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Klinik für Urologie
Direktor: Professor Dr. med. Kurt Miller

Habilitationsschrift

Über die Bedeutung biologischer Tumormarker bei der Diagnostik und Therapie des Harnblasenkarzinoms

zur Erlangung der Lehrbefähigung
für das Fach Urologie

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von

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INHALTSVERZEICHNIS

Abkürzungen

AUC	area under curve
BMI	Body Mass Index
bp	Basenpaare
Cis	Carcinoma in situ
D	Dalton
DNA	Desoxyribonukleinsäure
ECD	extrazellulare Domain
ECM	extrazelluläre Matrix
ECOG	Eastern Cooperative Oncology Group
EGF	epidermal growth factor
ELISA	enzyme linked immunosorbent assay
FISH	Fluorescent-In-Situ-Hybridization
GC	Gemcitabin, Cisplatin
HER	Human epidermal growth receptor
HRP	Horseradish Peroxidase
IHC	Immunhistochemie
LDH	Lactatdehydrogenase
MS-PCR	Methylierungs-spezifische Polymerase-Kettenreaktion
M-VAC	Methotrexat, Vinblastin, Adriamycin, Cisplatin
PCG	Paclitaxel, Cisplatin, Gemcitabin
PCR	Polymerase-Kettenreaktion
RLU	relative light unit
ROC	receiver operating characteristic
RT-PCR	Reverse Transkriptase Polymerase-Kettenreaktion
SEQ	Bisulfit-Sequenz
TGGE	Temperatur-Gradienten-Gel-Elektrophorese
TMB	Tetramethylbenzidin
TP53	Tumor-Suppressor-Gen p53
TPA	Tissue Polypeptide Antigen
uPA	Urokinase-Typ Plasminogen Aktivator
uPAR	Plasminogen Aktivator vom Urokinase-Typ-Rezeptor

1. Vorbemerkungen

Diese kumulative Habilitationsschrift beinhaltet Forschungsergebnisse, die ich an der Klinik für Urologie der Charité-Universitätsmedizin Berlin, Campus Charité Mitte (Direktor: Prof. Dr. med. S.A. Loening, aktuell Prof. Dr. med. Kurt Miller), im Bereich Laboratoriumsmedizin der Charité-Universitätsmedizin Berlin, Campus Charité Buch (damalige Leitung: Dr. rer. nat. Guntram Schulze), in der Molekularpathologie – Bereich Tumormarker am Centro Nacional de Investigaciones Oncológicas (CNIO) in Madrid, Spanien (Leitung: Dr. Marta Sánchez-Carbayo) und an der Klinik für Urologie des HELIOS Klinikum Bad Saarow (Leitung: Chefarzt Dr. med. Jürgen Ruttloff) erlangen konnte.

Seit meiner Dissertation, die ich während des Studiums im Jahr 1997 begonnen hatte, habe ich die wissenschaftliche Zusammenarbeit mit der Klinik für Urologie der Charité - Universitätsmedizin Berlin, Campus Charité Mitte gepflegt. Dort konnte ich auf dem Boden der Ergebnisse dieser Arbeit weitere Projekte zur Forschung an Tumormarkern beim Harnblasenkarzinom initiieren und meine während der Forschungsaufenthalte gewonnenen Kenntnisse einbringen.

Im Centro Nacional de Investigaciones Oncológicas (CNIO) in Madrid beschäftigte ich mich insbesondere mit der Entwicklung und Prüfung von epigenetischen Tumormarkern.

An den Untersuchungen beteiligten sich Doktoranden und wissenschaftliche Mitarbeiter, technische Mitarbeiterinnen und Mitarbeiter der Forschungsabteilungen der Kliniken für Urologie in Berlin und der Molekularpathologie – Sektion Tumormarker des CNIO in Madrid, Spanien, sowie die Kooperationspartner am Institut für Pathologie, Campus Charité Mitte und des HELIOS Klinikum Bad Saarow.

Arbeiten zu meinem Forschungsschwerpunkt konnte ich in den letzten Jahren in Peer-reviewed Journalen publizieren sowie auf nationalen und internationalen Kongressen vorstellen. Die durchgeführten Untersuchungen und Ergebnisse sind Gegenstand dieser kumulativen Habilitationsschrift und wurden in 5 Originalarbeiten

publiziert. Die Publikationen sind Bestandteil der Leitlinien für Tumormarker beim Harnblasenkarzinom.

Ziel dieser kumulativen Habilitationsschrift soll es sein, die gegenwärtige Bedeutung von Tumormarkern beim Harnblasenkarzinom unter Einbeziehung der eigenen Arbeiten darzulegen.

2. Einleitung

In den Industrieländern steigt die Anzahl an Neuerkrankungen und Todesfällen durch ein Harnblasenkarzinom stetig. Nicht allein durch die Inzidenz von 386300 und die Mortalität von 150200 im Jahre 2008 sollte die Erkrankung nachdenklich machen [1]. Das Harnblasenkarzinom ist auch ökonomisch bedeutsam, da es von allen bösartigen Tumorerkrankungen die höchsten Kosten pro Patient und insgesamt die fünfthöchsten Kosten verursacht [2, 3]. Durch routinemäßige Zystoskopien im Rahmen der Nachsorge werden alleine 13% der Kosten verursacht [4]. Durch einen gezielten Einsatz von Tumormarkern für das Harnblasenkarzinom könnten in Zukunft Kosten eingespart werden [5].

Etwa 80% der Blasentumorpatienten sind zwischen 50-79 Jahren alt [6]. Zum Zeitpunkt der Diagnose zeigen bereits 20-30% eine Muskelinfiltration, die Hälfte dieser Patienten ist dann bereits metastasiert [7]. Die Prognose für das metastasierte Harnblasenkarzinom ist nach wie vor schlecht, und die durchschnittliche Überlebenszeit des unbehandelten Patienten beträgt 3-6 Monate [8].

Ein umfassenderes Verständnis der Tumorbiologie wird in Zukunft ausschlaggebend sein, um Fortschritte in der Therapie des Harnblasenkarzinoms zu erreichen [9]. Die Entstehung und der Progress urothelialer Karzinome werden durch mindestens zwei Mechanismen verursacht, die auf zwei unterschiedlichen Vorgängen beruhen [10]. Ein Modell der urothelialen Tumorgenese und Progression ist in Abbildung 1 dargestellt. Nicht-invasive papilläre Blasentumore sind charakterisiert durch funktionsgewinnende Mutationen, bei denen *RAS* und *FGFR3,* sowie die Deletion von Chromosom 9q beteiligt sind. Das Carcinoma in situ und invasive Tumore sind durch funktionsverlierende Mutationen, welche die Tumorsuppressor-Gene *TP53*, *RB1* und *PTEN* betreffen, charakterisiert [11].

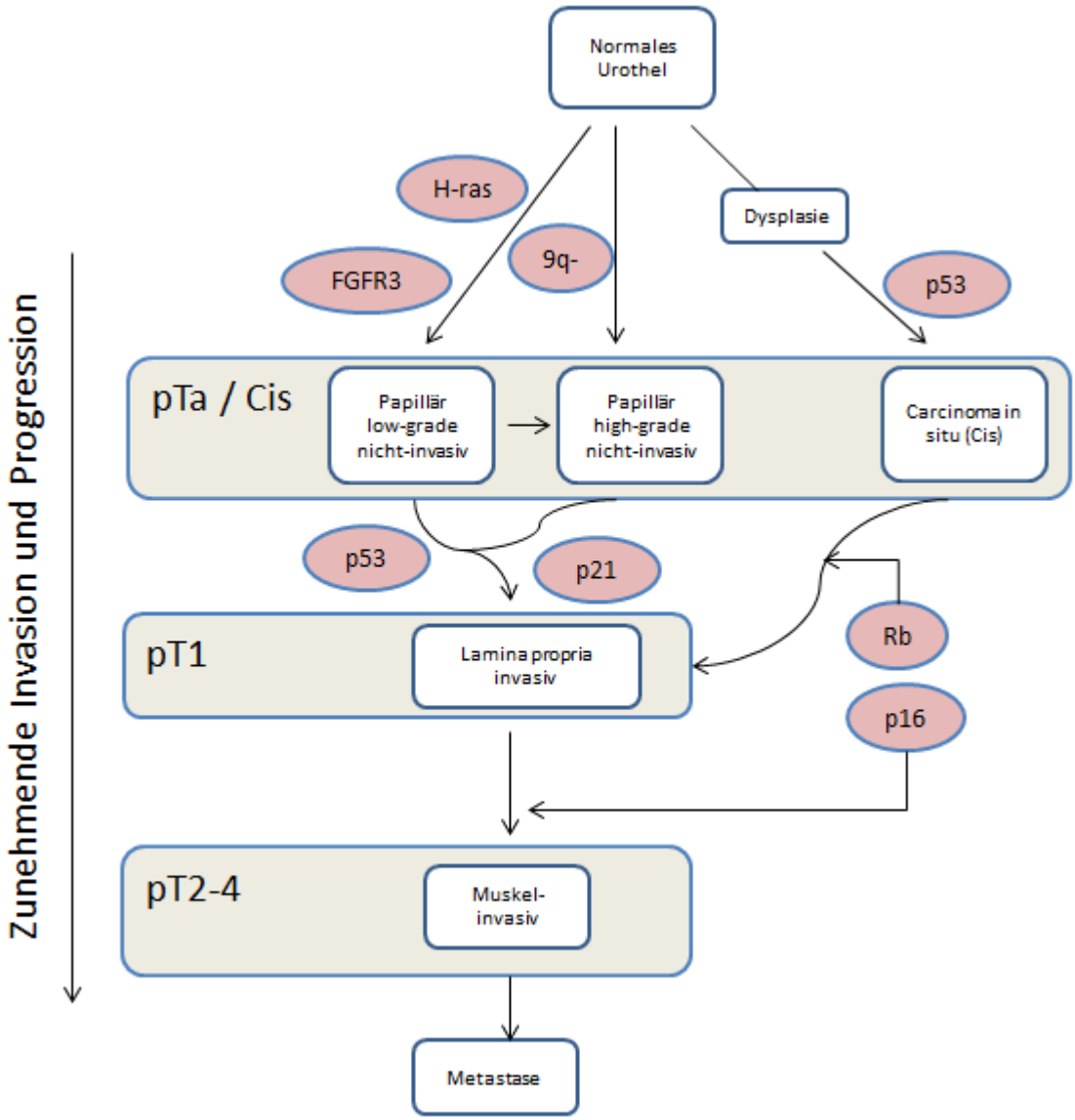

Abbildung 1: Modell der urothelialen Tumorgenese und Progression (modifiziert nach Mitra et al. [9])

Wegen dieser Heterogenität des Tumors sind neue Marker in Hinblick auf den Tumorprogress als klinische Parameter wichtig, die mehr Hinweise auf das klinische Verhalten des Tumors geben und bei der Auswahl der Therapie helfen können [12, 13]. Die gegenwärtige Systematisierung von Tumoren und die histologische Einteilung in unterschiedliche Stadien und Grade kann die Entwicklung von Tumoren jedoch nicht ausreichend beschreiben [14], wobei das histologische Grading der

WHO-Klassifikation von 2004 mehr auf die Aspekte der genetischen Stabilität eingeht [15, 16].

Malignome sind auf molekularer Ebene aber nicht nur durch genetische Läsionen wie Deletionen, Punktmutationen oder Translokationen gekennzeichnet. Epigenetische Faktoren spielen bei Entstehung und Progress von Tumoren eine zunehmende Rolle [17, 18]. So gesehen kann man das Harnblasenkarzinom sogar als molekulare Erkrankung bezeichnen, die von einer Mehrschritt-Akkumulation von genetischen und epigenetischen Faktoren gesteuert wird [19, 20].

Bei der Progression von Harnblasenkarzinomen sind insbesondere die Vorgänge der Hypermethylierung sowie eine veränderte Expression von Genen, die den Zellzyklus bzw. die Apoptose betreffen, zu nennen [21]. Die DNA-Hypermethylierung im Promotorbereich verschiedener Gene bewirkt eine Gen-Inaktivierung [22]. Dabei ist zu bemerken, dass die DNA-Hypermethylierung bei invasiven Tumoren häufiger als bei nicht-invasiven ist [23].

Unter pathophysiologischen Bedingungen findet eine Modifikation humaner DNA nur am Kohlenstoffatom Nr. 5 der Base Cytosin durch Addition einer Methylgruppe statt; dann entsteht 5‘-Methylcytosin. Das findet jedoch nur statt, wenn Cytosin von Guanosin gefolgt wird [21].

Derzeit erreichen die diagnostischen Tests nicht die optimalen Werte für Spezifität und Sensitivität. Die molekulare Heterogenität des Blasentumors macht es schwierig, dass ein einziges Molekül in der Lage sein wird, in fast allen Fällen einen Blasentumor von anderen Erkrankungen mit ähnlichem Charakter zu unterscheiden [24, 25].

In der vorliegenden Arbeit werden meine Arbeiten auf dem Gebiet der Tumormarker beim Harnblasenkarzinom zusammengefasst.

3. Untersuchte Tumormarker und methodische Grundlagen

3.1. Tumorsuppressor-Gen *TP53*

Etwa die Hälfte der malignen Prozesse zeigt eine Veränderung von *TP53*, so dass es nicht mehr seine Funktionen erfüllen kann [26, 27]. Eine wichtige Funktion von *TP53* besteht darin, die Apoptose von Zellen zu induzieren. Außerdem ist *TP53* ein Transkriptionsfaktor, der die Proliferation von Zellen unterdrückt. Eine Mutation von *TP53* kann dadurch zu einer unkontrollierten Zellproliferation führen [28]. Lokalisiert ist *TP53* auf dem kurzen Arm von Chromosom 17 [29]. Mutationen von *TP53* korrelieren häufig mit der Tumorentwicklung und Progression von Blasentumoren [9, 10].

Im Labor für Tumorgenetik der Klinik für Urologie an der Charité habe ich Mutationen von *TP53* mit folgenden Techniken untersucht: Isolierung von DNA aus frischem Gewebe und Urinsediment, Polymerase-Kettenreaktion (PCR), Temperatur-Gradienten-Gel-Elektrophorese (TGGE), sowie automatisierte DNA Sequenzierung. Die dabei benutzten Primer für die relevanten Exons 5-8 sind in Tabelle I aufgelistet.

Die PCR Produkte wurden nach Durchlaufen eine horizontalen Polyacrylamidgels unter definierten Temperaturbedingungen (TGGE, Qiagen, Hilden) dem Screening nach Mutationen aus den Abschnitten zugeführt, die aus dem Gel extrahierten wurden [30]. Mutationen wurden durch die automatisierte Sequenzanalyse von re-amplifizierten Gelbanden bestätigt (Amersham Pharmacia Biotech, Uppsala, Schweden) [31].

Tabelle I: Primer für die Exons 5-8 des *TP53* Gens

Exon 5	5´-(gC)TTC CTC TTC CTA CAg TAC TC	5'-CTg ggC AAC CAg CCC TgT CgT,
Exon 6	5´-(gC)ACg ACA ggg CTg gTT gCC CA	5´-AgT TgC AAA CCA gAC CTC Ag
Exon 7	5´-(gC)TCT CCT Agg TTg gCT CTg ACT g	5´-gCA AgT ggC TCC TgA CCT ggA
Exon 8	5´-CCT ATC CTg AgT AgT ggT AAT C	5´-(gC)CCg CTT CTT gTC CTg CTT gCT T

3.2. Tissue Polypeptide Antigen (TPA)

TPA wurde erstmals 1957 von Björklund und Björklund als potentieller Tumormarker beschrieben [32]. TPA ist ein im Kreislauf zirkulierender Komplex von Polypeptid-Fragmenten der Zytokeratine 8, 18 und 19. Es gilt als ein Indikator für eine erhöhte Zellproliferation und gehört als epithelialer Marker zu den tumorassoziierten Antigenen [33].

Methodik zum Messen von TPA in Serum und Urin: Die Konzentration von TPA wurde durch ein immunoluminometrisches Assay mit dem LIA-mat® TPA-M Prolifigen® (AB Sangtec Medical, Bromma, Schweden) gemessen. Der Tracer Antikörper und der immobilisierte Antikörper reagieren nach dem „Sandwich-Prinzip“ in einer Ein-Schritt-Reaktion mit dem TPA aus Proben und Standards. Das anti-TPA-Tracerkonjugat besteht aus dem Antikörper und einem kovalent gebundenen Isoluminolderivat. Der in der immunologischen Reaktion an der Wand gebundene Komplex aus Tracer und TPA wird dann in einer Lichtreaktion detektiert. Die daraufhin automatisch erfolgende Injektion von alkalischer Peroxidlösung und Katalysatorlösung in die Teströhrchen startet die Oxidation des Isoluminols. Da die Emission der Photonen sofort einsetzt und innerhalb weniger Sekunden wieder abklingt, wird die lichterzeugende Reaktion im Luminometer gestartet. Das bei der Reaktion entstehende Licht (425 nm) wird mit dem Photomultipler des Luminometers gemessen. Das in RLUs (relative light units) gemessene Lichtsignal ist direkt proportional zur TPA-Menge in Standard und Probe [34].

3.3. Plasminogen Aktivator vom Urokinase-Typ-Rezeptor (uPAR)

Der Plasminogenaktivator vom Urokinase-Typ-Rezeptor (uPAR) ist ein einkettiges Glykoprotein mit einem Molekulargewicht von 50 bis 60 kD. uPAR besteht aus drei Domains: Domain I ist unmittelbar an der Bindung an uPA beteiligt, während die Domains II und III dabei helfen, die uPAR Moleküle an der Zellmembran zu ordnen [35]. Das Vorhandensein eines zellulären Rezeptors für uPA wurde erstmals durch Vassalli et al. nachgewiesen [36], der eine spezielle Bindung von uPA zur Oberfläche der Monozyten beobachtete.

Der Urokinase-Typ Plasminogen Aktivator (uPA) ist eine Serinprotease, die bei der Aktivierung und Bindung zu seinem Rezeptor (uPAR) die Umwandlung von Plasminogen zu aktivem Plasmin katalysiert. Plasmin kann Kollagenase vom Typ IV aktivieren, welche dann die untere Membran von Kollagen Typ IV und Proteine der ECM einschließlich Fibrin, Fibronektin und Laminin abbaut [37-39]. Im Falle von Entzündungen und malignen Prozessen werden die Expressionen von uPA und uPAR hochgeregelt [40]. Es gibt einige Berichte über die Bedeutung von uPA beim Harnblasenkarzinom [41, 42] und seine Beziehung zur Tumorinvasion [41, 43].

Methodik zum Messen von uPAR: Für diese Studie wurde der IMUBIND® Total uPAR ELISA Kit (American Diagnostica Inc., Greenwich, USA) benutzt. Es handelt sich dabei um ein enzymgebundenes Immunoassay zur Quantifizierung von uPAR. Die Proben werden mit einem in einer vorbeschichteten Vertiefung und einem zweiten biotinylierten Antikörper inkubiert. Diese dienen dazu, die Grenze der uPAR-Moleküle zu erkennen. Nach Hinzufügung von mit Streptavidin verbundener Horseradish Peroxidase (HRP) ist die Formation des Antikörper-Enzym-Komplexes zur Detektion komplett. Die Hinzugabe eines Substrates aus Perborat / 3,3', 5,5'–Tetramethylbenzidin (TMB) und seine nachfolgende Reaktion mit HRP erzeugt eine blaue Lösung. Die Empfindlichkeit steigt durch Hinzugabe einer Stoplösung aus Schwefelsäure, die dann zu einer gelben Farbe wechselt. Das Gesamt-uPAR wird quantifiziert durch die Messung der Absorption der Lösung bei 450 nm.

3.4. Human epidermal growth receptor (HER-2/neu)

Das Onkogen *HER-2/neu* kodiert ein transmembranes Glykoprotein, welches ähnlich dem epidermal growth factor (EGF) Rezeptor ist; es ist auf Chromosom 17 lokalisiert [44]. Es ist assoziiert mit einer steigenden Rate der Zellproliferation, einem steigenden angiogenetischen Potential und einer reduzierten Zell-zu-Zell Adhäsion [44, 45]. Die Homologie in der Struktur mit dem EGF Rezeptor, die Tyrosinkinaseaktivität, sowie seine Möglichkeit, mitogenetische Antworten zu generieren, kennzeichnen den c-erbB-2 Rezeptor [46, 47]. Das Protein HER-2/neu ist ein sich in der Transmembran befindlicher Oberflächenwachstumsfaktor der Tyrosinkinase, der von normalen Epithelzellen exprimiert wird - in erhöhten Maßen von einigen Tumorzellen. Das Onkoprotein HER-2/neu hat ein Molekulargewicht von 185000 D und besteht aus drei Teilen: der Anteil der internen Tyrosinkinase, verantwortlich für intrazellulare Signalgebung, ein kurzer transmembraner Anteil und der Anteil der extrazellularen Domain (ECD), die mit den Wachstumsfaktoren interagiert. Es konnte gezeigt werden, dass die ECD von HER-2/neu von der Zelloberfläche als Glykoprotein mit einer Größe zwischen 97000 und 115000 D abgeschilfert wird. Die abgeschilferte ECD zirkuliert in besonders großen Mengen bei Malignompatienten.

Methodik zum Messen von HER-2/neu: HER-2/neu wurde mit dem Bayer Oncoprotein Test® (Bayer HealthCare, Leverkusen) gemessen. Mit Hilfe der Immunohistochemie (IHC) wird die volle Länge der HER-2 Moleküle bestimmt. Ein Immunoassays kann eine Abschätzung durch die zirkulierende ECD ermitteln. Der HER-2/neu ELISA ist ein nach dem sandwich-Prinzip aufgebautes enzymatisches Immumoassay, welches zwei monoklonale Antikörper benutzt, um die ECD des HER-2/neu Proteins im Serum zu quantifizieren.

3.5. Metastasen Suppressor-Gen *KISS-1* als Beispiel epigenetischer Marker

Im Rahmen der weiteren Experimente zur Detektion neuer epigenetischer Marker wurden insgesamt 30 Gene getestet, darunter *TP73, MSH6, VHL, RARB, ESR1, CDKN2A, PAX5A, PTEN, MGMT, MGMT2, PAX6, WT1, CD44, GSTP1, ATM, IGSF4, CHFR, BRCA2, RB1, RB12, THBS1, PYCARD, CDH13, TP53, BRCA1, STK11, GATA5, PMF1, KISS-1* und *MYO*. Diese Arbeiten erfolgten in der Abteilung für Tumormarker der Molekularpathologie am Centro Nacional de Investigaciones Oncológicas (CNIO) in Madrid. Da die Ergebnisse dieser Analysen nur am Rande dieser kumulativen Habilitationsschrift dargestellt werden, soll beispielgebend nur die Methodik eines Markers beschrieben werden.

Repräsentativ für die Analytik epigenetischer Marker wurde *KISS-1* ausgewählt, da die Methodik der Analytik aller oben genannten Marker prinzipiell ähnlich ist. Erste Ergebnisse von *KISS-1* wurden auf internationalen Kongressen und mit meiner Beteiligung von Cebrian et al. [48] publiziert.

KISS-1 befindet sich auf Chromosom 1q32 [49]; es ist eines der Gene, die auf Chromosom 6 reguliert werden. Experimentelle und klinische Studien haben seine Rolle als funktionell aktives Metastasen Suppressor-Gen bei einigen Tumoren gezeigt [49, 50]. Die Regulierung von Ereignissen unterhalb der Zellmatrix und deren Verbindungen, die in die Reorganisation des Zytoskeletts involviert sind, werden *KISS-1* zugeordnet [51, 52]. Die Mechanismen, durch die *KISS-1* in den Phänotyp von invasiven oder metastatischen Tumoren involviert ist, wurden bisher nicht sicher geklärt.

Eine unterschiedliche Expression von KISS-1 und ein Klon, der das regulatorische Gegenstück von KISS-1 auf Chromosom 6 repräsentiert [53, 54], wurden durch molekulare Profil-Analysen von Zelllinien aus Blasentumoren und Primärtumoren indentifiziert.

Die paraffin-eingebetteten Blasentumore wurden aus den Blöcken gestanzt [55]. Die Methylierung von *KISS-1* wurde durch zwei PCR der bisulfit-modifizierten DNA

analysiert, welche die chemische Konversion von Cytosin zu Uracil der unmethylierten, nicht aber der methylierten, durchführt. Zuerst erfolgte die Sequenzierung von beiden Strängen der *KISS-1* Promotorregion nach Behandlung der DNA mit Bisulfit. Eine zweite PCR mit primer-spezifischer methylierter oder modifiziert unmethylierter DNA wurde durchgeführt [56]. Beim Prinzip der Bisulfitkonversion wird durch die Behandlung einzelsträngige DNA mit Bisulfit Cytosin zur RNA-Base Uracil deaminiert, während 5‘-Methylcytosin vor solch einer Konversion geschützt ist. Nachfolgend wird Uracil als Thymidin amplifiziert [57, 58]. Die Primer-Sequenzen für die Bisulfit-Sequenzierung unmethylierter und methylierter Reaktionen wurden geplant, um den Startpunkt seiner Transkription 50 bp nach seinem ATG Start-Codon zu umfassen. DNA aus normalen Lymphozyten diente als Positivkontrolle für die un-methylierten Allele. Die PCR Produkte wurden nach Markierung mit Ethidiumbromid auf 2% Agarosegel geladen und unter einem UV Transilluminator sichtbar gemacht.

Die gesamte *KISS-1* codierende Sequenz wurde in Zelllinien von Harnblasenkarzinomen auf Mutationen untersucht. Die cDNA (1 µL) von *KISS-1* wurde sequenziert durch Verwendung folgender Techniken: PCR, Hochleistungs-Denaturierung, Chromatographie und Sequenzierung.

Die Amplifikation von PCR wurde in 30 µl Volumen enthaltenen Reaktionen durchgeführt; diese enthielten 0,2 µmol/l des jeweiligen Oligonukleotids, 3,5 µmol/l MgCl2, 200 µmol/l Deoxynucleotidtriphosphat, 1 Einheit EcoStart Taq Polymerase (Ecogen) und 1 µL cDNA als Template. Primersequenzen und PCR Bedingungen für *KISS-1* sind in Tabelle II dargestellt. Die PCR Produkte von *KISS-1* wurden direkt sequenziert.

Tabelle II: Primersequenzen und PCR Bedingungen für *KISS-1*

	Sense primer (5'-3')	Antisense primer (5'-3')	Produktgröße [bp]	Annealing-Temperatur [°C] (PCR Zyklen)
SEQ	TGGAAGGGGAATAGTTTTATTAG	TACAACTAAAACTCCTTCRACC	322	52 (40)
MS-PCR	CGGGTTGGAAGTTTTAGC	GCTTCGACAAACGAAAAAC	125	54 (37)
US-PCR	TTTTGGGTTGGAAGTTTTAGT	ACTTCAACAAACAAAAAACAAC	129	52 (37)
RT-PCR	ACTCACTGGTTTCTTGGCAGC	ACCTTTTCTAATGGCTCCCCA	183	60 (28)

4. Ergebnisse der Originalarbeiten

4.1. Originalarbeit 1

Ecke TH, Lenk SV, Schlechte HH, Loening SA.
Tissue Polypeptide Antigen (TPA) in Comparison with Mutations of Tumour Suppressor Gene P53 (*TP53*) in Patients with Bladder Cancer
Anticancer Res 2003; 23: 957-962

In dieser Arbeit habe ich Messungen von TPA in Serum- und Urinproben sowie Analysen von Mutationen von *TP53* durchgeführt. Zur Beurteilung der klinischen Einsatzmöglichkeiten von TPA wurden vor allem Sensitivität und Spezifität herangezogen. Eine weitere Frage, die durch diese Studie beantwortet werden sollte, war, ob sich TPA als Tumormarker beim Blasenkarzinom eignet und ob es Zusammenhänge zwischen Mutationen von *TP53* und TPA gibt. Außerdem sollte der Frage nachgegangen werden, ob sich Unterschiede bei verschiedenen Stadien und Graden des Harnblasenkarzinoms bei *TP53* und TPA ergeben.

Dazu wurden in dieser Studie wurden die Werte von TPA mit der Analyse der Mutationen von *TP53* bei tumorfreien Proben und Harnblasentumor-Patienten verglichen. 93 Patienten mit Harnblasenkarzinom, 24 Patienten mit gutartigen urologischen Erkrankungen sowie 18 gesunde Individuen waren Gegenstand dieser Arbeit.

Zunächst wurde nach einer ROC-Kurven Analyse der optimale Grenzwert bei 47 U/l für die Serumbestimmung und bei 60 U/mmol Kreatinin für die Urinbestimmung von TPA festgelegt. Die damit berechnete Sensitivität betrug 48,9% für die Serum- und 40,4% für die Urinbestimmung. Die Spezifität betrug 83% für Serum- und 100% für Urinwerte.

Im Serum der Blasentumorpatienten betrug die mediane TPA-Konzentration 46,0 U/l, bei den Patienten mit gutartigen urologischen Erkrankungen 32,5 U/l und bei der

gesunden Kontrollgruppe 27,9 U/l. Im Urin wurden für TPA in den drei Gruppen mediane Konzentrationen von 36,0, 13,3 und 5,9 U/mmol Kreatinin gemessen. Im Vergleich mit der Kontrollgruppe ist bei Patienten mit Harnblasenkarzinom TPA im Serum (p=0,012) und im Urin (p=0,002) signifikant höher. Demnach kann TPA sowohl im Serum als auch im Urin zwischen gutartigen und malignen Erkrankungen unterscheiden kann.

Pathologische Werte von TPA im Serum bei invasiven Harnblasentumoren wurden in 57,7% der Fälle gemessen; im Urin wurden mit 58,3% kaum mehr pathologische Werte gemessen. Bei den nicht-invasiven Harnblasenkarzinomen wurden erhöhte TPA-Werte in 45,5% der Fälle im Serum und in 36,1% der Fälle im Urin gefunden. Es wurde kein Unterschied der TPA-Werte bei nicht-invasiven und muskelinvasiven Harnblasentumoren bei der Berechnung einer statistischen Signifikanz gefunden.
In 81,8% der Fälle mit invasiven Harnblasenkarzinomen wurden Mutationen von *TP53* detektiert; bei den nicht-invasiven Karzinomen waren es 44,1%. *TP53* Mutationen bei Patienten mit Harnblasenkarzinomen korrelieren signifikant mit dem Tumorstadium (p=0,002).

Bei 66,4% der Patienten mit *TP53* Mutationen wurden erhöhte TPA-Werte im Serum gemessen; 66,7% der Patienten mit *TP53* Mutationen zeigten erhöhte TPA-Werte im Urin.
TPA ist im Serum bei Patienten mit Mutationen im Tumorsuppressor-Gen *TP53* im Vergleich zum Wildtyp signifikant erhöht (p=0,046); das gilt jedoch nicht für TPA im Urin (p=0,173) [59].

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Tissue Polypeptide Antigen (TPA) in Comparison with Mutations of Tumour Suppressor Gene P53 (*TP53*) in Patients with Bladder Cancer

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Abstract. *Backgound: Tissue polypeptide antigen (TPA) is a circulating complex of polypeptide fragments from cytokeratins 8, 18 and 19. It is a tumour-related protein. TPA is an indicator of higher cell proliferation. One function of TP53 is the suppression of apoptosis. TP53 mutations are frequently correlated with tumour development in bladder cancer. One function of TP53 is the suppression of apoptosis. We compared TPA expression and TP53 mutation analysis in tumour-free and bladder cancer patients. Materials and Methods: We examined 93 patients with bladder cancer, 24 patients with benign urological diseases and a control group of 18 healthy individuals. TPA concentration was measured by immunoluminometric assay with LIA-mat® TPA-M Prolifigen®. The normal cut-off value was defined at 47 U/l for serum and at 60 U/mmol for creatinine. Screening for TP53 mutations in tissue and urine sediment, amplification of the TP53 gene by polymerase chain reaction (PCR) for the exons 5, 6, 7 and 8 and temperature gradient gel electrophoresis (TGGE) were used to analyse the mutations. Statistical analysis included ROC, Mann-Whitney U-Test and Pearson's correlation. Results: For superficial bladder cancer the mutation frequency in TP53 was 44.8%. We found elevated TPA levels in 45.5% in serum and 36.1% in urine. For invasive bladder cancer the mutation frequency in TP53 was 79.2%. Elevated TPA levels were found in 57.7% in serum and in 58.3% in urine. TPA has a sensitivity of 48.9% in serum and 40.4% in urine; the specifity of TPA is 83% in serum and 100% in urine in comparison with healthy individuals. We found no correlation between TPA level and the inflammation status of the patient. Conclusion: This study demonstrated that TP53 mutation frequently occurs in higher stages of bladder tumours. There was no TPA level difference between superficial and invasive bladder cancer. TPA is significantly higher in serum ($p=0.012$) and in urine ($p=0.002$) in patients with bladder cancer in comparison with control group. TPA in serum is significantly higher in patients with mutation of TP53 ($p=0.046$) but not in urine ($p=0.173$) in comparison with patients with wild-type TP53.*

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Key Words: Bladder cancer, tissue polypeptide antigen, TPA, tumour marker, p53, tumour suppressor gene, mutation.

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TPA belongs as an epithelial marker to the tumour-associated antigens. TPA was identified by Björklund and Björklund as a potential tumour marker in 1957 (1, 2). It is a tumour-related protein originally isolated from extracts of pooled tumours. TPA is a component of the cytoskeleton of non-squamous epithelia. It is present in the proteolytic fragments of cytokeratins 8, 18 and 19, which are released into body fluids as a consequence of cell death (2, 21). In normal tissue TPA was found, too (12, 22). Higher values of TPA have been described for liver diseases and inflammatory processes. Some studies reported an increase of TPA serum concentration in tumour patients. The reason may be an increased cell destruction accompanied by invading tumour cells (20, 23, 27).

Mutations of turnoursuppressor gene *TP53* are frequently correlated with tumour development and progression in bladder cancer (14, 3 0). *TP53* is localized on the short arm of chromosome 17 (13, 17, 18). *TP53* has influence on cell cycle regulation, gene transcription, DNA repair, genome stability, chromosome segregation, angiogenesis and apoptosis. Genetic alteration is the most frequent reason for a change of function of *TP53.* Loss of function may also be due to binding with viral oncoproteins or cellular gene products or may be caused by dislocation of the protein to cytoplasma (11, 15, 32). A very important function of wild-type *TP53* is the induction of apoptosis (24).

It is known that the fragmentation of the cytokeratin filaments occurs during cell apoptosis (6). We do not know how quickly the soluble fragments of cytokeratins in rapidly growing and necrotic tumours are eliminated. The relationship between TPA and *TP53* is a less studied phenomenon, but it is very useful to know more about the association between apoptosis, TP53 and TPA (6).

We examined the following questions:

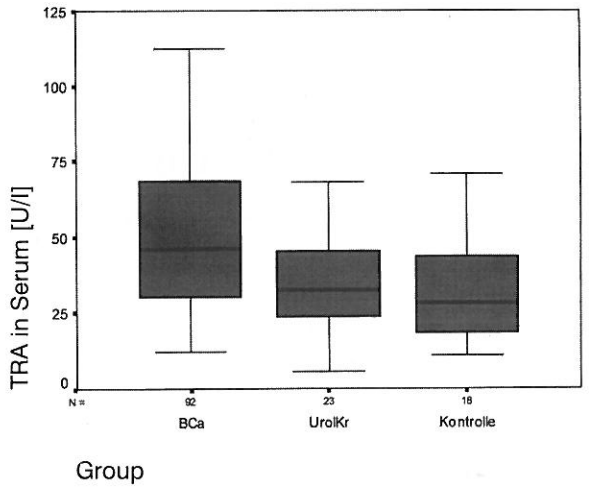

Figure 1. *TPA in serum.*

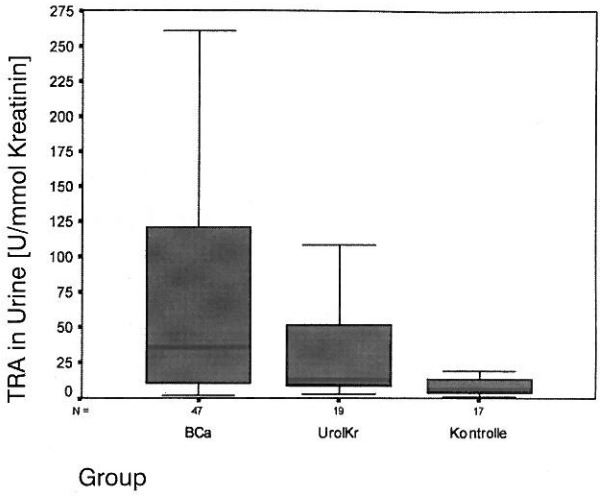

Figure 2. *TPA in urine.*

1. Is TPA higher in bladder cancer patients than in the control group? What are the values of sensitivity and specifity?
2. Are TPA and / or *TP53* mutation correlating with superficial and invasive bladder cancer?
3. What is the influence of inflammation on TPA values?
4. Is there a relationship between *TP53* und TPA?

Patients and Methods

We examined 93 patients with bladder cancer, 24 patients with benign urological diseases and a control group of 18 healthy individuals. One hundred and fifteen male patients and 21 female patients (average age 62.2 years) were included. The staging and grading of bladder cancer patients is shown Table I.

Measurement of TPA in serum und urine. Ten ml were centrifuged at 3000 rpm for 15 minutes. Fifty µl of Tween 20 were added per 10 ml of urine. For the assay, 50 µl of the pre-treated urine and 150 µl of kit diluent were added to the test tube. TPA concentration was measured by immunoluminometric assay with LIA-mat® TPA-M Prolifigen® (AB Sangtec Medical, Bromma, Sweden). The normal cut-off value was defined at 47 U/l for serum and at 60 U/mmol for creatinine. TPA-M LIA is a one-step luminometric immunoassay based on reaction tubes coated with monoclonal antibodies against cytokeratins 8, 18, 19 and soluble tracer antibodies conjugated with isoluminol (5,9).

Screening for TP53 mutations in tissue and urine sediment. Amplification of the *TP53* gene was accomplished by polymerase chain reaction (PCR) in four separate reactions for exons 5, 6, 7 and 8. Temperature gradient gel electrophoresis (TGGE) was used to analyze the mutations (28). Statistical analysis included ROC, Mann-Whitney *U*-test and Pearson's correlation.

Results

TPA levels in tumour patients and tumour-free patients. In the serum of bladder cancer patients, the median TPA concentration was 46.0 U/l. In the urine of the same bladder cancer patients, the median TPA was 36.0 U/mmol creatinine (Figures 1 and 2).

Table I. *Staging and grading of bladder cancer patients.*

Staging/ Grading	G1	G2	G3	Total
Ta	15	22	1	38
TI	4	16	7	27
T2	-	5	9	14
T3	-	I	11	12
T4	-	-	2	2
Total	19	44	30	93

In the serum of patients with benign urological diseases, the median TPA was 32.5 U/l. In the urine of these patients, the median TPA was 13.3 U/mmol creatinine. In the serum of healthy individuals, the median TPA was 27.9 U/l. In the urine of these patients, the median TPA was 5.9 U/mmol creatinine.

After ROC-curve analysis the optimal cut-off was 47 U/l for serum and 60 U/mmol creatinine for urine (Figures 3a and 3b).

The calculated diagnostic sensitivity was 48.9% for serum and 40.4% for urine. The calculated diagnostic specificity was 83% for serum and 100% for urine (Table II). For the comparison of bladder cancer patients with patients with

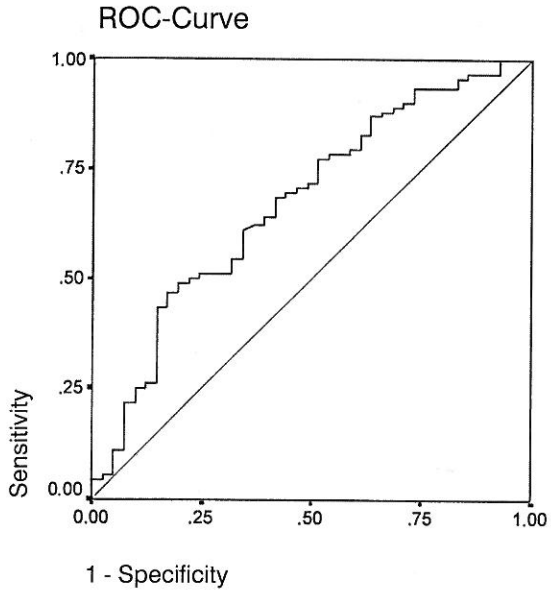

Figure 3a. *TPA in serum.*

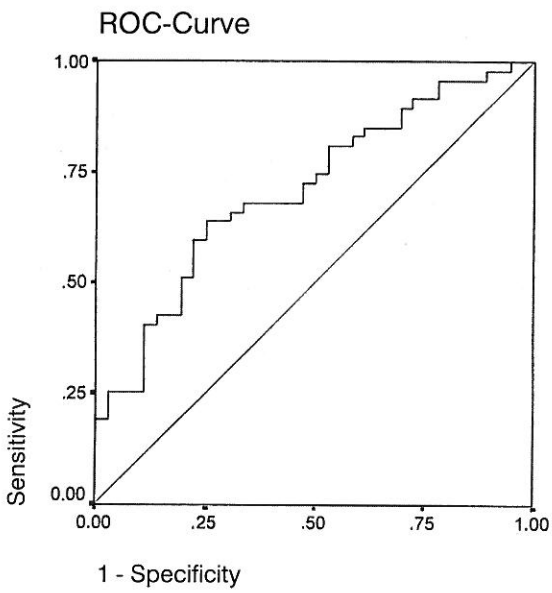

Figure 3b. *TPA in urine.*

Table II. *Sensitivicity and specificity for bladder cancer patients compared with healthy individuals.*

	TPA in serum	TPA in urine
Sensitivity	48.9%	40.4%
Specificity	83%	100%

Tabelle III. *TPA and TP53 in invasive and superficial bladder cancer.*

	pT24		pTa, pT I	
	TPA in serum	TPA in urine	TPA in serum	TPA in urine
* cut-off	11 42.3%	5 41.7%	36 54.5%	23 63.9%
* cut-off	15 57.7%	7 58.3%	30 45.5%	13 36.1%
Total	26	12	66	36

	pT2-4	pTa, pT I	Total
TP53 - Mutation	18 81.8%	26 44.1%	44 54.3%
TP53 - Wild-type	4 18.1 %	33 55.9%	37 45.7%
Total	22	59	81

benign urological diseases, we found a specifity of 78% for serum and 79% for urine.

Comparison of TPA levels and TP53 mutation for superficial and invasive bladder cancer. In this study we measured the pathological values of TPA in serum for invasive bladder cancer in 15 out of 26 cases (57.7%); in urine pathological levels were detected in 7 out of 12 cases (58.3%). For superficial bladder cancer we found elevated TPA levels in 30 out of 66 cases (45.5%) in serum and in 13 out of 36 cases (36.1 %) in urine.

TP53 mutation in patients with bladder cancer is significantly correlated with tumour staging. We detected mutations of *TP53* in invasive bladder cancer in 18 out of 22 cases (81.8%); in superficial bladder cancer we found 26 out of 59 cases (44.1%) with mutation of *TP53*. Pearson's significance with the Chi-Quadrat-Test between mutation of *TP53* and invasive bladder cancer was calculated as $p=0.002$ (Table III).

TPA and inflammation. In our study we could not specify whether inflammatory cells have more influence on TPA levels or on cancer cells. Therefore we excluded bladder cancer patients in this case. To find out if TPA levels are significantly higher in patients with an inflammation, we used only the patients with benign urological diseases. However the number of patients was too small to answer this question

Table IV. *TPA in patients with inflammation.*

	Inflammation	No Inflammation
Elevated TPA (serum)	0	5
TPA (serum) normal	5	13
Elevated TPA (urine)	1	3
TPA (urine) normal	4	12

Table V. *TPA and TP53 in patients with bladder cancer.*

	Mutation of *TP53*		Wild-type of *TP53*	
	TPA in serum	TPA in urine	TPA in serum	TPA in urine
< cut-off	17 38.6%	3 33.3%	24 64.9%	20 71.4%
> cut-off	27 61.4%	6 66.7%	13 35.1%	8 28.6%
Total	44	9	37	28

sufficiently. We found no statistical correlation between TPA and inflammations. Pearson's correlation was calculated as $p=0.183$ for serum and $p=1.000$ for urine (Table IV).

Correlations between TPA level and TP53-status. In 21 out of 44 patients (61.4%) with *TP53* mutation we found elevated TPA levels in serum. In 6 out of 9 patients (66.7%) with *TP53* mutation we found elevated TPA levels in urine.

Pearson's significance was calculated as $p=0.046$ for TPA in serum in comparison with *TP53* mutation; the calculation with TPA in urine was $p=0.173$ (Table V).

Discussion

In the reported experiments, TPA shows only an indifferent sensitivity but a very good specifity for bladder cancer. This is comparable with the reported 54.7% sensitivity and 100% specificity Maulard *et al.* for TPA in serum (16). Stieber *et al.* found 16% sensitivity and 95% specificity for TPA in serum (31). In the 1999 published study by Sanchez-Carbayo *et al.*, an 80.2% sensitivity and a 95% specificity was reported for TPA in urine (25).

Comparative cytokeratin analyses have shown cytokeratin 19 to be an essential part of TPA in bladder cancer (31). Pathological TPA serum concentration was found in 30 out of 66 patients (45.5%) with superficial bladder cancer in case of cut-off 47 U/l; elevated TPA-levels in urine were found in 13 out of 36 patients (36.1%) in case of cut-off 60 U/mmol creatinine. In invasive tumour stages (T2-T4) the pathological TPA concentrations were elevated both in serum (15 out of 26 = 57.7 %) and in urine (7 out of 12 = 58.3 %). We found no difference between superficial / invasive bladder cancer and elevated levels of TPA in serum / urine. This had already been reported by Filelia *et al.* (8) and by Casetta *et al.* (4).

The mutation frequency in the so-called high-risk exons 5-8 of the *TP53* gene is approximately 40% in bladder cancer tissue (28). Mutation of *TP53* might accelerate carcinogenesis especially by enhancement of cell proliferation, loss of apoptosis and by insufficient DNA repair (29). It has now been accepted that the p53 state plays a role in the progression of bladder tumours (7, 19, 26). In our study we found a mutation frequency of 81.8% for invasive bladder cancer and of 44.1% for superficial bladder cancer; these results confirm the published results of Schlechte *et al.* (28). Mutations in the *TP53* gene correlate with infiltrating bladder cancer and are qualified as a marker.

It is known that TPA is not specific for tumours and that an inflammation can influence the TPA levels (16,20-22). In our study we could not find a significance between elevated TPA levels and inflammation. Because the influence of inflammation is certain, we conclude that not enough patients were enrolled in this part of the study.

In our results the *TP53* mutation frequency was 61.4% in patients with elevated TPA level in the serum and 66.7% in patients with elevated TPA in the urine, respectively. We calculated Pearson's significance with $p=0.046$ for TPA in serum and $p=0.073$ for TPA in urine. This could be a sign that the TP53-status has a detectable influence on cytokeratin release. To date only a few results are known regarding the correlation between p53 and cytokeratin expression. Bodey and Kaiser have reported a coincidence of immunocytochemical detection between p53 and cytokeratin 19 among other factors in primary and metastatic cutaneous melanomas (3). However, a p53-induced release of cytokeratins into serum or urine has not yet been demonstrated. In human cell lines from non-small cell lung cancer and from neuroblastoma, aggregation and proteolytic cleavage of cytokeratins were shown during early apoptosis (33).

Conclusion

1. Pathological levels of TPA in serum ($p=0.012$) and in urine ($p=0.002$) are significantly higher in patients with bladder cancer in comparison to the control groups. Elevated TPA levels give a reference to malignant diseases. Further studies should observe TPA during follow-up.
2. Elevated TPA-levels have approximately the same frequency in invasive and superficial bladder cancer. The mutation frequency of *TP53* in invasive bladder cancer is

significantly higher than in superficial bladder cancer (p=0.002).

3. TPA in serum is significantly higher in patients with *TP53* mutation (P=0.046); we could not find this for TPA in urine (p=0.173). TPA analysis completes risk assessment of *TP53* status in bladder cancer.

4. We could not find a correlation between inflammation and elevated TPA-levels in serum (p=0.183) and urine (p=1.000). We recommend the determination of inflammatory markers in serum to exclude a possible influence of inflammation on TPA.

Acknowledgements

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4.2. Originalarbeit 2

Ecke TH, Schlechte HH, Schulze G, Lenk SV, Loening SA.
Four Tumour Markers for Urinary Bladder Cancer - Tissue Polypeptide Antigen (TPA), HER-2/neu (ERB B2), Urokinase-type Plasminogen Activator Receptor (uPAR) and *TP53* Mutation
Anticancer Res 2005; 25(1B): 635-642

Bei einem Teil der in der ersten Originalarbeit untersuchten Patienten war es möglich, weitere Tumormarker auf ihren Nutzen bei der Diagnostik des Harnblasenkarzinoms zu testen. Neben TPA und *TP53* Mutationen, wurden nun auch HER-2/neu und uPAR untersucht.

Diese Arbeit konzentrierte sich auf die Untersuchung, Sensitivität und Spezifität der einzelnen Marker in dieser Kohorte zu bestimmen und mit anderen Studien zu vergleichen. Daneben wurde auf Korrelationen der einzelnen Marker bei Patienten mit Harnblasenkarzinom und tumorfreien Individuen sowie der Korrelationen zwischen invasiven und nicht-invasiven Harnblasenkarzinomen.

Nach der ROC-Kurven Analyse wurden folgende optimale Grenzwerte festgelegt: TPA im Serum: 47 U/l, TPA im Urin: 60 U/mmol Kreatinin, HER-2/neu: 1610 HNU/ml, uPAR: 0,4 ng/ml. Die berechneten diagnostischen Sensitivitäten und Spezifitäten sind der Tabelle III zu entnehmen. Die entsprechenden AUC wurden wie folgt berechnet: 0,876 für TPA, 0,842 für uPAR und 0,863 für HER-2/neu. In Abbildung 2 sind die ROC-Kurven der untersuchten Tumormarker dargestellt.

Tabelle III: Sensitivität und Spezifität der untersuchten Tumormarker

	TPA im Serum	TPA im Urin	HER-2/neu	uPAR
Sensitivität	68,3%	33,3%	88,9%	79,5%
Spezifität	88,9%	100%	62,5%	71,4%

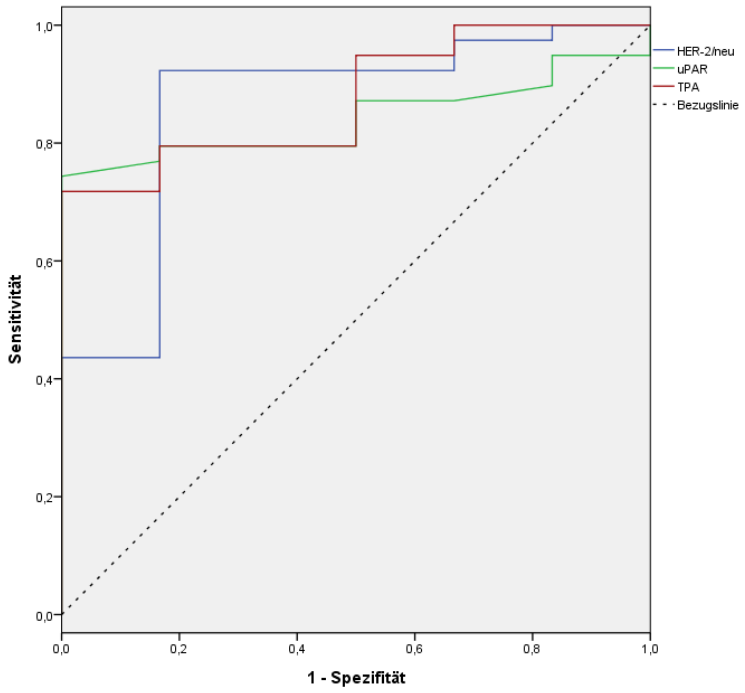

Abbildung 2: ROC-Kurve

Zunächst wurde der Frage nachgegangen, wie hoch die Konzentrationen der einzelnen Tumormarker waren. Bei den Blasentumorpatienten wurde eine mediane TPA-Konzentration von 54,5 U/l im Serum und von 37,4 U/mmol Kreatinin im Urin gemessen, die mediane HER-2/neu Konzentration betrug 1988,5 HNU/ml, die

mediane uPAR Konzentration 0,57 ng/ml. Im Serum von Patienten mit gutartigen urologischen Erkrankungen betrug die mediane TPA-Konzentration 37,2 U/l, während diese für TPA im Urin 10,8 U/mmol Kreatinin betrug, die mediane HER-2/neu Konzentration betrug 1563 HNU/ml, die mediane uPAR Konzentration 0,36 ng/ml.

Pearson's Signifikanz nach dem Chi-Quadrat-Test zeigte, dass pathologische Werte von TPA im Serum (p=0,001) und HER-2/neu (p=0,001) signifikant höher sind bei Patienten mit Harnblasenkarzinom im Vergleich zur Kontrollgruppe.

Weiterhin wurde geprüft, welcher Marker mit nicht-invasiven und invasiven Harnblasenkarzinomen korreliert. Bei invasiven Tumoren wurden pathologisch erhöhte Werte für TPA im Serum in 71,4% der Fälle, für HER-2/neu in 78,6% der Fälle und für uPAR in 87,5% der Fälle gemessen. Beim nicht-invasiven Harnblasenkarzinom wurden erhöhte Werte für TPA in 67,4% der Fälle, für HER-2/neu in 89,1% der Fälle und für uPAR in 77,4% der Fälle detektiert.
TP53 Mutationen bei Patienten mit Harnblasenkarzinom korrelierten signifikant mit dem Tumorstadium. Bei allen Proben von invasiven Harnblasenkarzinomen wurden Mutationen von *TP53* detektiert; bei den nicht-invasiven Harnblasenkarzinomen wurden zu 50% Mutationen von *TP53* gefunden. Pearson's Signifikanz nach dem Chi-Quadrat-Test wurde mit p=0,001 zwischen Mutation von *TP53* und der Muskelinvasivität der in dieser Arbeit untersuchten Harnblasenkarzinome berechnet.

Zusammenfassend war in der vorliegenden Studie HER-2/neu der Tumormarker mit den besten Eigenschaften. Für HER-2/neu wurde eine hohe Sensitivität (88,9%) und eine gute Spezifität (62,5%) berechnet. Allerdings konnte HER-2/neu nicht zwischen nicht-invasiven und invasiven Harnblasenkarzinomen unterscheiden; es wurden erhöhte Werte von HER-2/neu bei 89,1% der nicht-invasiven und bei 78,6% der muskelinvasiven Harnblasentumor Patienten festgestellt [60].

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Four Tumour Markers for Urinary Bladder Cancer - Tissue Polypeptide Antigen (TPA), HER-2/neu (ERB B2), Urokinase-type Plasminogen Activator Receptor (uPAR) and *TP53* Mutation

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Abstract. *Purpose: Tissue polypeptide antigen (TPA) is present in the proteolytic fragments of cytokeratins 8, 18 and 19 as a component of the cytoskeleton of nonsquamous epithelia. HER-2/neu protein is a transmembrane tyrosine kinase cell surface growth factor receptor that is expressed on normal epithelial and some cancer cells. The urokinase-type plasminogen activator receptor (uPAR) is a GPI-linked single-chain glycoprotein. Mutations of the tumour suppressor gene P53 (TP53) are frequently correlated with tumour development and progression. We compared TPA, HER-2/neu and uPAR, and TP53 mutation in tumour-free and bladder cancer patients. Materials and Methods: Clinical samples were used from 60 patients with tumours of the urinary bladder and from 9 patients with benign urological diseases. TPA was analyzed by the immuno-luminometric assay LIA-mat® TPA-M Prolifigen®. HER-2/neu was measured using the Bayer Oncoprotein test. uPAR was measured with the IMUBIND® Total uPAR ELISA Kit. Mutation status in TP53 exons 5, 6, 7 and 8 was analyzed by temperature gradient gel electrophoresis of exon-specific PCR products and by sequence analysis. Statistical analysis included ROC, Mann-Whitney U-test and Pearson's correlation. Results: Pathological concentrations of TPA, HER-2/neu and uPAR are detectable in the serum and in urine of bladder cancer patients. The calculated diagnostic sensitivity for TPA in serum was 68.3%, for TPA in urine 33.3%, for HER-2/neu 86.7% and for uPAR 79.5%. Pathological levels of TPA in serum ($p=0.001$) and HER-2/neu ($p=0.001$) were significantly higher in patients with bladder cancer in comparison to the control group. For superficial bladder cancer, the mutation frequency in TP53 was 50%, while for invasive bladder cancer the mutation frequency in TP53 was 100%. Elevated TPA, HER-2/neu and uPAR levels were associated with all grades and stages of bladder cancer. Conclusion: TPA, HER-2/neu or uPAR can differ between bladder cancer patients and the control group, but not between superficial and invasive bladder cancer. TP53 mutation frequently occurs in higher stages of bladder tumours.*

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Key Words: Bladder cancer, TPA, HER-2/neu, uPAR, tumour marker, TP53.

Tissue polypeptide antigen (TPA) belongs as an epithelial marker to the tumour-associated antigens. TPA was identified by Björklund and Björklund as a potential tumour marker in 1957 (1, 2). It has been reported that the main subunit is a 43 kD, single-chain polypeptide with an isoelectric point at pH 4.5 (3). It is a tumour-related protein originally isolated from extracts of pooled tumours. TPA is present in the proteolytic fragments of cytokeratins 8, 18 and 19, which are released into body fluids as a sign of cell death (2, 4). In normal tissue, TPA was found, too (4, 5). Higher values of TPA have been described for liver diseases and inflammatory processes (4, 6, 7). Some studies reported an increase of TPA serum concentration in tumour patients (6, 8-12). The reason may be increased cell destruction accompanied by invading tumour cells (7, 13). At present, it can be considered as a possible differentiation and / or proliferation marker of epithelial tissue (6).

The oncoprotein HER-2/neu (also known as c-erbB-2) encodes a transmembrane glycoprotein that is similar to the epidermal growth factor (EGF) receptor. Structural homology with the EGF receptor and possession of tyrosine kinase activity and its ability to generate mitogenetic responses all point to the c-erbB-2 receptor (14, 15). The HER-2/neu protein is a transmembrane tyrosine kinase cell

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Table I. *Staging and grading of bladder cancer patients.*

Staging / Grading	G1	G2	G3	Total
Ta	11	12	1	24 (2 Tis)
T1	4	14	4	22 (4 Tis)
T2	0	2	5	7 (1 Tis)
T3	0	2	3	5
T4	0	0	2	2
Total	15	30	15	60

surface growth factor receptor that is expressed on normal epithelial cells and overexpressed in some cancer cells. The full length HER-2/neu oncoprotein has a molecular weight of 185000 D and is composed of three parts: the internal tyrosine kinase portions, responsible for intracellular signalling, a short transmembrane portion and the extracellular domain (ECD), which is the portion that interacts with growth factors. The ECD of HER-2/neu has been shown to be shed from the cell surface as a glycoprotein between 97,000 and 115,000 D. The shed ECD circulates in normal individuals and has been found to circulate in abnormally high levels in cancer patients (26).

The urokinase-type plasminogen activator receptor (uPAR) is a GPI-linked single-chain glycoprotein having a molecular weight between 50 kD and 60 kD. uPAR is composed of three domains: Domain I is involved with uPA binding, while Domains II and III aid in orienting the uPAR molecule on the cell membrane (17). The presence of a cellular receptor for uPA was first demonstrated by Vassalli *et al.* (18), who observed a saturable specific binding of uPA to the surface of monocytes. uPAR bind both the enzymatically inactive single-chain pro-uPA and the enzymatically active two-chain HMw-uPA with high affinity (17).

Mutations of the tumour suppressor gene *TP53* are frequently correlated with tumour development and progression in bladder cancer (19). *TP53* is localized on the short arm of chromosome 17 (20). *TP53* has an influence on cell cycle regulation, gene transcription, DNA repair, genome stability, chromosome segregation, angiogenesis and apoptosis. Genetic alteration is the most frequent reason for a change of function of *TP53*. Loss of function may also be due to binding with viral oncoproteins or cellular gene products or may be caused by dislocation of the protein to cytoplasma (21, 22). A very important function of wild-type *TP53* is the induction of apoptosis (23).

In this study, we compared the results of measuring four different tumour markers for bladder cancer: TPA, HER-2/neu, uPAR and *TP53* mutations. We examined the following questions: Which marker has the highest sensitivity / specificity? Which marker correlates with superficial and invasive bladder cancer?

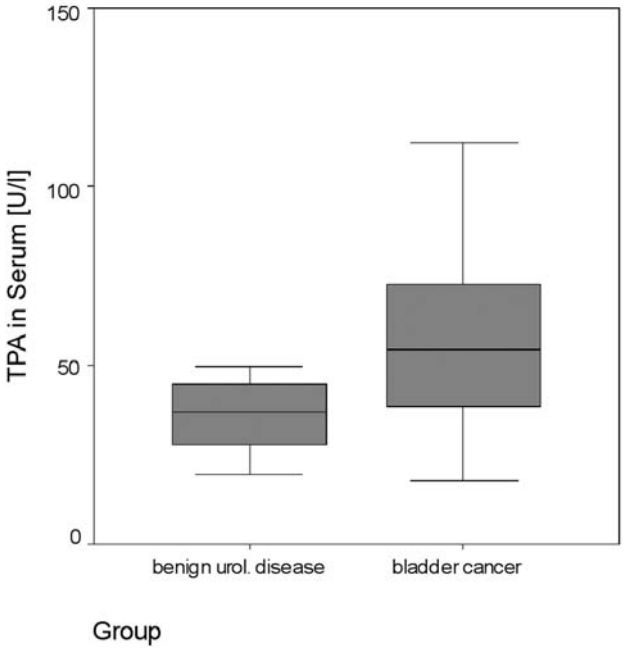

Figure 1. *Boxplot for TPA in serum.*

Materials and Methods

We used material from 60 patients with primary diagnosed tumours of the urinary bladder and from 9 patients with benign urological diseases. Fifty-seven male patients and 12 female patients (average age 63.8 years) were included. The staging and grading of the bladder cancer patients is shown in Table I. Genetic mutation analysis was carried out in urothelial samples and in urine. The TPA concentration was analyzed in serum and in urine. HER-2/neu and uPAR were analyzed in urine.

Measurement of TPA in serum and urine. Ten ml urine were centrifuged at 3000 rpm for 15 minutes. Fifty µl of Tween 20 were added per 10 ml of urine. For the assay, 50 µl of the pre-treated urine and 150 µl of kit diluent were added to the test tube (24). The TPA concentration was measured by immunoluminometric assay with LIA-mat® TPA-M Prolifigen® (AB Sangtec Medical, Bromma, Sweden). TPA-M LIA is a one-step luminometric immunoassay based on reaction tubes coated with monoclonal antibodies against cytokeratins 8, 18, 19 and soluble tracer antibodies conjugated with isoluminol (25).

Measurement of HER-2/neu. The currently most widely used method to analyse the HER-2/neu gene and protein are the Fluorescent-In-Situ-Hybridization (FISH) for DNA. Immunohistochemistry (IHC) detects the full-length HER-2 molecule and an immunoassay technique enables a quantitative estimate to be made of the circulating ECD. FISH and IHC are performed primarily on the tissue biopsy taken at the time of primary diagnosis and reflect the status of the gene or protein at that time point, whereas the ELISA method enables

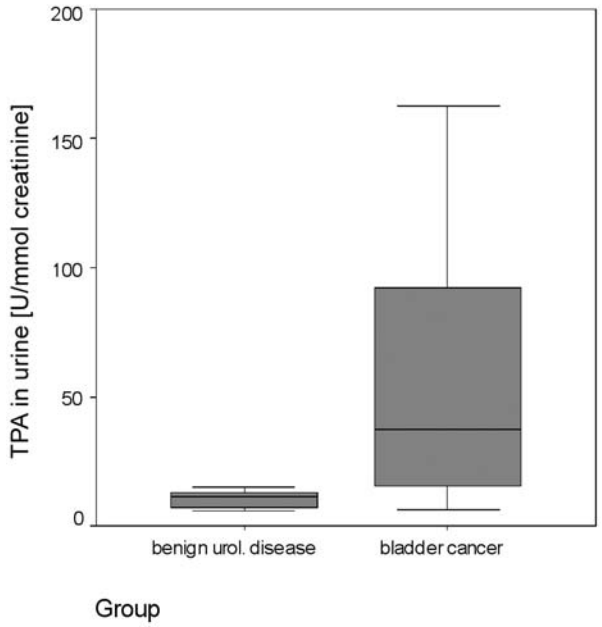

Figure 2. *Boxplot for TPA in urine.*

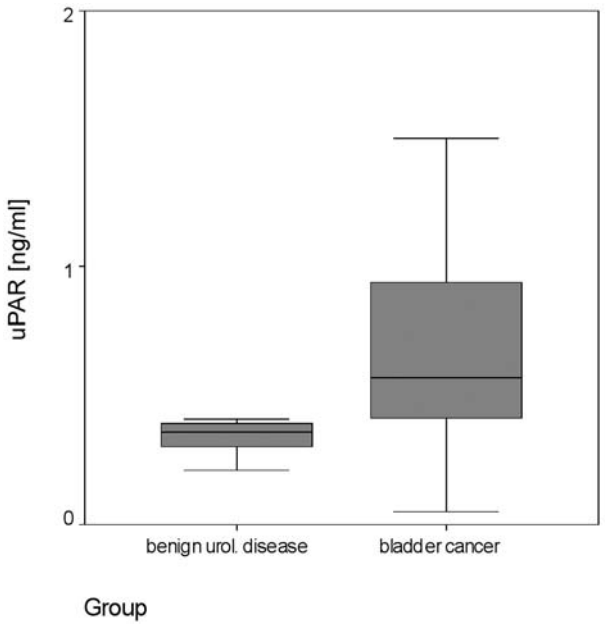

Figure 4. *Boxplot for uPAR.*

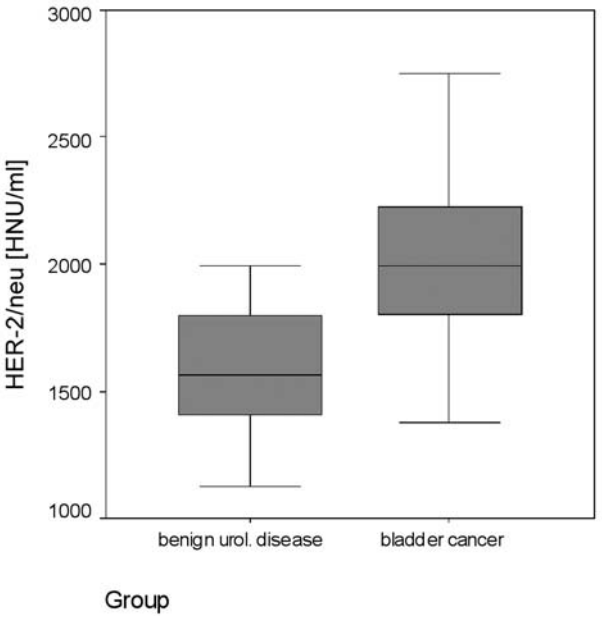

Figure 3. *Boxplot for HER-2/neu.*

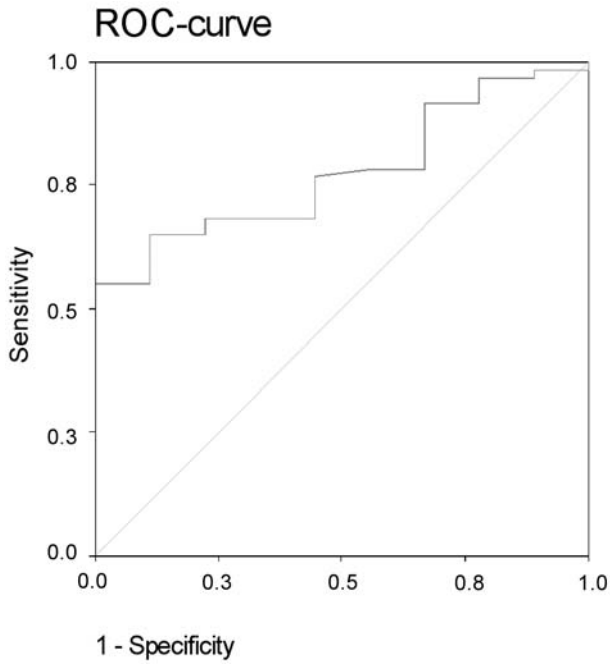

Figure 5. *ROC-curve for TPA in serum.*

subsequent measurement to be made, thereby allowing oncologists to follow the course of the disease. The HER-2/neu ELISA is a sandwich enzyme immumoassay that utilizes two monoclonal antibodies to quantitate the ECD of the HER-2/neu protein in serum (26).

Screening of uPAR. The IMUBIND® Total uPAR ELISA kit (American Diagnostica Inc., Greenwich, USA) is an enzyme-linked immunoassay for the quantitation of human urokinase-type plasminogen activator receptor (uPAR) in tissue extracts, human plasma and cell culture supernants. The lower detection limit of

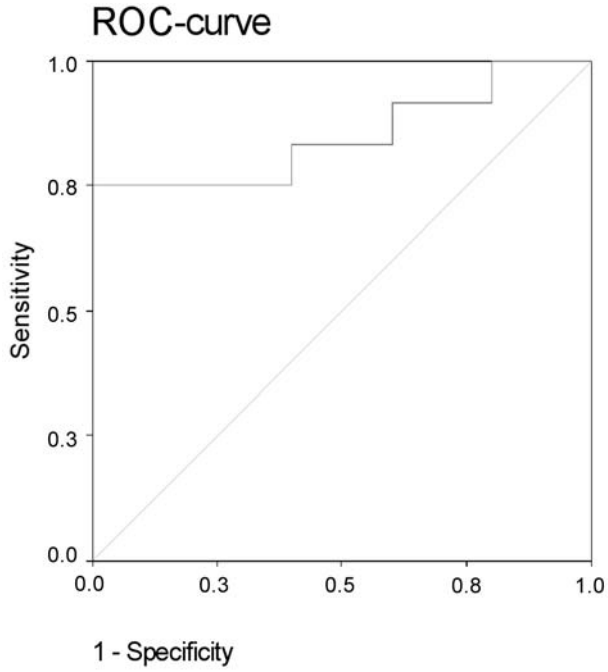

Figure 6. *ROC-curve for TPA in urine.*

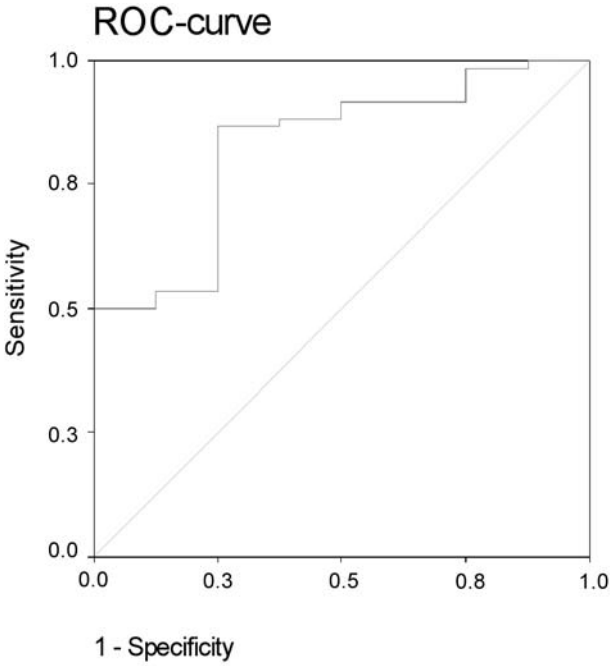

Figure 7. *ROC-curve for HER-2/neu.*

this assay is 0.1 ng total uPAR/mL of sample. Soluble, native and recombinant uPAR, as well as uPAR/uPA and uPAR/uPA/PAI-1 complexes, are all recognized by this assay. The IMUBIND® Total uPAR ELISA employs a rabbit polyclonal antibody against human

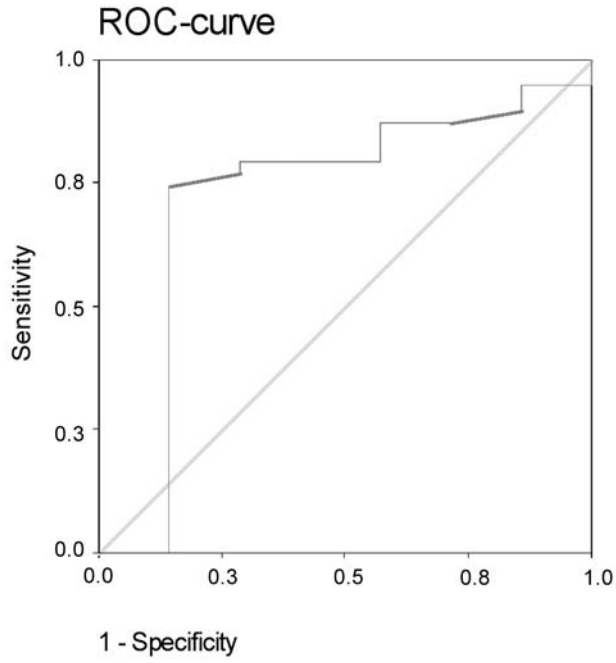

Figure 8. *ROC-curve for uPAR.*

Table II. *Sensitivity and specificity for measured tumour markers.*

	TPA in serum	TPA in urine	HER-2/neu	uPAR
Sensitivity	68.3%	33.3%	88.9%	79.5%
Specificity	88.9%	100%	62.5%	71.4%

uPAR as the capture antibody. Samples incubate in precoated microtest wells and a second, biotinylated antibody is used to recognize the bound uPAR molecules. Adding streptavidin conjugated horseradish peroxidase (HRP) completes the formation of the antibody-enzyme detection complex. The addition of a perborate / 3,3', 5,5' – tetramethylbenzidine (TMB) substrate, and its subsequent reaction with the HRP, creates a blue-coloured solution. Sensitivity is increased by addition of a sulfuric acid stop solution, yielding a yellow colour. Total uPAR levels are quantified by measuring solution absorbance at 450 nm and comparing the values with those of a standard curve.

Screening of TP53 mutations. DNA was isolated by phenol extraction from cellular urine sediments and tumour tissue. Amplification in separate reactions by polymerase chain reaction (PCR) of *TP53* exons 5, 6, 7 and 8 was performed with so-called GC-clamped primers (16). Mutation screening was performed by temperature gradient gel electrophoresis (TGGE). Technical details of the methods used are described in previous reports (27). Each TGGE experiment was carried out with a DNA-negative PCR-control sample and with a positive mutation control to rule

Table III. *Elevated levels of tumour markers in superficial and invasive bladder cancer.*

	TPA in serum	TPA in urine	HER-2/neu	uPAR
Elevated levels for pTa, pT1	31 out of 46 [67.4%]	4 out of 11 [36.4%]	41 out of 46 [89.1%]	24 out of 31 [77.4%]
Elevated levels for pT2-4	10 out of 14 [71.4%]	0 out of 1 [0%]	11 out of 14 [78.6%]	7 out of 8 [87.5%]

Table IV. *TP53 mutation in superficial and invasive bladder cancer.*

	pTa, pT1	pT2-4	Total
TP53 - Mutation	23 [50%]	13 [100%]	36 [61%]
TP53 - Wild-type	23 [50%]	0 [0%]	23 [39%]
Total	46	13	59

out potential contamination. A 4-band-pattern of silver staining in the TGGE was estimated as indicative of a mutation. Sequencing was done for either mutant and, for control, wild-type bands, excised from the silver-stained TGGE-gel, or directly on the isolated DNA: the specific bands or exons were reamplified with the same PCR primers as before, but instead of a GC-clamp, a biotinylated primer at the 5'-end was used (28).

Statistical analysis included ROC, Mann-Whitney *U*-test and Pearson's correlation.

Results

Tumour marker levels in bladder cancer patients and tumour-free patients. In serum of bladder cancer patients, the median TPA concentration was 54.5 U/l, while in the urine of bladder cancer patients the median TPA was 37.4 U/mmol creatinine. In serum of patients with benign urological diseases, the median TPA was 37.2 U/l, while in the urine of these patients the median TPA was 10.8 U/mmol creatinine (Figures 1 and 2). The median HER-2/neu concentration in the urine of bladder cancer patients was 1988.5 HNU/ml, and the median uPAR concentration was 0.57 ng/ml. In the urine of patients with benign urological diseases, the median HER-2/neu concentration was 1563 HNU/ml, and the median uPAR concentration in this group was 0.36 ng/ml (Figures 3 and 4).

After ROC-curve analysis the following optimal cut-offs were found: TPA in serum: 47 U/l (Figure 5), TPA in urine: 60 U/mmol creatinine (Figure 6), HER-2/neu: 1610 HNU/ml (Figure 7), uPAR: 0.4 ng/ml (Figure 8)

The calculated diagnostic sensitivity for TPA in serum was 68.3%, for TPA in urine 33.3%, for HER-2/neu 86.7% and for uPAR 79.5%. The calculated diagnostic specificity for TPA in serum was 88.9%, for TPA in urine 100%, for HER-2/neu 62.5% and for uPAR 71.4% (Table II).

Pearson's significance with the Chi-Quadrat-test showed that pathological levels of TPA in serum ($p=0.001$) and HER-2/neu ($p=0.001$) were significantly higher in patients with bladder cancer in comparison to the control group. Pathological levels of TPA in urine ($p=0.140$) and for uPAR ($p=0.006$) could not give such a high statistical significance.

For all analysed tumour markers the Mann-Whitney *U*-test was calculated. The significance for TPA in serum was $p=0.008$, for TPA in urine $p=0.027$, for HER-2/neu $p=0.003$ and for uPAR $p=0.064$. HER-2/neu had the highest sensitivity, and it could make the best statistically significant differentiation between patients with bladder cancer and tumour-free patients.

Which marker correlates with superficial and invasive bladder cancer? We measured pathological values of TPA in serum for invasive bladder cancer in 10 out of 14 cases (71.4%); in urine, pathological TPA levels were detected in 0 out of 1 case. Pathological levels for HER-2/neu for invasive bladder cancer were found in 11 out of 14 cases (78.6%), and for uPAR in 7 out of 8 cases (87.5%). For superficial bladder cancer, we found elevated TPA levels in 31 out of 46 cases (67.4%) in serum, and in 4 out of 11 cases (36.4%) in urine. Pathological levels for HER-2/neu for superficial bladder cancer were found in 41 out of 46 cases (89.1%), and for uPAR in 24 out of 31 cases (77.4%). These results are presented in Table III. The Chi-Quadrat-Test of Pearson's significance between the analysed tumour markers and invasive bladder cancer found no result $p<0.005$.

TP53 mutation in patients with bladder cancer is significantly correlated with tumour staging. We detected mutations of *TP53* in invasive bladder cancer in 13 out of 13 cases (100%); in superficial bladder cancer, we found 23 out of 46 cases (50%) with mutation of *TP53*. Pearson's significance with the Chi-Quadrat-Test between mutation of *TP53* and invasive bladder cancer was calculated with $p=0.001$ (Table IV).

Thus, mutation in *TP53* is the best marker to differentiate between invasive and superficial bladder cancer.

Discussion

In the reported experiments, TPA showed only a slight sensitivity (68.6% for TPA in serum and 33.3% for TPA in urine), but a very good specificity (88.9% for TPA in serum and 100% for TPA in urine) for bladder cancer patients. This is comparable with the 54.7 % sensitivity and 100 %

specificity as published by Maulard *et al.* (6) for TPA in serum. Stieber *et al.* (29) found 16% sensitivity and 95% specificity for TPA in serum. In the study published by Sanchez-Carbayo *et al.* (12), an 80.2% sensitivity and a 95% specificity was reported for TPA in urine. Menendez Lopez *et al.* (11) found a specificity of 73% and a sensitivity of 45%. All these reported results correlate with our results.

Comparative cytokeratin analyses have shown cytokeratin 19 to be an essential part of TPA in bladder cancer (29). The analysis in non-invasive tumours seemed to be difficult. Pathological TPA serum concentration was found in 31 out of 46 patients (67.4%) with superficial bladder cancer; elevated TPA levels in urine were found in 4 out of 11 patients (36.4%). In invasive tumour stages (pT2 – pT4), the pathological TPA concentrations were elevated in serum (10 out of 14 = 71.4%). A missing correlation of TPA level with tumour grading in bladder cancer, confirming our results, has already been reported (8, 10). We found no statistically significant difference between superficial / invasive bladder cancer and elevated levels of TPA in serum / urine. This has already been reported by Filella *et al.* (9) and by Casetta *et al.* (30).

It is known that TPA is not specific for tumours and that an inflammation can influence the TPA levels (4, 6, 7). The number of patients in the present study was insufficient to reach a conclusion regarding the influence of inflammation.

In this study, HER-2/neu showed a very high sensitivity (88.9%) and a good specificity (62.5%). HER-2/neu was the best of all the analysed tumour markers of this study. However, HER-2/neu could not differentiate between superficial and invasive bladder cancer, since we found elevated HER-2/neu levels in 89.1% of superficial and in 78.6% of invasive bladder cancer patients. The results of Lonn *et al.* (31) show that elevated erb-b2 levels correlate with tumour grade of superficial bladder cancer. HER-2/neu and the other analysed tumour markers were not specific for bladder cancer. Neumann *et al.* (26) measured elevated levels of HER-2/neu in breast cancer.

The sensitivity for uPAR was high at 79.5%, while the specificity was 71.4%. Our results do not differ from other studies (17). Shariat *et al.* (32) found that elevated uPAR levels are significantly higher in bladder cancer patients than in healthy individuals. Casella *et al.* (33) tested uPAR and uPA before cystoscopy and showed that uPAR could help to find high-risk patients for bladder cancer.

The mutation frequency in the so-called high-risk exons 5 - 8 of the *TP53* gene is approximately 40 % in bladder cancer tissue (34, 35). Mutation of *TP53* might accelerate carcinogenesis, especially by enhancement of cell proliferation, loss of apoptosis and by insufficient DNA repair (36). It has now been widely accepted that the p53 state plays a role in the progression of bladder tumours (37, 38). In our study, we found a mutation frequency of 100% for invasive bladder cancer and of 50% for superficial bladder cancer, confirming already published results (35). Mutations in the *TP53* gene are correlated with infiltrating bladder cancer and qualify as a marker.

Conclusion

1. For all analysed tumour markers, pathological concentrations were detectable in serum and / or in urine of bladder cancer patients.
2. Pathological levels of TPA in serum ($p=0.001$) and HER-2/neu ($p=0.001$) were significantly higher in patients with bladder cancer in comparison to the control group. Elevated TPA levels in serum and elevated HER-2/neu levels give a reference to malignant diseases.
3. HER-2/neu had the highest sensitivity of all analysed tumour markers.
4. Elevated levels of TPA, HER-2/neu and uPAR had approximately the same frequency in invasive and superficial bladder cancer. The mutation frequency of *TP53* in invasive bladder cancer was significantly higher than in superficial bladder cancer ($p=0.001$).

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4.3. Originalarbeit 3

Ecke TH, Bartel P, Koch S, Ruttloff J, Theissig F.
Chemotherapy with gemcitabine, paclitaxel, and cisplatin in the treatment of patients with advanced transitional cell carcinoma of the urothelium
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Diese nicht-randomisierte retrospektive Studie wurde entworfen, um die Durchführbarkeit, Toxizität, und Wirksamkeit der Dreifach-Chemotherapie von Gemcitabin, Paclitaxel und Cisplatin bei Patienten mit fortgeschrittenem Urothelkarzinom zu prüfen. Daneben dienten die Ergebnisse dieser Arbeit der Beschreibung einer Kohorte, an der weiterführende Untersuchungen auf Tumormarker durchgeführt wurden und werden.

Dosismodifikation, Einschlusskriterien, statistische Analysen, Evaluation vor der Behandlung, Beurteilung der Wirksamkeit und Definition des Therapieansprechens sind der Originalarbeit im Anhang detailliert zu entnehmen.

Zwischen August 2000 und November 2005 wurden insgesamt 59 Patienten in diese Auswertung eingeschlossen, 45 Männer und 14 Frauen (medianes Alter 70,0 Jahre). Zusammenfassend wurden 24 Patienten (41%) mit fortgeschrittenem Urothelkarzinom ohne Lymphknoten- oder viszerale Metastasen eingeschlossen, 26 Patienten (44%) hatten ein fortgeschrittenes Urothelkarzinom und Lymphknotenmetastasen ohne viszerale Metastasierung, neun Patienten (15%) hatten ein fortgeschrittenes Urothelkarzinom und viszerale Metastasen. Der ECOG Performance Status zum Zeitpunkt des Beginns der Chemotherapie war bei 24 Patienten (41%) gleich 0, bei 26 Patienten (44%) gleich 1 und bei neun Patienten (15%) gleich 2.

Alle Patienten erhielten an den Therapietagen 1 und 8 intravenös Gemcitabin in einer Dosierung von 1000 mg/m² sowie intravenös Paclitaxel in einer Dosierung von 80

mg/m²; Cisplatin wurde am Therapietag 2 in einer Dosierung von 50 mg/m² intravenös appliziert. Dieses Schema wurde alle 21 Tage wiederholt.

Eine Neutropenie von Grad 3 oder 4 war eine der häufigsten Nebenwirkungen während dieser Therapie (39%), dreizehn Patienten benötigten aufgrund von neutropenischem Fieber eine stationäre Behandlung (22%). In keinem Fall kann über eine ernsthafte Thrombozytopenie oder Blutung berichtet werden.

Die partielle Remissionsrate betrug 81%, komplette Remissionsrate 56%. Das mediane Gesamtüberleben betrug 22,0 Monate, die jeweiligen 1- und 2-Jahres-Überlebensraten betrugen 68% und 39%. Nach einem medianen follow-up von 17,5 Monaten, waren 29 Patienten am Leben, 25 Patienten waren ohne Progress. Das mediane progressionsfreie Überleben für die gesamte Gruppe betrug 10,0 Monate. Das mediane Überleben für Patienten mit ECOG Status 0, 1 und 2 betrug 37,5, 17,0 und 12,0 Monate.

Die Dreifach-Kombination aus Gemcitabin, Paclitaxel und Cisplatin wurde insgesamt gut toleriert und war hocheffektiv als Erstlinien-Therapie für das fortgeschrittene Urothelkarzinom, was durch die Ansprechraten, die mediane Zeit bis zum Progress und das mediane Überleben noch unterstrichen wird.

Diese Therapie sollte trotz der etwas höheren Toxizitäten im Vergleich zur Zweifach-Kombination mit Gemcitabin und Cisplatin als mögliche Option für weitere prospektive Evaluierungen in Betracht gezogen werden. Auch diese Arbeit konnte nachweisen, dass der ECOG Performance Status ein wichtiger prädiktiver Faktor für das Überleben ist [61].

Die Ergebnisse dieser Arbeit und die weitere Auswertung der vorliegenden Daten werden in eine Multicenter-Studie integriert werden, die unter Führung von Professor Matt Galsky vom Mount Sinai Hospital in New York, USA, erstmals auf dem ASCO 2012 vorgestellt wurde und der weitere Publikationen folgen werden.

Chemotherapy with gemcitabine, paclitaxel, and cisplatin in the treatment of patients with advanced transitional cell carcinoma of the urothelium

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Abstract. Chemotherapeutic agents are active in advanced bladder cancer, and various combinations have shown promising results. The objective of this study was to evaluate the efficacy of combination chemotherapy with gemcitabine, paclitaxel, and cisplatin in patients with advanced urothelial carcinoma. Fifty-nine patients with metastatic or locally advanced transitional cell carcinoma of the urothelium were treated between 2000 and 2005. No patient had received any previous systemic chemotherapy. All patients received chemotherapy intravenously with gemcitabine at a dose of 1000 mg/m^2 on days 1 and 8, paclitaxel at a dose of 80 mg/m^2 on days 1 and 8, and cisplatin at a dose of 50 mg/m^2 on day 2. Treatment courses were repeated every 21 days. After completion of four to six courses in this regimen an intravenous application of gemcitabine was repeated every 28 days at a dose of 1000 mg/m^2. Fifty-nine patients were treated between 2000 and 2005. Nine patients (15%) had ≥1 visceral site of metastases, and no patient had received any previous systemic chemotherapy. Forty-eight patients (81%) achieved objective responses to treatment (56% complete responses). The median actuarial survival was 22 months, and the actuarial 1-year and 2-year survival rates were 68% and 39%, respectively. After a median follow-up of 17.5 months, 29 patients remained alive and 25 were free of disease progression. The median progression-free survival for the entire group was 10 months. The median survival time for patients with an Eastern Cooperative Oncology Group (ECOG) status of 0, 1, and 2 was 37.5, 17, and 12 months, respectively. Grade 3-4 neutropenia occurred in 39% of the patients. The combination of gemcitabine, paclitaxel, and cisplatin is a highly effective and tolerable regimen for patients with advanced urothelial carcinoma. This treatment should be considered as a suitable option that deserves further prospective evaluation. The ECOG performance status is an important predictive factor for survival.

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Key words: cisplatin, chemotherapy, gemcitabine, paclitaxel, urothelial cell cancer

Introduction

Transitional cell carcinoma (TCC) of the urothelium has a high prevalence among the elderly. Approximately 80% of those who are affected are between 50-79 years of age (1). When diagnosed, 20-30% of bladder carcinomas display muscle infiltration, and 50% of these metastasize (2-4). The prognosis for metastasized urothelial carcinomas is poor and the average survival rate for untreated patients is 3-6 months (5).

Advanced transitional cell carcinoma of the urothelium is moderately sensitive to chemotherapy, and there are a number of agents that produce response rates in the 10-40% range (6-8). Cisplatin is one of the most effective single-agent treatments for metastatic urothelial cancer, and forms the cornerstone of combination chemotherapy.

In the 1980s the chemosensitivity of urothelial cell cancer was recognized when phase II studies demonstrated the activity of cisplatin, methotrexate, adriamycin, vinblastine and 5FU in advanced and/or metastatic disease. The next step in developing a more effective therapy was to combine these known effective agents into two-, three-, and four-drug combinations (9). In 1985 investigators from the Memorial Sloan Kettering Cancer Center reported a four-drug regimen of cisplatin, doxorubicin, methotrexate and vinblastine (MVAC) (10). The initial study on 24 patients gave an overall response rate of 71%. In an updated report on a larger series of 121 evaluable patients, the overall response rate to MVAC was 72%, with a clinical complete response rate to chemotherapy alone of 18% and with an additional 11% of the patients being rendered disease free with post chemotherapy surgery (11). Unfortunately, MVAC therapy is associated with significant morbidity. Also the prognosis of patients with metastatic transitional cell carcinoma remains poor, with a median survival of only 12-14 months (6,11-13). Furthermore, the MVAC regimen is relatively toxic, and it is difficult to administer to elderly patients and to patients who have a poor performance status.

In the past decade, several new chemotherapeutic agents have shown activity against advanced urothelial bladder

cancer, including the taxanes paclitaxel and docetaxel (14-18), and gemcitabine (19-22). Gemcitabine (Gemzar®; Eli Lilly & Co., Indianapolis, IN, USA) is a pyramidine antimetabolite that has preclinical and clinical activity against urothelial cancer with an overall response rate of 30% in previously treated and untreated patients (19,23). Gemcitabine offers proven activity against a range of solid tumors (20,24-26). In particular, in metastatic urothelial cancer, gemcitabine alone yielded response rates of 23-29% with a complete response rate of 4-13%, in both previously treated and untreated patients (5). The good activity and toxicity profile of single-agent gemcitabine and its synergism with cisplatin in pre-clincal models (27) have led to the development of this combination in advanced TCC. Von der Maase *et al* (13) reported on a large multinational phase III trial comparing the M-VAC regimen with gemcitabine plus cisplatin (GC), with a total of 405 patients accrued. The final results showed that the two regimens were similar in terms of response rate, time to progression and survival. However, the GC combination provided a better safety profile with decreased toxicity. Based on these data, the GC combination proved to be a standard alternative treatment in patients with advanced TCC.

Paclitaxel (Taxol®) is an anti-microtubule agent that stabilizes microtubules, induces G2/M cell cycle arrest, as well as the phosphorylation of the anti-apoptotic protein bcl-2 (28), and has preclinical and clinical activity against TCC (16,29). Initial phase II studies of two-drug combinations of docetaxel, or paclitaxel, with cisplatin, have shown activity in untreated patients, with response rates that are in the same range as obtained with MVAC (30-35).

In view of the activity of gemcitabine and the taxanes, the partially non-overlapping toxicities of these agents and their different mechanisms of action, the next logical step was to combine these compounds, and incorporate platinum as the back-bone in a three-drug regimen. Investigators in Spain conducted phase I/II trials of the triplet combination of paclitaxel, cisplatin, and gemcitabine (PCG) in a total of 61 patients (36). The investigators began their study in a formal phase I setting, escalating the dose levels of both paclitaxel and gemcitabine, administered weekly, on days 1 and 8, with a fixed dose of 70 mg/m^2 cisplatin, on day 1, every three weeks. At dose level 4, with paclitaxel at 90 mg/m^2 weekly, grade 3 asthenia was determined to be the dose-limiting toxicity. With this schedule and dose intensity, asthenia, as well as myelotoxicity were manageable. G-CSF was used only for secondary prophylaxis in patients in whom either neutropenic fever or grade 4 neutropenia had been observed in the preceding cycle. The phase I study (15 patients) and phase II study (46 evaluable patients) together gave an overall response rate of 78% and a median survival time for the phase I and the phase II part of the study of 24 and 16 months, respectively. In view of these results, several international groups of oncology have begun the largest randomized trial ever designed in metastatic bladder cancer to compare the three-drug design regimen of PCG with the two-drug combination of GC. A total of 610 patients will be needed to detect a difference in survival of 4 months (from 14 to 18 months). The study is restricted to patients with a good clinical performance status [an Eastern Cooperative Oncology Group (ECOG) status of 0 or 1] and a creatinine clearance of at least 60 ml/min.

Since information about the predictive factors for response and survival is needed for the selection of patients who are likely to benefit from new combinations and for stratification purposes in randomized trials, an analysis of the predictive factors for response and survival with the regimen containing PCG was performed (37). The pretreatment characteristics analyzed were age, gender, ECOG performance status (38), histopathology, visceral metastasis (bone, liver, lung), number of sites of disease, LDH and hemoglobin. The factors that were associated with decreased survival in the univariate analysis were a performance status of >0, the presence of visceral metastasis, and more than one site of malignant disease.

In our study we also included patients with an ECOG status of 2 and a lower creatinine clearance with the application of a reduced dose. This nonrandomized retrospective study was designed to evaluate the feasibility, toxicity, and efficacy of this three-drug chemotherapy regimen in patients with advanced urothelial carcinoma. Previous experience with either gemcitabine plus platinum regimens had produced response rates of 41-56%, with median survivals in the range of 10-14 months. Therefore, the achievement of a response rate of >60% or a median survival of >14 months with this regimen would be indicative of sufficient activity to proceed with further development (39).

Patients and methods

Eligibility. Patients who were eligible for this trial were required to have histologically confirmed transitional cell carcinoma of the urothelial tract (bladder, ureter, or renal pelvis) that was either metastatic or locally advanced and unresectable. Patients with locally advanced, unresectable bladder carcinoma who had any possibility of curative therapy with combined-modality treatment were excluded from this trial. All patients were required to have measurable disease. Patients were not allowed to have received a previous systemic chemotherapy regimen. Previous intravesical treatment was allowed if the most recent intravesical therapy was completed >1 month prior to the study enrolment. Previous radiation therapy was not allowed.

Additional eligibility requirements included the following: An ECOG performance status of 0 to 2, a leukocyte count of ≥3,000/μl, a platelet count of ≥100,000/μl, serum bilirubin <1.5 mg/dl, serum creatinine ≥2.5 mg/dl, and age >18 years. Patients with other active malignancies or with any other serious or active medical conditions were excluded. Pregnant or lactating females were ineligible. All patients were required to provide written informed consent prior to the study enrolment.

Pretreatment evaluation. Prior to enrolling in this trial, all patients were required to have a complete history, physical examination, complete blood counts, differential, chemistry profile, and urinalysis. In addition, patients underwent computed tomography scans of the chest, abdomen, and pelvis with appropriate tumor measurements.

Treatment. All patients received treatment with the following regimen: Gemcitabine at a dose of 1,000 mg/m^2 as a 30-min

intravenous infusion followed by paclitaxel at a dose of 80 mg/m^2 as a 1-h intravenous infusion on days 1 and 8. On day 2 cisplatin was applied at a dose of 50 mg/m^2 as an intravenous infusion and hydration with 2,000 ml NaCl 0.9%. The regimen was repeated every 21 days. Patients received standard paclitaxel premedication and antiemetic prophylaxis.

After completion of four to six courses in this regimen a further therapy followed: Gemcitabine at a dose of 1,000 mg/m^2 as a 30-min intravenous infusion. The regimen was repeated every 28 days.

For the patients who had rapid progress after completing the three-drug regimen with gemcitabine, paclitaxel, and cisplatin or during gemcitabine monotherapy, we used the common three-drug chemotherapy with methotrexate, epirubicine, and cisplatin. The regimen of the courses was as follows: Methotrexate at a dose of 30 mg/m^2 as a very short intravenous infusion on days 1 and 15. On day 2 cisplatin was applied at a dose of 70 mg/m^2 as an intravenous infusion and hydration with 2,000 ml NaCl 0.9% followed by epirubicine at a dose of 30 mg/m^2 as a short intravenous infusion. The regimen was repeated every 21 days. Patients received standard premedication and anti-emetic prophylaxis.

Dose modifications. All patients received full doses of all 3 agents on day 1 of the first course of treatment. Subsequent doses were based on the hematological and nonhematological toxicity observed. Dose modifications for myelosuppression were determined by the blood counts measured on the day of scheduled treatment. Nadir blood counts were not used as a basis for dose reduction.

On day 1 of each course, full doses of all drugs were administered if the leukocyte count was ≥3,000/μl and the platelet count was >100,000/μl. If the leukocyte count was <3,000/μl or the platelet count was <100,000/μl, then treatment was delayed for one or two days.

All patients with an ECOG performance status of 2, or with renal insufficiency in the stage of compensated retention received reduced doses of 50% to 70%. In the case of good tolerance of the therapy we applied higher doses for the following cycles.

Assessment of treatment efficacy. Patients were evaluated for response to treatment after the completion of 4 courses (12 weeks). Re-evaluation included a repeat of all previously abnormal radiological studies with a repeat of objective tumor measurement. Patients who achieved an objective response (complete or partial) or stable disease after the completion of four courses of therapy continued treatment with this regimen. Re-evaluations were performed after the completion of each additional courses of therapy. Treatment was continued for a total of six courses. Patients who completed 6 courses and remained in remission were given further treatment with a single dose of gemcitabine. This additional treatment was continued for at least two years. Patients who completed these two years of gemcitabine monotherapy and remained in remission did not receive any further specific treatment and were re-evaluated for ongoing response at 3-month intervals including a clinical examination, ultrasound of the abdomen and computed tomography scans of the chest, abdomen, and pelvis with appropriate tumor measurements.

Table I. Histological staging and grading of patients.

Staging/ grading	G1	G2	G3	Total
T1	1	1	2	4
T2	1	3	11	15
T3-4	0	6	26	32
T4	0	0	8	8
Total	2	10	47	59

Response definitions. Responses were defined using the World Health Organization Response criteria. A complete response required the total disappearance of all clinically and radiographically detected tumors for at least 4 weeks. Patients had partial response if treatment produced a reduction of ≥50% in the size of measurable lesions, as measured by the product of the greatest perpendicular dimensions, with no evidence of new disease if measurable lesions were reduced by <50% or increased by <25%, as determined by the measurement of the products of the greatest perpendicular dimensions, with no new lesions appearing. Patients who had any appearance of new lesions or who had an increase of >25% in the size of any existing lesions had progressive disease.

Progression-free survival was defined as the interval between the date of the first treatment and the date of documented tumor progression.

Statistical analysis. Survival was measured from the time of the initiation of chemotherapy until death or the last follow-up. Actuarial survival curves were constructed using the Kaplan-Meier method (40). Statistical analysis included the Chi-square test after Pearson's correlation was computed and performed by the computer program SPSS 12.0.1. All patients who received at least two doses of treatment were included in the toxicity analysis. Toxicity was evaluated using the National Cancer Institute Common Toxicity Criteria.

Results

Patient population. From August 2000 to November 2005 a total of 59 patients were included in the trial. Forty-five male patients and 14 female patients (average age 69.7 years, median age 70 years) were included. The pathohistological stage and grade is shown in Table I. The patient characteristics are summarized in Table II. The majority of patients (35 out of 59 patients = 59%) had metastatic disease. Four patients (7%) were treated with radical tumor surgery and came to progress (lymph node or visceral metastases) >1 year after the first treatment. Twenty-seven patients (46%) had lymph node or visceral metastases just after radical tumor surgery. Six (10%) patients had lymph node or visceral metastases before surgery and no surgical treatment was done before chemotherapy. Nineteen patients (32%) had neither lymph node nor visceral metastases just after radical tumor surgery. Three (5%) patients had neither lymph node nor visceral

Table II. Patient characteristics (n=59 patients).

Characteristic	No. of patients (%)
Age (years)	
Median	70
Range	52-82
Gender	
Male	45 (76)
Female	14 (24)
TNM stage	
Any T, N0, M0	24 (41)
Any T, N^+, M0	26 (44)
Any T, any N, M1	9 (15)
ECOG performance status	
0	24 (41)
1	26 (44)
2	9 (15)
Previous treatment	
Radical tumor surgery	48 (81)
Site of tumor	
Locoregional/lymph node only (pN1)	6 (10)
pN2 only	20 (34)
Visceral metastases	9 (15)

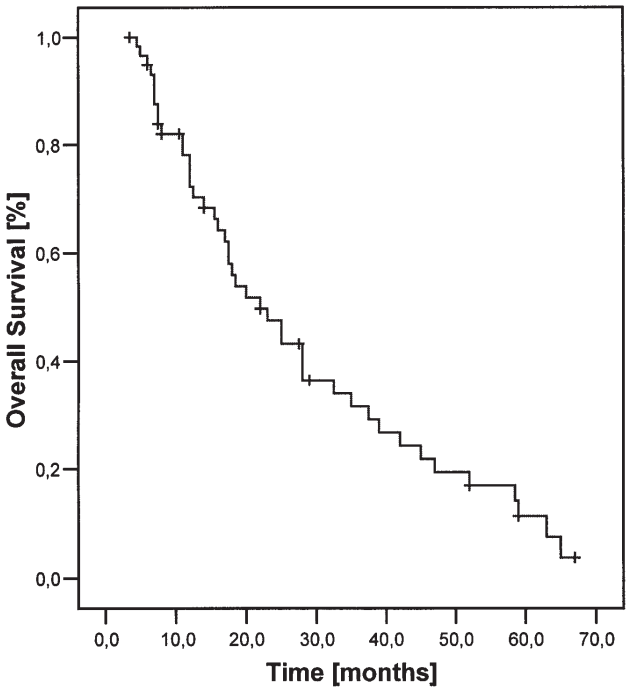

Figure 1. Overall survival for the entire cohort of patients (n=59).

metastases before surgery and no surgical treatment was done before chemotherapy because of non-compliance for surgical therapy. In summary, we included 24 patients (41%) with advanced urothelial cancer without lymph node or visceral metastases, 26 patients (44%) had advanced urothelial cancer and lymph node metastases without visceral metastases, and 9 patients (15%) had advanced urothelial cancer and visceral metastases (Table II). The ECOG performance status at the point of starting chemotherapy was 0 for 24 patients (41%), 1 for 26 patients (44%), and 2 for 9 patients (15%). Before the beginning of chemotherapy 48 patients (81%) had radical tumor surgery: Radical cystectomy or nephroureterectomy. No patient had received previous chemotherapy or radiation.

Treatment schedule. All 59 patients received at least two courses of treatment and were evaluated for response. Thirty-four patients received gemcitabine monotherapy. Five patients received the second line chemotherapy (MEC) because of rapid progression after the three-drug regimen with gemcitabine, paclitaxel, and cisplatin or during gemcitabine monotherapy.

The median number of treatment courses received in the first line three-drug regimen with gemcitabine, paclitaxel, and cisplatin was five (range, two to seven courses). A reduced dose of the three drugs was administered to 38 patients (64%). This high number of reduced doses given is because 25 patients (42%) had an ECOG performance score of 1, and 10 patients (17%) had an ECOG performance score of 2. Therefore, the large majority of dose modifications in this clinical trial were on the basis of a bad performance score and parallel diseases such as renal insufficiency, coronary heart disease, bronchial asthma, and other common diseases of older patients. Fifty-five patients (93%) received monotherapy with gemcitabine after the three-drug regimen. The median number of treatment courses received in the monotherapy with gemcitabine was two (range, 0-33 courses).

Treatment efficacy. Forty-eight out of 59 patients (81%) had major responses to treatment. The median survival of all patients was 22 months (Fig. 1), and the actuarial 1-year and 2-year survival rates were 68% (19 out of 59 patients died) and 39% (36 out of 59 patients died), respectively. Thirty-three patients (56%) achieved complete responses. After a median follow-up of 17.5 months, 29 patients remained alive and 25 were free of disease progression. The median progression-free survival for the entire group was 10 months (Fig. 2); for patients with objective responses, the median progression-free survival was 14 months (Fig. 3). The median survival time was very different according to the ECOG performance status (Fig. 4): The median survival time for patients with an ECOG status of 0 was 37.5 months, for patients with a status of 1, 17 months, and for patients with a status of 2, 12 months.

Toxicity. Neutropenia was the most common grade 3 or 4 toxicity produced by this treatment regimen (Table III). Twenty-three patients (34%) experienced grade 3-4 neutropenia, and 13 patients required hospitalization for the treatment of neutropenia and fever. No case of severe thrombocytopenia and no bleeding episodes were reported.

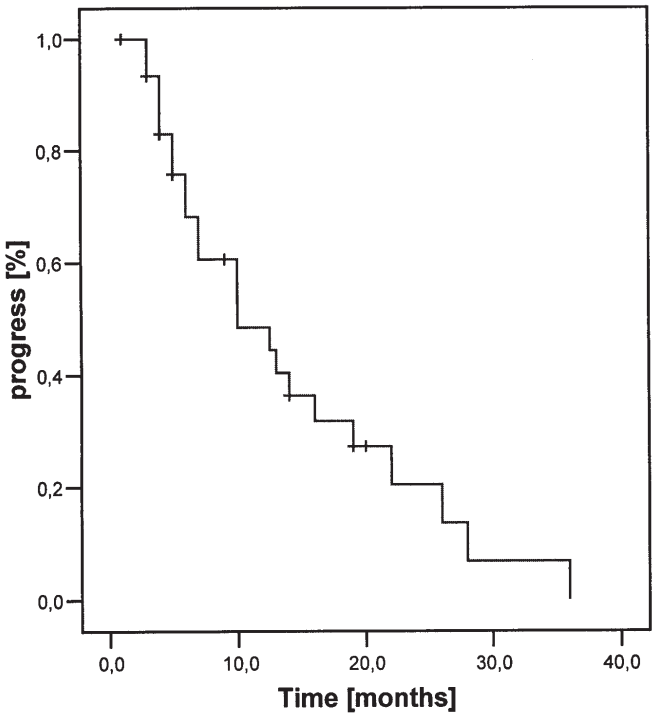

Figure 2. Time-to-progress for all the patients.

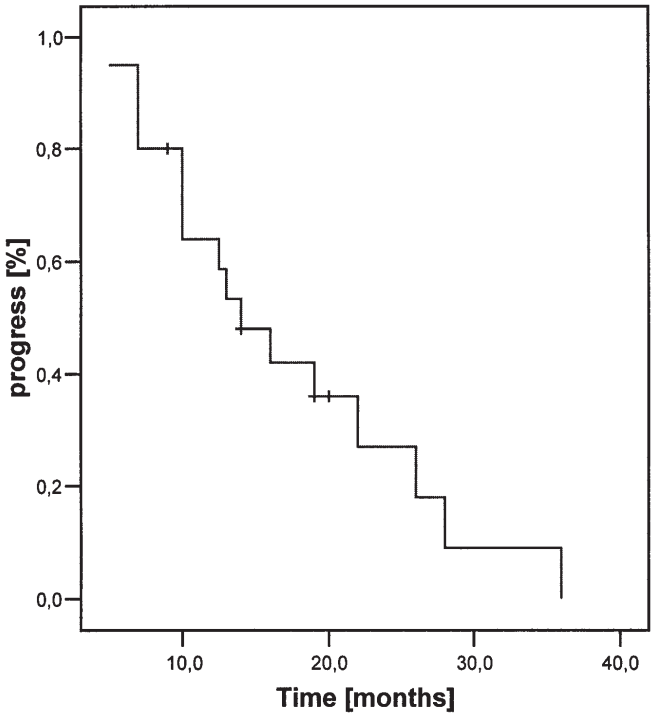

Figure 3. Time-to-progress for patients with objective response.

Severe nonhematological toxicity was uncommon with this treatment regimen. Twenty patients (34%) experienced treatment-related fatigue; emesis was reported in 14 cases (24%). More severe was the treatment-related nausea in 22 cases (37%).

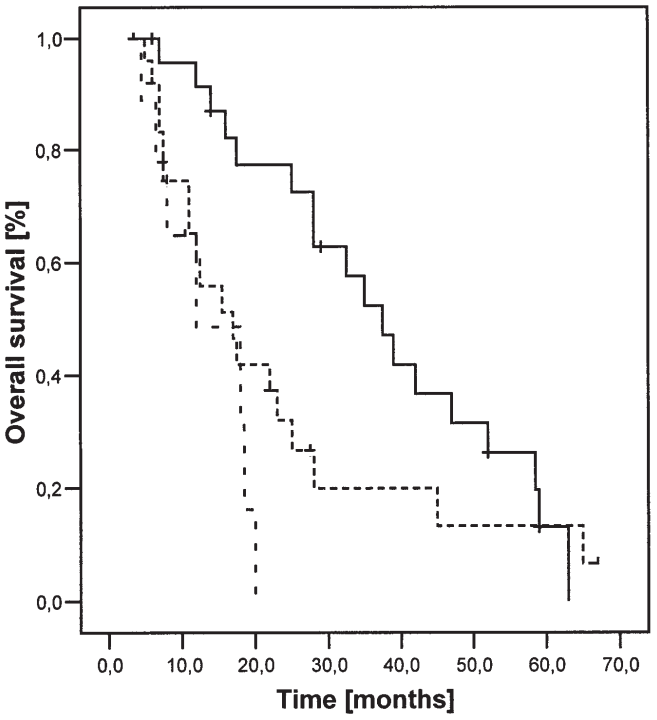

Figure 4. Overall survival for the entire cohort of patients (n=59) according to the ECOG performance score (0, ——, 1, - - - - -, 2, - - - - -).

Table III. Treatment-related toxicity (n=59 patients/284 courses).

Toxicity	No. of patients (%)
Hematological	
Neutropenia (grade 3)	10 (17)
Neutropenia (grade 4)	13 (22)
Anemia	14 (24)
Thrombocytopenia	0 (0)
Nonhematological	
Nausea	22 (37)
Emesis	14 (24)
Fatigue	20 (34)

Discussion

This three-drug regimen with a combination of gemcitabine, paclitaxel, and cisplatin was well tolerated and highly effective as a first-line treatment for advanced urothelial carcinoma, as demonstrated by the response rate, the median time to progression and median survival.

Cisplatin-based chemotherapy represents the most effective treatment for advanced urothelial cancer. Nevertheless, the majority of these patients are elderly, have impaired renal function, or have a poor performance status that make them unfit for such treatment. We therefore used the effective three-drug regimen of gemcitabine, paclitaxel and cisplatin with dose modifications for the unfit patients.

Table IV. Main patient characteristics and prognostic factors of the two gemcitabine containing triplet studies and our results.

	Hussain *et al* (41) P carbo G study (n=49)	Bellmunt *et al* (36) PCG study (n=61)	Lorusso *et al* (42) PCG (n=42)	Our results PCG (n=59)
Median age	63	66	69	70
ECOG status 0/1/2	24/20/5	21/34/6	18/16/8	24/26/9
Metastatic %	80	82	76	59
Visceral metastases %	49	36	55	15
Overall response (OR) %	68	78	43	81
Complete response (CR) %	32	28	12	56
Median survival	14.3	24	15.3	22

In the three reported gemcitabine triplets (36,41,42) responses were seen at all sites, including visceral metastases, and the median survival in the cohort of patients with visceral disease was still 14.3, 11.4, and 15.3 months. Table IV shows patient characteristics and the main prognostic factor distributions for these studies in comparison with our results. The overall response rate in the patients with visceral metastases was 77.6% for the cisplatin-based regimen of Bellmunt *et al* (36), and 68% for the carboplatin-based therapy of Hussain *et al* (41). Lorusso *et al* found an overall response rate of only 43% (42). The historical data from the MVAC series showed response rates as low as 20% in patients with visceral disease (10,12,43). Also the median survival time for patients who received the PCG-triplet regimen was with 14.3 months compared to that observed with the MVAC series. In our study 81% had major responses to treatment. The median survival of all the patients was very high with 22 months and very close to the median survival time of 24 months from Bellmunt *et al* (36). Thirty-three patients (56%) achieved complete responses. The median progression-free survival for the entire group was 10 months; for patients with objective responses, the median progression-free survival was 14 months. As it is already known that the ECOG performance status is a predictive factor for survival and response (44) we found a different median survival time according to this factor: The median survival time for patients with an ECOG status of 0, 1, and 2 was 37.5, 17 and 12 months, respectively. These results are in general agreement with the results of Bellmunt *et al* (36). The lower percentage of patients with metastasis (59%), and of patients with visceral metastasis (15%) could be the reason for the better response to therapy in comparison to the results of Bellmunt *et al*, and Hussain *et al* (36,41,45). It is also possible however, that the supportive therapy has improved with the years. Androulakis *et al* treated a group of 26 patients with the three-drug regimen (46). They had 16.7% complete response, and 33.3% partial response; because of the short follow-up they could not give a median survival time.

New promising regimes include combinations of chemotherapeutical drugs. During investigations of new drugs and drug combinations in advanced urothelial cancer, it should be emphasized that patients with good prognostic features have a 15-20% possibility of long-term survival and potential cure by use of effective chemotherapy (44,47). It is extremely important that these patients are offered effective and not suboptimal chemotherapy (4).

Our treatment was designed on the basis that paired combinations of paclitaxel, gemcitabine and cisplatin, which represent three of the most active single agents in advanced urothelial cancer, and these combinations have been shown to achieve higher response rates than any of the three agents alone. Combination chemotherapy offers the potential to optimise response rates and survival by using agents with complementary mechanisms of action (48).

Besides the development of new chemotherapeutic drug regimes, another approach is the integration of biologically targeted agents into combined modality treatment. Similarly research within molecular biology in bladder cancer may improve our possibilities in selecting patients for different treatment regimes based on new and more specific markers than the ones we have today (49-51).

The addition of paclitaxel to the gemcitabine/cisplatin regimen was found to increase toxicity without any evidence of improved efficacy. Continued clinical analysis of current regimens as well as the development of novel combinations, will be necessary for the continued improvement in the treatment of patients with advanced urothelial carcinoma.

In conclusion, the partial response rate was 81%, and the complete response rate was 56%. The median actuarial survival was 22 months, and the actuarial 1-year and 2-year survival rates were 68% and 39%, respectively. After a median follow-up of 17.5 months, 29 patients remained alive and 25 were free of disease progression. The median progression-free survival for the entire group was 10 months. The combination of gemcitabine, paclitaxel, and cisplatin is a highly effective and tolerable regimen for patients with advanced urothelial carcinoma. This treatment should be considered as a suitable option deserving further prospective evaluation. The ECOG performance status is an important predictive factor for survival.

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4.4. Originalarbeit 4

Ecke TH*, Sachs MD*, Lenk SV, Loening SA, Schlechte HH.
TP53 gene mutations as an independent marker for urinary bladder cancer progression
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In dieser Arbeit wurde genauer auf den Einfluss von *TP53* beim Harnblasenkarzinom eingegangen. Dabei wurde dieser vor allem in Hinblick auf die Rezidivrate und die Progression von Harnblasentumoren untersucht.

Es gibt wenige Publikationen, die molekulargenetische Analysen von *TP53* mit der Progression von Harnblasenkarzinomen verglichen haben. In der vorliegenden Arbeit wurden die Ergebnisse der TGGE und Daten der Sequenzierung von Patienten mit einem follow-up von bis zu 95 Monaten vorgestellt.

Tumorgewebe wurde von 75 Patienten aus transurethralen Harnblasentumorresektionen gewonnen. Das mediane Alter der Patienten betrug 66,3 Jahre. Die patho-histologische Diagnose ergab pTa in 46 Fällen, pT1 in 27 Fällen und ein isoliertes Carcinoma in situ (Cis) bei zwei Patienten. Fünf Patienten hatten ein Cis in Kombination mit einem pT1 Urothelkarzinom.
Kaplan-Meier und Cox-Regression Analysen wurden mit folgender Definition der Endpunkte berechnet: Tumorprogress als Invasion des subepithelialen Gewebes unterhalb der muscularis mucosae (Progress von pTa zu pT1), Muskelinvasivität bei zuvor nicht-invasiver Erkrankung (Progress zu pT2 oder höher) oder die Entwicklung einer Metastasierung.

Allgemeine Ergebnisse
Bei 34,7% der Patienten wurden eine oder mehr *TP53* Mutationen mittels TGGE im Tumorgewebe gesichert. Die Mutationsfrequenz betrug 23,9% bei pTa-Tumoren und 55,6% bei pT1-Tumoren. Von den 26 in *TP53* mutierten Tumoren wurden sechs Mutationen in Exon 5, sechs in Exon 6, 15 in Exon 7 und vier in Exon 8 gefunden.

Zusammenhang zwischen *TP53* und der Rezidivrate

Die Gesamt-Rezidivrate in dieser Population betrug 76,0% (57/75). Die mittlere Zeit bis zum Auftreten eines Rezidivs betrug 32,5 Monate. Von den 49 *TP53*-wildtyp Tumoren rezidivierten 69,4% nach einer medianen Zeit von 35,6 Monaten. Von den 26 in *TP53* mutierten Tumoren rezidivierten 88,5% nach einer medianen Zeit von 27,8 Monaten.

Die Kaplan-Meier Analysen für die Tumorrezidive zeigte keinen statistisch signifikanten Unterschied zwischen Tumoren mit und ohne *TP53* Mutationen.

Zusammenhang zwischen *TP53* und der Progressionsrate

Die gesamte Tumorprogressionsrate betrug 18,7% (14/75). Die mediane Zeit bis zum Tumorprogress betrug 19,8 Monate. 38,5% (10/26) der Patienten mit *TP53* Mutationen hatten einen Tumorprogress innerhalb von 14,4 Monaten. 8,2 % (4/49) der wildtyp Tumoren verhielten sich progressiv innerhalb von 33,3 Monaten. Das progressionsfreie Überleben in der Patientengruppe mit einer nachgewiesenen Mutation war signifikant kürzer (Log Rank: p=0,0031, Breslow: p=0,0009). Bei den 29 Hochrisiko Tumoren betrug die Tumorprogressionsrate 27,6% (8/29). Die mittlere Zeit bis zur Tumorprogression von diesen 8 Patienten betrug 23,5 Monate.

Die Frequenz der Tumorprogression war signifikant höher in mutierten verglichen mit wildtyp Tumoren. Cox-Regression Analysen zeigten einen signifikanten und unabhängigen Einfluss von *TP53* Mutationen auf die Tumorprogression im Vergleich mit Tumorgrad, Stadium und Vorkommen früherer Blasentumore in der Anamnese.

Die Berechnung des Einflusses von exon-spezifischen Mutationen auf die Tumorprogression zeigte, dass nur Mutationen im Exon 8 eine statistische Signifikanz in diesem Punkt erreichten. Wie bei den meisten anderen Tumoren ist die Mutationsfrequenz von 6,1% im Exon 8 geringer als die Mutationsfrequenz in anderen Exons. Getrennte Prüfungen von Exon 8 Mutationen innerhalb der Hochrisiko-Gruppe zeigten einen statistisch signifikanten Unterschied (Log Rank: p=0,0021; Breslow: p=0,0037). Die geringe Anzahl von Proben in dieser Studie mag verantwortlich dafür sein, dass in den Mutationen der Exons 5, 6 und 7 bei separater Analyse keine Signifikanz in Hinblick auf *TP53* als Progressionsfaktor erreicht wurde; wurden alle Mutationen zusammen analysiert, zeigte sich eine hohe Signifikanz. Auf

der anderen Seite erscheint es interessant, dass Mutationen im Exon 8 eine so hohe statistische Signifikanz zeigen.

Zehn der elf sequenzierten Mutationen verursachen vermutlich biologische Effekte: Darunter sind drei von diesen „nonsense“ Mutationen. Besonders bemerkenswert erscheint die intron 8-Mutation C → G in Position 14594 auf Map 6; diese befindet sich außerhalb der Exon 8 Grenze. Codon 245 ist eine bekannte hotspot-Mutation beim Harnblasenkarzinom und einigen anderen Tumoren.

Zusammenfassend zeigen unsere Ergebnisse, dass Mutationen von *TP53* einen unabhängigen prognostischen Faktor für ein erniedrigtes progessionsfreies Überleben bei nicht-invasiven Harnblasenkarzinomen darstellen. Darüber hinaus scheinen Mutationen an bestimmten Stellen des *TP53* Gens, besonders im Exon 8, mit einer schlechteren Prognose vergesellschaftet zu sein. Mutationen in definierten strukturellen und funktionellen Domains von *TP53* können deshalb als molekularbiologische Marker für Prognose und Behandlungsstrategien von nicht-invasiven Harnblasenkarzinomen herangezogen werden [62]. Diese Resultate sind umso wertvoller, seitdem der Nachweis von *TP53* Mutationen auch im Urinsediment als nicht-invasive Methode der Diagnostik möglich ist [63, 64].

TP53 gene mutations as an independent marker for urinary bladder cancer progression

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Abstract. This study evaluates the influence of the *TP53* genetic status on tumour recurrence and progression with a highly effective electrophoretic technique. DNA from tissue of 75 non-invasive urinary bladder cancers was PCR amplified in the *TP53* exons 5-8 and run on horizontal polyacrylamide gels under defined temperature conditions to yield specific gel shifts. Kaplan-Meier and Cox-Regression analysis were performed with tumour progression. The overall tumour recurrence in our patient population was 76.0% (57/75). Tumour recurrence frequency was 69.4% (34/49) in patients with *TP53* wild-type, and 88.5% (23/26) in patients with *TP53* mutation. There was no statistically significant difference with regard to recurrence frequency and time to recurrence. The progression-free survival was significantly shorter in patients with *TP53* mutations, and the frequency of tumour progression was significantly higher in mutated as compared to wild-type tumours. Cox-Regression analysis showed a significant and independent influence of *TP53* mutation on tumour progression in comparison with tumour grade, stage and history of prior bladder cancer. If segregated by exons, mutations in the DNA binding region of exon 8 seem to have a particular high influence on tumour progression. We conclude that genetic analysis of *TP53* can select patients at high risk of bladder tumour progression that should be followed closely and may benefit from early radical surgical procedures.

Introduction

With an estimated 61,000 newly diagnosed cases and approximately 13,180 deaths in the United States in 2007, carcinoma of the bladder is the second most common cancer of the urogenital tract (1). The incidence of urinary bladder cancer has increased in the last decades. Bladder cancer has a high rate of recurrence and a significant number of non-invasive tumours will progress to muscle-invasive disease. Due to the heterogeneity of the tumour, new markers for tumour progression are clearly needed as clinical parameters, such as tumour grade and stage are not accurate in predicting the biological behaviour and thus guiding the choice of treatment, especially in high risk cases (2-5).

TP53 mutations are the most frequent genetic alterations in human malignancies. Bladder tumours (40%) are *TP53* mutated (6). A strong association of p53 protein overexpression with a higher rate of progression and recurrence of bladder cancer has been shown (7). We have previously shown that genetic analysis of *TP53* gene can provide valuable information in regards to tumour progression and recurrence and that such analysis is possible in the urine sediment (8,9).

In this study we demonstrate with a highly effective electrophoretic technique that mutations of the *TP53* gene are a statistically significant and independent indicator for early tumour progression.

Materials and methods

Tumour tissue was obtained from 75 patients undergoing transurethral resection of urinary bladder tumour. Twenty-five of the patients had a history of prior non-invasive bladder cancer. The mean age of patients (64 males, 11 females) was 66.3 (range 44-88) years. The histological diagnosis according to the 1997 TNM classification was Ta in 46 cases, T1 in 27 cases, isolated Carcinoma *in situ* (Cis) in 2 patients and 5 patients had Cis combined with synchronous T1 carcinomas. Tumour grade was G1 in 21 cases, G2 in 47 (4 combined with Cis) cases and G3 in 5 (1 combined with Cis) cases.

Three of the 75 patients were treated with intravesical bacillus Calmette Guerin (BCG) after surgery. One patient had a mutation in exon 6, 1 in exon 7, 1 was *TP53* wild-type. One patient was treated with mitomycin C (wild-type), 1 with BCG and mitomycin C (wild-type). None of these 5 patients had progression. The other cases did not receive additional intravesical treatment after the samples were taken.

Total-DNA was isolated from frozen tumour tissues (in four cases from paraffine samples) and amplified by PCR as

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Key words: bladder cancer, progression, p53, mutation, tumour suppressor gene

Table I. Patients with non-invasive bladder cancer and tumour progression.

No.	Age (years)	Sex	Primary histopathology	*TP53*-genotype	Affected gene map position	Codon altered	Time-to-progression (months)	Progress to
1	65	M	TaG1	Exon 6	13397 C→T	213 Arg→opal-Stop	15	T1G3M0
2	55	M	TaG1	Exon 7	Insertion 14008 C	228 Asp→Ser Frameshift	18	T3G2M0
				Exon 8				
3	66	M	T1G2	Exon 7			6	T2G2M0
4	75	M	Cis	Exon 6	13399 A→G	213 Arg Silent	8	T2G3M0
5	77	M	T1G3	Exon 7	14029 C→A		29	T3G3M0
				Exon 8	14510 G→C	281 Asp→His		
				Intron 8	14594 C→G			
6	59	M	T1G3	Exon 7	14070 G→T	248 Arg→Leu	10	T1G3M1 + Cis
7	74	M	TaG1	Exon 8	14514-15 GG→AT	282 Arg→His	15	T1G3M1 + Cis
8	58	M	T1G2	Exon 8	14501 C→G	278 Pro→Ala	5	T2G3M0 + 'Cis
9	58	F	TaG2	Exon 5	13106 G→A	143 Val→Met	33	T2G3M0
10	64	M	TaG2	Exon 7	14060-1 GG→CT	245 Gly→Leu	5	T1G3M0 + Cis
11	63	M	TaG2	Wild-type			70	T3G3M1
12	67	M	T1G2	Wild-type			6	T1G3M0
13	61	M	TaG2	Wild-type			30	T1G2M0
14	77	M	T1G2	Wild-type			27	T2G3M0

described before (10). Briefly, the critical exons 5-8 of the *TP53* gene were amplified using the following primers: exon 5, 5'-(gC)TTC CTC TTC CTA CAg TAC TC and 5'-CTg ggC AAC CAg CCC TgT CgT; exon 6, 5'-(gC)ACg ACA ggg CTg gTT gCC CA and 5'-AgT TgC AAA CCA gAC CTC Ag; exon 7, 5'-(gC)TCT CCT Agg TTg gCT CTg ACT g and 5'-gCA AgT ggC TCC TgA CCT ggA; and exon 8, 5'-CCT ATC CTg AgT AgT ggT AAT C and 5'-(gC)CCg CTT CTT gTC CTg CTT gCT T [gC refers to a 40-bp-GC-rich sequence according to Metzger *et al* (11)].

The PCR products were run on horizontal polyacrylamide gels under defined temperature conditions (TGGE, Qiagen, Hilden, Germany) to yield specific gel shifts as a screening for mutations (10). Mutations were confirmed in some cases by automated sequence analysis (Amersham Pharmacia Biotech, Uppsala, Sweden) of re-amplified TGGE bands using the nested dye-labelled primers (12).

The statistical evaluation was performed using the software SPSS for windows, version 15.0. Kaplan-Meier and Cox-Regression analysis were calculated with tumour progression defined as invasion of subepithelial connective tissue beyond the muscularis mucosae (progression from Ta to T1), muscle invasion of former non-invasive disease (progression to T2 or higher), or development of metastatic disease as the end-point. All 14 patients with tumour progression are indicated in Table I. For further analysis patients were subdivided into a 'low risk' (TaG1, TaG2) and a 'high risk' (all T1, all G3 and all Cis) subgroup of tumour progression according to their histopathological classification.

Results

In 26 of 75 patients (34.7%) one or more *TP53* mutations were detected by TGGE in tumour tissue. The mutation frequency was 23.9% (11/46) in Ta-tumours, and 55.6% (15/27) in T1-tumours. One of two tumours with isolated Cis was mutated, as were three of five T1-tumours with associated Cis.

In the 26 *TP53* mutated tumours 6 mutations were found in exon 5, 6 in exon 6, 15 in exon 7 and 4 in exon 8. Five patients had mutations in two *TP53* exons, always a combination of an exon 7 mutation with other mutated exons (one in combination with exon 5, two with exon 6, two with exon 8). In this double mutation subgroup only the 2 patients with exon 7 and exon 8 mutation suffered from tumour progression. Successful sequence analyses results of 9 from 10 patients with mutations in the TGGE and tumour progression are shown in Table I.

The overall tumour recurrence in our patient population was 76.0% (57/75). The mean time to recurrence was 32.5 months (range 3-95, median 27.0). Of the 49 *TP53* wild-type tumours 69.4% (n=34) recurred after a mean of 35.6 months (range 3-94, median 28.5). Of the 26 *TP53* mutated tumours 88.5% (n=23) recurred after a mean of 27.8 months (range 5-95, median 18.0).

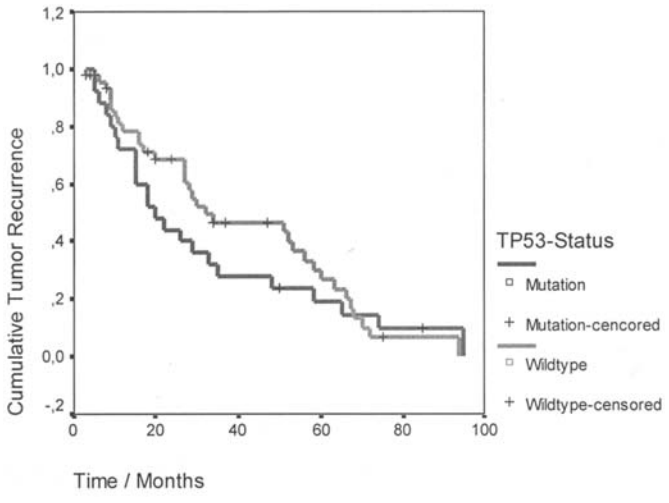

Figure 1. Kaplan-Meier analysis of tumour recurrence in non-invasive bladder cancers (*TP53* wild-type and mutation).

Kaplan-Meier analysis for tumour recurrence showed no statistically significant difference between tumours with *TP53* wild-type and mutation (statistic tests: log-rank, p=0.4487; Breslow, p=0.1289) as shown in Fig. 1. The mean time to recurrence (27.8 months in *TP53* mutation, 35.6 months in *TP53* wild-type) did not differ significantly, either.

The overall tumour progression frequency was 18.7% (14/75). The mean time to tumour progression was 19.8 months (range 5-70); 38.5% (10/26) of the *TP53* mutation patients had a tumour progression within 14.4 months (range 5-33), and 8.2% (4/49) of the wild-type tumours progressed within 33.3 months (range 6-70). The progression-free survival was significantly shorter in the mutation group (statistic tests: log-rank, p=0.0031; Breslow, p=0.0009).

Of the 29 high risk tumours (27 T1G3-tumours and 2 Cis) the tumour progression was 27.6% (8/29). The mean time to tumour progression of these 8 patients was 23.5 months (range 5-70) months. Six of the 46 low risk tumours (13.0%) progressed after a mean time of 14.8 months (range 5-30).

The number of tumours that progressed was significantly different in patients with *TP53* wild-type versus mutation if calculated for the whole study population (Fig. 2A) and if calculated separately for the low risk group (statistic tests: log-rank, p=0.0107; Breslow, p=0.0140) as it can be seen in Fig. 2B. However, in the high risk subgroup the Kaplan-Meier technique did not show a significant difference of tumour progression between patients with *TP53* wild-type and mutation (statistic tests: log-rank, p=0.1869; Breslow, p=0.0788) as shown in Fig. 2C.

The calculation of the influence of exon-specific mutations on tumour progression showed that only mutations in exon 8 reached a specific significance on tumour progression (Fig. 3). Separate evaluation of exon 8 mutations within the high risk subgroup showed a significant difference (statistic tests: log-rank, p=0.0021; Breslow, p=0.0037; figure not shown).

Examination of mutations in single exons showed different frequencies in patients with tumour progression: exon 5, 1/10; exon 6, 2/10; exon 7, 5/10; and exon 8, 4/10. This is outlined in the Kaplan-Meier computations (Fig. 3), and in the Cox-Regression analysis showing exon 8-mutations as an independent progression factor in patients of the high risk subgroup (Table II).

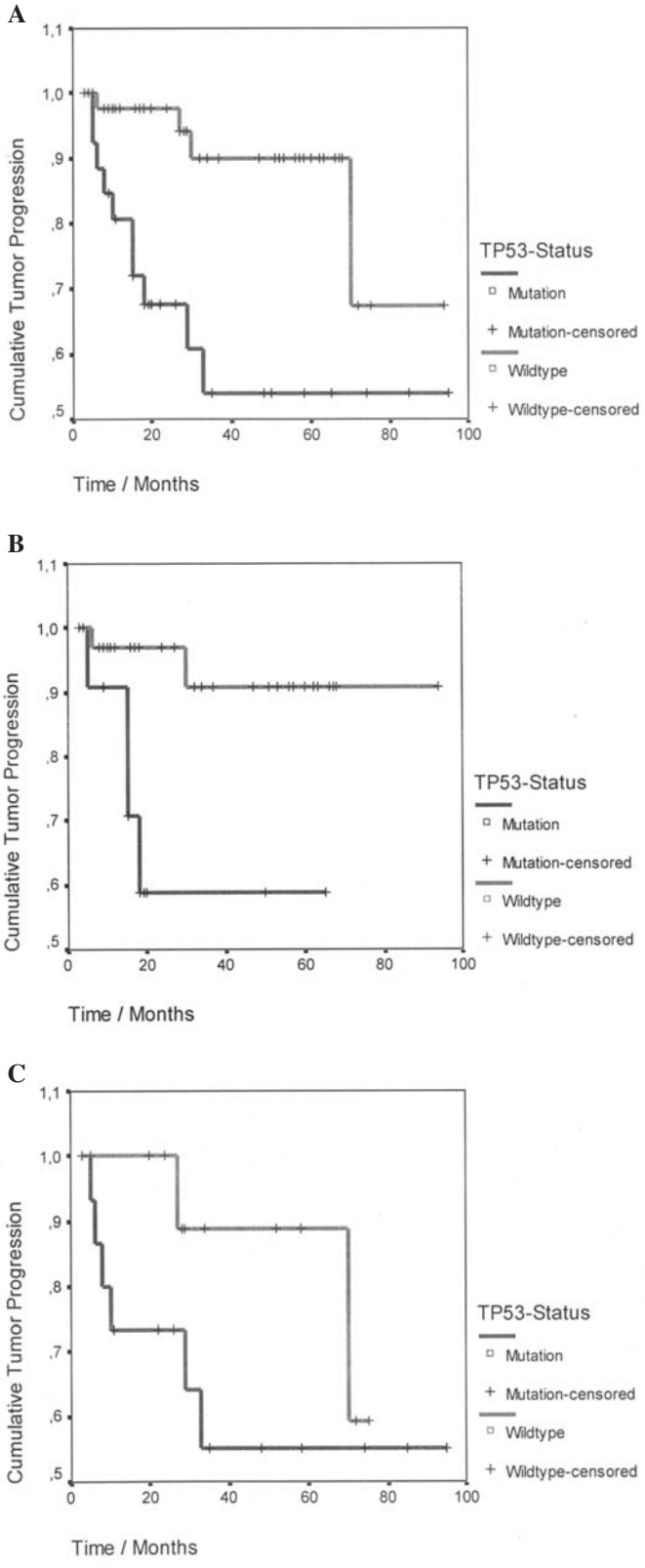

Figure 2. (A) Kaplan-Meier analysis of tumour progression for all patients (*TP53* wild-type and mutation). (B) Kaplan-Meier analysis of tumour progression for low risk tumours (*TP53* wild-type and mutation). (C) Kaplan-Meier analysis of tumour progression for high risk tumours (*TP53* wild-type and mutation).

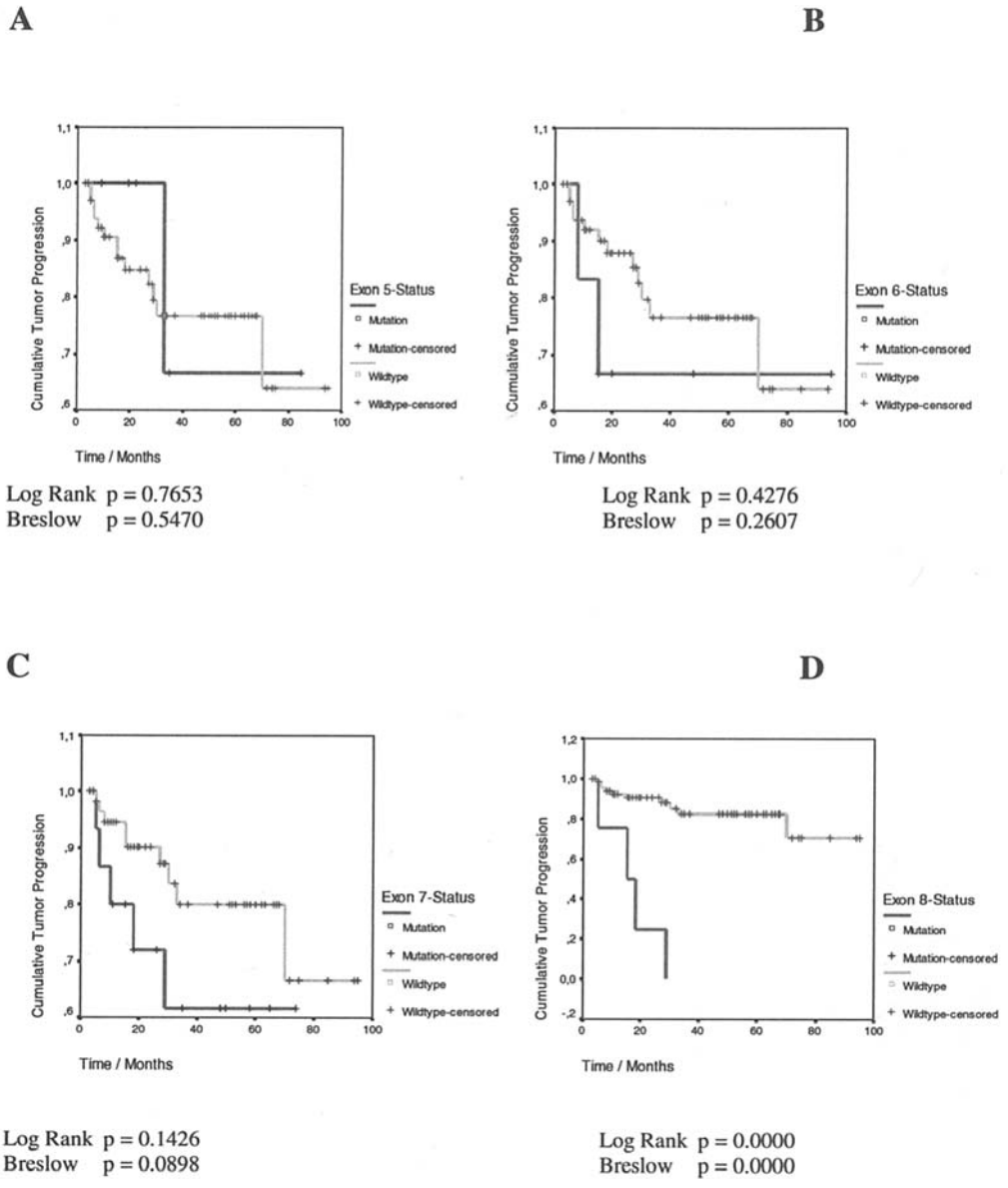

Figure 3. Kaplan-Meier analysis of tumour progression - exon-specific mutations of the *TP53* gene (A-D, exons 5-8).

Interestingly, the multivariate Cox-Regression analysis of tumour stage, grade, history of previous bladder cancer, *TP53*-status, patient age and gender as progression factors revealed, that the *TP53*-status reached statistical significance in the initial block of the Cox-Regression technique. Calculations of the exon 8-status showed stronger significance as the analysis of all 75 patients (Table II, calculatation nos. 1 and 2) or the low and high risk subgroups. The variable G2 does not reach significance in all calculations of the initial block and was excluded in the next step of the Cox-Regression analysis. The term Exp(B) in Table II refers to the increase of probability to suffer from tumour progression in case of a mutation. Exon 7 status has no influence on tumour progression with our data.

If tumour progression was defined as muscle invasion or metastases the number of cases with progression would be reduced to 10 (primary tumours: 2 Ta, 7 T1 and 1 Cis; 8 with *TP53* mutations, 2 with wild-type). In statistic tests a high statistical significance is computed (log-rank, p=0.0009; Breslow, p=0.0002). Kaplan-Meier analysis in Fig. 4 and

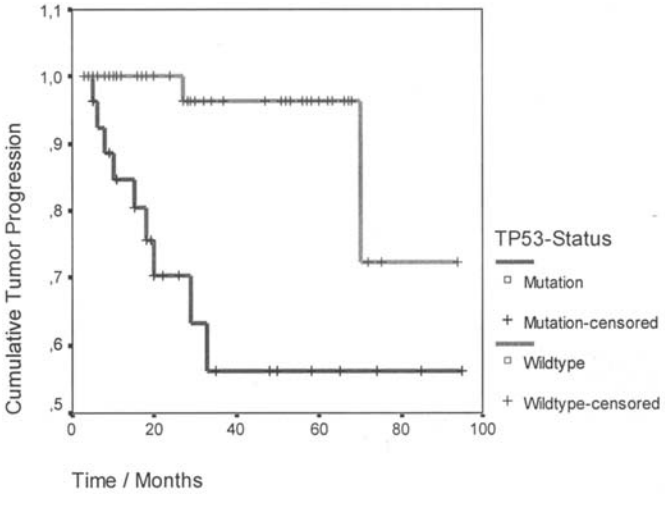

Figure 4. Kaplan-Meier analysis for tumour progression between tumours with *TP53* wild-type and mutation for progression to muscle invasion and/or metastatic disease.

Table II. Cox-Regression analysis of bladder cancer progression variables are *TP53* status, exon 8 status, patients age and sex, tumour stages, tumour grades.

Computation No.	No. of patiens	Variables	Significance in beginning block	Exp(B)	95% CI[a] of Exp(B)
1	75	*TP53*-status	0.003	4.869	1.522-15.578
		Age	0.999		
		Sex	0.514		
		Stage	0.393		
		Ta	0.257		
		T1	0.333		
		Grade	0.269		
		G1	0.825		
		G2	0.062		
		G3	0.717		
2	75	Exon 8	0.000	12.020	3.490-41.396
		Grade	0.272		
3	75	Exon 7	0.133		
		Grade	0.236		
4	46 low risk	Exon 8	0.015	22.314	1.837-271.088
		Grade	0.603		
5	29 high risk	Exon 8	0.014	8.519	1.535-47.277
		Grade	0.551		
6	Tumour progression to invasive tumours, 75	*TP53*-status	0.016	7.109	1.431-35.326
		Grade	0.337		
		G1	0.609		
		G2	0.081		
		G3	0.588		

[a]CI, confidence interval.

Cox-Regression analysis (Table II, calculation no. 6) show significance for *TP53* status and exon 8 status.

Discussion

In this study we show our experiments and calculations supporting *TP53* mutations as a tumour progression factor superior to and independent of tumour grading and staging, patient age and gender, in non-invasive bladder cancer. Only within the high risk subpopulation, if evaluated separately, this relationship was not statistically significant, which supports the results of Peyromaure *et al*, who found no prognostic value of p53 overexpression in T1G3 bladder tumours that were treated with BCG therapy (13). In our cohort 5 patients received intravesical therapy, 2 of them with *TP53* wild-type. We think that this number is too low to influence the statistical significance of our data. The low number of patients receiving intravesical therapy is certainly a point of discussion and might be explained by the fact that most patients were treated outside the study institution and that by the time this study was initiated, intravesical therapy was not yet generally accepted as a standard procedure. On the other hand, our patient population enables an evaluation independent of additional treatments and therefore a more precise definition of the role of *TP53* gene mutations.

Our data do not show a significant relationship between *TP53* mutation and rate of tumour recurrence. Although the recurrence rate in our study is at the higher end of previously published results it is still within their limits and could also be explained by the low percentage of patients who received intravesical therapy (14-16).

Llopis *et al* described, that p53 protein expression has prognostic value for survival and progression in T1 bladder tumours and can be used for early detection of T1 bladder tumours with poor prognosis (17).

Until now, no definitive molecular evidence proving or disproving the progression from non-invasive to invasive bladder tumours has been reported (18). *TP53* mutations have been shown in non-invasive stages of bladder cancer in the range of 35% with increasing frequencies of up to 70% in invasive stages (10). Interestingly, in those tumours that have not directly inactivated *TP53* it is suspected that the functionality is hampered by mutated components of signaling pathways that activate p53 (19). In an *in vitro* study it was shown that organisms with multiple *TP53* genes are tumour resistant (20).

Several groups have presented results of p53 to be a tumour progression factor in bladder cancer and several other malignancies (17,21-37). So far, the role of p53 as a prognostic indicator has been contradictory. Immunological detection of p53 overexpression has been interpreted as mutation. For example, Wu *et al* found p53 overexpression in 70% of non-invasive bladder tumours, but only the ki-67 index was a significant and independent predictor of recurrence and progression (38). They used immunohistochemical detection of p53 overexpression with a cut-off of 20% of nuclei staining positive.

Immunological detection of p53 overexpression and bladder cancer progression was presented by Kuczyk *et al* (26). A number of reports have shown, however, that despite good concordance between *TP53* mutation and p53 overexpression there is not a direct causal relationship between mutation and protein accumulation and that apparently, other events than mutation can trigger p53 stability (28,39). Dahse *et al* found that *TP53* mutations seem to occur more often in higher malignant bladder tumours with a higher tendency of recurrence and progression, although their results were not statistically significant (40). Furthermore, *TP53* mutation or p53 overexpression precedes chromosome 9 defects in Cis as a precursor for invasive cancer (41,42). Prognostic implications of *TP53* gene mutations in bladder tumours were discussed by Lorenzo-Romero *et al* (43). Many studies have analyzed p53 in bladder cancer; the prevalence of p53 alterations increases with stage and grade (6,44-46), but there is no definite evidence that p53 overexpression is an independent prognostic factor (45).

There are less publications combining molecular genetic *TP53* analysis with progression in bladder cancer. In this study we present TGGE and sequencing data of patients with a follow-up of up to 95 months. Our *TP53* exon specific mutation frequencies are in the same range as in some other urological tumours. As in most other tumours, the mutation frequency of 6.1% (4/66) of exon 8 in bladder cancer is lower than other exon mutation frequencies. The overall *TP53* mutation frequency of urothelial tissue without tumour verification is in the range of 13% (47).

The small sample number in this study probably accounts for the non-significance of exons 5-, 6- and 7-mutations, if analyzed separately, while all mutations taken together showed a high significance as a progression factor. On the other hand, it seems interesting, that exon 8 mutations reach statistical significance. This result is supported by prior work by Huang *et al* (41), who found that mutations in exon 8 were more useful indicators of prognosis for non-small cell lung cancer than mutations in other *TP53* exons. They suggested, that the worse overall survival of the patients with mutations in exon 8 was associated with mutations in codon 273 and between codons 280 and 285, which are included into the H2 alpha helix. The abnormal conformation of H2 might play an important role not only in the loss of normal function but also in the acquisition of tumourigenesis (41). Also, Skaug *et al* found exon 8 mutations were related to even still poorer lung cancer-related prognosis than mutations at other locations within *TP53* (27).

The possible function of *TP53* defects for tumour progression should be further elucidated. Some doubts exist about the loss of DNA repair capabilities in case of p53 defects. Huang *et al* (41) have outlined the 3'→5' exonuclease function of p53 wild-type protein for proofreading function of DNA-polymerase α.

Ten of eleven sequenced mutations in our study (Table I) are suspected to cause strong biological effects; three of them are non-sense mutations. The mentioned intron 8 mutation C→G in position 14594 maps 6 bases outsite the exon 8 border, therefore an influence on splicing may be possible. Codon 245 is a hotspot mutation in bladder cancer and several other tumours. All other identified mutation sequences are in regions of special functional activity of p53. The exon 8 region has DNA binding properties. Missense mutations of codons 245, 278, 281 and 282 encompass conserved regions of *TP53* (48). Codon 248 is a well known mutational hotspot in bladder cancer. Codon 143 mutations may result in p53 overexpression and increased cell proliferation (49).

In summary, our results show, that *TP53* genetic mutations are independent prognostic factors for poor progression free survival in non-invasive bladder cancer. Furthermore, mutations at certain sites of the *TP53* gene, particularly at exon 8, can cause even poorer prognosis as these sites involve the biological function of p53. Mutations in defined structural and functional domains of p53 may therefore be useful molecular biological markers for prognosis and treatment strategies of non-invasive transitional cell carcinomas. This finding is even more valuable, since *TP53* mutations can be analyzed in urine cells by non-invasive methods (8,39,50). *TP53* analyses in tumour tissue or urine cells might guide the clinician towards a more aggressive therapy, such as radical cystectomy for high risk T1G3 or Cis tumours which could otherwise undergo bladder sparing procedures and close surveillance. With newer and faster techniques for genetic analysis, this might be included into the daily routine in the future.

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4.5. Originalarbeit 5

Ecke TH, Schlechte HH, Gunia S, Lenk SV, Loening SA.
Body mass index (BMI) and mutations of tumor suppressor gene p53 (*TP53*) in patients with urinary bladder cancer
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Adipositas erreicht in Europa und den Vereinigten Staaten von Amerika den Stand einer Epidemie und wird zu einem sehr wichtigen Gesundheitsrisiko [65]. Der Body mass index (BMI) ist ein indirektes Maß für Adipositas und ist definiert als Quotient aus Gewicht in kg geteilt durch Körpergröße in m². Obwohl der BMI nicht zwischen Fettgewebe und magerer Körpermasse unterscheiden kann, gibt es eine gute Korrelation zwischen BMI und dem Anteil des Körperfettes, wie diese durch Unterwassergewicht und andere Techniken gemessen werden kann [66]. Die WHO definiert Normalgewicht mit einem BMI unter 25 kg/m², Übergewicht mit einem BMI zwischen 25 und 29.9 kg/m² und Fettleibigkeit mit einem BMI über 30 kg/m² [66].

In dieser Arbeit wurde untersucht, ob es eine Beziehung zwischen dem Risikofaktor eines pathologischen BMI und der Mutationsfrequenz von *TP53* gibt.

Die Messungen von Gewicht und Größe einschließlich der Berechnung des BMI wurden zu dem Zeitpunkt der ersten Diagnosestellung eines Harnblasenkarzinoms vorgenommen.

In der Gruppe der Patienten mit pathologischem BMI wurde zunächst auffällig, dass eine höhere Rate von Komorbiditäten wie KHK, COPD, Diabetes mellitus und anderen Erkrankungen des Gefäßsystems gefunden wurde.

Nach der Definition der WHO hatten 50,7% der Patienten einen BMI unter 24,9 kg/m². In dieser Gruppe hatten 68,4% eine *TP53* Mutation. In der Gruppe der übergewichtigen Patienten (38,7%) fanden wir eine *TP53* Mutationsfrequenz von

44,8%. Nur 10,7% der Patienten waren adipös; in dieser Gruppe fanden wir eine Mutationsfrequenz in *TP53* von nur 25%.
Zunehmendes Körpergewicht oder Körpergröße waren also nicht mit einer höheren *TP53* Mutationsfrequenz assoziiert. Zusammenfassend konnte in dieser Studie kein statistisch signifikanter Zusammenhang zwischen *TP53* Mutationsfrequenz und BMI gefunden werden (p>0,05).

In der vorliegenden Arbeit wurde eine Mutationsfrequenz von 84,2% bei invasiven und von 44,6% bei nicht-invasiven Harnblasenkarzinomen gefunden.

Obwohl Hafron et al. keine signifikante Beziehung zwischen hohem BMI und gesamtem oder krankheitsspezifischem Überleben bei Patienten mit Komorbiditäten bei Patienten mit invasivem Harnblasenkarzinom, die zur Zystektomie geplant waren, scheint es einen Trend gegenüber eines besseren krankheitsspezifischen Überleben bei Normalgewicht zu geben [67]. Das könnte durch die höhere Rate von anderen Erkrankungen bei Patienten mit höherem BMI zu begründen sein. In der großen prospektiven Studie von Holick et al. konnten ihre Ergebnisse nicht die Rolle des BMI in der Karzinogenese des Harnblasenkarzinoms unterstützen [68].

In dieser relativ kleinen Gruppe konnte kein Zusammenhang zwischen der Mutationsfrequenz von *TP53* und einem pathologisch erhöhten BMI gefunden werden; dennoch sollten diese Daten nach Sichtung der Literatur in die Erhebung von Risikoprofilen für Harnblasenkarzinome eingeschlossen werden [69].

Die Angaben von Körpergröße und Gewicht fanden auch Eingang in den Blasentumor Fragebogen, der 2011 in das Projekt „WHO Global Network of Collaborating Centres in Occupational Health“ integriert wurde [70].

ELSEVIER

Urologic Oncology: Seminars and Original Investigations 26 (2008) 470–473

UROLOGIC ONCOLOGY

Original article

Body mass index (BMI) and mutations of tumor suppressor gene p53 (*TP53*) in patients with urinary bladder cancer

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Abstract

Objective: Obesity is estimated to account for up to 20% of all cancer deaths. Mutations of *TP53* are frequently correlated with tumor development and progression. We evaluated the effect of body mass index (BMI) and mutation status of tumor suppressor gene p53 (*TP53*) on patients with urinary bladder cancer.

Materials and methods: Clinical samples were used from 75 patients with tumors of the urinary bladder. Mutation status in *TP53* exons 5, 6, 7, and 8 was analyzed by temperature gradient gel electrophoresis of exon-specific PCR products and by sequence analysis. Statistical analysis included Pearson's correlation.

Results: For noninvasive bladder cancer, the mutation frequency in *TP53* was 44.6%, while for invasive bladder cancer the mutation frequency in *TP53* was 84.2%. Normal weight, overweight, and patients with obesity had a *TP53* mutation frequency of 68.4%, 44.8%, and 25%, respectively ($P < 0.05$).

Conclusions: *TP53* mutation frequently occurs in higher stages of bladder tumors. Body mass index is not associated with a higher *TP53* mutation frequency in our study, but BMI should be included for collecting data of bladder cancer risk profile.

Keywords: Body mass index; Bladder cancer; *TP53*; Mutation

1. Introduction

In Western countries, urinary bladder cancer is the fourth most common cancer in men and the ninth most common in women. The incidence of urinary bladder cancer has increased in the last decade. There is still a lack of tumor markers for urinary bladder cancer to improve optimal therapy and outcome [1–5]. Tumor stage and grade are still the best established prognostic factors in urinary bladder cancer. Because of their limited prognostic value, the biological heterogeneity of the disease, and the different clinical course, the analyses of cytogenetic and molecular changes have vastly expanded and offer the possibility of higher risks of tumor progression [6].

Obesity is reaching epidemic levels in Europe and in the United States, and is becoming a very important health risk [7]. Body mass index (BMI) is an indirect measure of adiposity that is determined by weight in kg divided by height in m^2. Although BMI does not distinguish between adipose tissue and lean body mass, there is good correlation between BMI and the percent of body fat, as measured by underwater weight and other techniques [8]. The WHO defines BMI as normal weight—less than 25 kg/m^2, overweight—25 to 29.9 kg/m^2, and obesity—30 kg/m^2 or greater [8].

Mutations of the tumor suppressor gene *TP53* are frequently correlated with tumor development and progression in bladder cancer [9]. *TP53* is localized on the short arm of chromosome 17 [10,11]. *TP53* has influence on cell cycle

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Table 1
Staging and grading of bladder cancer patients

Staging/grading	Low-grade	High-grade	Total
Ta	36	1	37
T1	15	4	19
T2	2	7	9
T3	1	7	8
T4	0	2	2
Total	54	21	75

regulation, gene transcription, DNA repair, genome stability, chromosome segregation, angiogenesis, and apoptosis. Genetic alteration is the most frequent reason of a change of function of *TP53*. Loss of function may also be due to binding with viral oncoproteins or cellular gene products, or may be caused by dislocation of the protein to cytoplasma [12–14]. A very important function of wild-type *TP53* is the induction of apoptosis [15].

The first detection of *TP53* mutations in cellular urine sediment was in 1991 [16]. We analyzed the effect of the *TP53* state in urinary bladder cancer by genetic analysis of tumor tissue and urine sediment with a highly effective electrophoretic technique. *TP53* mutation frequency in urinary bladder cancer estimated by temperature gradient gel electrophoresis (TGGE) is around 40% [17]. TGGE analyses show that about 80% of urinary bladder cancer tissue mutations can be detected in cellular urine sediments [18].

Some studies have been conducted to assess the association between p53 overexpression/mutations and risk factors for a variety of cancers [19]. The relationship between obesity and bladder cancer is less well defined to its impact on cancer related mortality [20–24], and only little attention has been given to its relationship to *TP53* mutation frequency. We wanted to find out if there is an association between the risk factor of pathological BMI and a mutation of *TP53*.

2. Materials and methods

We used material from 75 patients with primary diagnosed tumors of the urinary bladder, 56 noninvasive and 19 muscle invasive tumors. Sixty-two male patients and 13 female patients (average age 65.3 years) were included. The staging and grading of the bladder cancer patients is shown in Table 1. Genetic mutation analysis was carried out in urothelial samples and in cellular urine sediment.

The data of weight and height of the patients was measured at the point where the first samples for *TP53* mutation screening were taken. Any change of weight during a progressive disease of urinary bladder cancer was not included in the analyses. The measurement of weight and height including the BMI calculation was made at the point of the first time the diagnosis of bladder cancer was made.

2.1. Screening of TP53 mutations

Isolation of DNA by phenol extraction from tumor tissue were made in all cases. Additionally, in some cases we made also an isolation of DNA from cellular urine sediments. Amplification in separate reactions by polymerase chain reaction (PCR) of *TP53* exons 5, 6, 7, and 8 with so-called GC-clamped primers were done [25]. Mutation screening by temperature gradient gel electrophoresis (TGGE): technical details of the methods used as described in previous reports [17]. Each TGGE experiment was carried out with a DNA-negative PCR-control sample and with a positive mutation control to rule out potential contaminations. A 4-band-pattern of silver staining in the TGGE was estimated as indicative of a mutation. Sequencing of either mutant and, for control, wild-type bands, excised from the silver-stained TGGE-gel, or directly of the isolated DNA: the specific bands or exons were re-amplified with the same PCR primers as before, but instead of a GC-clamp, a biotinylated primer at the 5′-end was used [26].

Statistical analysis included χ^2-test after Pearson's correlation computed and performed by the computer program SPSS ver. 12.0.1 (SPSS Inc., Chicago, IL).

3. Results

3.1. Characteristics of patients

We included 75 bladder cancer patients. The details of staging and grading are shown in Table 1. Twenty-five percent of the patients were aged less than 60 years, 75% were aged 60 years or older; 83% were males, and all included patients were white (Table 2).

In the group of patients with pathological BMI, we found a higher rate of comorbidities such as coronary heart disease, obstructive lung disease, diabetes mellitus, and other diseases of the vascular system. Seven out of 48 patients with pathological BMI (14.6%) had 1 or more comorbidities; 3 out of 27 patients with normal BMI (11.1%) had 1 or more comorbidities. At this point we found no statistical significance ($P < 0.05$); nevertheless, comorbidities are higher in patients with a higher BMI.

Table 2
TP53 mutation and demographic factors

	No *TP53* mutation	*TP53* mutation	Percent positive	*P*-value
Age				
<60	8	11	57.9	
≥60	26	30	53.6	0.744
Sex				
Female	5	8	61.5	
Male	29	33	53.2	0.584

Table 3
TP53 mutation and risk factors

	No *TP53* mutation	*TP53* mutation	Percent positive	*P*-value
Weight (kg)				
⩽58	1	7	87.5	
59–68	2	9	81.8	0.737
69–77	9	15	62.5	0.253
⩾78	22	10	31.3	0.020
Height (cm)				
⩽157	1	7	87.5	
158–170	11	13	54.2	0.092
⩾171	22	21	48.8	0.676
BMI (kg/m^2)				
⩽24.9	12	26	68.4	
25–29.9	16	13	44.8	0.052
⩾30	6	2	25	0.312

3.2. Weight, height, and body mass index (BMI) and TP53 mutation frequencies

Looking on the WHO definition, 38 out of 75 patients (50.7%) had a BMI less than 24.9 kg/m^2. In that group we had 68.4% of *TP53* mutation frequency. In the group of overweight patients (29 out of 75 patients = 38.7%) we found a *TP53* mutation frequency of 44.8%. Only 8 patients (8 out of 75 patients = 10.7%) had obesity; there we found a *TP53* mutation frequency of only 25%. All details of this part are shown in Table 3.

Increased body weight or height was not associated with a higher *TP53* mutation frequency. In general, in our study no significant relationship between *TP53* mutation frequency and BMI could be found ($P < 0.05$).

3.3. Differences between noninvasive and invasive bladder cancer

TP53 mutation in patients with bladder cancer is significantly correlated with tumor staging. We detected mutations of *TP53* in invasive bladder cancer in 16 out of 19 cases (84.2%); in noninvasive bladder cancer, we found 25 out of 56 cases (44.6%) with mutation of *TP53*. Pearson's significance with the χ^2 test between mutation of *TP53* and invasive bladder cancer was calculated with $P = 0.003$ (Table 4). Thus, mutation in *TP53* is a good marker to differentiate between invasive and superficial bladder cancer.

We detected pathological BMI in invasive bladder cancer in 9 out of 19 cases (47.4%); in noninvasive bladder cancer, we found 39 out of 56 cases (69.6%) with pathological BMI. Pearson's significance with the Chi-Quadrat-Test between pathological BMI and invasive bladder cancer was calculated with $P = 0.081$.

4. Discussion

The mutation frequency in the so-called high-risk exons 5 to 8 of the *TP53* gene is approximately 40% in bladder cancer tissue [27,28]. Mutation of *TP53* might accelerate carcinogenesis, especially by enhancement of cell proliferation, loss of apoptosis, and by insufficient DNA repair [29]. It id now widely accepted that the *TP53* state plays a role in the progression of bladder tumors [30]. In our study, we found a mutation frequency of 84.2% for invasive bladder cancer and of 44.6% for superficial bladder cancer, confirming already published results [28]. Mutations in the *TP53* gene are correlated with infiltrating bladder cancer and qualify as a marker.

There are conflicting reports as to whether accumulation of p53 protein as measured by immunohistochemical staining always equates to the actual frequency of *TP53* genomic mutation [31]. The accuracy of detecting *TP53* mutations, as determined by IHC, was 90.3% in human bladder cancer [32,33].

Our study of bladder cancer patients is disadvantaged by the fact that much information on other risk factors was unavailable because the study was retrospective, involving abstraction data from the medical record and analysis of banked tissue specimens. Information abstracted from medical charges may be less reliable than from conventional interview studies. However, since the information on risk factors was collected by surgeons or physicians who interviewed patients using a standard admission history form, the potential for misclassification may be similar to conventional interview or questionnaire studies [19]. In general, the limitations of the study are retrospective in nature, small population size, a moderate rate of comorbidities, and patient selection bias inherent in single institutional studies. Although Hafron et al. have not found a significant association between increasing BMI and overall or disease-specific survival in comorbid patients undergoing cystectomy for invasive bladder cancer, there may be a trend toward better disease-specific survival in normal weight [34]. This could be caused by a higher rate of other diseases in patients with a higher BMI. In the big 2006 prospective study by Holick et al., their findings could not support a role for BMI in bladder carcinogenesis [24].

It is known that mutation analysis is an important functionality of the protein as a tumor suppressor, but *TP53* is not the only factor in cancer, and the multifactorial nature of carcinogenesis is just as complex as that of obesity. To improve the conclusion of the work, *TP53* should be compared with leptin, for example, as a single factor.

Although it could not be shown in this small group that there is a relationship between *TP53* mutation frequency and a pathological BMI, it should be a field of further

Table 4
TP53 mutation in superficial and invasive bladder cancer

	pTa, pT1	pT2-4	Total	*P*-value
TP53 mutation	25 (44.6%)	16 (84.2%)	41 (64.7%)	
TP53 wild-type	31 (55.4%)	3 (15.8%)	34 (45.3%)	
Total	56	19	75	0.003

studies to include these data to find a risk profile for bladder cancer patients.

5. Conclusions

1. The mutation frequency of *TP53* in invasive bladder cancer is significantly higher than in noninvasive bladder cancer ($P = 0.003$).
2. Body mass index is not associated with a higher *TP53* mutation frequency.
3. Body mass index should be included for collecting data of bladder cancer risk profile.

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5. Zusammenfassende Diskussion und Ausblick

In den dargestellten Experimenten in Originalarbeit 1 [59] zeigte TPA nur eine mäßige Sensitivität (48,9% im Serum und 40,4% im Urin), aber eine sehr gute Spezifität (83% im Serum und 100% im Urin). In Originalarbeit 2 [60] wurde die Sensitivität mit 68,3% im Serum und 33,3% im Urin, die Spezifität mit 88,9% im Serum und 100% im Urin berechnet. In der Literatur werden für TPA im Serum Sensitivitäten zwischen 16% und 54,7% und Spezifitäten von 95% und 100% beschrieben [71, 72]. In der Studie von Sánchez-Carbayo et al. wurde für TPA im Urin jedoch eine deutlich höhere Sensitivität von 80,2% bei einer Spezifität von 95% gemessen [73].

Vergleichende Analysen wiesen Zytokeratin 19 als einen essentiellen Teil von TPA beim Harnblasenkarzinom nach [72]. Eine fehlende Korrelation zwischen TPA und Tumorgrad beim Harnblasenkarzinom wurde in einer anderen Studie berichtet [74]. Ein statistisch signifikanter Unterschied zwischen invasiven / nicht-invasiven Harnblasenkarzinomen und erhöhten Werten von TPA konnte in den Originalarbeiten 1 und 2 nicht nachgewiesen werden [59, 60]. Zu ähnlichen Ergebnissen kamen die Arbeitsgruppen von Filella et al. [75] und Casetta et al. [76].

Einen großen Anteil an dieser Arbeit haben die Untersuchungen auf Mutationen von *TP53* beim Harnblasenkarzinom. In den folgenden Abschnitten erfolgt eine zusammenfassende Diskussion dieser Ergebnisse.
In Originalarbeit 1 wurde eine Mutationsfrequenz von 81,8% bei den muskelinvasiven und von 44,1% bei den nicht-invasiven Blasentumoren festgestellt [59]. In Originalarbeit 2 betrugen die entsprechenden Werte 100% und 50%, in Originalarbeit 5 wurden 84,3% und 44,6% berechnet [60]. Diese Ergebnisse sind übereinstimmend mit bereits publizierten Ergebnissen, in denen die Mutationsfrequenz der sogenannten high-risk Exons 5-8 von *TP53* mit etwa 40% im Tumorgewebe angegeben wird. [77, 78]. Mutationen von *TP53* steigen im Rahmen der Karzinogenese an, besonders durch den gesteigerten Zellumsatz, Verlust der Apoptose und durch ungenügende Reparaturen der DNA [79].

Die Frequenz der *TP53* Mutationen wurde bei Patienten mit erhöhten TPA-Werten im Serum zu 61,4%, bei Patienten mit erhöhten TPA-Werten im Urin zu 66,7% festgestellt. Pearson's Signifikanz wurde mit p=0,046 für TPA im Serum und mit p=0,173 für TPA im Urin berechnet. Das könnte ein Hinweis sein, dass der *TP53*-Status einen Einfluss auf die Zytokeratin-Ausschüttung hat. Eine p53-induzierte Ausschüttung von Zytokeratinen in Serum oder Urin konnte bis jetzt nicht nachgewiesen werden. In menschlichen Zelllinien des nicht-kleinzelligen Bronchialkarzinoms und des Neuroblastomas wurden jedoch eine Anhäufung und proteolytische Teilung von Zytokeratinen während der frühen Apoptose gezeigt [80].

Es ist bekannt, dass die Fragmentierung der Zytokeratin-Filamente während der Apoptose der Zelle auftritt [81]. Wir wissen jedoch nicht, wie schnell die löslichen Fragmente der Zytokeratine in schnell wachsenden und nekrotischen Tumoren eliminiert werden.

Die Sensitivität für uPAR in Originalarbeit 2 war mit 79,5% hoch, während die Spezifität 71,4% betrug [60]. Diese Ergebnisse unterscheiden sich nicht von denen anderer Studien [35]. Casella et al. [82] haben uPAR und uPA vor der Durchführung der diagnostischen Zystoskopie gemessen und zeigten, dass uPAR helfen könnte, Patienten mit besonders hohem Risiko für ein Harnblasenkarzinom zu finden. Shariat et al. [83] fanden in einer späteren Arbeit heraus, dass erhöhte Werte von uPAR signifikant höher bei Patienten mit Harnblasenkarzinom sind im Vergleich zu gesunden Individuen.
Es ist bekannt, dass uPA an seiner Oberfläche seinen Rezeptor uPAR gebunden haben muss, damit eine Tumorzellinvasion erfolgen kann [84-86]. uPAR bindet an pro-uPA, welches dann in seine aktive Form umgewandelt wird. Diese steigert dann die Produktion von an der Oberfläche gebundenem Plasmin, welches durch α2-Antiplasmin vor einer Inaktivierung geschützt ist [85]. uPAR wurde als führende Grenze bei der Tumorinvasion bezeichnet, welche bei einigen Tumorarten als Faktor für eine schlechte Prognose gehalten wird [84, 87]. Für das Harnblasenkarzinom konnte das in der Studie von Bhuvarahamurthy et al. allerdings nur in einem Fall bestätigt werden. Die Überexpression von uPA und uPAR wurde in dieser Arbeit als Kennzeichen einer fortgeschrittenen Erkrankung gesehen [88].

In Originalarbeit 2 wurde für HER-2/neu eine Sensitivität von 88,9% und eine Spezifität von 62,5% berechnet [60]. HER-2/neu konnte jedoch keinen statistisch signifikanten Unterschied zwischen nicht-invasiven und invasiven Harnblasenkarzinomen nachweisen.
Im Vergleich dazu zeigten die Ergebnisse von Lonn et al. [89], dass erhöhte Werte von HER-2/neu zumindest mit dem Tumorgrad von nicht-invasiven Harnblasenkarzinomen korrelieren. In der IHC wurde die HER-2/neu Überexpression bei 37-50% der urothelialen Karzinome beschrieben [90].
Eine Arbeit von Tsai et al. untersuchte den prognostischen Wert von HER-2/neu und p53 ebenfalls mit der Technik der IHC, allerdings nur beim invasiven Harnblasenkarzinom [91]. Dabei zeigte p53 eine weitaus bessere prognostische Signifikanz als HER-2/neu. Ein wichtiger Punkt dieser Studie war der Nachweis, dass eine p53 und HER-2/neu Co-Expression mit dem Lymphknotenstatus und der Zeit bis zum Progress korrelierten. Erklärend dafür ist die Tatsache, dass eine EGFR Überexpression das Wachstum des Blasentumors fördern kann und ein Cis in einen Hochrisiko-Tumor umwandeln kann [92]. Die Hypothese, dass eine p53 und HER-2/neu Co-Expression eine schlechtere Prognose zur Folge haben und widerstandsfähiger gegenüber Cisplatin-basierter Chemotherapie sind, konnte von Tsai et al. jedoch nicht bewiesen werden [91].
Im Hinblick auf die Weiterentwicklung von Tumormarkern wird neben einer prognostischen Information der Ausblick auf therapeutische Option immer wichtiger. In der Arbeit von Jarvinen et al. wurde gezeigt, dass p53 und HER-2/neu mit dem Target-Gen Topoisomerase IIα assoziiert sind [93]. Einige Autoren nehmen sogar an, dass die Widerstandsfähigkeit gegenüber Chemotherapeutika in Zellen mit HER-2/neu Amplifikation mit einer genetischen Veränderung der Topoisomerase IIα assoziiert ist; zusätzlich kann p53 die Promotor Region der menschlichen Topoisomerase IIα regulieren und ihre katalytische Aktivität durch Verbesserung der Rate der ATP Hydrolyse stimulieren [94, 95].

Besonders beim Tumormarker HER-2/neu, welcher beim Mammakarzinom eine immense Bedeutung hat, ist die Frage interessant, ob es Medikamente gibt, die durch die Hemmung des ErbB-Rezeptors etablierte Therapien begünstigen könnten.

In einer Studie an Zelllinien von Harnblasenkarzinomen konnte gezeigt werden, dass der Inhibitor des ErbB-Rezeptors Lapatinib die Wirkung der Zytostatika Gemcitabin, Paclitaxel und Cisplatin verbessert. Der ebenfalls von dieser Gruppe untersuchte Wert von p53 war zwei Zelllinien (RT112 und J82) ähnlich. Dieser war weder durch Lapatinib oder die Dreifachkombination der Chemotherapie beeinträchtigt [96].
Die Gruppe von Jimenez et al. zeigte, dass nahezu alle HER-2 positiven Primärtumore auch HER-2 positive Metastasen entwickelt hatten. Die HER-2/neu Überexpression in Primärtumor oder Metastasen war jedoch nicht mit dem Überleben assoziiert ist [97]. In einer anderen Arbeit wiesen Kruger et al. nach, dass der HER-2 Status ein unabhängiges Anzeichen für das krankheitsbedingte Überleben ist [98].
In der Arbeit von Hussain et al. wurde in einer Multicenter-Studie untersucht, welche Bedeutung HER-2/neu bei Patienten unter Therapie mit Trastuzumab, Paclitaxel, Carboplatin und Gemcitabin hat. Dabei konnte festgestellt werden, dass HER-2/neu positive Patienten mehr viszerale Metastasen hatten als HER-2/neu negative Patienten [99].

Vor dem Hintergrund der Bedeutung von Tumormarkern als Instrument mit prognostischer Aussagekraft, ist die Originalarbeit 3 ein wichtiges Element bei der Akquirierung von Daten, anhand derer bekannte Tumormarker geprüft oder neue Tumormarker entwickelt werden können [61].

Die Prognose von Patienten mit metastasiertem Urothelkarzinom bleibt schlecht mit einem medianen Überleben von nur 12-14 Monaten [7]. Fortgeschrittene Urothelkarzinome sind nur mäßig sensitiv auf eine Chemotherapie; es gibt eine Reihe von Wirkstoffen, die Ansprechraten zwischen 10 und 40% zeigen [7, 100]. Im letzten Jahrzehnt wurden einige neue Chemotherapeutika angewandt, die eine hohe Aktivität gegen fortgeschrittene Urothelkarzinome zeigten, darunter auch die Taxane Paclitaxel und Docetaxel [101, 102], sowie Gemcitabin (Gemzar®; Eli Lilly and Company, Indianapolis, IN) [103, 104].
Eine große multinationale Phase III Studie hat von der Maase [100] mit insgesamt 405 Patienten veröffentlicht; dabei wurde das M-VAC Schema mit der Kombinationstherapie aus Gemcitabin und Cisplatin (GC) verglichen. Im

Endergebnis zeigte sich, dass beide Schemata in Hinblick auf Ansprechraten, Zeit bis zum Progress und Überleben ähnlich sind. Die GC-Kombination zeigte jedoch weniger Nebenwirkungen und eine geringere Toxizität.
Die Aktivitäten von Gemcitabin und der Taxane, die teilweise nicht überlappenden Toxizitäten dieser Substanzen und ihre unterschiedlichen Wirkmechanismen führten dazu, diese zu kombinieren; Cisplatin als Rückgrat in eine Dreifach-Kombinationstherapie einzubinden war der nächste logische Schritt. Eine Gruppe von Bellmunt et al. initiierte eine Phase I/II Studie dieser Dreifach-Kombination aus Paclitaxel, Cisplatin und Gemcitabin (PCG), in welche insgesamt 61 Patienten eingeschlossen wurden [105].
Tumormarker und andere klinische und laborchemische Parameter sind als prädiktive Faktoren für das Ansprechen und das Überleben zur Selektion der Patienten, die von neuen Kombinationstherapien profitieren, erforderlich. Solche Stratifizierungen wurden in randomisierten Studien durchgeführt, eine Analyse der prädiktiven Faktoren für Ansprechen und Überleben für die PCG-Polychemotherapie wurde ebenfalls von Bellmunt et al. durchgeführt [106]. Die vor der Therapie erfassten Daten umfassten Alter, Geschlecht, ECOG Performance Status [107], Histopathologie, das Vorhandensein viszeraler Metastasen (Knochen, Leber, Lunge), die Anzahl der betroffenen Regionen, LDH und Hämoglobin. Die Faktoren, die mit einem verminderten Überleben in einer univariaten Analyse assoziiert waren, beinhalteten Performance Status > 0, das Vorhandensein viszeraler Metastasen und mehr als eine Region der malignen Erkrankung.

Neben der von mir als Originalarbeit 3 [61] vorgelegten Arbeit gibt es drei weitere Berichte dieser Therapie [105, 108, 109]. Das mediane Überleben in der Kohorte der Patienten mit viszeralen Metastasen lag bei 14,3, 11,4 und 15,3 Monaten. Die Gesamtüberlebensrate bei Patienten mit viszeraler Metastasierung betrug 77,6% bei der cisplatin-basierten Therapie von Bellmunt et al. [105], 68% bei Hussain et al. [108]. Lorusso et al. fanden nur eine Gesamtüberlebensrate von 43% [109]. Wie aus den Daten der M-VAC Serien bekannt ist, wurden dort Ansprechraten um 20% bei Patienten mit viszeralen Metastasen berichtet [110, 111]. Demnach war auch das mediane Überleben der Patienten, die nach dem PCG-Schema behandelt wurden mit 14,3 Monaten besser als das in den M-VAC Serien. In der Originalarbeit 3 lag die

Ansprechrate bei 81%. Das mediane Überleben aller Patienten war mit 22,0 Monaten sehr hoch und sehr nah an der von Bellmunt et al. [105] berichteten medianen Überlebenszeit von 24 Monaten. 33 Patienten (56%) erreichten eine komplette Remission. Das mediane progressionsfreie Überleben für die gesamte Gruppe betrug 10,0 Monate; für Patienten mit objektivem Ansprechen auf die Therapie, betrug das mediane progressionsfreie Überleben 14,0 Monate. Wie in anderen Studien beschrieben, ist der ECOG Performance Status ein prädiktiver Faktor für Überleben und Ansprechen [112]. Die Zeiten für das mediane Überleben für Patienten mit ECOG Status von 0, 1, und 2 betrugen 37,5, 17,0, und 12,0 Monate. Diese Ergebnisse sind ebenfalls mit den Ergebnissen von Bellmunt et al. [105] vergleichbar. Der geringere Anteils an Patienten mit Metastasen (59%) und mit viszeralen Metastasen (15%) könnte ein Grund für die besseren Ansprechraten im Vergleich zu den Ergebnissen von Bellmunt et al. und Hussain et al. [105, 108]. Aber es ist auch möglich, dass die Verbesserungen der Supportivtherapie in den letzten Jahren eine Rolle spielen.

Unsere Therapie wurde auf der Basis entwickelt, dass die Kombination von Paclitaxel, Gemcitabin und Cisplatin, welche die drei Medikamente mit der höchsten Aktivität als Einzelsubstanzen darstellen, höhere Ansprechraten erzielen als diese drei Substanzen alleine. Kombinationen von Chemotherapeutika bieten das Potential, Ansprechraten und medianes Überleben zu optimieren durch die Anwendung von Medikamenten, die sich in ihrem Wirkmechanismus ergänzen [113].

Die Originalarbeit 4 hatte zum Ziel, weitere Einflüsse von *TP53* Mutationen auf die Rezidivrate und progressives Verhalten von Blasentumoren in Erfahrung zu bringen [62].

Ein weiterer wichtiger Punkt der Untersuchungen von *TP53* Mutationen ist das Ergebnis, dass der Nachweis der Mutationen sowohl im Tumorgewebe als auch im Urinsediment gelang. Das ist ein gemeinsames Ergebnis der Originalarbeiten 1, 2 und 5 [59, 60, 69]. Erstmals konnten Sidransky et al. 1991 *TP53* Mutationen im Urin von drei Patienten mit einem invasiven Blasentumor finden [27]. Diese waren identisch mit den *TP53* Mutationen der dazugehörigen Gewebeproben.

Viele präklinische Studien haben gezeigt, dass die Überexpression von Signalpfaden, Angiogenese, Überleben und Proliferation steuern, sowie eine Assoziation mit einem schlechten Endergebnis haben [114-116]. Die Akkumulation von p53 im Nukleus, die mittels IHC detektiert werden kann, korreliert stark mit den Mutationen in *TP53* [117, 118]. Bemerkenswert ist weiterhin, dass die Promotor Region von *TP53* beim muskelinvasiven Harnblasenkarzinom praktisch nie methyliert ist [119].

Llopis et al. beschrieben, dass die Expression von p53 Protein einen prognostischen Wert für das Überleben und den Progress bei pT1 Harnblasenkarzinomen hat und für eine frühe Detektion von pT1 Harnblasenkarzinomen mit schlechter Prognose benutzt werden kann [120].

TP53 Mutationen zeigten nicht-invasiven Stadien von Harnblasenkarzinomen eine Häufigkeit von 35% mit steigenden Anteilen bis zu 70% bei invasiven Stadien [10, 30, 118]. Interessanterweise wurde in solchen Tumoren, die nicht direkt *TP53* inaktiviert hatten, vermutet, dass die Funktionalität durch mutierte Komponenten des Signalpfades behindert wird, die p53 aktivieren [10]. In einer in-vitro Studie konnte gezeigt werden, dass Organismen mit multiplen *TP53* Genen tumorresistent sind [121]. Grundsätzlich sind jedoch nicht alle mutierten p53 Proteine funktionslos [122].

Einige Gruppen stellten Ergebnisse von p53 als Faktor der Tumorprogression beim Harnblasenkarzinom vor [120, 123-126]; Patienten mit *TP53* Mutation haben eine höhere Wahrscheinlichkeit der Progression und ein schlechtere Prognose [127, 128]. Mutationen von *TP53* oder eine Überexpression von p53 führen zu einem Defekt im Chromosom 9 und gelten beim Cis als Vorbote für ein invasives Karzinom [129].

Die Gruppe von Huang et al. [130] konnte nachweisen, dass Mutationen im Exon 8 ein nützlicherer Indikator für den Progress beim nicht-kleinzelligen Bronchialkarzinom waren als Mutationen in anderen Exons von *TP53*. Sie suggerierten, dass das schlechte Gesamtüberleben der Patienten mit Mutationen im Exon 8 assoziiert war mit Mutationen im Codon 273 und zwischen Codon 280 und 285, welche die H2 alpha Helix beinhalten. Die abnormale Konformation von H2 könnte nicht nur im Verlust der normalen Funktion eine Rolle spielen, sondern auch für die Aufnahme der Tumorgenese [130]. Ebenso fand die Gruppe von Skaug et al., dass Mutationen in Exon 8 eine Beziehung schlechteren Prognose beim Bronchialkarzinom als Mutation anderer Lokalisation innerhalb von *TP53* zeigten [131].

Die Exon 8 Region hat DNA bindende Eigenschaften. Mutationen der Codons 245, 278, 281 und 282 umfassen die erhaltenen Regionen von *TP53* [132]. Codon 248 ist ein bekannter veränderlicher hotspot beim Harnblasenkarzinom. Codon 143 Mutationen haben eine p53 Überexpression und eine zunehmende Zellproliferation zur Folge [133].
Aufgrund der doppelten Rolle des wildyps von p53 als Initiator der Reparatur von zerstörter DNA und als Trigger der zellulären Apoptose, ist die p53-assoziierte Sensitivität auf Chemotherapeutika unterschiedlich. Funktionslosem, mutiertem p53, dem die Fähigkeit fehlt, den Zellzyklus zur Reparatur von DNA anzuhalten, könnte den zytotoxischen Effekt von DNA-vernetzender Chemotherapie, wie Cisplatin, verbessern. Auf der anderen Seite kann funktionsloses mutiertes p53 durch seine Instabilität zur Induktion der Apoptose die Chemosensitivität zu Blockern der DNA-Synthese wie Fluorouracil vermindern [91].
In Originalarbeit 4 konnte nachgewiesen werden, dass Untersuchungen auf *TP53* Mutationen als Parameter für den Tumorprogress einen von Tumorgrad und –stadium, Patientenalter und Geschlecht unabhängigen Faktor beim nicht-invasiven Harnblasenkarzinom darstellen. Diese Ergebnisse bestätigen die Resultate von Peyromaure et al., der keinen prognostischen Wert bei mit BCG behandelten Patienten mit T1G3 Harnblasenkarzinom für eine p53 Überexpression finden konnte [134].
Einige Studien hatte jedoch gezeigt, dass trotz guter Konkordanz zwischen der *TP53* Mutation und der Überexpression von p53 keine direkte kausale Beziehung zwischen Mutation und Proteinakkumulation besteht und dass offensichtlich andere Ereignisse als eine Mutation die Stabilität von p53 beeinflussen können [135, 136].

Einige Studien wurden eingeführt, um die Verbindung zwischen der Überexpression von p53 und Risikofaktoren zu beurteilen [137]. Die Beziehung zwischen Adipositas und Harnblasenkarzinom ist kaum definiert und seine Wirkung auf die tumorbedingte Sterblichkeit [68, 138-140].

Epigenetische Faktoren rücken bei der Suche nach geeigneten Markern immer mehr in den Vordergrund [21]. Die Arbeitsgruppe von Moore et al. konnte nachweisen, dass die DNA-Hypermethylierung von Leukozyten mit einem ansteigenden Risiko

verbunden ist, ein Harnblasenkarzinom zu entwickeln; darüber hinaus ist diese Beziehung unabhängig vom Nikotinabusus und anderen Risikofaktoren [141].

Aus der Arbeit über die Auswertung der Dreifach-Chemotherapie beim fortgeschrittenen und metastasierten Urothelkarzinom werden weitere Arbeiten hervorgehen, die Marker hervorbringen könnten, welche eventuelle prognostische Aussagen erlauben werden. Einige Auswertungen epigenetischer Marker erfolgten bereits in Zusammenarbeit mit dem Centro Nacional de Investigaciones Oncológicas (CNIO) in Madrid. Erste Ergebnisse wurden in der Arbeit von Cebrian et al. demonstriert [48].
Niedrige Werte von KISS-1 wurden bei Harnblasenkarzinomen gefunden, verglichen mit nicht-invasiven Tumoren, bieten diese Ergebnisse Informationen zur Prognose. Die geringere Expression dieses Genes wurde auch in Zellen gefunden, die sich von fortgeschrittenen Harnblasenkarzinomen ableiten lassen [55]. Die Analyse der Expression von KISS-1 durch in situ Hybridisation auf Mikroarrays bestätigte den Verlust von KISS-1 in der Progression der Erkrankung; weiterhin zeigte sich eine Assoziation zu Tumorstadium, Grad, und Überleben [24, 142, 143].

6. Zusammenfassung und abschließende Wertung

Die Ergebnisse dieser Arbeit werfen auch die Frage nach möglichen Therapien des Harnblasenkarzinoms in Zukunft auf. Obwohl bereits auf Moleküle zielgerichtete Therapien für das Harnblasenkarzinom entwickelt wurden, ist es aufgrund der Karzinogenese der Erkrankung nicht möglich, ein einziges Molekül als Therapieziel heranzuziehen. Um optimale therapeutische Ergebnisse zu erzielen, sollten Synergismen, die den unterschiedlichen Entstehungswegen der Erkrankung Tribut zollen, der nächste Schritt in einem sinnvollen Umgang mit der Weiterentwicklung von Therapien sein [9].

Sonpavde hat zum Ausdruck gebracht, dass nach wie vor nicht klar ist, ob prognostische Marker entscheidende Faktoren sind, die zielgerichtet zu einer wesentlichen Änderung in der Diagnostik und Therapie des fortgeschrittenen Urothelkarzinom der Harnblase führen werden, oder ob sie Beobachter oder nur Effekte von dahinterliegenden Ereignissen sind, die andere Methoden benötigen [144]. Dennoch gibt die vorliegende Arbeit Anlass zur Hoffnung, dass durch die Fortführung der Forschungsarbeit auf dem Gebiet der Tumormarker für das Harnblasenkarzinom in Zukunft neben Aussagen zur Prognose und der Wahrscheinlichkeit eines Ansprechens auf medikamentöse Therapien auch Ziele gefunden werden, die direkt bei der Heilung dieser Erkrankung eine Rolle spielen.

Die in dieser kumulativen Habilitationsschrift vorgelegten Originalarbeiten haben größtenteils Einfluss auf andere Arbeiten auf diesem Gebiet.

Die Ergebnisse der Originalarbeit 3 [61] und die weitere Auswertung der vorliegenden Daten erfolgt in einer Multicenter-Studie, die unter Führung von Professor Matt Galsky vom Mount Sinai Hospital in New York, USA, erstmals auf dem ASCO 2012 vorgestellt wird und der weitere Publikationen folgen werden.

Die Originalarbeit 4 [62] hat neben der von mir verfassten Übersichtsarbeit [145] über Blasentumormarker Eingang in die offiziellen Leitlinien der National Academy of Clinical Biochemistry (NACB) für den Gebrauch von Tumormarkern für das

Harnblasenkarzinom gefunden, bei deren Erstellung ich selbst als Co-Autor beteiligt war [146].

Die Bedeutung von Körpergröße und Gewicht, sowie BMI wurde durch die Originalarbeit 5 deutlich gemacht [69]. Diese Angaben fanden auch Eingang in den Blasentumor Fragebogen, der unter anderem durch meine Mitwirkung entstanden ist und der 2012 veröffentlicht werden wird. Dieser Fragebogen wird in das Projekt „WHO Global Network of Collaborating Centres in Occupational Health“ integriert werden [70].

Darüber hinaus geht diese Arbeit auch auf die Entwicklung neuer Marker aus dem Bereich der Epigenetik ein, die sicherlich das Bild des „profiling“ von Tumoren in Zukunft prägen wird. Weiterführende Arbeiten werden die Kohorte der PCG-Polychemotherapie aus der Originalarbeit 3 [61] auf Marker mit prognostischer Aussagekraft untersuchen.

In der Hoffnung, dass diese Habilitationsschrift die Bedeutung von Tumormarkern beim Harnblasenkarzinom darstellt, soll sie einen Anstoß für die wichtige Weiterentwicklung auf diesem Gebiet geben.

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ERKLÄRUNG

§4 Abs. 3 der HabOMed der Charité

Hiermit erkläre ich, dass

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