany).

Statistical analyses

Statistical analyses were performed using SPSS 20 (SPSS, Chicago, IL).

Results

MED15 nuclear expression in PCa tissues and cell lines

Immunhistochemical staining (IHC) shows MED15 nuclear expression in distant metastatic CRPC ($CRPC^{MET}$), local recurrent CRPC ($CRPC^{LOC}$), androgen-sensitive PCa which includes non-metastasised primary PCa (PCa^{N0}), lymph node metastasised primary PCa with corresponding lymph node metastases (PCa^{N1}), and in an independent primary PCa validation cohort (PCa^{VAL}) (Figs. 1a and 1c). On the other hand, benign prostatic tissues lacked MED15 nuclear expression, and exhibited only a cytoplasmic expression. Defining overexpression as expression >3 Remmele score, we found MED15 to be overexpressed at much higher frequency in CRPC compared to androgen-sensitive PCa (Fig. 1d).

In the PCa cell lines, IHC shows MED15 nuclear expression in metastatic androgen-sensitive (LNCaP and VCaP) and $CRPC^{MET}$ (PC3 and DU145) as well as benign prostate cells (BPH1) (Supporting Information Fig. 1a). We further confirmed these observations by immunofluorescence (Supporting Information Fig. 1c) and Western blot (Figs. 5b and 5c and Supporting Information Fig. 1d).

MED14 lack of nuclear expression in PCa tissues and cell lines

IHC staining shows a lack of a nuclear expression of MED14 in androgen-sensitive PCa as well as CRPC (Fig. 1b). In the PCa cell lines, IHC staining showed a lack of MED14 nuclear expression (Supporting Information Fig. 1b).

Copy number and rearrangement status of MED15

Using fluorescence in situ hybridization (FISH) assays, we found MED15 to be amplified in only one $CRPC^{MET}$ case (Supporting Information Figs. 2a–2d) and non-rearranged (Supporting Information Figs. 2e and 2f).

MED15 overexpression and clinical outcome

Using the Kaplan–Meier analysis, we estimated the effect of MED15 nuclear expression on overall survival of PCa^{LOC} overexpressing MED15. We found MED15 nuclear expression to correlate with worse clinical outcome and thus represent a highly lethal phenotype (Fig. 2a).

MED15 overexpression and AR in PCa tissues

In contrast to low/lack of MED15 expression, we observed that MED15 overexpression significantly associates with AR overexpression and amplification in PCa (Chi-Square $p < 0.001$, $p < 0.001$, respectively) (Supporting Information Table 1).

MED15 overexpression and ERG rearrangement in CRPC

We found MED15 to be overexpressed in 76% of $CRPC^{MET}$, whereas ERG rearrangement in 26% of cases. Furthermore, MED15 expression inversely correlates with ERG rearrangement in PCa (Pearson $R = -0.175$, $p < 0.01$) (Supporting Information Table 1).

MED15 overexpression and TGF-β signaling in PCa tissues

Immunhistochemical staining of p-SMAD3 as an indicator of TGF-β signaling was performed on PCa tissues (Figs. 2b and 2c). In contrast to low/lack of MED15 expression; we found MED15 overexpression to significantly associate with p-SMAD3 in PCa (Chi-Square $p < 0.001$).

TGF-β signaling activation and MED15 expression in PCa cells

Treatment of PC3 cells with recombinant TGF-β3 leads to the activation of TGF-β signaling as indicated by the increased transcriptional levels of TGF-β target gene PAI (Fig. 3a) and increased protein expression of p-SMAD3 (Fig. 3b). TGF-β signaling activation also results in increased levels of MED15 at both the mRNA and protein levels

(Figs. 3d–3f).

Effect of MED15 upon TGF-β signaling and TGF-β-enhanced proliferation in PCa cells

MED15 knockdown effects the phosphorylation (Figs. 4a–4d) and shuttling (Figs. 4a–4c) of p-SMAD3 to the nucleus. Knockdown of MED15 reduces TGF-β-enhanced cellular proliferation in PC3 cells (Figs. 4e and 4f). Briefly, to show that TGF-ß results in increased proliferation and that MED15 knockdown leads to a reduction in TGF-β-enhanced proliferation, the absorbance was normalized to one in the MTT assay of cells not treated with TGF-β with or without MED15 knockdown. This then illustrates the relative enhancement of proliferation in TGF-β treated cells with and without MED15 knockdown, respectively (Figs. 4e and 4f).

MED15 and proliferation in PCa tissues and cell lines

MED15 overexpression correlates with high proliferative activity in PCa tissues as indicated by the tumor proliferation markers Ki67 and pHH3 (Fig. 5a). Knockdown of MED15 decreased proliferation in both androgen-dependent dihydrotestosteron (DHT) treated LNCaP and -independent $CRPC^{MET}$ DU145 and PC3 cells (Figs. 5d−5k).

Sanger sequencing of MED15 in CRPC

We performed Sanger sequencing of the entire MED15 (18 exons) in a series of seven $CRPC^{MET}$ samples, which revealed a three base-pair insertion (CAG) in 2 patients in exon 7 (rs361923), and several base-pair substitutions in introns 5, 12, 14 and 15 in four patients (Supporting Information Fig. 3). The CAG insertion was found to be inframe thus does not result in an amino acid change.

Discussion

As the link between various mediator subunits and human diseases is emerging,[3] we investigated in this study whether the mediator complex subunit MED15 which is essential for TGF-β/activin/nodal/Smad2/3 signal transduction[4] is implicated in castration-resistant prostate cancer (CRPC).

Strikingly, we found MED15 to be overexpressed in 76% of distant metastatic CRPC

($CRPC^{MET}$) and 70% of local-recurrent CRPC ($CRPC^{LOC}$), in contrast to low frequencies in androgen-sensitive PCa, and no expression in benign prostatic tissue (Figs. 1a, 1c and 1d). We further found that MED15 overexpression is a specific to MED15, and is not part of a general overexpression of the entire tail module, as MED14, a subunit of the tail module directly neighboring MED15 exhibited a lack of nuclear expression (Fig. 1b). In support of our tissue findings, MED15 shows a very strong nuclear expression in PCa cell lines (Supporting Information Figs. 1a and 1c), whereas MED14 lacks a nuclear expression (Supporting Information Fig. 1b). Prompted by the high frequency of MED15 overexpression in CRPC, we investigated and found that MED15 overexpression was not a result of gene amplification (Supporting Information Figs. 2a–2d) or rearrangement (Supporting Information Figs. 2e and 2f).

Interestingly, we found MED15 overexpression to correlate with worse clinical outcome in PCa^{LOC}, indicating that PCa^{LOC} overexpressing MED15 represents a highly lethal phenotype (Fig. 2a). Based on these findings, MED15 expression seems to be of a prognostic value, as patients with a low and medium MED15 expression had a significantly higher median survival (4.7 years) as compared to patients with MED15 overexpression (2.5 years ($p \bullet < \bullet 0.02$). Moreover, the 5-year survival rate was around 50% for patients with low and medium MED15 expression as compared to 20% for patients with MED15 overexpression. This observation suggests that a high MED15 expression defines an even more aggressive CRPC phenotype.

Notably, in contrast to low/lack of MED15 expression, MED15 overexpression significantly associates with AR overexpression and amplification in PCa (Supporting Information Table 1). Furthermore, as the rearrangement of ERG is common in PCa,[9] we found that MED15 overexpression is even more common in $CRPC^{MET}$ than the ERG rearrangement (76% vs. 26%) and that MED15 expression inversely correlates with ERG rearrangement in PCa (Supporting Information Table 1).

As MED15 has been reported to essential for TGF-ß signal transduction,[4] we assessed the activation of the TGF-β signaling as indicated by SMAD3 phosphorylation levels (p-SMAD3) in relation to MED15 expression status in PCa (Figs. 2b and 2c). In contrast to low/lack of MED15 expression, MED15 overexpression significantly associates with p-SMAD3 in PCa. Of note, SMAD3 has been reported to be overexpressed in human PCa tissues and to be necessary for progressive growth of

PCa cells in nude mice.[10]

As the above clinical findings indicate that the activation of TGF-β signaling may lead to the overexpression of MED15, we investigated whether the activation of TGF-β signaling leads to MED15 overexpression in PCa cell lines. As amongst all PCa cell lines PC3 cells has been shown to express TGF-β receptors and the highest expression of TGF-ß3, which was shown to be significantly more potent than TGF-β1 in increasing migration and invasion in PC3 cells,[11] we treated PC3 cells with recombinant TGF-β3 protein. We found that TGF-β3 treatment leads to the activation of the TGF-β signaling as indicated by increased transcriptional level of the TGF-β target gene PAI (Fig. 3a) and protein expression of p-SMAD3 (Fig. 3b). We further show that such a TGF-β signaling activation leads to a high increase in MED15 protein level (Figs. 3d–3f), but a marginal increase at the mRNA level (Fig. 3c). In support of our findings, Zhao et al. reported recently that in breast cancer cells, MED15 could be upregulated at both mRNA as well as protein levels by TGF-β treatment.[12] A close examination of Zhao et al.,' results seem to show a marginal increase in MED15 at the RNA level in comparison to the protein level. Interestingly, Zhao et al. further showed that the regulation of MED15 by TGF-β signaling is not at the transcriptional level, thus suggested that MED15 is likely an indirect target of TGF-β. However, the authors suggested that activated TGF-β may demand for higher MED15 protein levels to transduce signaling to the nucleus.

Furthermore, we investigated the impact of MED15 upon TGF-β signaling in PCa cells, and found that MED15 knockdown effects the phosphorylation and shuttling of p-SMAD3 to the nucleus (Figs. 4a – 4d). In support of our results, Zhao et al. showed in breast cancer, MED15 deficiency reduces the phosphorylation and nuclear accumulation of SMAD2/3 and affects the nucleocytoplasmic shuttling of Smad2/3. Zhao et al. were also able to show that SMAD3 interacts with MED15, and that the interaction is important for SMAD3 transcriptional activation, along with MED15 knockdown reducing the stability of p-SMAD2.[12]

Our results from prostate cancer cells combined with Zhao et al.' findings in breast cancer cells suggest that activated TGF-β demand for higher MED15 protein levels to transduce signaling to the nucleus, and that MED15 via its interaction with SMAD3 influences the receptor downstream intracellular signaling by acting upon the phosphorylation and shuttling of p-SMAD3 to the nucleus.

The above findings then led us to examine whether activation of TGF-β signaling promotes cellular proliferation in PC3 cells, and whether the knockdown of MED15 may affect TGF-β-enhanced cellular proliferation. Interestingly, we found that activation of TGF-β signaling promotes proliferation in PC3 cells (Fig. 4e). It is worth noting that other studies have observed that TGF-β treatment has no effect upon proliferation in PC3 cells.[13, 14] In these studies, cells were first serum-starved and then treated with different concentrations of TGF-β (1 or 10 ng mL^{-1}) in the presence of just 5% FCS [13, 14] whereas in our study, we plated the cells in normal medium containing 10% FCS and treated with a 40 ng mL^{-1} TGF-β (Fig. 4e). Additionally, it has been reported that TGF-β inhibits proliferation only when cells were plated in serum-free medium [15]. It was observed that in the presence of basic fibroblast growth factor, TGF-β did not inhibit proliferation.[15] Moreover, we found that knockdown of MED15 reduces TGF-β-enhanced cellular proliferation in PC3 cells (Figs. 4e and 4f).

Furthermore, we found MED15 overexpression to correlate with high proliferative activity in PCa tissues (Fig. 5a), and that knockdown of MED15 decreases proliferation in both androgen-dependent-DHT treated cells as well as -independent cells (Figs. 5d−5k).

To have a more comprehensive analysis of MED15, we performed Sanger sequencing of MED15 in 7 $CRPC^{MET}$ patients, and found only an insertion residing in exon 7 in 3/7 $CRPC^{MET}$ that was inframe and does not result in an amino acid change, as well as several intronic substitutions (Supporting Information Fig. 3).

Taken together, our findings implicate MED15 in CRPC as a model of therapeutic-resistant diseases. As MED15 is conserved, it is likely to emerge as a phenotype in other therapeutic-resistant diseases, and not restricted to our disease model.

References

Kelleher RJ, III, Flanagan PM, Kornberg RD. A novel mediator between activator proteins and the RNA polymerase II transcription apparatus. Cell 1990;61:1209–15.

Fondell JD, Ge H, Roeder RG. Ligand induction of a transcriptionally active thyroid hormone receptor coactivator complex. Proc Natl Acad Sci USA 1996;93:8329–33.

Malik S, Roeder RG. The metazoan mediator co-activator complex as an integrative hub for transcriptional regulation. Nat Rev 2010;11:761–72.

Kato Y, Habas R, Katsuyama Y, et al. A component of the ARC/mediator complex required for TGF beta/Nodal signaling. Nature 2002;418:641–6.

Amaral TM, Macedo D, Fernandes I, et al. Castration-resistant prostate cancer: mechanisms, targets, and treatment. Prostate Cancer 2012;2012:327253.

Braun M, Scheble VJ, Menon R, et al. Relevance of cohort design for studying the

frequency of the ERG rearrangement in prostate cancer. Histopathology 2011;58:1028–36.
Direct Link:
Perner S, Demichelis F, Beroukhim R, et al. TMPRSS2:ERG fusion-associated deletions provide insight into the heterogeneity of prostate cancer. Cancer Res 2006;66:8337–41.
Remmele W, Stegner HE. Recommendation for uniform definition of an immunoreactive score (IRS) for immunohistochemical estrogen receptor detection (ER-ICA) in breast cancer tissue. Pathologe 1987;8:138–40.
Tomlins SA, Rhodes DR, Perner S, et al. Recurrent fusion of TMPRSS2 and ETS transcription factor genes in prostate cancer. Science (New York, NY) 2005;310:644–8.
Lu S, Lee J, Revelo M, et al. Smad3 is overexpressed in advanced human prostate cancer and necessary for progressive growth of prostate cancer cells in nude mice. Clin Cancer Res 2007;13:5692–702.
Walker L, Millena AC, Strong N, et al. Expression of TGFbeta3 and its effects on migratory and invasive behavior of prostate cancer cells: involvement of PI3-kinase/AKT signaling pathway. Clin Exp Metastasis 2013;30:13–23.
Zhao M, Yang X, Fu Y, et al. Mediator MED15 modulates transforming growth factor beta (TGFbeta)/Smad signaling and breast cancer cell metastasis. J Mol Cell Biol 2013;5:57–60.
Vo BT, Cody B, Cao Y, et al. Differential role of Sloan-Kettering Institute (Ski) protein in Nodal and transforming growth factor-beta (TGF-beta)-induced Smad signaling in prostate cancer cells. Carcinogenesis 2012;33:2054–64.
Walker L, Millena AC, Strong N, et al. Expression of TGFbeta3 and its effects on migratory and invasive behavior of prostate cancer cells: involvement of PI3-kinase/AKT signaling pathway. Clin Exp Metastasis 2012;30:13–23.
Morton DM, Barrack ER. Modulation of transforming growth factor beta 1 effects on prostate cancer cell proliferation by growth factors and extracellular matrix. Cancer Res 1995;55:2596–602.

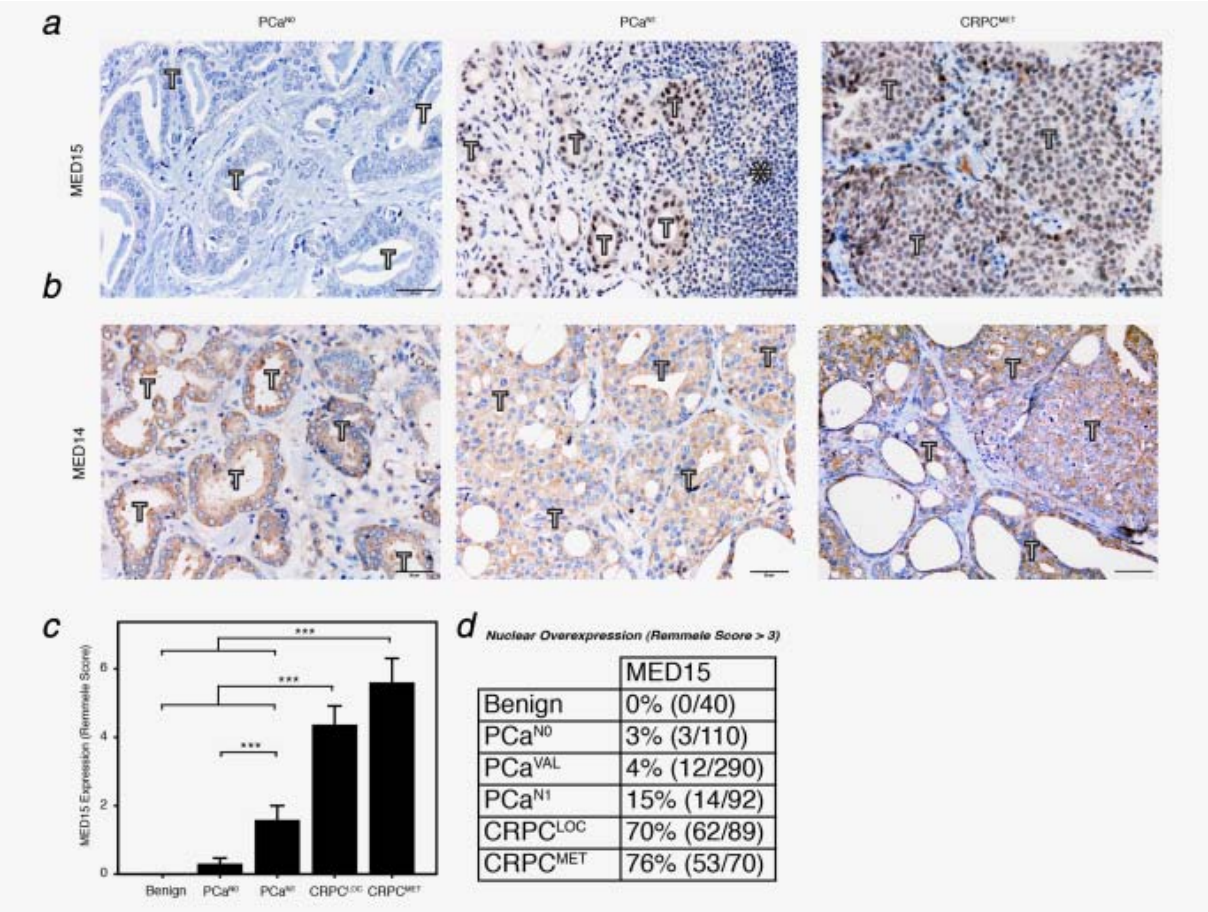


Figure 1. MED15 and MED14 expression in prostate cancer. Immunhistochemical staining of PCa^{N0}, PCa^{N1} and $CRPC^{MET}$ for MED15 (*a*) and MED14 (*b*). "T": prostate cancer; asterisks (*): lymphoid tissue. Scale bar, 20 µm. MED15 nuclear expression in $CRPC^{LOC}$ and $CRPC^{MET}$, as compared to androgen-sensitive PCa and benign tissues. ***p < 0.001, Kruskal–Wallis test (ANOVA) (*c*). Table summarizing the percentage of cases with *MED15* nuclear overexpression (expression > 3 Remmele score) (*d*).

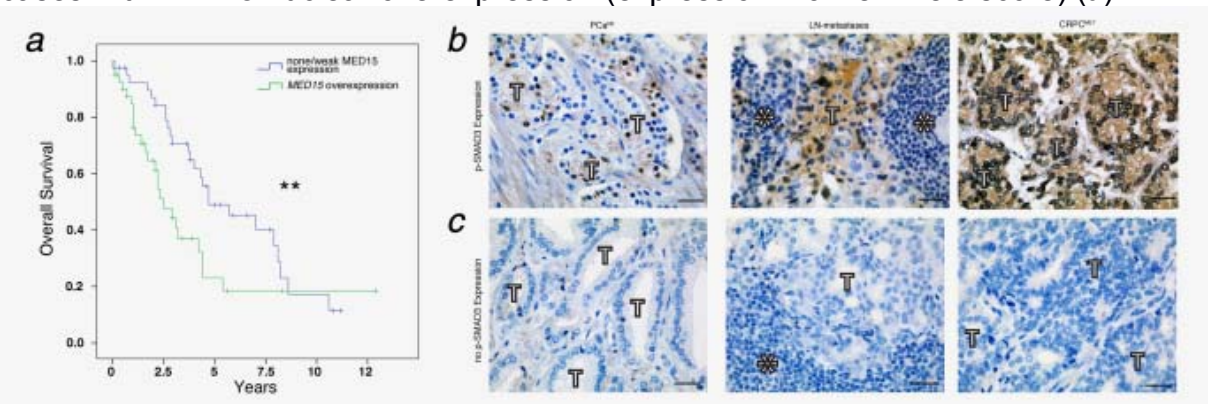


Figure 2. Correlation of MED15 with clinical outcome and association with TGF-β signaling. Kaplan–Meier estimates the effect of MED15 nuclear expression on overall survival in patients with PCa^{LOC} (*a*) ** p < 0.02, log-rank test. Immunhistochemical staining of p-SMAD3 in PCa^{N0}, LN-metastases and $CRPC^{MET}$ (*b*,*c*). "T": prostate cancer;

asterisks (*): lymphoid tissue. Scale bar, 10 μm

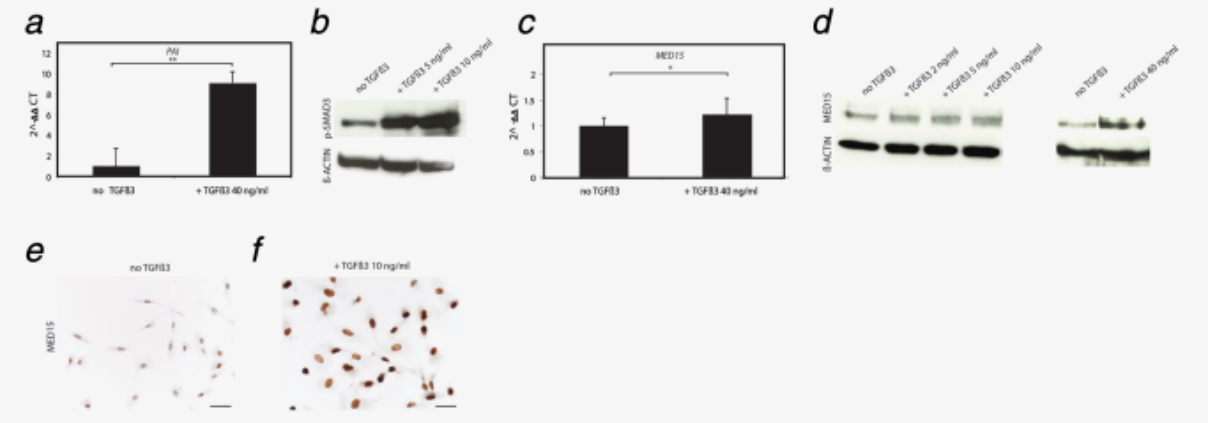


Figure 3. Effect of TGF-ß signaling upon MED15 expression. qRT-PCR of TGF-β target gene *PAI* (*a*) and *MED15* (*c*) of PC3 cells treated with recombinant TGF-β3 for 24 hr. Western blot of p-SMAD3 (*b*) and MED15 following treatment with recombinant TGF-β3 of PC3 cells for 72 hr (*d*). Immunohistochemistry of MED15 following treatment with recombinant TGF-β3 in PC3 cells for 72 hr (*e*,*f*). *p□<□0.05, **p□<□0.01, ***p□<□0.001. Scale bar, 10 μm.

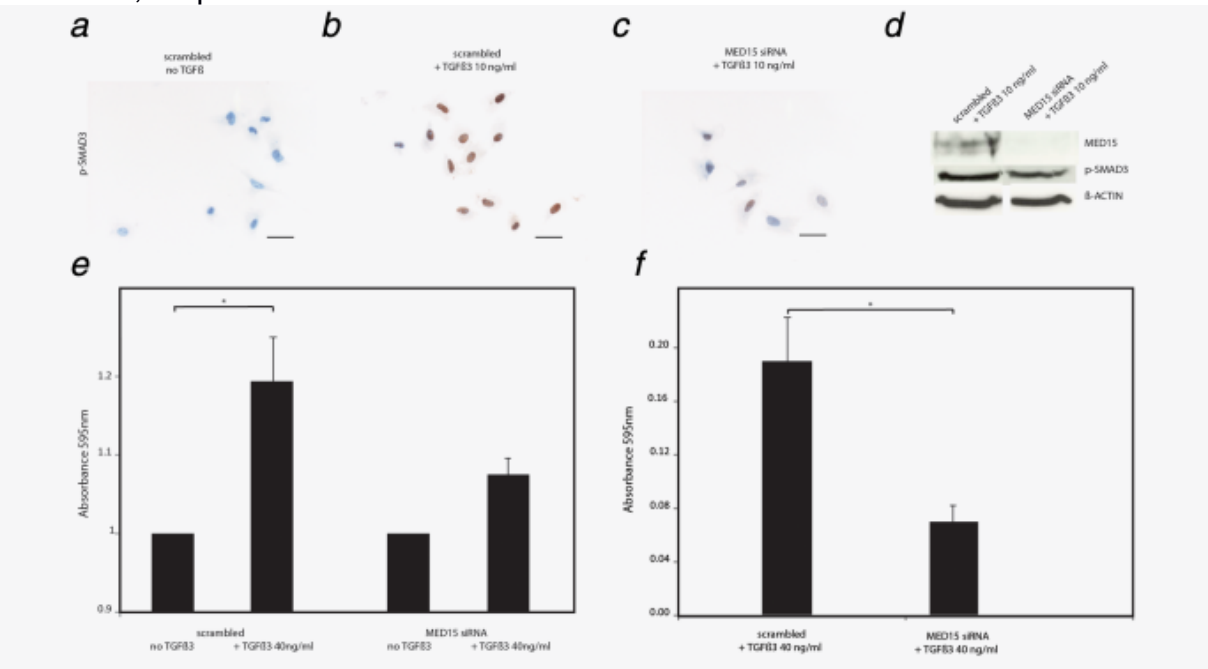


Figure 4. Effect of MED15 on TGF-β signaling and TGF-β-enhanced proliferation. Immunohistochemistry of p-SMAD3 in untreated (*a*) PC3 cells and following treatment with recombinant TGF-β3 of PC3 cells treated before with scrambled siRNA (*b*) or siRNA specific for *MED15* (*c*), as well as Western blotting (*d*). MTT proliferation assay of untreated and TGF-β3 treated PC3 cells for 72 hr with or without MED15 knockdown. Absorbance of untreated cells was normalized to one. (*e*). Relative enhancement of

absorbance after TGF-β treatment in control and MED15 knockdown PC3 cells (*f*) *p□<□0.05, **p□<□0.01, ***p□<□0.001. Scale bar, 10 μm.

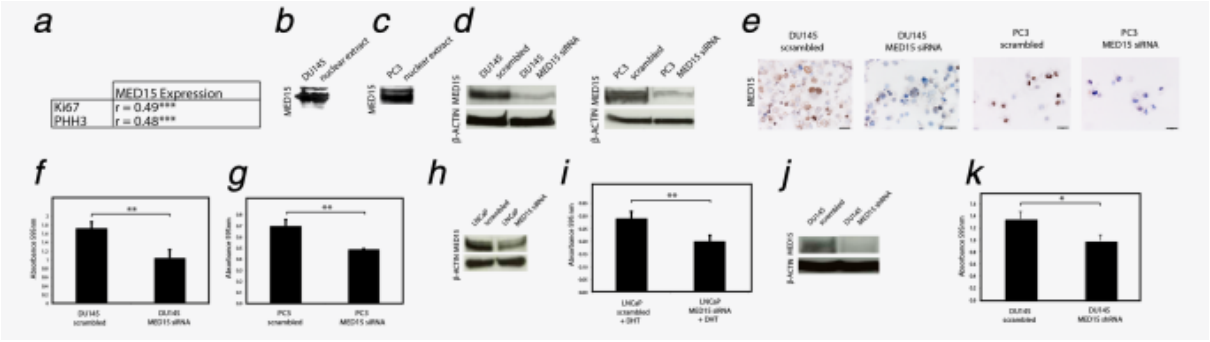


Figure 5. MED15 knockdown and proliferation in PCa cells. Correlation of MED15 expression with tumor proliferation markers Ki67 and PHH3 (*a*). Expression of MED15 in PC3 (*b*) and DU145 (*c*) nuclear extracts by Western blot. SiRNA mediated *MED15* knockdown in DU145 and PC3 cell lines by Western blot (*d*) and IHC (*e*). MTT proliferation assay of PC3 cells (*f*). *p□<□0.05, **p□<□0.01, ***p□<□0.001. Similarly, proliferation assay of DU145 cells (*g*). *p□<□0.05, **p□<□0.01, ***p□<□0.001. SiRNA mediated *MED15* knockdown in LNCaP cell line (*h*). Proliferation assay of LNCaP cells treated with DHT (*i*). *p□<□0.05, **p□<□0.01, ***p□<□0.001. ***p□<□0.001, Pearson. ShRNA stable knockdown of *MED15* in the DU145 cell line (*j*). Proliferation assay of DU145 cells with stable knockdown of *MED15* compared to the scrambled control (*k*). *p□<□0.05, **p□<□0.01, ***p□<□0.001. Scale bar, 10 μm.

Endocr Relat Cancer. 2014 Aug;21(4):663-75. doi: 10.1530/ERC-14-0171. Epub 2014 Jun 17.

MED12 overexpression is a frequent event in

castration-resistant prostate cancer

Zaki Shaikhibrahim1,2,*, Anne Offermann1,2,*, Martin Braun1,2, Roopika Menon1,2, Isabella Syring1,2,9, Michael Nowak1,2, Rebecca Halbach1,2, Wenzel Vogel1,2, Christian Ruiz3, Tobias Zellweger4, Cyrill A Rentsch5, Maria Svensson6, Ove Andren6,7, Lukas Bubendorf3, Saskia Biskup8, Stefan Duensing10, Jutta Kirfel2 and Sven Perner1,2

1 Department of Prostate Cancer Research, 2 Institute of Pathology, University Hospital of Bonn, Sigmund-Freud Strasse 25, 53127 Bonn, Germany
3 Institute for Pathology, University Hospital Basel, Basel, Switzerland
4 Department of Urology, St. Claraspital, Basel, Switzerland
5 Department of Urology, University Hospital Basel, Basel, Switzerland
6 Department of Urology, University Hospital of O¨ rebro, O¨ rebro, Sweden
7 School of Health and Medical Sciences, O¨ rebro University, O¨ rebro, Sweden
8 Center for Genomics and Transcriptomics, CeGaT GmbH, Tuebingen, Germany
9 Clinic for Urology and Pediatric Urology, University Hospital of Bonn, Sigmund-Freud Strasse 25, 53127 Bonn, Germany
10 Section of Molecular Urooncology, Department of Urology, School of Medicine, University of Heidelberg, Heidelberg, Germany

*(Z Shaikhibrahim and A Offermann contributed equally to this work)

Abstract

In a recent effort to unravel the molecular basis of prostate cancer (PCa), Barbieri and colleagues using whole-exome sequencing identified a novel recurrently mutated gene, MED12, in 5.4% of primary PCa. MED12, encoding a subunit of the Mediator complex, is a transducer of Wnt/b-catenin signaling, linked to modulation of hedgehog signaling and to the regulation of transforming growth factor beta (TGFb)-receptor signaling. Therefore, these studies prompted us to investigate the relevance of MED12 in PCa. Expression of MED12, SMAD3 phosphorylation, and proliferation markers was assessed by immunohistochemistry on tissue microarrays from 633 patients. siRNA-mediated knockdown of MED12 was carried out on PCa cell lines followed by cellular proliferation assays, cell cycle analysis, apoptosis assays, and treatments with recombinant TGFb3. We found nuclear overexpression of MED12 in 40% (28/70) of distant metastatic castration-resistant prostate cancer (CRPCMET) and 21% (19/90) of local-recurrent CRPC (CRPCLOC) in comparison with frequencies of less than 11% in androgen-sensitive PCa, and no overexpression in benign prostatic tissues. MED12 expression was significantly correlated with high proliferative activity in PCa tissues, whereas knockdown of MED12 decreased proliferation, reduced G1- to S-phase transition, and increased the expression of the cell cycle inhibitor p27. TGFb signaling activation associates with MED12 nuclear overexpression in tissues and results in a strong

increase in MED12 nuclear expression in cell lines. Furthermore, MED12 knockdown reduced the expression of the TGFb target gene vimentin. Our findings show that MED12 nuclear overexpression is a frequent event in CRPC in comparison with androgen-sensitive PCa and is directly implicated in TGFb signaling.

Introduction

Prostate cancer (PCa) is a clinically heterogeneous disease and a leading cause of cancer death worldwide, thus unraveling the molecular basis of this disease is of great importance. Therefore, a notable effort has been made recently by Barbieri et al. (2012) in which new recurrently mutated genes were identified in primary PCa through whole exome sequencing. Among the recurrently mutated genes identified, MED12, encoding a subunit of the Mediator complex, is mutated in 5.4% of primary PCa (Barbieri et al. 2012). Interestingly, in an earlier study, MED12 has been reported to be mutated at a high frequency in uterine leiomyomas (Makinen et al. 2011). MED12 is part of the multi-protein complex Mediator, which is essential for the transcription of protein-coding genes, and serves as hub for diverse signaling pathways (Malik & Roeder 2010). The division of the Mediator complex into four distinct modules, namely head, middle, tail, and kinase, reflects different functions of distinct Mediator subunits (Cai et al. 2009). MED12 is a subunit of the kinase module and has been shown to
function as a transducer of Wnt/b-catenin signalling (Kim et al. 2006), linked to the modulation of hedgehog signaling (Zhou et al. 2006) and to the regulation of transforming growth factor beta (TGFb) receptor signalling (Huang et al. 2012). TGFb signaling regulates prostate growth by inhibiting proliferation and inducing apoptosis, and thus serves as a tumor-suppressor in normal prostatic tissue and early stages of PCa (Tang et al. 1999). Loss or mutations of components of TGFb pathway (Tang et al. 1999), as well as altered TGFb signaling, promote cell invasion, metastasis, angiogenesis, and epithelial mesenchymal transition, indicative of an oncogenic role of TGFb signaling during prostate tumorigenesis and progression. In addition, activation of TGFb signaling has been shown to increase the invasive and migratory behavior of PCa cells (Walker et al. 2013), and inhibition of TGFb signaling reduced tumor growth and/or invasiveness in vivo (Gaspar et al. 2007, Moore et al. 2008). Furthermore, a study in 2007 has shown that SMAD-dependent TGFb signaling is critical for PCa growth and

progression in nude mice (Lu et al. 2007). The response of metastatic PCa cell lines to TGFb depends
on the cellular microenvironment. In a recent study, we observed increased proliferation of the PCa cell line PC3 in
response to exogenous TGFb3 when cells were grown in the presence of all growth factors (Shaikhibrahim et al. 2013). Other studies showed that TGFb treatment had no effect upon proliferation in PC3 cells when cells were plated in serum-free medium (Vo et al. 2012). Prompted by the reports about mutations in MED12 in PCa tissues, and the critical role of MED12 in signalling pathways that have been reported to be involved in PCa (Yardy & Brewster 2005, Chen et al. 2011), in this study, we investigated the relevance of MED12 in PCa and its relationship with TGFb signaling.

Materials and methods

The study was approved by the Internal Review Board of the University Hospital of Bonn and performed in accordance with the Declaration of Helsinki.

Cohorts

The study cohort comprised 656 tissue samples, consisting of 40 benign tissue samples, 405 non-metastasized primary PCa samples, 91 lymph node metastasized primary PCa samples or lymph node metastases, and 160 castration-resistant prostate cancer (CRPC) samples (90 local recurrent CRPC and 70 distant metastatic CRPC). These samples were obtained from patients at the University Hospital of Bonn, Basel and Örebro. We collected both primary tumor and corresponding lymph
node metastases from 32 patients. We assessed the expression of pSMAD3 in 110 primary PCa, 78 lymph node metastases, and 40 CRPC samples.

Tissue microarrays

The tissue microarrays were constructed as described previously (Braun et al. 2011).

Immunohistochemistry

The following primary antibodies were used (dilutions, clones, and manufacture): anti-MED12 rabbit polyclonal (1:50, A300-774A, Bethyl Laboratories, Montgomery, TX, USA),

anti-PHH3 rabbit polyclonal (1:100, CMC36911010; Cell Marque, Rocklin, CA, USA), ready-to-use anti-Ki-67 rabbit monoclonal (1:100, 30-9, Ventana, Tucson, AZ, USA), anti-pSMAD3 rabbit monoclonal (1:50, D12E11, Cell Signaling, Danvers, MA, USA), anti-p27 Kip1 rabbit monoclonal (1:100, D69C12 XP, Cell Signaling), anti-p21 mouse monoclonal (1:100, DCS60.2, Emergo Europe, Hamburg, Germany), and anti-Vimentin rabbit monoclonal (1:100, D21H3, 3932, Cell Signaling) antibody. Negative controls for antibodies were performed using the same immunohistochemistry (IHC) protocol without primary antibody. Protein expression was quantified based on the immunoreactivity score (IRS; Remmele & Stegner 1987). IRS takes into account both the amount of immunoreactive cells and the intensity of the staining and is calculated as the percentage of immunoreactive tumor cells (0, 0%; 1, < 10%; 2, 10–50%; 3, 51–80%; and 4 > 80%) x staining intensity (from 0, no expression to 3, strong expression). An IRS with a value of 0 was considered as none or as a very weak expression, whereas an IRS of 1–2 was considered as weak to medium expression. Furthermore, an IRS of 3 and above was considered as high expression (overexpression, IRS R3). These categories were decided upon before the analyses to avoid the risk of 'data overfitting'. We chose a score of 3 or higher, as it reflects cases which were either visibly expressing MED12 in virtually all cancer cells or harbor at least a marked or strong expression in 11–50% (or more) of cancer cells. Notably, the statistical correlations were performed with the full-range MED12 IRS and not with the dichotomized categories (i.e. overexpression vs weak-to-medium expression), thus the distinction between overexpressing and nonoverexpressing cases had no effect on these analyses. The association between MED12 and TGFb signaling activation was examined dichotomizing the full-range of MED12 IRS in overexpressing and nonoverexpressing cases. For each tissue sample, we analyzed 3 scores and calculated the mean IRS, considering all 3 scores or at least 2 scores excluding outliers.

Cell lines

All cell lineswerepurchasedfromtheAmericanTypeCulture Collection (ATCC, Manassas, VA, USA) and were grown in a 5% CO2 incubator at 37°C and 85% humidity. Monolayer cultures of PC3 and BPH1 cells were maintained in RPMI 1640 medium (Biochrom, Berlin, Germany) containing 10% heat-inactivated FCS (Sigma), 1% streptomycin–

penicillin antibiotics (Gibco), and 1% glutamine. Furthermore, for the cell lines LNCaP and DU145, the medium mentioned earlier was supplemented with 25 mM HEPES buffer (PAA Laboratories GmbH, Pasching, Austria) and 1%NEAA (Gibco). The cell lines were authenticated usingMultiplex Cell Authentication by Multiplexion (Heidelberg, Germany), as described previously (Castro et al. 2013).

Immunofluorescence

Immunofluorescence (IF) was carried out using anti- MED12 rabbit polyclonal antibody (1:50, A300-774A, Bethyl Laboratories) on BPH1, DU145, PC3, and LNCaP cells as described by Cell Signaling Technology, Inc.

siRNA-mediated MED12 knockdown

For MED12 knockdown,we used SMARTpool–ON-TARGETplus MED12 siRNA (Thermo Scientific, Darmstadt, Germany) and as control, we used ON-TARGETplus nontargeting pool (Thermo Scientific). DU145 and LNCaP were transfected with 100 nmol/l siRNA using Screenfect A (Genaxxon Bioscience GmbH, Ulm, Germany). The MTT proliferation assay was carried out using siRNA-transfected cells in 96-well plates. For immunocytochemistry (ICC), western blotting analysis, cell cycle analysis, and apoptosis assay, siRNA transfection was carried out in six-well plates; after 72 h, the cells were plated on glass slides for ICC or harvested for protein extraction or functional assays. Quantitative analysis of p27, p21, and Vimentin expression was carried out using Tissue Studio (Definiens Developer XD 2.0, Definiens AG, Munich, Germany).

QRT-PCR

RNA was isolated using the RNeasy Mini Kit (Qiagen) and reverse transcribed using an iScript cDNA synthesis kit, according to manufacturer's instructions (Bio-Rad). PCRs were carried out using a Power SYBR Green Kit (Thermo Fisher Scientific) according to the manufacturer's instructions using Light-Cycler 480 II (Roche). For each sample in a given experiment, technical duplicate reactions were carried out using b-actin as an housekeeping gene. Fold changes were calculated using the formula 2KDDCT. Primer pairs used for MED12 (Applied Biosystems): MED12: F, 50 attcaagcagctatgggattcaa-30 and MED12: R, 50-ctggacgaagatcgcgtctg-30.

Western blotting

For the preparation of whole-protein cell lysates, cell pellets were washed with ice-cold PBS and re-suspended in an extraction buffer containing 1% dithiothreitol, phosphatise inhibitor, protease inhibitor, and the phenylmethanesulfonylfluoride for 60 min. The lysates were then centrifuged for 30 min at 13 000 *g* at 4 8C. The supernatant with whole-protein lysate was harvested, and the protein concentration was measured using the bicinchoninic acid–Protein Assay Kit (Thermo Scientific). Thereafter, whole-cell extracts were fractionated by SDS– PAGE and transferred to a PVDF membrane using a transfer apparatus according to the manufacturer's protocols (Bio-Rad). After incubation with 5% nonfat milk in TBST (10 mMTris, pH 8.0, 150 mMNaCl, and 0.5% Tween 20) for 30 min, the membrane was incubated with anti- MED12 rabbit polyclonal (1:50, A300-774A, Bethyl Laboratories), anti-phospho (S423CS425)-SMAD3 rabbit MAB (1:1000, EP823Y, Abcam, Cambridge, UK), antib-actin MAB (1:5000, A1978, Sigma Aldrich, St Louis, MO, USA), and anti-Vimentin rabbit monoclonal (1:1000, 3932, Cell Signaling) primary antibodies at 4 8C overnight. The membranes were washed three times with TBST for 10 min and incubated with a 1:5000 dilution of HRP-conjugated anti-mouse or anti-rabbit antibodies for 1 h. The blots were washed with TBST three times and developed with the ECL System (GE Healthcare Life Science, Freiburg, Germany) according to the manufacturer's protocol.

TGFb treatment

PC3 cells were serum starved in a medium without FCS for 48 h, and then treated with recombinant TGFb3 (Immuntools, Friesoythe, Germany) at a concentration of 10 ng/ml for 1 h, followed by preparation of wholeprotein lysate. For ICC, PC3 cells were seeded on slides in a medium containing 10% FCS. When cells were attached, they were grown in serum-free medium for 48 h, and then treated with serum-starved medium with or without 10 ng/ml TGFb3 for 1 or 24 h. The slides were then washed with PBS and cells were fixed in paraformaldehyde (PFA) overnight. For detection of pSMAD3 after 1 h of TGFb3 treatment, ICC was carried out using a primary antibody against pSMAD3. ICC using a primary antibody against MED12 was carried out on 24 h-treated cells. pSMAD3 and MED12 cytoplasmic and nuclear expression were analyzed by two independent

pathologists. For Vimentin analysis, PC3 cells treated with scrambled or MED12 siRNA were plated on slides 72h after siRNA transfection and grown in a medium containing 10% FCS and 20 ng/ml recombinant TGFb3 for 48 h. The slides were then washed with PBS and cells were fixed in 4% PFA overnight. For detection of vimentin, ICC was carried out using a primary antibody against vimentin.

MTT cell proliferation assay

The cells were analyzed for proliferation using the MTT assays according to the manufacturer's protocol (Roche) after siRNA transfection. DU145 and LNCaP cells were grown in a medium containing 10% FCS with physiological levels of androgens (1–10 nM dihydrotestosterone). Cellular proliferation was measured 3, 4, and 5 days after siRNA transfection. Each experiment was independently repeated twice in triplicates.

Cell cycle analysis

For cell cycle analysis, we performed propidium iodide (PI) DNA staining followed by FACS according to standard protocols (Sigma–Aldrich) as well as ICC for cell cycle markers p21 and p27. In more detail, 72 h (LNCaP cells) or 144 h (DU145 cells) after siRNA transfection, the cells were harvested and then plated on slides for ICC or used for FACS analysis. For FACS, the cells were washed with ice-cold PBS followed by resuspension in 200 ml staining buffer (Tris-buffered saline, Nonidet p-40, 1:1000) containing 7.5 mg/ml PI (Sigma–Aldrich) and ribonuclease A (Sigma–Aldrich) at a concentration of 1:1000. After 30 min incubation at room temperature, PI staining of cells was analyzed using FACSCanto II Cell Analyzer.

FlowJo Software Package (Treestar, OR, USA) was used for analyzing flow cytometry data. For ICC, the cells were grown overnight on slides, washed with PBS, and fixed with PFA. ICC staining was carried out using antibodies against p21 and p27 and analyzed by two independent pathologists.

Annexin V/PI apoptosis assay

For apoptosis assay, the cells were stained with Annexin V and PI, and evaluated for apoptosis by flow cytometry according to the manufacturer's protocol (eBioscience, San Diego, CA, USA). Briefly, cells were washed twice with PBS and binding buffer, stained

with 5 ml of Annexin V–FITC and 2.5 ml of PI in 1x binding buffer for 15 min at room temperature protected from light. Apoptotic cells were determined using FACSCanto II Cell Analyzer. Analysis of apoptotic cells included both, early apoptotic (Annexin V-positive and PI-negative) and late apoptotic (Annexin V-positive and PI-positive) cells.

Statistical analyses

Statistical analyses were performed using Microsoft Excel 2010 and SPSS 20 (SPSS).

Results

Nuclear expression and overexpression of MED12 in PCa tissues

IHC showed that nuclear expression of MED12 was significantly higher in 70 distant metastatic CRPC samples (CRPCMET) and 90 local recurrent CRPC samples (CRPCLOC) in comparison with androgen-sensitive PCa and benign prostatic tissues (Fig. 1a and b). Androgen-sensitive PCa consisted of 110 analyzed non-metastasized primary PCa (PCaN0) as well as 91 lymph-node-metastasized primary PCa or lymph node metastases (PCa N1). An independent PCa validation cohort (PCa Val), comprising 295 primary PCa tissue samples, was analyzed and included in our results. Nuclear overexpression of MED12 is defined as expression IRS R3, and thus MED12 is overexpressed more frequently in CRPC in comparison to androgensensitive PCa (Fig. 1c).

Nuclear expression of MED12 in prostate cell lines

ICC and IF indicated a medium to strong MED12 nuclear expression in metastatic CRPC cells (PC3 and DU145) and metastatic androgen-sensitive LNCaP cells, compared with medium expression of MED12 in benign prostatic BPH1 cells (Fig. 2a and b).

MED12 effects proliferative activity of PCa cells

IHC results for the proliferation markers Ki67 and pHH3 were assessed for 110 primary PCa, 91 lymph-nodemetastasized primary PCa with corresponding lymph node metastases, and 40 CRPC samples. MED12 expression was found to correlate significantly with the expression of the tumor proliferation markers Ki67 and pHH3 (Pearson correlation, P!0.01). To assess the effect ofMED12 on proliferative activity in

PCa cell lines, we carried out siRNA-mediated MED12 knockdown followed by MTT cell proliferation assay. siRNA treatment led to decreased MED12 expression at the mRNA level and protein level in DU145 and LNCaP cells, as illustrated by qRT-PCR and western blotting respectively (Fig. 3a and b). The MTT proliferation assay indicates significantly reduced cell proliferation 5 days after MED12- siRNA transfection in both androgen-independent DU145 and androgen-dependent LNCaP cells (Fig. 3c).

Knockdown of MED12 inhibits cell cycle progression in PCa cells

To assess the effect of MED12 upon cell cycle distribution, we carried out FACS analysis using PI DNA staining in DU145 and LNCaP cells. Cell cycle distribution revealed that DU145 cells with MED12 knockdown accumulated in G0/G1 phase (46%) in comparison with control cells (11%), and that the percentage of cells in G2/M phase was reduced upon MED12 knockdown (17 vs 48% in control cells) 6 days after siRNA transfection (Fig. 3d). The proportion of LNCaP cells in S phase of the cell cycle decreased upon MED12 knockdown (9 vs 28% of control cells), whereas the percentage of cells in G2/M phase increased to 29% upon MED12 knockdown compared with control cells (11%) 72 h after siRNA transfection (Fig. 3e). We next carried out expression analysis for the cyclin dependent kinase inhibitors (CDKi) p21 and p27 in control and MED12-knockdown cells. Immunocytochemical staining revealed increased p27 protein expression and nuclear localization of p27 upon MED12 knockdown in DU145 (Fig. 4a) and LNCaP (Fig. 4b) cells. To quantify the expression of p27 in control and MED12 knockdown cells, we analyzed the immunocytochemical staining intensity (Fig. 4c and d). In DU145 and LNCaP cells with MED12 knockdown, p27 protein expression was increased 2.9- and 1.3-fold compared with control cells respectively (Fig. 4c and d). In contrast, DU145 control and MED12 knockdown cells exhibit no difference in p21 protein expression profile (Supplementary Fig. 1a, see section on supplementary data given at the end of this article), whereas p21 is slightly more expressed in LNCaP cells upon MED12 knockdown when compared with control cells (Supplementary Fig. 1b).

MED12 knockdown has no effect upon apoptosis rate in PCa cells

To examine whether reduced cell proliferation upon MED12 knockdown is a result of

increased apoptosis, we carried out Annexin V–PI staining assay. We observed no significant difference in the apoptosis rate between DU145 control (4.2%) and MED12 knockdown cells (5.9%) (Supplementary Fig. 2a, see section on supplementary data given at the end of this article). The percentage of apoptotic cells was slightly increased upon MED12 knockdown in LNCaP cells, indicated by 5.7% Annexin V-positive MED12 knockdown cells compared with 1.8% Annexin V-positive control cells (Supplementary Fig. 2b).

MED12 is implicated in TGFb signaling in PCa cells

In order to examine whether TGFb signaling activation is associated with MED12 nuclear overexpression in PCa tissues, we carried out IHC for pSMAD3 on 110 primary PCa, 78 lymph node metastases, and 40 CRPC samples. In contrast to low/absent MED12 expression, MED12 nuclear overexpression was significantly associated with pSMAD3 expression in PCa tissues (Fig. 5a and Supplementary Table 1, see section on supplementary data given at the end of this article). To investigate whether TGFb signaling activation effects expression and cellular localization of MED12, PC3 cells were treated with recombinant TGFb3 followed by ICC or MED12. Treatment of PC3 cells with recombinant TGFb3 leads to the activation of TGFb signalling as indicated by the increased expression and nuclear localization of pSMAD3 (Fig. 5b and c). TGFb signalling activation results in a strong increase in MED12 nuclear expression, and a decrease in the cytoplasmic expression (Fig. 5d). Next, we investigated the effect of MED12 on the expression of TGFb target genes. ICC (Fig. 5e) as well as western blotting analysis (Fig. 5g) showed reduced Vimentin expression in response to exogenous TGFb3 in MED12-knockdown PC3 cells compared with control cells. Analysis of the immunocytochemical staining intensity revealed that control cells expressed twofold higher Vimentin levels compared with MED12 knockdown cells (Fig. 5f).

Discussion

In our study, we found MED12 nuclear expression to be significantly higher in distant metastatic CRPC (CRPCMET) and local recurrent CRPC (CRPCLOC) as compared with androgen-sensitive PCa and benign prostatic tissues (Fig. 1a and b). MED12 was

overexpressed in 40% of CRPCMET and 21% CRPCLOC (Fig. 1c). In the majority of cases, we observed a heterogeneous expression pattern for MED12 within the samples from single patients of primary PCa and lymph node metastases. The focal heterogeneity in staining profiles concerned both, the amount of immunoreactive cells as well as the intensity of the staining. This result may be due to the known heterogeneous and multifocal nature of PCa (Karavitakis et al. 2011). Interestingly, MED12 expression was more homogeneous in CRPC tissues compared with primary PCa and lymph node metastasis. The reduced heterogeneity of MED12 expression in advanced stages may be indicative of an important role of MED12 in the progression of PCa to castration resistance.

In light of our findings from tissue samples, we examined the expression of MED12 in benign prostatic BPH1 as well as metastatic androgen-sensitive LNCaP cells and PCa cells with dispensed androgen signaling (PC3 and DU145). In support of our findings from tissue samples, we found that MED12 exhibits medium to strong nuclear expression in PCa cells, compared with medium expression in benign prostatic cells (Fig. 2a and b). As MED12 is part of the Mediator complex, a co-activator of the general transcription machinery (Malik & Roeder 2010), it is expressed in nonmalignant cells. Consistent with that, we observed a weak staining for MED12 in benign prostate tissues (Fig. 1a and b).

Our observation that MED12 nuclear overexpression is a frequent event in CRPC in comparison with androgensensitive PCa, prompted us to examine whether MED12 expression was correlated with high proliferative activity in PCa. MED12 expression correlated significantly with the proliferation markers Ki67 and pHH3 in PCa tissues. Based upon these results, we found that knockdown of MED12 decreased proliferation in both androgen-independent CRPC DU145 cells and androgen-dependent LNCaP cells (Fig. 3b and c). In order to examine whether the reduced proliferation of MED12 knockdown cellsmay be affected by cell cycle arrest and/or increased apoptosis, we carried out cell cycle assays as well as apoptosis assays in DU145 and LNCaP cells. While we found no effect of MED12 knockdown upon the percentage of apoptotic cells (Supplementary Fig. 2), we observed significant differences in the cell cycle distribution between control and MED12-knockdown DU145 and LNCaP cells (Fig. 3d and e). In DU145 cells, we found that MED12-knockdown cells being significantly arrested in

G0/G1 phase (Fig. 3d), and in LNCaP cells we observed that the percentage of cells in S phase was reduced upon MED12 knockdown (Fig. 3e). These results are indicative of a role of MED12 in the G1- to S-phase transition. To get further support for MED12-knockdown-induced cell cycle arrest at G0/G1 phase, we analyzed the expression levels of G1- to S-phase negative regulators p21 and p27. These proteins are able to block the kinase activity of CDKs at the G1- to S-phase checkpoint during cell cycle, and their upregulation has been shown to cause growth inhibition (Sherr & Roberts 1999). At the same time, reduced p27 expression has been associated with a more aggressive phenotype and poor survival (Doganavsargil et al. 2006). Increased p27 expression in MED12-knockdown DU145 and LNCaP cells (Fig. 4a and b) may provide evidence that MED12 is a negative regulator of p27 expression, which is consistent with the observation that MED12-knockdown cells showed reduced proliferation in MTT assays and decreased G1- to S-phase transition in cell cycle analysis. Differences in p21 expression upon MED12 knockdown were only slightly detectable in LNCaP cells (Supplementary Fig. 1b), and could not been observed in DU145 cells (Supplementary Fig. 1a). However, studies uncovered important differences between several cell types with regard to the regulation of CDK inhibitors such as p21 and p27 (Sherr & Roberts 1999) which could explain our different results in DU145 and LNCaP cells for p21 expression. Furthermore, several reports indicate that p21 and p27 have different functions (Martin-Caballero et al. 2004, Munoz-Alonso et al. 2005), and can be differentially expressed in cancer cells (Sherr & Roberts 1999). Interestingly,MED12 knockdown may affect signaling pathways, including b-catenin signaling, whose inhibition has been shown to cause G1 arrest in colorectal cancer cells (van de Wetering et al. 2002). In addition to reduced G1- to S-phase transition in MED12-knockdown cells, in LNCaP cells we observed that the percentage of cells in G2/M phase was increased upon MED12 knockdown (Fig. 3e). Notably, we found MED12 expression in PCa tissues was correlated with the expression of the M-phase marker pHH3, which is upregulated during G2- to M-phase transition (Hesse et al. 2012). Togetherwith our results indicating that LNCaP cells with MED12 knockdown exhibited reduced cell viability by MTT assay, we propose that MED12 may be involved in the checkpoint regulation during G2- to M-Phase transition. Interestingly, in malignant melanoma cells, the loss of CDK8 activity, which requires MED12 (Knuesel et al. 2009), reduced proliferation mediated by G2/M-

phase arrest (Kapoor et al. 2010). Previous studies revealed fundamental differences in cell cycle regulator patterns between LNCaP and DU145 cells, and specific cell-cycle-regulating pathways in the two cell lines (Mad'arova et al. 2002, Cifuentes et al. 2003, Benavides et al. 2010). Results from studies indicated that the androgen receptor and p53 status might determine different responses to CDK inhibitors and cell-cycle-modulating agents between LNCaP and DU145 cells (Mad'arova et al. 2002, Benavides et al. 2010). Our results show different effects of MED12 knockdown upon cell cycle regulation in LNCaP and DU145 cells, and may support previous observations described earlier. Further experiments are needed to unravel the molecular basis of MED12-mediated cell cycle regulation.

Recent studies have reported that SMAD3 is overexpressed in PCa tissues and is necessary for progressive growth of PCa cells in nude mice (Lu et al. 2007). On the basis of these results and results from other studies showing that MED12 is implicated in TGFb-receptor regulation (Huang et al. 2012) and our findings that MED12 has increased expressed in castration-resistant PCa tissues, we aimed to investigate whether MED12 is implicated in TGFb signaling in PCa. We found that the activation of TGFb signaling was associated with MED12 nuclear overexpression in PCa tissue (Fig. 5a and Supplementary Table 1). Results of recent studies have indicated that the TGFb isoform 3 is highly expressed in PCa and androgenindependent PCa cells (Karan et al. 2002). Furthermore, TGFb3 is more potent in increasing motility and invasive behavior in PCa cells (Walker et al. 2013), as well as in endometrial cancer cells compared with other isoforms (Van Themsche et al. 2007). Therefore, we used TGFb3 to activate TGFb signaling in PC3 cells and found a strong increase in MED12 nuclear expression and a decrease in the cytoplasmic expression (Fig. 5d). Our observations indicate that the nuclear MED12 overexpression is a response to the activation of TGFb signaling in PCa. Based on these observations, we aimed to investigate the role of MED12 in TGFb-regulated gene expression. TGFb signaling activation leads to increased expression of vimentin, a crucial event during PCa progression and metastasis (Zavadil & Bottinger 2005). We observed reduced Vimentin expression in PC3 cells with MED12 knockdown grown under TGFb3 stimulation, when compared with control cells (Fig. 5e and f). We suggest that MED12, as part of the Mediator complex, is required for TGFb-regulated gene expression, and that MED12 is therefore shuttled into the nucleus in response to

TGFb signaling activation. Recently, a study by Huang and colleagues revealed that MED12 has an additional function in the cytoplasm distinct from its role within the Mediator complex, and there it negatively regulates TGFb receptor signaling. In contrast, the aim of our study was to investigate the role of MED12 as part of the Mediator complex, which acts in the nucleus as a co-activator for the transcription machinery. In addition, Huang et al. (2012) carried out MED12 knockdown experiments under conditions without activation of TGFb signaling. In contrast, we investigated the effect of MED12 knockdown upon TGFb signaling in response to TGFb pathway activation.

However, further studies are required to investigate the underlying mechanisms. It has to be considered that p27 is also a TGFb-responsive gene in prostate cell lines (Robson et al. 1999), and that TGFb-regulated p27 expression leads to growth inhibition in different cell lines (Polyak et al. 1994). Interestingly, recent studies have demonstrated DU145 cells to be refractory to growth arrest by TGFb (Cipriano & Chen 1998), and that TGFb treatment of

DU145 cells led to increased p21 expression, but had no effect on p27 expression (Park et al. 2000). Furthermore, several studies have reported that LNCaP cells are insensitive to activation of TGFb signaling (Zhang et al. 2005), thus DU145 and LNCaP cells have been described as being insensitive to TGFb-regulated p27 expression. In this study, we found increased p27 expression in MED12-knockdown DU145 and LNCaP cells and decreased Vimentin expression in MED12-knockdown PC3 cells.

Recently, we have found similar expression patterns in PCa tissues and functional results in cell lines for the Mediator tail subunit MED15 (Shaikhibrahim et al. 2013). The interplay between different subunits of the Mediator complex provides the basis for the function of the Mediator as a hub between signaling pathways and the transcription of specific genes. Concordantly, studies showed that the simultaneous inactivation of multiple Mediator subunits has different effects compared with single knockdown of individual subunits (Larsson et al. 2013). In addition, some subunits require the activity of other subunits of the Mediator for specific signalling (Knuesel et al. 2009). Therefore, it is likely that a simultaneous upregulation of MED12 and MED15 is required for the activation of pathways and the expression of specific genes. Interestingly, in PCa tissues, we found that MED12 overexpression only occurs when MED15 is overexpressed (data not shown). This result may provide evidence that high MED15

expression levels lead directly or indirectly to higher MED12 expression. Further experiments are needed to explore whether the activity of specific subunits influences the expression of other Mediator subunits, or whether the activation of specific pathways may lead to the simultaneous upregulation of different subunits.

Finally, it is worth noting that while Barbieri et al. (2012) performed whole-exome sequencing of primary PCa samples and found MED12 to be mutated in six out of 111 samples, Stoehr et al. (2013) reported a lack of

evidence for MED12 mutations using Sanger sequencing of PCa samples from a small subgroup of patients. Taken together, our findings indicate that MED12 expression clearly plays a role in PCa and is directly implicated in TGFb signaling in PCa.

Declaration of interest

The University of Bonn has filed a patent which includes and is not limited to the use of MED12 in prostate cancer, on which S Perner, Z Shaikhibrahim, R Menon, and M Braun are co-inventors.

Funding

The study was supported by a grant from the German Research Foundation (Deutsche Forschungsgemeinschaft (DFG), Emmy-Noether-Program, (PE1179/2-1), the Rudolf-Becker-Foundation, and the Wilhelm-Sander- Foundation (2011.077.1), to S Perner, a medical doctoral fellowship grant (BONFOR) of the Medical Faculty of the University of Bonn to A Offermann, the Ferdinand Eisenberger Fellowship of the German Society of Urology (DGU) to I Syring, and the Medical Faculty Heidelberg of the University of Heidelberg to S Duensing.

Author contribution statement

S Perner and Z Shaikhibrahim study concept and approach. S Perner, Z Shaikhibrahim, A Offermann, and M Braun designed experiments. A Offermann, R Menon, I Syring, W Vogel, M Braun, M Nowak, R Halbach, and Z Shaikhibrahim carried out the experiments. L Bubendorf, C A Rentsch, O Andren, and M Svensson provided tissues. S Perner, Z Shaikhibrahim, A Offermann, and M Braun analyzed and interpreted data. S Perner, Z

Shaikhibrahim, and A Offermann wrote manuscript. S Duensing, L Bubendorf, S Biskup, J Kirfel, C Ruiz, O Andren, and M Svensson revised manuscript.

References

Barbieri CE, Baca SC, Lawrence MS, Demichelis F, BlattnerM, Theurillat JP, White TA, Stojanov P, Van Allen E, Stransky N et al. 2012 Exome sequencing identifies recurrent SPOP, FOXA1 and MED12 mutations in prostate cancer. Nature Genetics 44 685–689. (doi:10.1038/ng.2279)

Benavides MA, Hagen KL, Fang W, Du P, Lin S, Moyer MP, Yang W, Bland KI, Grizzle WE & Bosland MC 2010 Suppression by L-methionine of cell cycle progression in LNCaP and MCF-7 cells but not benign cells. Anticancer Research 30 1881–1885.

Braun M, Scheble VJ, Menon R, Scharf G, Wilbertz T, Petersen K, Beschorner C, Reischl M, Kuefer R, Schilling D et al. 2011 Relevance of cohort design for studying the frequency of the ERG rearrangement in prostate cancer. Histopathology 58 1028–1036. (doi:10.1111/j.1365-2559.2011.03862.x)

Cai G, Imasaki T, Takagi Y & Asturias FJ 2009 Mediator structural conservation and implications for the regulation mechanism. Structure 17 559–567. (doi:10.1016/j.str.2009.01.016)

Castro F, DirksWG, Fahnrich S, Hotz-Wagenblatt A, Pawlita M & Schmitt M 2013 High-throughput SNP-based authentication of human cell lines. International Journal of Cancer 132 308–314. (doi:10.1002/ijc.27675)

Chen M, Carkner R & Buttyan R 2011 The hedgehog/Gli signalling paradigm in prostate cancer. Expert Review of Endocrinology &Metabolism 6 453–467. (doi:10.1586/eem.11.24)

Cifuentes E, Croxen R, Menon M, Barrack ER & Reddy GP 2003 Synchronized prostate cancer cells for studying androgen regulated events in cell cycle progression from G1 into S phase. Journal of Cellular Physiology 195 337–345. (doi:10.1002/jcp.10317)

Cipriano SC & Chen YQ 1998 Insensitivity to growth inhibition by TGF-b1 correlates with a lack of inhibition of the CDK2 activity in prostate carcinoma cells. Oncogene 17 1549–1556. (doi:10.1038/sj.onc.1202069)

Doganavsargil B, Simsir A, Boyacioglu H, Cal C & Hekimgil M 2006 A comparison of p21 and p27 immunoexpression in benign glands, prostatic intraepithelial neoplasia and prostate adenocarcinoma. BJU International 97 644–648. (doi:10.1111/j.1464-410X.2006.06054.x)

Gaspar NJ, Li L, Kapoun AM, Medicherla S, Reddy M, Li G, O'Young G, Quon D, Henson M, Damm DL et al. 2007 Inhibition of transforming growth factor b signaling reduces pancreatic adenocarcinoma growth and invasiveness. Molecular Pharmacology 72 152–161. (doi:10.1124/mol.106.029025)

Hesse M, Raulf A, Pilz GA, Haberlandt C, Klein AM, Jabs R, Zaehres H, Fugemann CJ, Zimmermann K, Trebicka J et al. 2012 Direct visualization of cell division using high-resolution imaging of M-phase of the cell cycle. Nature Communications 3 1076. (doi:10.1038/ncomms2089)

Huang S, HolzelM, Knijnenburg T, Schlicker A, Roepman P, McDermott U, Garnett M, Grernrum W, Sun C, Prahallad A et al. 2012 MED12 controls the response to multiple cancer drugs through regulation of TGF-b receptor signaling. Cell 151 937–950. (doi:10.1016/j.cell.2012.10.035)

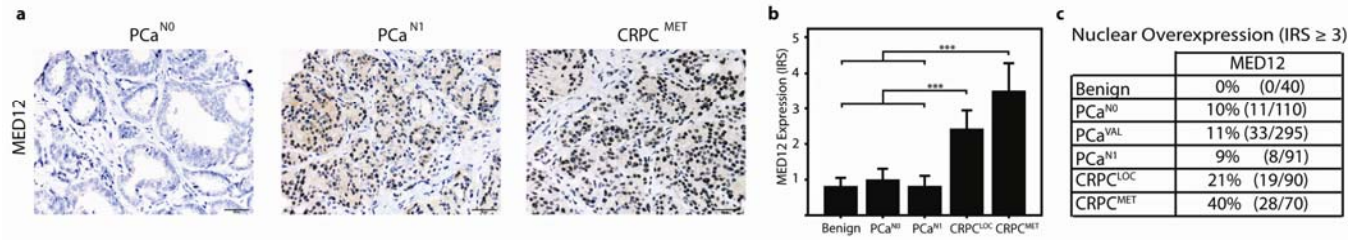
Kapoor A, Goldberg MS, Cumberland LK, Ratnakumar K, Segura MF, Emanuel PO, Menendez S, Vardabasso C, LeroyG,Vidal CI et al. 2010 The histone variant macroH2A suppresses melanoma progression through regulation of CDK8. Nature 468 1105–1109. (doi:10.1038/nature09590)
Karan D, Kelly DL, Rizzino A, Lin MF & Batra SK 2002 Expression profile of differentially-regulated genes during progression of androgen-independent growth in human prostate cancer cells. Carcinogenesis 23 967–975. (doi:10.1093/carcin/23.6.967)
Karavitakis M, Ahmed HU, Abel PD, Hazell S & Winkler MH 2011 Tumor focality in prostate cancer: implications for focal therapy. Nature Reviews. Clinical Oncology 8 48–55. (doi:10.1038/nrclinonc.2010.190)
Kim S, Xu X, Hecht A & Boyer TG 2006 Mediator is a transducer of Wnt/ b-catenin signaling. Journal of Biological Chemistry 281 14066–14075. (doi:10.1074/jbc.M602696200)
Knuesel MT, Meyer KD, Donner AJ, Espinosa JM & Taatjes DJ 2009 The human CDK8 subcomplex is a histone kinase that requires Med12 for activity and can function independently of mediator. Molecular and Cellular Biology 29 650–661. (doi:10.1128/MCB.00993-08)
Larsson M, Uvell H, Sandstrom J, Ryden P, Selth LA & Bjorklund S 2013 Functional studies of the yeast med5, med15 and med16 mediator tail subunits. PLoS ONE 8 e73137. (doi:10.1371/journal.pone.0073137)
Lu S, Lee J, Revelo M, Wang X, Lu S & Dong Z 2007 Smad3 is overexpressed in advanced human prostate cancer and necessary for progressive growth of prostate cancer cells in nude mice. Clinical Cancer Research 13 5692–5702. (doi:10.1158/1078-0432.CCR-07-1078)
Mad'arova J, Lukesova M, Hlobilkova A, Strnad M, Vojtesek B, Lenobel R, Hajduch M, Murray PG, Perera S & Kolar Z 2002 Synthetic inhibitors of CDKs induce different responses in androgen sensitive and androgen insensitive prostatic cancer cell lines. Molecular Pathology 55 227–234. (doi:10.1136/mp.55.4.227)
Makinen N, Mehine M, Tolvanen J, Kaasinen E, Li Y, Lehtonen HJ, Gentile M, Yan J, Enge M, Taipale M et al. 2011 MED12, the mediator complex subunit 12 gene, is mutated at high frequency in uterine leiomyomas. Science 334 252–255. (doi:10.1126/science.1208930)
Malik S & Roeder RG 2010 The metazoan Mediator co-activator complex as an integrative hub for transcriptional regulation. Nature Reviews. Genetics 11 761–772. (doi:10.1038/nrg2901)
Martin-Caballero J, Flores JM, Garcia-Palencia P, Collado M & Serrano M 2004 Different cooperating effect of p21 or p27 deficiency in combination with INK4a/ARF deletion in mice. Oncogene 23 8231–8237. (doi:10.1038/sj.onc.1207863)
Moore LD, Isayeva T, Siegal GP & Ponnazhagan S 2008 Silencing of transforming growth factor-b1 in situ by RNA interference for breast cancer: implications for proliferation and migration in vitro and metastasis in vivo. Clinical Cancer Research 14 4961–4970. (doi:10.1158/1078-0432.CCR-07-4604)
Munoz-Alonso MJ, Acosta JC, Richard C, Delgado MD, Sedivy J & Leon J 2005 p21Cip1 and p27Kip1 induce distinct cell cycle effects and differentiation programs in myeloid leukemia cells. Journal of Biological Chemistry 280 18120–18129. (doi:10.1074/jbc.M500758200)
Park BJ, Park JI, Byun DS, Park JH & Chi SG 2000 Mitogenic conversion of transforming growth factor-b1 effect by oncogenic Ha-Ras-induced activation of the

mitogen-activated protein kinase signaling pathway in human prostate cancer. Cancer Research 60 3031–3038.
Polyak K, Kato JY, Solomon MJ, Sherr CJ, Massague J, Roberts JM & Koff A 1994 p27Kip1, a cyclin–Cdk inhibitor, links transforming growth factorb and contact inhibition to cell cycle arrest. Genes and Development 8 9–22. (doi:10.1101/gad.8.1.9)
RemmeleW& Stegner HE 1987 Recommendation for uniform definition of an immunoreactive score (IRS) for immunohistochemical estrogen receptor detection (ER-ICA) in breast cancer tissue. Pathologe 8 138–140.
Robson CN, Gnanapragasam V, Byrne RL, Collins AT & Neal DE 1999 Transforming growth factor-b1 up-regulates p15, p21 and p27 and blocks cell cycling in G1 in human prostate epithelium. Journal of Endocrinology 160 257–266. (doi:10.1677/joe.0.1600257)
Shaikhibrahim Z, Menon R, Braun M, Offermann A, Queisser A, Boehm D, VogelW, Ruenauver K, Ruiz C, Zellweger T et al. 2013 MED15, encoding a subunit of the mediator complex, is overexpressed at high frequency in castration-resistant prostate cancer. International Journal of Cancer 135 19–26. (doi:10.1002/ijc.28647)
Sherr CJ & Roberts JM 1999 CDK inhibitors: positive and negative regulators of G1-phase progression. Genes and Development 13 1501–1512. (doi:10.1101/gad.13.12.1501)
Stoehr R, Taubert H, Gaisa NT, Smeets D, Kneitz B, Giedl J, Ruemmele P, Wieland WF, Rau TT & Hartmann A 2013 Lack of evidence for frequent MED12 p.L1224F mutation in prostate tumours from Caucasian patients. Journal of Pathology 230 453–456. (doi:10.1002/path.4208)
Tang B, de Castro K, Barnes HE, Parks WT, Stewart L, Bottinger EP, Danielpour D & Wakefield LM 1999 Loss of responsiveness to transforming growth factor b induces malignant transformation of nontumorigenic rat prostate epithelial cells. Cancer Research 59
4834–4842.
Van Themsche C, Mathieu I, Parent S & Asselin E 2007 Transforming growth factor-b3 increases the invasiveness of endometrial carcinoma cells through phosphatidylinositol 3-kinase-dependent up-regulation of X-linked inhibitor of apoptosis and protein kinase c dependent induction of matrix metalloproteinase-9. Journal of Biological Chemistry
282 4794–4802. (doi:10.1074/jbc.M608497200)
Vo BT, Cody B, Cao Y & Khan SA 2012 Differential role of Sloan-Kettering Institute (Ski) protein in Nodal and transforming growth factor-b (TGF-b)-induced Smad signaling in prostate cancer cells. Carcinogenesis 33 2054–2064. (doi:10.1093/carcin/bgs252)
Walker L, Millena AC, Strong N & Khan SA 2013 Expression of TGFb3 and its effects on migratory and invasive behavior of prostate cancer cells: involvement of PI3-kinase/AKT signaling pathway. Clinical & Experimental Metastasis 30 13–23. (doi:10.1007/s10585-012-9494-0)
van de Wetering M, Sancho E, Verweij C, de Lau W, Oving I, Hurlstone A, van der Horn K, Batlle E, Coudreuse D, Haramis AP et al. 2002 The
b-catenin/TCF-4 complex imposes a crypt progenitor phenotype on colorectal cancer cells. Cell 111 241–250.
Yardy GW & Brewster SF 2005 Wnt signalling and prostate cancer. Prostate Cancer and Prostatic Diseases 8 119–126. (doi:10.1038/sj.pcan.4500794)
Zavadil J & Bottinger EP 2005 TGF-b and epithelial-to-mesenchymal transitions. Oncogene 24 5764–5774. (doi:10.1038/sj.onc.1208927)
Zhang Q, Rubenstein JN, Jang TL, Pins M, Javonovic B, Yang X, Kim SJ, Park I & Lee

Zavadil J & Bottinger EP 2005 TGF-b and epithelial-to-mesenchymal transitions. Oncogene 24 5764–5774. (doi:10.1038/sj.onc.1208927)
Zhang Q, Rubenstein JN, Jang TL, Pins M, Javonovic B, Yang X, Kim SJ, Park I & Lee C 2005 Insensitivity to transforming growth factor-b results from promoter methylation of cognate receptors in human prostate cancer cells (LNCaP). Molecular Endocrinology 19 2390–2399.
Zhou H, Kim S, Ishii S & Boyer TG 2006 Mediator modulates Gli3- dependent sonic hedgehog signaling. Molecular and Cellular Biology 26 8667–8682.

Received in final form 11 June 2014
Accepted 16 June 2014
Made available online as an Accepted Preprint
17 June 2014

Fig. 1



	MED12
Benign	0% (0/40)
PCa^{N0}	10% (11/110)
PCa^{VAL}	11% (33/295)
PCa^{N1}	9% (8/91)
$CRPC^{LOC}$	21% (19/90)
$CRPC^{MET}$	40% (28/70)

Figure 1. MED12 expression in prostate cancer tissue. Immunhistochemical staining of PCaN0, PCaN1, and CRPCMET for MED12 (a). Scale bars, 20 mm. Mean IRS score for MED12 expression in benign, PCaN0, PCaN1, and CRPCLOC and CRPCMET respectively (b). Percentage and number of cases with MED12 nuclear overexpression (defined as expression RIRS score 3) (c). Bars and error bars indicate mean IRSGS.D. Independent t-test, ***P<0.005.

Fig. 2

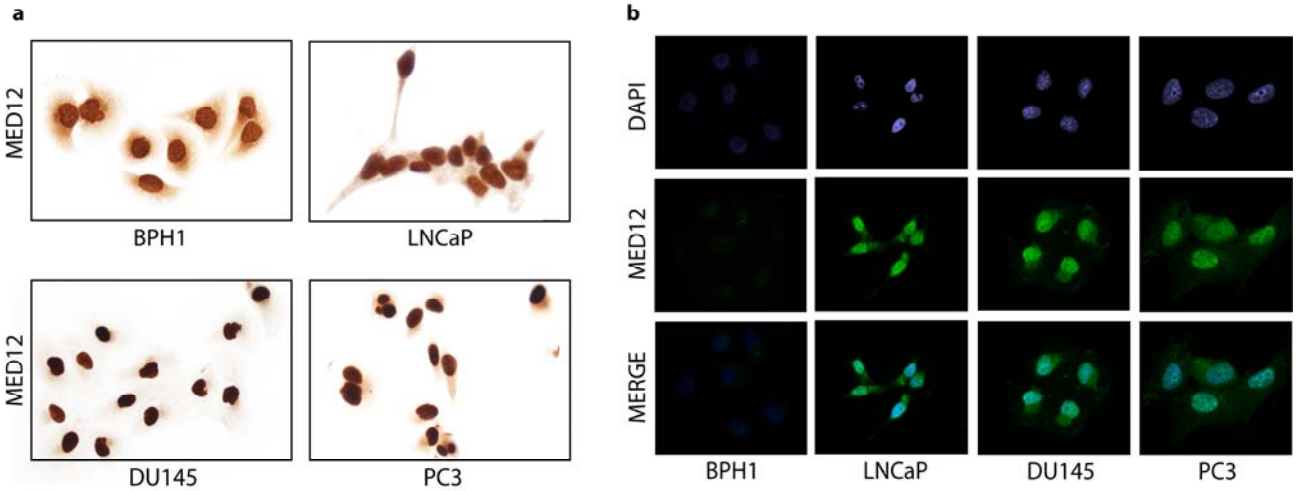


Figure 2. MED12 expression in prostatic cell lines. Immunocytochemical staining (a) and immunfluorescence (b) of MED12 in benign prostate cell line BPH1 and prostate cancer cell lines LNCaP, DU145, and PC3.

Fig. 3

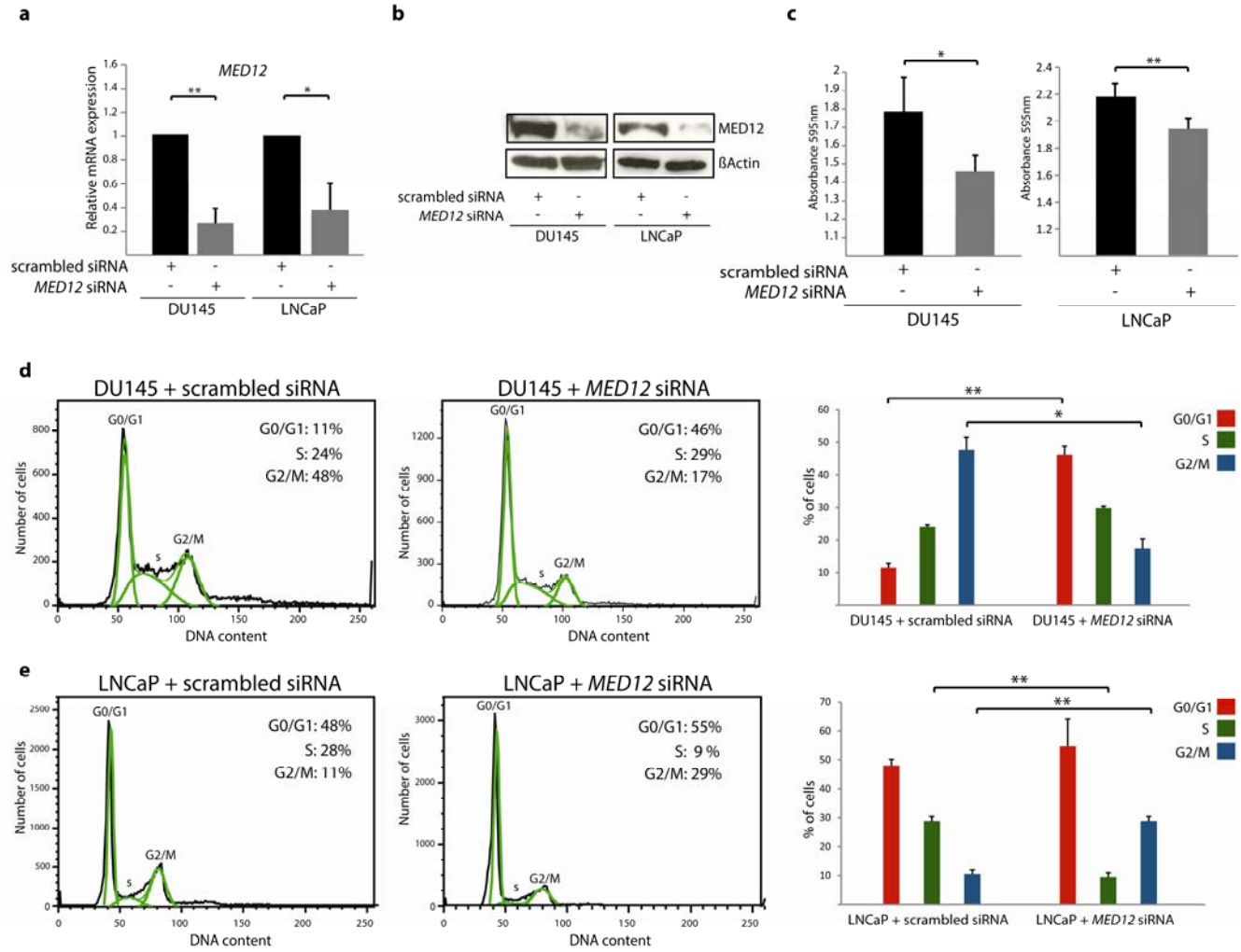


Figure 3. Effect of MED12 upon proliferation and cell cycle distribution in prostate cancer cells. siRNA-mediated MED12 knockdown in DU145 and LNCaP cell lines shows reduced MED12 expression by qRT-PCR (a) and western blotting (b) 72 h after siRNA transfection. MED12 mRNA expression in cells treated with scrambled siRNA was normalized to one. Reduced proliferation of MED12-knockdown cells compared with cells treated with scrambled siRNA in DU145 and LNCaP cells 5 days after siRNA transfection by MTT proliferation assay (c). Bars and error bars indicate the mean of the absorbance at 595 nm +/- SD. The percentages of cells in different phases of the cell cycle are shown for DU145 (d) and LNCaP (e) cells 6 days (DU145) or 72 h (LNCaP) after transfection with scrambled or MED12-specific siRNA. Bars and error bars indicate mean percentages of cells in each phase +/- SD. Independent t-test, *P<0.05 and **P<0.01.

Fig. 4

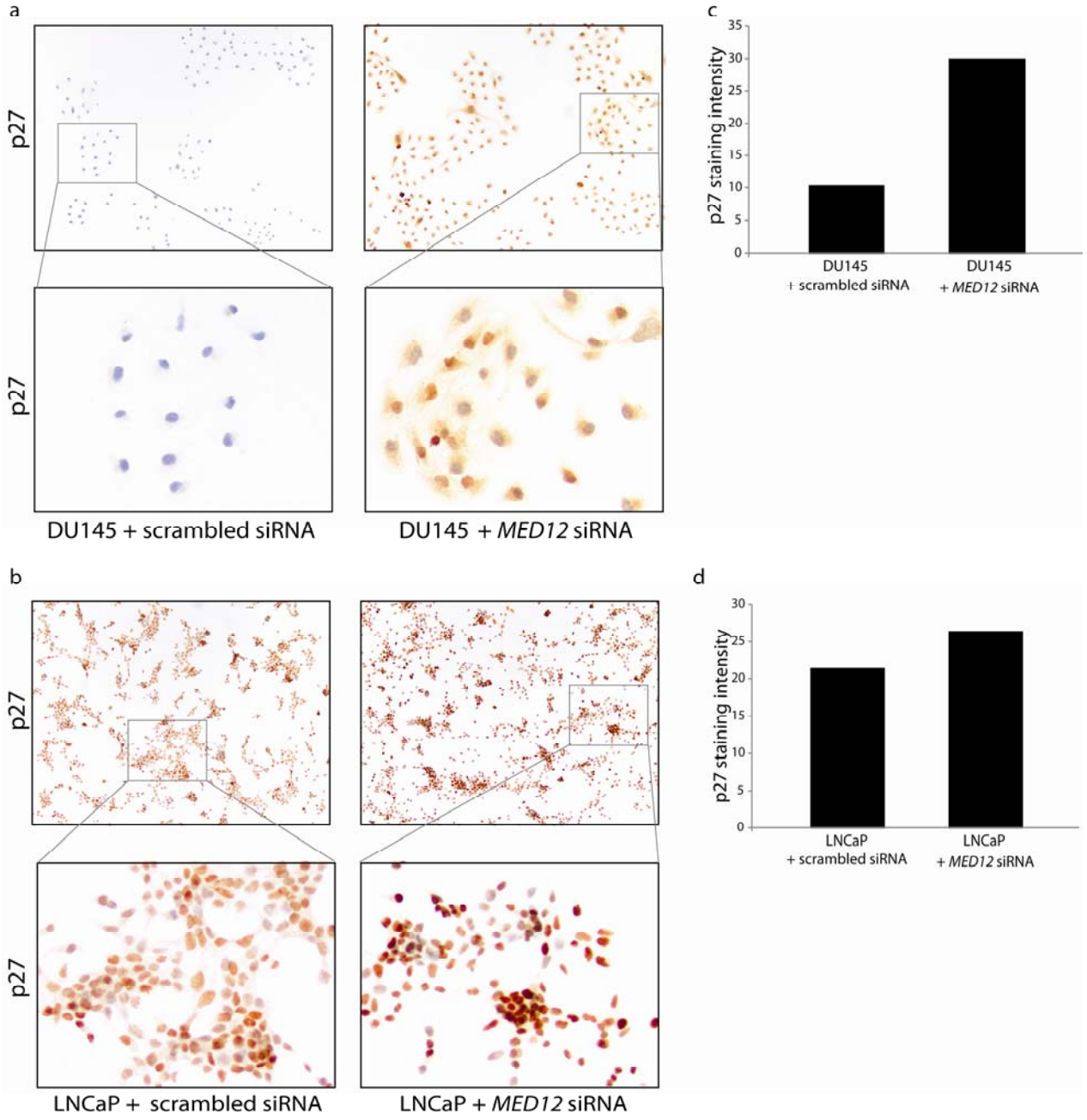


Figure 4. MED12 knockdown effects p27 protein expression. Immuncytochemical staining (a and b) and staining intensity analyses (c and d) show increased p27 expression in DU145 (a and c) and LNCaP (b and d) cells with MED12 knockdown compared with control cells.

Fig. 5

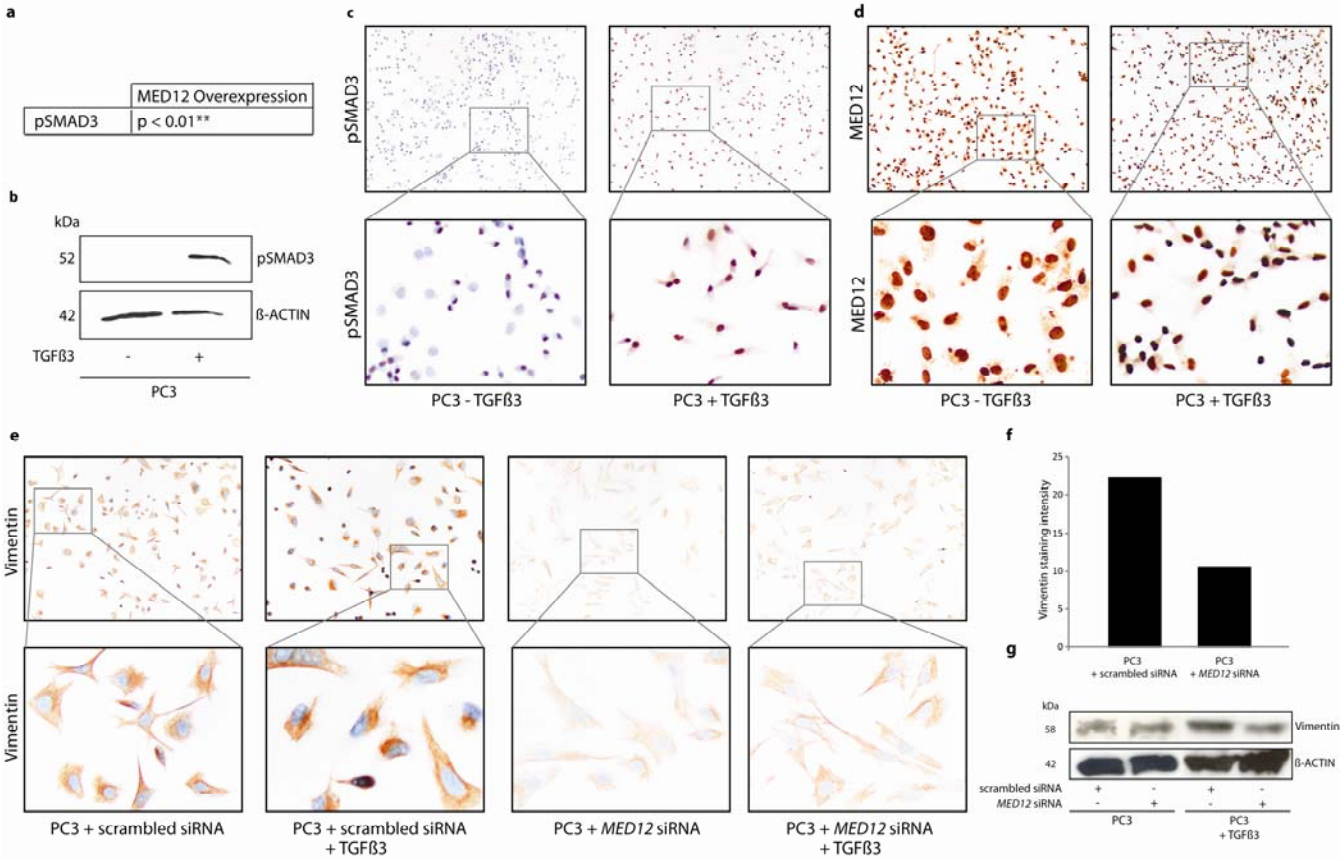


Figure 5. MED12 is implicated in TGFb signaling. Expression of pSMAD3 is associated significantly with MED12 nuclear overexpression in PCa tissues (a). *c*2, **P<0.01. Phosphorylation of SMAD3 in PC3 cells following treatment with 10 ng/ml recombinant TGFb3 shown by western blotting (b). Immunocytochemical staining showed increased pSMAD3 after 1 h (c) and increased expression and nuclear localization of MED12 (d) in PC3 cells treated with TGFb for 24 h compared with untreated cells. Immunocytochemical staining (e) showed reduced vimentin expression in PC3 cells with MED12 knockdown compared with control cells grown in a medium with or without 20 ng/ml recombinant TGFb3 for 48 h. Bar graph shows reduced vimentin staining intensity in MED12-knockdown cells compared with control cells (f). Western blotting analysis revealed reduced vimentin expression in PC3 cells with MED12-knockdown comparedwith control cells grown in medium with or without 20 ng/ml recombinant TGFb3 for 48 h (g).

Am J Pathol. 2015 Apr;185(4):1114-22. doi: 10.1016/j.ajpath.2014.12.010. Epub 2015 Mar 16.

Clinical and molecular implications of MED15 in Head and Neck Squamous Cell Carcinoma

Zaki Shaikhibrahim*[1,2], Anne Offermann*[1,2], Rebecca Halbach[1,2], Wenzel Vogel[1,2], Martin Braun[1,2], Glen Kristiansen[2], Friedrich Bootz[3], Jörg Wenzel[4], Ralf Mikut[5], Claudia Lengerke[6], Markus Reischl[5], Andreas Schröck[3], Sven Perner[1,2]

* These authors contributed equally.

[1] Department of Prostate Cancer Research, [2] Institute of Pathology, University Hospital of Bonn, Bonn, Germany

[3] Department of Otorhinolaryngology, University Hospital of Bonn, Bonn, Germany

[4] Department of Experimental Dermatology, University Hospital of Bonn, Bonn, Germany

[5] Karlsruhe Institute of Technology, Institute for Applied Computer Science, Karlsruhe, Germany

[6] Department of Biomedicine, University Hospital Basel, Basel, Switzerland

Department of Prostate Cancer Research, Institute of Pathology, University Hospital of Bonn, Sigmund-Freud Strasse 25, 53127 Bonn, Germany

Abstract

Head and Neck Squamous Cell Carcinoma (HNSCC) progression depends on various dysregulated pathways. Regulation of diverse pathways is mediated by the Mediator complex. The Mediator subunit MED15 is essential for TGFß signaling and involved in breast and prostate cancers. We investigated whether MED15 is implicated in HNSCC. We performed immunohistochemistry (IHC) for MED15 on 324 tissue samples and assessed Ki67 and pSMAD3 as TGFß marker. MED15 knockdown followed by proliferation and migration assays, as well as TGFß1 treatment followed by MED15 analysis was performed in cells. We found MED15 to be overexpressed in 35% of primary tumors, 30% of lymph node metastases, and 70% of recurrences in contrast to no/low expression in benigns. MED15 overexpression in primaries from patients who developed recurrences was associated with higher mortality rates and occurred at highest frequency in oral cavity/oropharyngeal tumors. Furthermore, MED15 expression correlates between primaries and corresponding lymph node metastases. MED15 correlates with proliferative activity in tissues, and MED15 knockdown reduces

proliferation and migration. We observed an association between MED15 and TGFß activity in tissues, as TGFβ activation leads to increased MED15 expression and pSMAD3 is reduced in MED15 knockdown cells. Taken together, our results implicate MED15 in HNSCC, and hint that MED15 overexpression is a clonal event during HNSCC progression. Our findings further suggest that MED15 may serve as prognostic marker for recurrence, and therapeutic target for HNSCC patients.

Introduction

Head and neck cancer includes tumors of the oral cavity, oropharynx, hypopharynx and larynx, which histopathologically are classified as squamous cell carcinomas (SCCs) in about 90% of all head and neck tumors [1]. With approximately 600,000 new cases diagnosed each year, head and neck squamous cell carcinomas (HNSCCs) rank as the sixth most common cancer worldwide [2] leading to approximately 350,000 deaths per year [3]. Surgical resection alone or in combination with radiation and chemotherapy is the most common treatment option for HNSCC patients [4]. However, high rates of recurrence and early metastatic disease [5], [1] lead to a poor prognosis of HNSCC patients [6]. Furthermore, diagnosis at advanced stages [7] and lack of available targeted therapeutic approaches result in a survival rate of only 50% [2]. The main risk factors for HNSCC include lifestyle behavior such as tobacco use [8] and alcohol consumption [4] as well as chronic infections with oncogenic human papillomavirus (HPV) [9]. Increased knowledge about the underlying genetic alterations [10] and dysregulated pathways [11] in HNSCC has led to the identification of promising key targets for therapy in a subset of patients [12]. The evidence that multiple aberrant pathways account for the progression of HNSCC [11] calls for a much deeper understanding of the effect of molecules involved in these signaling pathways upon HNSCC progression. Such knowledge may provide the basis for the development of novel prognostic, predictive and therapeutic targets for this disease [11].

Interestingly, the regulation of diverse signaling pathways is mediated by a multi-protein complex, called Mediator [13]. Through interactions with regulatory transcription factors on one hand and the Pol II-transcription machinery on the other hand, Mediator is a key for the regulation of protein-coding genes [13]. The Mediator complex consists of 30 subunits

in humans, which are either part of the head, the middle or tail modules, or the kinase module [14]. Altered expression and function of Mediator subunits have been implicated in various diseases [15]. For example, MED15, a subunit within the tail module [14], serves as a target for different signaling pathways [16-18] and was shown to play pathophysiological roles in several human diseases [19, 20] including breast cancer [21]. Recently, we have reported the implication of MED15 in castration-resistant prostate cancer and its direct link to TGFß signaling in this disease [22]. Notably, TGFß signaling is involved in HNSCC [23], and a large study reported alterations in SMAD dependent TGFß signaling at high frequency and showed that patients with active SMAD signaling exhibited worse survival rates [24]. Moreover, abnormalities in SMAD interacting proteins were described to contribute to a dysregulated TGFß signaling [21, 22, 25].

Therefore, we aimed to investigate whether MED15 is implicated in HNSCC, and thus further contribute to the understanding of the molecular basis of this disease.

Materials and Methods

This study was approved by the Internal Review Board of the University Hospital of Bonn in accordance with the Declaration of Helsinki.

Cohort Design

The cohort used in this study consists of tissues from 119 patients with HNSCC. Briefly, the cohort contained 113 primary tumor tissues, 85 lymph node metastases, 30 recurrent tumor tissues and 20 samples of normal squamous epithelium from the head and neck area (benign). For each patient, corresponding tissues were available. Out of these, from 82 patients primary tumors with corresponding lymph node metastases were available and 36 patients developed recurrent tumors. Origins of tissues include oral cavity, oropharynx, hypopharynx and larynx. “Recurrent tumors” included locally recurrent tumors in the site of the previous resection as well as recurrent tumors distant from primary tumor. Clinicopathological data were available for 108 patients and are provided in **Supplementary Table 1**. For correlation with clinical data of patients who developed recurrent tumors, we included the 36 patients with relapses from our cohort, as well as additional primary tumors from 72 patients who all developed recurrent

tumors. Clinicopathological data of patients who all developed recurrent tumors for these 108 patients are provided in **Supplementary Table 2**. “Mortality” was defined as outcome endpoint after a follow-up 1 to 12 years after first diagnosis. All patients used in this study were diagnosed and treated at the University Hospital Bonn, Germany.

Immunohistochemistry (IHC)

IHC was performed as described previously [22]. Briefly, staining was performed on sections of paraffin embedded tissues using Ventana XT immunostainer (Ventana, Tuscon, AZ). The following primary antibodies were used (dilution, clone, company): anti-MED15 rabbit monoclonal (1:50, 11566-1-AP, Proteintech, Chicago, IL), ready-to-use anti-Ki-67 rabbit monoclonal (clone 30-9, Ventana,AZ), anti-pSMAD3 rabbit monoclonal (1:50, EP823Y, Abcam, Cambridge, UK). Positive and negative controls for MED15- and pSMAD3 antibodies were performed using same IHC protocol without primary antibodies. Quantitative analysis of MED15 and Ki67 expression was performed using Tissue Studio (Definiens Developer XD 2.0) as described previously [26]. Nuclear staining level of MED15 is reflected as an expression score, which is calculated by multiplication of the staining intensity and the index of immunoreactive cells. MED15 expression levels were upfront defined by dividing the expression score into the following groups: no expression (score < 0.07), low expression (score ≥ 0.07 < 0.2) or overexpression (score ≥ 0.2) **(Supplementary Figure 1).** For correlation studies, the full score for MED15 was used. The expression score for Ki67 expression in tissues reflects the index of proliferating cells. The intensity of positive staining for pSMAD3 was assessed semi-quantitatively and scored as either 0 (negative) or 1 (positive). For IHC staining of SCC-25 and HSC-3 cell lines, cells were grown on glass slides, fixed with 4% Paraformaldehyde (PFA) overnight and IHC was performed according the published protocol for cultured cell lines by Cell Signaling Technology (Danvers, MA) using an anti-MED15 rabbit polyclonal antibody (1:50, 11566-1-AP, Proteintech, Chicago, IL) or an ready-to-use anti-Ki-67 rabbit monoclonal antibody (clone 30-9, Ventana, AZ).

Immunfluorescense (IF)

HSC-3 and SCC-25 cells were grown on glass slides and fixed with 4% paraformaldehyd (PFA) overnight. IF was performed according to the manufacturer´s

protocol (Cell Signaling Technology, Danvers, MA) using an anti-MED15 rabbit polyclonal antibody (1:50, 11566-1-AP, Proteintech, Chicago, IL).

Cell lines and culture conditions

SCC-25 cells were purchased from DSMZ (German Collection of Microorganisms and Cell Cultures, Germany) and HSC-3 cells from JCRB (Japanese Collection of Research Bioresources Cell Bank, Japan). Cells were maintained in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% Fetal Calf Serum (FCS) and 1% Penicillin/Streptomycin. Cell lines were authenticated in 2014 using Multiplex Cell Authentication by Multiplexion (Heidelberg, Germany) as described recently[27]. The SNP profiles matched known profiles or were unique.

siRNA transfection

To obtain a transient MED15 knockdown, HSC-3 cells and SCC-25 were transfected with 50 nM of siRNA specific for MED15 using Screenfect A (Genaxxon, Germany) according to manufactor´s instruction. Sequences for MED15 specific siRNA pool: 5´CCAAGACCCGGGACGAAUA,GGGUGUUGUUAGAGCGUCU3´,5´GGUCAGUCAAA UCGAGGAU3´,5´CCGGACAAGCACUCGGUCA3´. Scrambled siRNA pool was used as negative control. Cells were used for further experiments 48 hours after transfection.

Proliferation Assay

Cell proliferation was assessed using MTT Proliferation Assay (Roche, Mannheim, Germany). 48 hours after siRNA transfection, HSC-3 and SCC-25 cells were seeded in 96-well plates and proliferation was measured immediately and after additional 48, 72 and 96 hours. For each experiment, data are shown from three independent experiments performed in technical triplicates.

Western Blot

Cells were washed with ice-cold Phosphate-Buffered Saline (PBS), lysed in protein extraction buffer and centrifuged at 13,000rpm for 30 min. Protein concentration was measured by BCA protein assay (Pierce, Rockford, IL), subjected to SDS-PAGE

analysis and transferred to polyvinylidene difluoride (PVDF) membranes. Membranes were incubated with primary antibodies specific for MED15 (rabbit monoclonal, 1:200, 11566-1-AP, Proteintech, Chicago, IL), for ßACTIN (mouse monoclonal, 1:5000, A1978, St.Louis, MO) or for pSMAD3 (rabbit monoclonal, 1:1000, EP823Y, Abcam, Cambridge, UK) overnight as previously described [22, 28, 29]. Then, membranes were incubtaed with horseradish peroxidase (HRP)-conjugated secondary antibodies at room temperature for 1 hour. Staining was detected using ECL Plus chemiluminescence sytem (GE Healthcare Bio-Sciences, Pittsburgh, US).

Migration Assay

48 hours after siRNA transfection, SCC-25 cells were seeded in 6-well plates and grown until confluence. Cells were treated with Mitomycin C (Sigma Aldrich, MO, US) (10µg/ml) for 2 hours to prevent cell proliferation. A wound healing assay was performed in which a scratch was then made across the center of each well using a sterile 200µl pipette tip and non-adherent cells were washed off with PBS. Images were taken immediately and after 24, 48, 72 and 96 hours.

TGFß1 Treatment

HSC-3 and SCC-25 cells were seeded in 6-well plates in growth medium to attach overnight followed by serum starvation for 24 hours with medium containing 2% Fetal Calf Serum (FCS). Cells were then treated with 10 or 20 ng/ml recombinant TGFß1 (Immunotools, Friesoythe, Germany) or with distilled water as control in medium containing 2% FCS for 1, 2 or 6 hours. Thereafter, cells were harvested for protein analysis. SCC25 cells were transfected with siRNA, after 96 hours cells serum starved for 24 hours with medium containing 2% FCS following treatment with 10 or 20ng/ml TGFß1 for 1 hour.

Statistical analysis

Statistical analysis was performed using SPSS Statistics 17.0 (SPSS, Chicago, IL).

Results

MED15 expression in head and neck cancer tissues (HNSCC) and cell lines

IHC shows MED15 to be overexpressed in 35% of primary tumors, 30% of lymph node metastases, and 70% of recurrences, in contrast to no/low expression in all benign tissues (**Fig. 1a-d**). In particular, we observed that 20% of primary tumors exhibited no expression of MED15, whereas 45% harbored low levels of MED15. Furthermore, 15% of lymph node metastases exhibited a lack of MED15 expression, and 55% showed low expression levels. In recurrent tumor tissues, MED15 is expressed at low levels in 23% of samples, whereas a subset of 7% exhibited no MED15 expression. To illustrate the increase in MED15 expression during HNSCC progression, we compared the median of the MED15 expression score among the different stages **(Fig. 1e)**.

In order to examine the protein expression level of MED15 in HNSCC cells, we performed IHC (**Fig. 2a**) and IF (**Fig. 2b**) in the squamous cell carcinoma (SCC) cells HSC-3 and SCC-25, which are both known to exhibit invasive and metastatic behavior [30]. Our results show that both cell lines harbor a strong nuclear MED15 expression **(Fig. 2a, b)**.

Correlation between MED15 and clinicopathological features

We found MED15 overexpression to associate with heavy alcohol consumption (Chi-Square, $^{**}p \leq 0.01$). Correlation analysis with age, sex, tobacco consumption, survival of patients as well as with pathological features as tumor localization, T-state, presence of lymph node or distant metastases at time of diagnosis and HPV-status revealed no significant association. Clinicopathological characteristics of patients and the corresponding MED15 overexpression status are provided as **Supplementary Table 1.**

MED15 overexpression associates with high mortality rate

To investigate whether MED15 overexpression associates with clinicopathological data of a specific subset of patients, we performed IHC on primary tumor tissues from 108 patients who all developed recurrent tumors. As a result, we found a significant association between MED15 overexpression and high mortality rate (78%) of patients

(Chi-Square *p ≤ 0.05) **(Fig. 3a)**. Upon comparing the expression score of MED15 in tumors of different localizations, we found a significantly higher expression of MED15 in tumors originating from the oral cavity or oropharynx in comparison with tumors from the hypopharynx or larynx (independent samples t-test **p ≤ 0.01) **(Fig. 3b)**. Interestingly, the mortality rate of patients harboring tumors in the oropharynx or oral cavity was significantly higher than of patients harboring the tumor in the hypopharynx or larynx (Chi-Square **p ≤ 0.01) **(Fig. 3c)**. Furthermore, we found MED15 overexpression to associate with heavy alcohol consumption of patients (Chi-Square *p ≤ 0.05). Correlation analysis to other clinical data revealed no significance. Clinicopathological characteristics of patients who developed recurrent disease and the corresponding MED15 overexpression status are provided as **Supplementary Table 2.**

MED15 overexpression is a clonal event during HNSCC progression

To investigate whether MED15 overexpression may be a clonal event during cancer progression, we compared the expression of MED15 among patients for which we had matched tissues from both primary tumors and their corresponding lymph node metastases. We found the vast majority of patients to show a concordance for the MED15 overexpression or lack of it between primary tumors and their matched corresponding lymph node metastases **(Fig. 4a)**. Furthermore, we observed a significant correlation between the expression scores of MED15 in the lymph node metastases and the corresponding primary tumors (Pearson correlation, **p ≤ 0.01) (**Fig. 4b**). Each dot represents the expression score of the primary tumor (x-axis) and the corresponding lymph node (y-axis) for one patient.

Effect of MED15 upon proliferation in HNSCC

IHC staining for the proliferation marker Ki67 in HNSCC tissues revealed a significant correlation between MED15 expression and high proliferative activity (Pearson correlation **p ≤ 0.01). To investigate whether MED15 affects proliferation, we performed siRNA mediated knockdown of MED15 in the HNSCC cell lines HSC-3 and SCC-25 (**Fig. 5a**). Thereafter, we compared the measured proliferative activity of control and MED15 knockdown cells by MTT proliferation assay as well as by IHC for Ki67. We observed a significant reduction in cellular proliferation in MED15 knockdown cells as

compared to control cells after 72 hours (**Fig. 5b**). Additionally, SCC-25 cells with MED15 knockdown exhibited a reduction in the number of Ki67 positive cells in comparison to control cells (**Fig. 5c**).

MED15 effects migration in HNSCC cells

To investigate the effect of MED15 knockdown upon migration potential of HNSCC cells, we used the wound healing assay. After 48 hours, SCC-25 control cells colonized the wound at faster rate than MED15 knockdown cells (**Fig. 6a**). In contrast to MED15 knockdown cells, control cells covered the wound completely after 96 hours (**Fig. 6a**).

MED15 expression is linked to TGFß signaling

IHC for pSMAD3 as an activation marker for TGFß signaling revealed an association between TGFß signaling activation and MED15 expression (Fisher´s exact test *$p \leq 0.05$). To investigate whether MED15 is implicated in TGFß signaling in the HNSCC cell lines HSC-3 and SCC-25, we first analyzed the basal activity status of TGFß signaling as well as the concomitant expression of MED15 **(Fig. 6b)**. Western blot analysis showed that MED15 and pSMAD3 were co-expressed in HSC-3 and SCC-25 cells (**Fig. 6b**). To examine whether hyper-activated TGFß signaling leads to increased MED15 protein expression, we treated serum-starved HSC-3 and SCC-25 cells with recombinant TGFß1. Phosphorylation of SMAD3 following TGFß1 treatment for 1 hour indicates successful activation of TGFß signaling by the recombinant protein treatment (**Fig. 6c**). In HSC-3 and SCC-25 cells, we observed an increased expression of MED15 at protein level in TGFß1 treated cells in comparison to untreated cells after 6 and 2 hours, respectively (**Fig. 6d**).

The observation that high TGFß activity correlates with strong MED15 expression prompted us to investigate whether MED15 knockdown affects the activity of TGFß signaling. We found that SCC-25 cells with MED15 knockdown expressed lower pSMAD3 levels following TGFß treatment compared to control cells by Western blot analysis **(Fig. 6e)** as well IHC (data not shown).

Discussion

To our knowledge, this study provides the first evidence that a Mediator complex subunit is expressed and functionally implicated in HNSCC. We found the Mediator subunit MED15 to be overexpressed in HNSCC in contrast to no or low expression in benign epithelium **(Fig. 1a-d)**. MED15 is overexpressed in 35% of primary tumors, 30% of lymph node metastases, and interestingly in 70% of recurrent tumors. Additionally, we observed an increased level of MED15 protein expression during progression of the disease **(Fig. 1e)**. Observing that MED15 is overexpressed at high frequency in recurrent tumor tissues, prompted us to investigate whether MED15 is implicated in the survival of patients who developed relapses. Even though there is a high mortality rate in patients who suffered from recurrences (58%), we found MED15 overexpression to associate significantly with an even higher mortality rate (78%) of the patients **(Fig. 3a)**. It is worth noting that survival rates have been reported to differ between HNSCC patients and oral/oropharyngeal tumors are generally associated with worse outcome than hypopharyngeal/laryngeal tumors [7]. Consistent with that, we observed that tumors occurring in the oropharynx or oral cavity are associated with higher mortality rates in comparison with tumors in the hypopharynx or larynx **(Fig. 3c)**. Interestingly, we found a significantly higher expression score of MED15 in tumors originating in the oral cavity or oropharynx in comparison to tumors from the hypopharynx or larynx **(Fig. 3b)**.

There was a strong correlation between the expression levels of MED15 in primary tumors and their corresponding lymph node metastasis from individual patients **(Fig. 4b)**, as well as concordance in 74% of cases for presence or absence of MED15 overexpression **(Fig. 4a).** Such observation indicates that MED15 overexpression is likely to be a clonal event in the progression of HNSCC **(Fig. 4a)**. Although the majority of patients shows concordance, we observed discordance in a subset of patients **(Fig. 4a)**. In the case of patients harboring MED15 overexpression in the primary tumors, but lack overexpression in the corresponding lymph node metastasis, it is plausible that such observation is due to the fact that the lymph node metastases which we accessed have developed from another primary tumor clone lacking MED15 overexpression, combined with our inability to have access to all of the lymph node metastases associated with these patients. In the few cases in which the lymph node metastasis

exhibits a MED15 overexpression, but lack overexpression in the primary tumor, such observation might be due the heterogeneity within the primary tumor. Multiple clones within a tumor are known to generate intratumoral heterogeneity in HNSCC [31], and several primary clones may have led to metastasis. As we had no access to all of the primary clones, we may have matched primary tumors and lymph node metastases from different cell clones, thus observing different levels of MED15 expression.

These findings regarding MED15 overexpression is particularly significant, as genetic alterations which provide cells with growth advantages and metastatic potential may be present only in subpopulation of cells in the primary tumor, but increase in tissue from metastases and relapsed HNSCC tumors [32]. Recurrence and metastatic disease are reasons for poor survival rates of patients with HNSCC and remain a challenge to treat [33]. Finding molecular markers that may predict the risk for development of tumor recurrence and presence of metastases might be helpful for early diagnosis and treatment decisions of HNSCC relapse. Results of HNSCC genetic analyses suggest a clonal relationship between recurrent primary tumor and the initial primary tumor [34]. Interestingly, overexpression of MED15 is detectable in 70% of recurrent HNSCC tumors **(Fig. 1e)**, and our correlation analysis provides evidence that MED15 overexpression is likely to be a clonal event during HNSCC progression **(Fig. 4a)**. Based on our observations, MED15 overexpression may have the potential to predict the likelihood of the development of recurrences occurring when detected in cancer cells of the primary HNSCC tumors. In support of our tissue findings, we found MED15 to be highly expressed in the HNSCC malignant cell lines HSC-3 and SCC-25 **(Fig. 2a, b)**. Furthermore, we found MED15 expression to correlate with high proliferative activity in HNSCC tissues, and knockdown of MED15 reduced both proliferation **(Fig. 5b, c)** and migration **(Fig. 6a)** in functional assays. These findings combined with the high frequency of MED15 overexpression in recurrent HNSCC tumors, indicate that targeting MED15 may serve as a potential therapeutic option especially efficient in patients displaying advanced disease and tumor recurrence. The development of a small-molecule inhibitor specific for MED15 which blocks the activity of MED15 might be a possible strategy to target intracellular MED15 in patients with HNSCC. Alternatively, a small-molecule inhibitor can be used to disrupt the interaction between MED15 and

pSMAD3. As MED15 is localized in the cytoplasm and nucleus of cancer cells, small-molecule inhibitors which reach intracellular sites through diffusion present the most effective way of targeting MED15.

Furthermore, our results provide evidence for a direct link between MED15 expression and TGFß signaling in HNSCC **(Fig. 6b, d)**. In HNSCC tissues, we observed a significant association between pSMAD3 and MED15 expression. We found increased MED15 expression in response to hyper-activation of TGFß signaling in HSC-3 and SCC-25 cell lines **(Fig. 6d)**. In support of these functional findings, similar observations have been reported in breast [21] and prostate cancer [22] cells. Findings in breast [21] and prostate [22] cancers have shown that MED15 is required for TGFß transcriptional activation [17, 21] and TGFß regulated downstream effects [17, 21, 22]. In this study, we found MED15 and pSMAD3 expression to associate in HNSCC tissue, and that cases lacking MED15 exhibited no pSMAD3 expression. Collectively, these findings indicate that MED15 effects TGFß signaling activation. In many cancer types, including HNSCC, dysregulated TGFß signaling promotes an epithelial-mesenchymal transition- (EMT) like phenotype through several mechanisms [23]. This includes regulation of the expression of transcription factors which are involved in EMT [35], and interestingly, MED15 is known to be essential for TGFß activated gene expression [17, 21]. Several studies revealed that TGFß induced EMT in cancer cells is SMAD dependent [36, 37], and expression of activated SMAD in HNSCC tissues correlates with worse survival [24]. Our observation that pSMAD3 is reduced upon MED15 knockdown in SCC25 cells is consistent with similar findings in breast [21] and prostate [22] carcinoma cells. Furthermore, TGFß1 stimulation of HNSCC cells has been shown to increase the migration ability in wound healing assays [35], and our results reveal a reduced migration of HNSCC cells as well as decreased levels of pSMAD3 in response to TGFß stimulation upon MED15 knockdown **(Fig. 6a, e)**. As TGFß signal transduction plays a critical role in HNSCC progression [24, 38], targeting MED15 based on our results may have a major impact on the progression of HNSCC.

Taken together, our findings implicate the Mediator complex through its subunit MED15 for the first time in HNSCC. We further present evidence that MED15 overexpression is a clonal event during HNSCC progression and associates with high mortality in patients

suffering from recurrences. Our findings further suggest that MED15 may serve as a prognostic marker for recurrence, and as therapeutic target in HNSCC patients suffering from recurrences.

Disclosure/Conflict of interest

We declare no conflict of interest.

References

[1] Barnes L, Eveson, J.W., Reichart, P., Sidransky, D.: Pathology and Genetics of Head and Neck Tumours, WHO Classification of Tumours.
[2] Warnakulasuriya S: Global epidemiology of oral and oropharyngeal cancer. Oral Oncol 2009, 45:309-16.
[3] Kamangar F, Dores GM, Anderson WF: Patterns of cancer incidence, mortality, and prevalence across five continents: defining priorities to reduce cancer disparities in different geographic regions of the world. J Clin Oncol 2006, 24:2137-50.
[4] Forastiere A, Koch W, Trotti A, Sidransky D: Head and neck cancer. N Engl J Med 2001, 345:1890-900.
[5] Woolgar JA, Triantafyllou A: A histopathological appraisal of surgical margins in oral and oropharyngeal cancer resection specimens. Oral Oncol 2005, 41:1034-43.
[6] Boehm A, Wichmann G, Mozet C, Dietz A: [Current therapy options in recurrent head and neck cancer]. HNO 2010, 58:762-9.
[7] Jemal A, Siegel R, Ward E, Murray T, Xu J, Thun MJ: Cancer statistics, 2007. CA Cancer J Clin 2007, 57:43-66.
[8] Hecht SS: Tobacco carcinogens, their biomarkers and tobacco-induced cancer. Nat Rev Cancer 2003, 3:733-44.
[9] Benson E, Li R, Eisele D, Fakhry C: The clinical impact of HPV tumor status upon head and neck squamous cell carcinomas. Oral Oncol 2013.
[10] Stransky N, Egloff AM, Tward AD, Kostic AD, Cibulskis K, Sivachenko A, Kryukov GV, Lawrence MS, Sougnez C, McKenna A, Shefler E, Ramos AH, Stojanov P, Carter SL, Voet D, Cortes ML, Auclair D, Berger MF, Saksena G, Guiducci C, Onofrio RC, Parkin M, Romkes M, Weissfeld JL, Seethala RR, Wang L, Rangel-Escareno C, Fernandez-Lopez JC, Hidalgo-Miranda A, Melendez-Zajgla J, Winckler W, Ardlie K, Gabriel SB, Meyerson M, Lander ES, Getz G, Golub TR, Garraway LA, Grandis JR: The mutational landscape of head and neck squamous cell carcinoma. Science 2011, 333:1157-60.
[11] Molinolo AA, Amornphimoltham P, Squarize CH, Castilho RM, Patel V, Gutkind JS: Dysregulated molecular networks in head and neck carcinogenesis. Oral Oncol 2009, 45:324-34.
[12] Du Y, Peyser ND, Grandis JR: Integration of molecular targeted therapy with radiation in head and neck cancer. Pharmacol Ther 2013.
[13] Malik S, Roeder RG: The metazoan Mediator co-activator complex as an integrative hub for transcriptional regulation. Nat Rev Genet 2010, 11:761-72.
[14] Lewis BA, Reinberg D: The mediator coactivator complex: functional and physical roles in transcriptional regulation. J Cell Sci 2003, 116:3667-75.

[15] Napoli C, Sessa M, Infante T, Casamassimi A: Unraveling framework of the ancestral Mediator complex in human diseases. Biochimie 2012, 94:579-87.
[16] Yang F, Vought BW, Satterlee JS, Walker AK, Jim Sun ZY, Watts JL, DeBeaumont R, Saito RM, Hyberts SG, Yang S, Macol C, Iyer L, Tjian R, van den Heuvel S, Hart AC, Wagner G, Naar AM: An ARC/Mediator subunit required for SREBP control of cholesterol and lipid homeostasis. Nature 2006, 442:700-4.
[17] Kato Y, Habas R, Katsuyama Y, Naar AM, He X: A component of the ARC/Mediator complex required for TGF beta/Nodal signalling. Nature 2002, 418:641-6.
[18] Thakur JK, Arthanari H, Yang F, Pan SJ, Fan X, Breger J, Frueh DP, Gulshan K, Li DK, Mylonakis E, Struhl K, Moye-Rowley WS, Cormack BP, Wagner G, Naar AM: A nuclear receptor-like pathway regulating multidrug resistance in fungi. Nature 2008, 452:604-9.
[19] Xiaoli, Yang F: Mediating lipid biosynthesis: implications for cardiovascular disease. Trends Cardiovasc Med 2013, 23:269-73.
[20] Berti L, Mittler G, Przemeck GK, Stelzer G, Gunzler B, Amati F, Conti E, Dallapiccola B, Hrabe de Angelis M, Novelli G, Meisterernst M: Isolation and characterization of a novel gene from the DiGeorge chromosomal region that encodes for a mediator subunit. Genomics 2001, 74:320-32.
[21] Zhao M, Yang X, Fu Y, Wang H, Ning Y, Yan J, Chen YG, Wang G: Mediator MED15 modulates transforming growth factor beta (TGFbeta)/Smad signaling and breast cancer cell metastasis. J Mol Cell Biol 2013, 5:57-60.
[22] Shaikhibrahim Z, Menon R, Braun M, Offermann A, Queisser A, Boehm D, Vogel W, Ruenauver K, Ruiz C, Zellweger T, Svensson M, Andren O, Kristiansen G, Wernert N, Bubendorf L, Kirfel J, Biskup S, Perner S: MED15, encoding a subunit of the mediator complex, is overexpressed at high frequency in castration-resistant prostate cancer. Int J Cancer 2013.
[23] White RA, Malkoski SP, Wang XJ: TGFbeta signaling in head and neck squamous cell carcinoma. Oncogene 2010, 29:5437-46.
[24] Xie W, Aisner S, Baredes S, Sreepada G, Shah R, Reiss M: Alterations of Smad expression and activation in defining 2 subtypes of human head and neck squamous cell carcinoma. Head Neck 2013, 35:76-85.
[25] Iyer NG, Ozdag H, Caldas C: p300/CBP and cancer. Oncogene 2004, 23:4225-31.
[26] Braun M, Kirsten R, Rupp NJ, Moch H, Fend F, Wernert N, Kristiansen G, Perner S: Quantification of protein expression in cells and cellular subcompartments on immunohistochemical sections using a computer supported image analysis system. Histol Histopathol 2013, 28:605-10.
[27] Castro F, Dirks WG, Fahnrich S, Hotz-Wagenblatt A, Pawlita M, Schmitt M: High-throughput SNP-based authentication of human cell lines. Int J Cancer 2013, 132:308-14.
[28] Litzenburger BC, Kim HJ, Kuiatse I, Carboni JM, Attar RM, Gottardis MM, Fairchild CR, Lee AV: BMS-536924 reverses IGF-IR-induced transformation of mammary epithelial cells and causes growth inhibition and polarization of MCF7 cells. Clin Cancer Res 2009, 15:226-37.
[29] Ding Z, Wu CJ, Chu GC, Xiao Y, Ho D, Zhang J, Perry SR, Labrot ES, Wu X, Lis R, Hoshida Y, Hiller D, Hu B, Jiang S, Zheng H, Stegh AH, Scott KL, Signoretti S, Bardeesy N, Wang YA, Hill DE, Golub TR, Stampfer MJ, Wong WH, Loda M, Mucci L, Chin L, DePinho RA: SMAD4-dependent barrier constrains prostate cancer growth and metastatic progression. Nature 2011, 470:269-73.

[30] Erdem NF, Carlson ER, Gerard DA, Ichiki AT: Characterization of 3 oral squamous cell carcinoma cell lines with different invasion and/or metastatic potentials. J Oral Maxillofac Surg 2007, 65:1725-33.
[31] Mroz EA, Tward AD, Pickering CR, Myers JN, Ferris RL, Rocco JW: High intratumor genetic heterogeneity is related to worse outcome in patients with head and neck squamous cell carcinoma. Cancer 2013, 119:3034-42.
[32] Bockmuhl U, Schluns K, Schmidt S, Matthias S, Petersen I: Chromosomal alterations during metastasis formation of head and neck squamous cell carcinoma. Genes Chromosomes Cancer 2002, 33:29-35.
[33] Ho AS, Kraus DH, Ganly I, Lee NY, Shah JP, Morris LG: Decision making in the management of recurrent head and neck cancer. Head Neck 2014, 36:144-51.
[34] Califano J, Westra WH, Meininger G, Corio R, Koch WM, Sidransky D: Genetic progression and clonal relationship of recurrent premalignant head and neck lesions. Clin Cancer Res 2000, 6:347-52.
[35] Saito D, Kyakumoto S, Chosa N, Ibi M, Takahashi N, Okubo N, Sawada S, Ishisaki A, Kamo M: Transforming growth factor-beta1 induces epithelial-mesenchymal transition and integrin alpha3beta1-mediated cell migration of HSC-4 human squamous cell carcinoma cells through Slug. J Biochem 2013, 153:303-15.
[36] Schiller M, Dennler S, Anderegg U, Kokot A, Simon JC, Luger TA, Mauviel A, Bohm M: Increased cAMP levels modulate transforming growth factor-beta/Smad-induced expression of extracellular matrix components and other key fibroblast effector functions. J Biol Chem 2010, 285:409-21.
[37] Porsch H, Bernert B, Mehic M, Theocharis AD, Heldin CH, Heldin P: Efficient TGFbeta-induced epithelial-mesenchymal transition depends on hyaluronan synthase HAS2. Oncogene 2013, 32:4355-65.
[38] Prime SS, Davies M, Pring M, Paterson IC: The role of TGF-beta in epithelial malignancy and its relevance to the pathogenesis of oral cancer (part II). Crit Rev Oral Biol Med 2004, 15:337-47.

Fig. 1

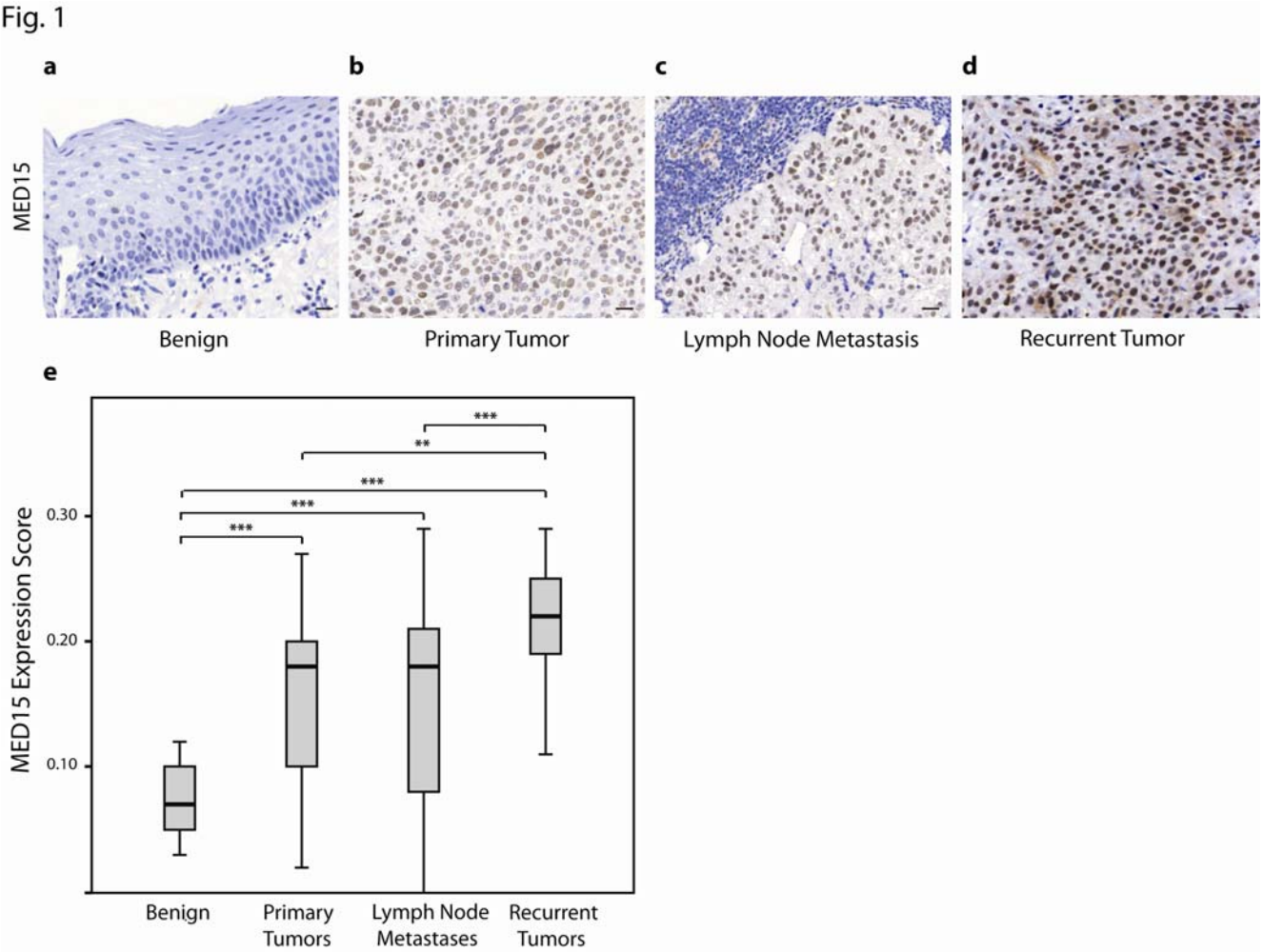


Figure 1. MED15 expression in head and neck tissues. Immunohistochemical staining reveals expression of MED15 in benign epithelium (A), primary tumor (B), lymph node metastasis (C), and recurrent tumor tissues (D). E: Box plots of the median MED15 protein expression reveal increased MED15 expression during cancer progression. **P < 0.01, ***P < 0.001, independent-sample t-test.
Scale bar = 20 μm (A-D).

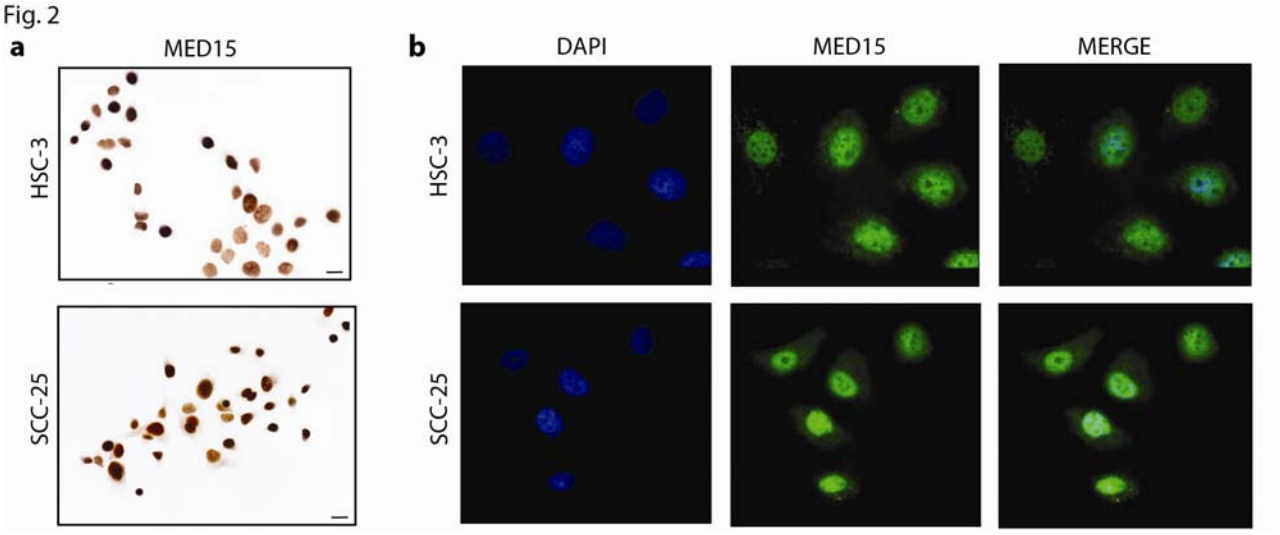


Figure 2. MED15 expression in head and neck squamous cell carcinoma (HNSCC) cells. Immunohistochemical (A) and immunofluorescent (B) staining reveals nuclear MED15 expression in the HNSCC cells HSC-3 and SCC-25. Scale bars: 20 µm (A); 10 µm (B).

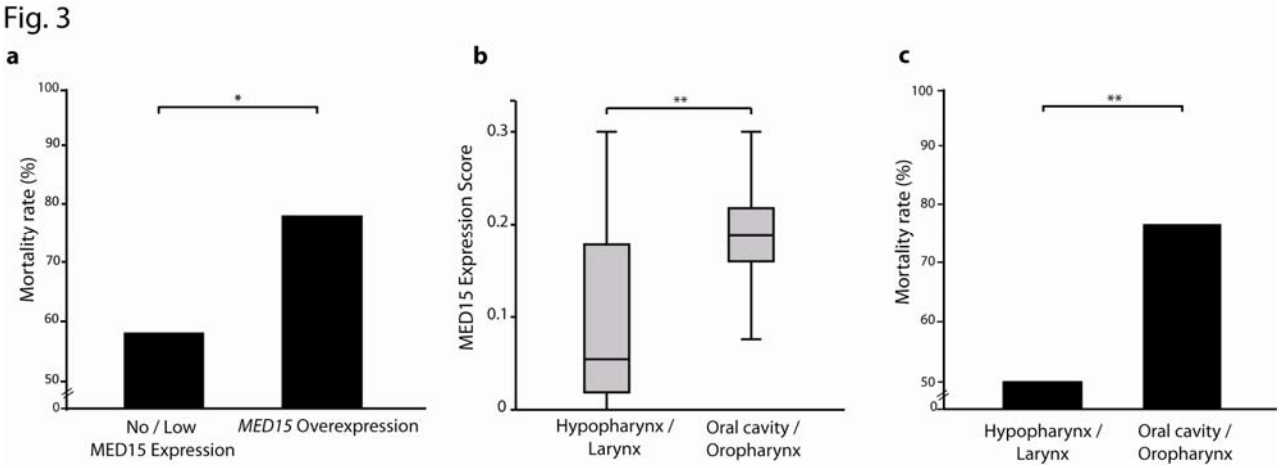


Figure 3 Association of MED15 with mortality rates and site-specific expression. A: Mortality rates of patients harboring MED15 overexpression in primary tumor (78%) compared with patients with no or low MED15 expression (58%). B: MED15 expression in tumors localized in the oral cavity or oropharynx compared with the hypopharyngeal or laryngeal tumors. C: Mortality rates of patients with tumors in the oral cavity or oropharynx (77%) compared with patients with hypopharyngeal or laryngeal tumors (50%). *P < 0.05, x2-test (A); **P ≤ 0.01, independent-sample t-test (B) and x2-test (C).

Fig. 4

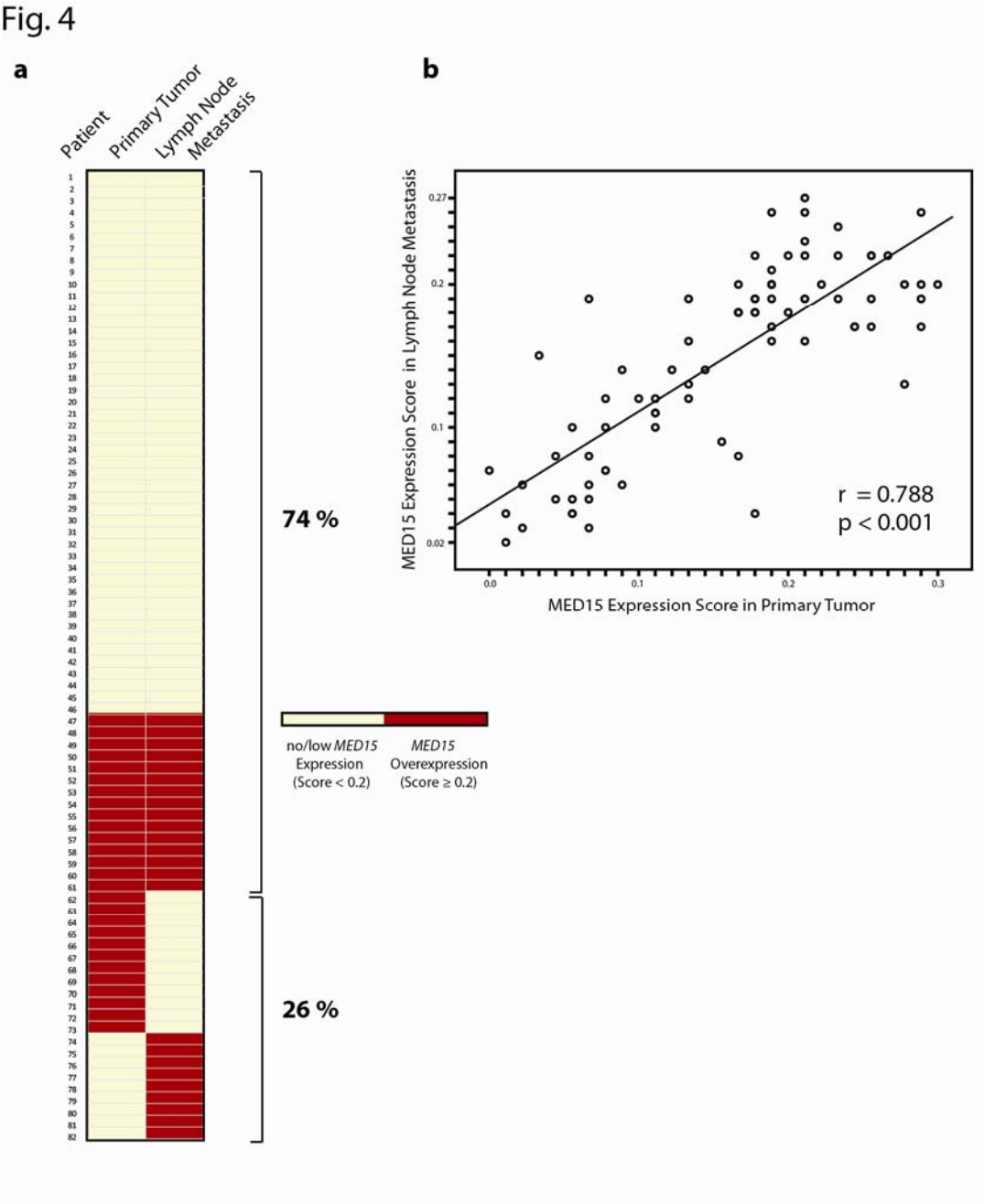


Figure 4. MED15 expression in primary tumors and matched corresponding lymph node metastases. A: Heat map illustrating concordance for MED15 overexpression in primary tumors and their matched corresponding lymph node metastases in 74% of patients. B: Scatterplot showing a significant correlation between the expression score of MED15 in primary tumors and corresponding lymph node metastases (r=0.788). ***P ≤ 0.001, Pearson correlation (B).

Fig. 5

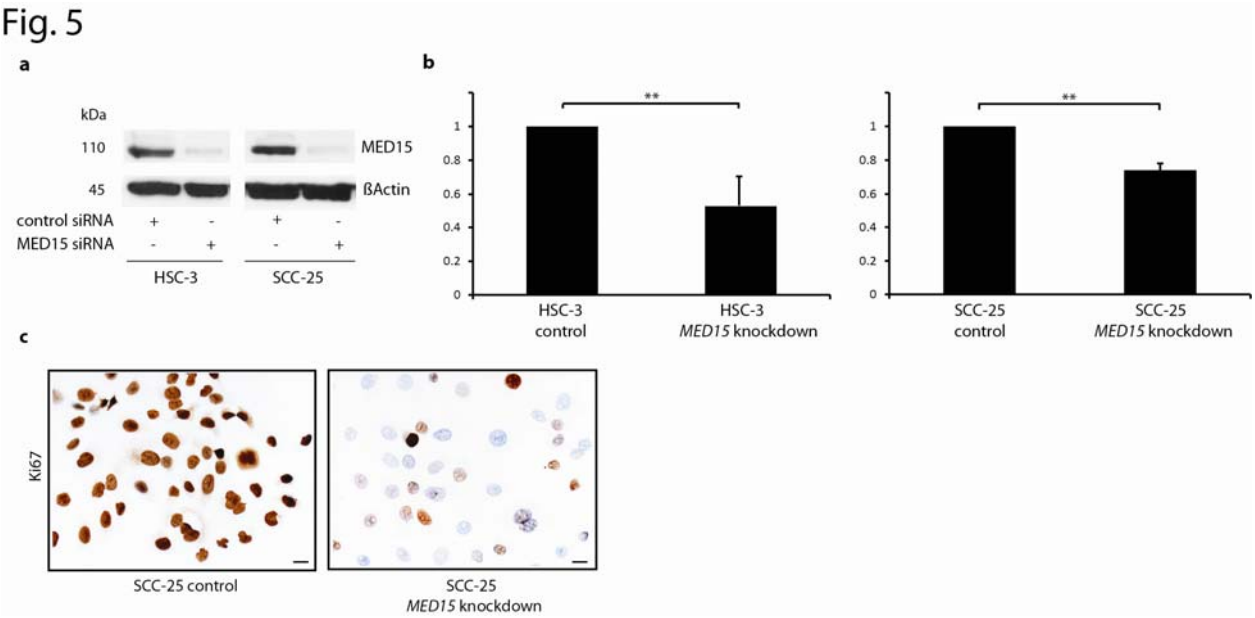


Figure 5 Correlation between MED15 expression and proliferation in head and neck squamous cell carcinoma (HNSCC) tissues and the effect of MED15 knockdown on HNSCC cell proliferation. A: Western blot showing siRNA-mediated MED15 knockdown in HSC-3 and SCC-25 cells. B: MTT proliferation assay of HSC-3 and SCC-25 cells reveals reduced proliferation in MED15 knockdown cells compared with control cells after 72 hours. Absorbance at 595 nm was normalized to control cells. C: Immunohistochemical staining reveals decreased Ki-67 expression in SCC-25 cells with MED15 knockdown compared with control cells 96 hours after siRNA transfection. **P ≤ 0.01, one-sample t-test (B). Scale bar = 20 µm (C).

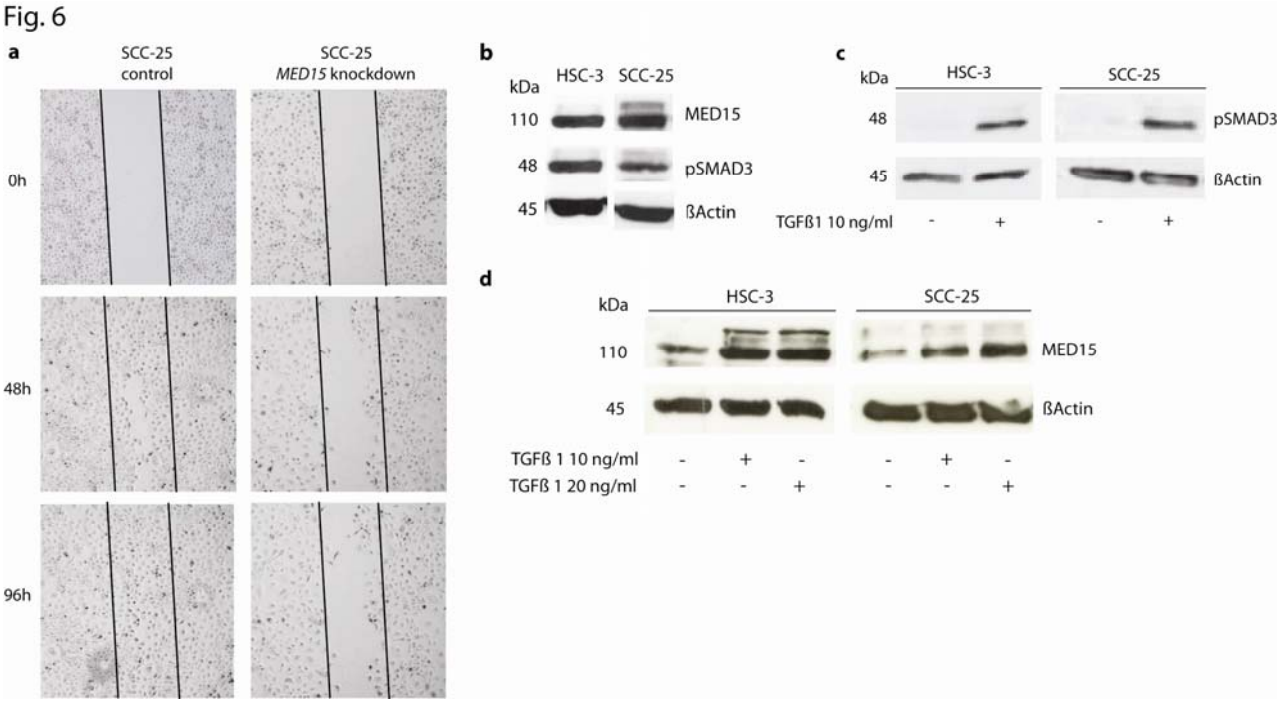
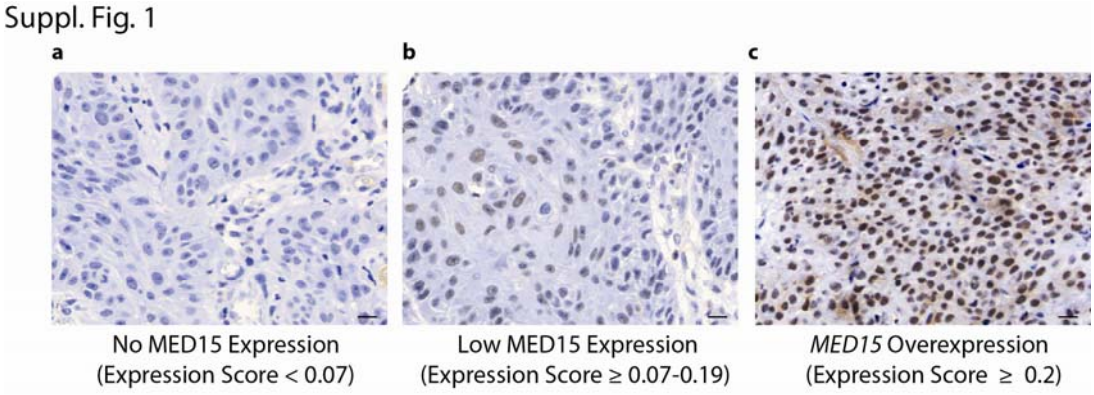


Figure 6 Effect of MED15 on migration and association between MED15 and transforming growth factor (TGF)-b signaling. A: Wound healing scratch assay in SCC-25 cells reveals reduced migration of MED15 knockdown cells compared with control cells. Images of cells were taken immediately (0 hour) and at indicated hours after the scratch. B: Western blot of HSC-3 and SCC-25 cell protein lysates exhibiting co-expression of MED15 and pSMAD3. C: Western blot of protein lysates prepared from serum-starved HSC-3 and SCC-25 cells that were treated with 10 ng/mL of TGF-b1 have increased phosphorylation of SMAD3 after 1 hour. D: Western blot of HSC-3 and SCC-25 cells that were treated with 10 or 20 ng/mL of TGF-b1 reveals increased MED15 expression level after 6 hours in HSC-3 cells and after 2 hours in SCC-25 cells. E: Western blot of SCC-25 cells that were treated with scrambled or MED15 siRNA followed by TGF-b treatment for 1 hour reveals reduced pSMAD3 expression in MED15 knockdown cells. Scale bar = 40 µm (A).



Supplementary Figure 1. MED15 expression levels in HNSCC tissues. MED15 expression levels based on the expression score: **(a)** no expression (score < 0.07), **(b)** low expression (score ≥ 0.07-0.19) or **(c)** overexpression (score ≥ 0.2).

Suppl. Table 1. MED15 Expression in primary tumors and clinicopathological data of patients with corresponding cancer tissues.

		Number	No/Low MED15 Expression	*MED15* Overexpression	p Value
Age	< 60 years	108	31	15	0.514
	≥ 60 years		38	24	
Gender	Male	108	55	31	0.978
	Female		14	8	
Site of Origin	Oral cavity/Oropharynx	107	42	30	0.108
	Hypopharynx /Larynx		26	9	
Tobacco	Nonsmoker	86	11	3	0.308
	Smoker		48	24	
Alcohol	Non-Drinker/Occasional	80	42	11	
	Heavy Consumption		13	14	0.005
Pathological features					
T-Status	T1-T2	105	33	24	0.169
	T3-T4		34	14	
Lymph Node Metastasis (at time of presentation)	Negative	105	16	9	0.892
	Positive		50	30	
Distant Metastasis (at time of presentation)	Negative	108	67	36	0.248
	Positive		2	3	
HPV Status	Negative	108	59	36	0.235
	Positive		10	3	
Survival	Survived	98	37	18	0.485
	Death		26	17	

Suppl. Table 2. MED15 Expression in primary tumors and clinicopathological data of patients who developed recurrent tumors.

		Number	No/Low MED15 Expression	*MED15* Overexpression	p Value
Age	< 60 years	108	27	13	0.616
	≥ 60 years		49	19	
Gender	Male	108	60	22	0.258
	Female		16	10	
Site of Origin	Oral cavity/Oropharynx	106	35	25	0.003
	Hypopharynx /Larynx		39	7	
Tobacco	Nonsmoker	86	8	1	0.235
	Smoker		54	23	
Alcohol	Non-Drinker/Occasional	80	39	9	
	Heavy Consumption		19	13	0.032
Pathological features					
T-Status	T1-T2	103	45	19	0.698
	T3-T4		26	13	
Lymph Node Metastasis (at time of presentation)	Negative	105	42	13	0.892
	Positive		29	19	
Distant Metastasis (at time of presentation)	Negative	108	74	30	0.363
	Positive		2	2	
HPV Status	Negative	108	72	29	0.428
	Positive		4	3	
Survival	Survived	108	32	7	
	Death		44	25	0.046

3. Danksagung

Besonders bedanken möchte ich bei Herrn Prof. Dr. med. Sven Perner, der mir die Durchführung meiner Doktorarbeit ermöglicht hat und mein Interesse an der medizinischen Wissenschaft geweckt, gefördert und immer unterstützt hat. Dank seiner Motivation und Zustimmung hatte ich die Möglichkeit, mich durch ein Stipendium des SciMed-Promotionskollegs uneingeschränkt auf die Doktorarbeit zu konzentrieren und erste Einblicke in die Wissenschaft zu erlangen. Ich danke Sven Perner für die wertvolle, unvergessliche Zeit während meiner Doktorarbeit, sein Vertrauen in meine Fähigkeiten und die persönlichen Erfahrungen, die ich dadurch machen konnte.

Zudem gilt mein besonderer Dank Herr PD Dr. med. Zaki Shaikhibrahim für die ausgezeichnete Betreuung und besonders für seine uneingeschränkte Motivation während unserer Zusammenarbeit. Dank seiner tiefgründigen Einarbeitung in die Thematik, unserer intensiven Diskussionen und seines Vertrauens konnte ich eigene Ideen für unser Projekt entwickeln und umsetzen. Gerne erinnere ich mich an unermüdliche Diskussionen zu jeder Zeit und eine freundliche Zusammenarbeit.

Weiterhin möchte mich bei der gesamten Arbeitsgruppe bedanken, vor allem bei Wenzel Vogel für die Hilfe im Labor und bei jeglichen Fragen, und bei Martin Braun, Roopika Menon, Angela Queisser und Diana Böhm für die praktische Einarbeitung und ständige Hilfe im Labor. Danke an Kerstin Fuchs für die geduldige Einführung in die Zellkultur.

Ich bedanke mich bei der BONFOR-Forschungskommission für das Promotionsstipendium, wodurch ich mich uneingeschränkt auf die Erarbeitung meiner Doktorarbeit konzentrieren konnte.

Danke an meine Familie und meine Freunde, die mich auf dem ganzen Weg dieser Arbeit begleitet und mir stets verständnisvollen Rückhalt gegeben haben. Besonders meinen Eltern möchte ich für die uneingeschränkte Unterstützung und ihr Vertrauen danken.

4. Kongressbeiträge und wissenschaftliche Veröffentlichungen

Kongressbeiträge als päsentierender Autor

MED12 is a Potential Target for Therapeutic Intervention in Castration Resistant Prostate Cancer. **Offermann A,** Shaikhibrahim Z, Braun M, Menon R, Syring I, Nowak M, Halbach R, Vogel W, Ruiz C, Zellweger T, Rentsch CA, Svensson M, Andren O, Bubendorf L, Biskup S, Duensing S, Kirfel J, Perner S
Kongress der United States and Canadian Academy of Pathology (USCAP), San Diego, 2014 Plattformpräsentation

MED15 is Overexpressed at High Frequency in Head and Neck Squamous Cell Carcinoma and is Implicated in TGFβ Signaling. **Offermann A,** Shaikhibrahim Z, Halbach R, Vogel W, Braun M, Kristiansen G, Bootz F, Mikut R, Lengerke C, Reischl M, Schröck A, Perner S
Kongress der United States and Canadian Academy of Pathology (USCAP), San Diego, 2014 Posterpräsentation

MED12 Overexpression is a frequent event in prostate cancer. **Offermann A,** Shaikhibrahim Z, Braun M, Menon R, Syring I, Nowak M, Halbach R, Vogel W, Ruiz C, Zellweger T, Rentsch CA, Svensson M, Andren O, Bubendorf L, Biskup S, Duensing S, Kirfel J, Perner S
Kongress der Deutschen Gesellschaft für Pathologie (DGP), Berlin, 2014
Plattformpräsentation

MED15 is Overexpressed at High Frequency in Head and Neck Squamous Cell Carcinoma and is Implicated in TGFβ Signaling. **Offermann A,** Shaikhibrahim Z, Halbach R, Vogel W, Braun M, Kristiansen G, Bootz F, Mikut R, Lengerke C, Reischl M, Schröck A, Perner S
Kongress der Deutschen Gesellschaft für Pathologie (DGP), Berlin, 2014
Plattformpräsentation

MED12 Overexpression is a frequent event in prostate cancer. **Offermann A,** Shaikhibrahim Z, Braun M, Menon R, Syring I, Nowak M, Halbach R, Vogel W, Ruiz C, Zellweger T, Rentsch CA, Svensson M, Andren O, Bubendorf L, Biskup S, Duensing S, Kirfel J, Perner S
European Association of Cancer Research (EACR), München, 2014
Posterpräsentation

MED15 is Overexpressed at High Frequency in Head and Neck Squamous Cell Carcinoma and is Implicated in TGFβ Signaling. **Offermann A,** Shaikhibrahim Z, Halbach R, Vogel W, Braun M, Kristiansen G, Bootz F, Mikut R, Lengerke C, Reischl M, Schröck A, Perner S
European Association of Cancer Research (EACR), München, 2014
Posterpräsentation

Mutational landscape of the Mediator complex across human cancers. **Offermann A,** Shaikhibrahim Z, Boehm D, Deng M and Perner S
European Association of Cancer Research (EACR), München, 2014
Posterpräsentation

MED15 overexpression arises during androgen deprivation therapy via PI3K/mTOR signaling. **Offermann A**, Shaikhibrahim Z, Braun M, Syring I, Vogel W, Ruiz C, Zellweger T, Rentsch CA, Bubendorf L and Sven Perner
Kongress der United States and Canadian Academy of Pathology (USCAP), Boston, 2015
Posterpräsentation

MED15 Überexpression im kastrations-resistenten Prostatakarzinom vermittelt durch Aktivierung des PI3K/AKT/mTOR-Signalweges. **Offermann A**, Shaikhibrahim Z, Braun M, Syring I, Vogel W, Ruiz C, Zellweger T, Rentsch CA, Bubendorf L and Sven Perner
Kongress der Deutschen Gesellschaft für Pathologie (DGP), Frankfurt, 2015
Plattformpräsentation

Wissenschaftliche Veröffentlichungen

Menon R, Deng M, Rüenauver K, Queisser A, Peifer M, **Offermann A,** Boehm D, Vogel W, Scheble V, Fend F, Kristiansen G, Wernert N, Oberbeckmann N, Biskup S, Rubin MA, Shaikhibrahim Z, Perner S. Somatic copy number alterations by whole-exome sequencing implicates YWHAZ and PTK2 in castration-resistant prostate cancer. **J Pathol**. 2013; 231: 505-516

Shaikhibrahim Z, Menon R, Braun M, **Offermann A**, Queisser A, Boehm D, Vogel W, Rüenauver K, Ruiz C, Zellweger T, Svensson M, Andren O, Kristiansen G, Wernert N, Bubendorf L, Kirfel J, Biskup S, Perner S. MED15, encoding a subunit of the mediator complex, is overexpressed at high frequency in castration-resistant prostate cancer. **Int J Cancer** 2014; 135: 19-26

Shaikhibrahim Z*, **Offermann A*,** Braun M, Menon R, Syring I, Nowak M, Halbach R, Vogel W, Ruiz C, Zellweger T, Rentsch CA, Svensson M, Andren O, Bubendorf L, Biskup S, Duensing S, Kirfel J, Perner S. MED12 overexpression is a frequent event in castration-resistant prostate cancer. **Endocr Relat Cancer** 2014; 21: 663-675

Shaikhibrahim Z*, **Offermann A*,** Halbach R, Vogel W, Braun M, Kristiansen G, Bootz F, Mikut R, Lengerke C, Reischl M, Schröck A, Perner S. Clinical and molecular implications of MED15 in Head and Neck Squamous Cell Carcinoma. **Am J Pathol.** 2015; 185: 1114-1122

Klümper N, Syring I, **Offermann A**, Shaikhibrahim Z, Vogel W, Müller SC, Ellinger J, Strauß A, Radzun HJ, Ströbel P, Brägelmann J, Perner S, Bremmer F. Differential expression of Mediator complex subunit MED15 in testicular germ cell tumors. **Diagn Pathol.** 2015 Sep 17;10:165.

Syring I, Klümper N, **Offermann A**, Braun M, Deng M, Boehm D, Queisser A, von Mässenhausen A, Brägelmann J, Vogel W, Schmidt D, Majores M, Schindler A, Kristiansen G, Müller SC, Ellinger J, Shaikhibrahim Z, Perner S. Comprehensive

analysis of the transcriptional profile of the Mediator complex across human cancer types. **Oncotarget**. 2016 Mar 30.

Sonstige Veröffentlichungen

Shaikhibrahim Z, **Offermann A**, Perner S. Words of wisdom: Re: Punctuated evolution of prostate cancer genomes. **Eur Urol.** 2014; 65: 666-66

Preise und Auszeichnungen

2015: Promotionpreis der Deutschen Gesellschaft für Pathologie (DGP)