Genetische Veränderungen im p53-Tumorsuppressorpathway und deren prognostische Relevanz in Weichteilsarkomen und Ovarialkarzinomen

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von Herrn Dr. rer. nat. Frank Bartel

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Gutachter:

1. Prof. Dr. Roland Moll (Marburg)
2. C. James Kirkpatrick, MD, Ph.D. (Mainz)
3. Prof. Dr. Thorsten Stiewe (Marburg)

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Referat und Bibliographisches Verzeichnis

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Den Schwerpunkt der vorliegenden Arbeit bildete die Aufklärung der Mechanismen, die zur Fehlregulation des p53-MDM2-MDMX-Pathways in zwei völlig unterschiedlichen Tumorarten, den Weichteilsarkomen und den Ovarialkarzinomen, führen. Dies umfasste die Analyse von Alterationen wie z.B. Mutationen und Polymorphismen, Genamplifikationen, Überexpression der mRNA bzw. des Proteins sowie die Expression von Spleißvarianten und die Frage, ob diese Veränderungen können mit dem Krankheitsverlauf bzw. dem individuellen Todesrisiko betroffener Patienten korrelieren. In 28 % der von uns untersuchten Weichteilsarkomproben konnte ein amplifiziertes MDM2-Gen nachgewiesen werden. Dies war mit einer erhöhten Expression der MDM2-mRNA assoziiert. Interessanterweise korrelierten sowohl die MDM2-Genamplifiation als auch eine leicht erhöhte MDM2-mRNA-Expression mit einer guten Prognose bei Weichteilsarkompatienten. Patienten ohne MDM2-Genamplifikation wiesen ein 4,5-fach erhöhtes Risiko auf, am Tumor zu versterben. Demgegenüber war jedoch für Patienten, deren Tumor durch eine MDMX-Genamplifikation charakterisiert war, das relative Risiko um das 2,8-fache erhöht. Das MDMX-Gen war in 27 % der Weichteilsarkome amplifiziert. Neben dem MDMX-Genamplifikationsstatus wurde auch die Expression der „full-length"-MDMX-mRNA und der MDMX-S-Spleißvariante untersucht. Es stellte sich heraus, dass das Verhältnis „full-length" MDMX/MDMX-S einen unabhängigen Prognosefaktor für Weichteilsarkome darstellt. Patienten, deren Tumor eine Überexpression der MDMX-S-Spleißvariante aufwiesen, verstarben durchschnittlich 13 Monate nach Diagnosestellung, während Patienten, bei denen das Verhältnis zu Gunsten der FL-MDMX-mRNA verschoben war, 53 Monate überlebten. Bei der Sequenzanalyse des p53-sensitiven MDM2-P2-Promotors, die in Zusammenarbeit mit A.J. Levine und G.L. Bond (Princeton, USA) durchgeführt wurde, konnte ein Einzelnukleotid-polymorphismus (T>G; SNP309) identifiziert werden. Das G-Allel war in Weichteilsarkompatienten mit einem 12 Jahre früheren Tumorauftreten assoziiert. Weitere Untersuchungen zeigten, dass das G-Allel die Tumorentwicklung vor allem in pre-menopausalen Frauen, nicht jedoch bei Männern, beschleunigen konnte. Bei den Ovarialkarzinomen konnte die Assoziation des G-Allels mit einem früheren Alter der Tumorentstehung nur bei einer Überexpression des Östrogen-Rezeptors beobachtet werden. Diese Daten stützen die Hypothese, dass Geschlechtshormone (hier: Östrogen), die Entwicklung von Tumoren bei Individuen mit einem G/G-Genotyp beschleunigen können. Bei einem SNP im 3'-UTR des MDMX-Gens zeigte sich ebenfalls eine Abhängigkeit von Östrogen-Rezeptor. Bei den von uns untersuchten Ovarialkarzinomen konnten wir ebenfalls zahlreiche Veränderungen des p53-MDM2-MDMX-Pathways detektieren, die einen Einfluss auf das rezidiv-freie und Gesamtüberleben der Patientinnen haben. Interessant war der Befund, dass Patientinnen, deren Tumor das Wildtyp-p53-Protein überexpremierte, die kürzeste Überlebenszeit aufwiesen. Dies zeigt deutlich, dass die isolierte Betrachtung des p53-Gen- bzw. Proteinexpressionsstatus nicht aussagekräftig ist. Des Weiteren zeigen unsere Arbeiten die Bedeutung der Östrogenrezeptorexpression beim Ovarialkarzinom. Obwohl der ER-Status selbst keine prognostische Aussagekraft besitzt, jedoch war das G-Allel des SNP309 bei Patientinnen mit einem ER-positiven Tumor mit einem signifikant früheren Auftreten des Ovarialkarzinoms verbunden.

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Abkürzungen

Abb.	Abbildung
AS	antisense
amol	attomol
ATCC	American Type and Tissue Collection – Amerikanische Zell- und Gewebesammlung
bidest.	bidestilliertes Wasser
bp	Basenpaar
cDNA	komplementäre DNA
DEPC	Diethylpyrocarbonat
dmin	- „double minute chromosomes“ – kurze Doppelchromosomen ohne Zentromer
DNA	Desoxyribonukleinsäure
ds	Doppelstrang
dsODN	Doppelstrangoligodesoxynukleinsäure
EDTA	Ethylendiamintetraessigsäure
FL	„full length“ – in der Arbeit als Synonym für das regulär gespleißte MDM2- bzw. MDMX-Transkript verwendet
g	Gramm
HE	Haematoxylin-Eosin (-Färbung)
IHC	Immunhistochemie
kDa	Kilodalton
LMS	Leiomyosarkom
LOH	„loss of heterozygosity“ – Verlust der Heterozygotie
MBO	“mixed backbone oligonucleotid” – Oligonukleotid, das aus DNA und RNA besteht
MDM2	„murine double minute gene 2“ – in der Arbeit für das humane Homolog HDM2 verwendet; in der Literatur werden beide Bezeichnungen parallel verwendet
MFH	malignes fibröses Histiozytom
MM	Mismatch
mRNA	“messenger RNA” – Boten-RNA
mt	Mutante, mutiert
n.b.	nicht bestimmt
nt	Nukleotid
OD	optische Dichte
ODN	Oligodesoxyribonukleinsäure
PBS	“phosphate buffered saline” – Phosphat-gefufferte Kochsalzlösung
PCR	„polymerase chain reaction“ – Polymerasekettenreaktion
RMS	Rhabdomyosarkom
rpm	„revolutions per minute“ – Umdrehungen pro Minute
RT	reverse Transkription, Raumtemperatur
SE	sense
wt	Wild-Typ
WTS	Weichteilsarkom
zmol	zeptomol

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Die Ergebnisse der vorliegenden Arbeit entstanden während meiner Tätigkeit als Leiter der Juniorgruppe und als Laborleiter am Institut für Pathologie der Martin-Luther-Universität Halle-Wittenberg. Ich möchte an dieser Stelle zunächst die Gelegenheit nutzen, um mich bei den zahlreichen Unterstützern und Begleitern während meiner Forschungen auf dem Gebiet der molekularen Charakterisierung von Tumoren bedanken.

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1 Einleitung

1.1 Der p53-MDM2-MDMX-Pathway und seine Bedeutung im Tumorprozess

Die Kontrolle und das Aufrechterhalten der genomischen Integrität sowie eine strikte Regulation des Zellzyklus sind unerlässlich für die Tumorsuppression, aber auch für das Ansprechen auf eine Chemotherapie und/oder Bestrahlung. Ein Schlüsselprotein für die Wirksamkeit einer Vielzahl von Chemotherapeutika ist der Tumorsuppressor p53. Nach einer Schädigung der DNA, z.B. durch Carboplatin, aktiviert p53 verschiedene Signalwege, die einerseits zum Zellzyklusarrest führen andererseits Apoptose auslösen können. Obwohl p53 in etwa 50 % der der malignen Tumoren KEINE Mutationen aufweist, ist der p53-Tumorsuppressor-Pathway in seiner Funktion gestört, z.B. aufgrund der Überexpression von negativen Regulatoren. Dazu zählen unter anderem die Proteine MDM2 und MDMX.

Im Rahmen meiner Forschungstätigkeit habe ich mich intensiv mit der Aufklärung der Mechanismen, die zur Fehlregulation des p53-MDM2-MDMX-Pathways in Tumoren führen, beschäftigt. Dies schließt Alterationen wie z.B. Mutationen und Polymorphismen, Genamplifikationen, Überexpression der mRNA bzw. des Proteins sowie die Expression von Spleißvarianten ein. Alle diese Veränderungen können eng mit dem Krankheitsverlauf und dem individuellen Progression betroffener Patienten korrelieren. Des Weiteren stand die Aufgabe im Mittelpunkt, neue molekulare Targets für alternative und/oder ergänzende Tumortherapien zu identifizieren. Untersuchungsobjekte waren die seltene, histologisch und tumorbiologisch äußerst heterogene Gruppe der Weichteilsarkome und die vergleichsweise häufigen, klinisch jedoch ebenso problematischen Ovarialkarzinome.

1.2 Weichteilsarkome

Alle malignen Tumoren des nichtepithelialen und extraskeletalen Gewebes sowie neuroektodermale Malignome des peripheren und autonomen Nervensystems mit Ausnahme des retikuloendothelialen Systems, der Glia und des Stützgewebes parenchymatöser Organe werden unter dem Begriff Weichteilsarkome (WTS) zusammengefasst [29]. Der Anteil der WTS an malignen Geschwülsten im Erwachsenenalter beträgt ca. 1 % [29,61]. Für die Bundesrepublik Deutschland bedeutet dies, dass jährlich etwa 800 bis 1500 Neuerkrankungen auftreten [39].

WTS stellen eine sehr heterogene Tumorgruppe dar. Man unterscheidet etwa 150 Entitäten und Subtypen. Die Histogenese der WTS ist weitgehend ungeklärt. Es wird angenommen, dass sich WTS aus undifferenzierten, mesenchymalen Stammzellen entwickeln. Die

Tumorbezeichnung spiegelt nicht deren Histogenese wider, sondern vielmehr den durch Differenzierungs- bzw. Dedifferenzierungsprozesse erreichten Phänotyp [53,60]. Ein Liposarkom ist demnach ein WTS mit Differenzierungsmerkmalen, die denen des Fettgewebes ähneln. Etwa 15 % aller WTS lassen sich keiner bestimmten Entität zuordnen [29].

Neben der Einteilung in die verschiedenen histologischen Subklassen dient die Definition des Malignitätsgrades (Grading) als wichtigstes Kriterium dem Ziel, das biologische Verhalten eines Tumors möglichst genau vorauszusagen. Bei der Bestimmung der einzelnen Faktoren zur Ermittlung des Gradings kommt es sowohl bei einfachen als auch bei komplexen Systemen zu individuellen Begutachtungsschwankungen. Die Einteilung von Tumoren in verschiedene Stadien („Staging") beruht neben dem Grading auch auf der Tumorgröße, der Tumorlokalisation, dem Verhalten zur Umgebung, dem Lymphknotenbefall sowie dem Vorliegen von Fernmetastasen [9].

Ein weiteres charakteristisches Merkmal von WTS, das ebenfalls zur besonderen klinischen Problematik dieser Tumorgruppe beiträgt, ist ihre ausgeprägte Lokalisationsvielfalt. Dies führt zusammen mit einem oftmals beschwerdefreien Wachstum häufig zu einer späten Diagnosestellung, so dass bis zu 20 % der Patienten zu diesem Zeitpunkt bereits nachweisbare Fernmetastasen haben [29,60]. Die Prognose der WTS ist vergleichsweise ungünstig. So beträgt die 5-Jahres-Überlebensrate für alle WTS-Entitäten 55 %, die 10-Jahres-Überlebensrate wird mit 38 % angegeben [58,109]. Als Hauptgründe für diese schlechte Prognose werden die bereits erwähnte späte Diagnosestellung mit dem daraus folgenden hohen Stadium sowie eine inadäquate Erstbehandlung, aber auch besondere tumorbiologische, nur molekular erfassbaren Eigenschaften genannt. Eine frühe und umfassende Ausnutzung der diagnostischen Möglichkeiten ist daher für eine erfolgreiche Therapieplanung und die Senkung der Mortalitätsrate unerlässlich [36].

Die radikale chirurgische Entfernung des Tumors (R0-Resektion) bildet die Hauptsäule der Therapie von WTS [153], da diese Tumoren oft eine ausgeprägte Resistenz gegenüber Strahlen- und Chemotherapie zeigen. Diese Therapieformen spielen bei der Planung des Gesamttherapiekonzepts deswegen eine untergeordnete Rolle. Die Anwendung der Chemotherapie zeigt daher oftmals nur eine geringe Verbesserung des Gesamtüberlebens [120].

Für WTS-Patienten eine individualisierte Prognose zu erstellen oder eine prädiktive Aussage über den Effekt einer Therapie zu treffen, gestaltet sich äußerst schwierig. WTS gelten im Vergleich zu anderen Tumorarten wegen ihrer Charakteristika als Sonderfälle der Onkologie und der Chirurgie.

Als Konsequenz der genannten Besonderheiten ist es notwendig, zusätzlich zu den bisherigen klinischen und histomorphologischen Prognosemarkern, WTS intensiv molekulargenetisch zu charakterisieren und Gene bzw. Proteine zu identifizieren, die maßgeblich an der Genese von WTS beteiligt sind und exaktere prognostische Vorhersagen erlauben. Daraus leiten sich schließlich möglicherweise alternative Therapieverfahren ab, die den Tumor für eine Bestrahlung oder Chemotherapie sensibilisieren oder einen für den Tumor essentiellen Signalweg beeinflussen.

1.3 Ovarialkarzinome

Das Ovarialkarzinom ist in vielen westlichen Ländern nach dem Zervix- und dem Endometriumkarzinom das dritthäufigste weibliche Genitalkarzinom. Es weist jedoch von allen gynäkologischen Malignomen die höchste Sterblichkeitsrate auf. In Deutschland erkranken jährlich mehr als 8000 Frauen an einem Ovarialkarzinom. Aufgrund des relativ beschwerdefreien Wachstums befinden sich zum Zeitpunkt der Diagnosestellung etwa 75 % der Tumoren bereits in einem fortgeschrittenen Stadium [22]. Jährlich versterben mehr als 6000 Patientinnen an einem Ovarialkarzinom. Damit steht es an fünfter Stelle bei den Krebstodesursachen der Frauen. Die Sterberate hat sich seit vielen Jahrzehnten nicht verringert. Das Manifestationsalter liegt meist zwischen dem 50. und 60. Lebensjahr. Ovarialkarzinome können jedoch auch bei Mädchen und jungen Frauen auftreten. Die Überlebensraten für Patientinnen mit Ovarialkarzinomen sind gering. Sie betragen ein Jahr nach der Diagnose 66 %, 45 % nach drei Jahren sowie 37 %, in spezialisierten Zentren 45 %, fünf Jahre nach Diagnosestellung [21].

Trotz der Verbesserungen in der Medizin sind die Langzeitüberlebenschancen für Patientinnen mit einem fortgeschrittenen Ovarialkarzinom nach wie vor sehr schlecht. Deswegen wäre es hier von besonders großer Bedeutung, die Tumoren bereits im Frühstadium zu erkennen. Da auch molekulare Ursachen weitestgehend unbekannt sind, ist es wichtig, epidemiologische Risikofaktoren zu kennen und diese in die Prävention und Früherkennung einzubeziehen. Zu diesen Risikofaktoren zählen u.a. das Alter der Frau sowie hormonelle Einflüsse. Eine frühe Menarche, Kinderlosigkeit, fehlende Stillzeiten wirken sich ungünstig aus. Die Einnahme hormoneller Ovulationshemmer, Schwangerschaften sowie Laktation sind hingegen mit einem verminderten Risiko verbunden [106,126,136,146]. Die Ursache der protektiven Wirkung von oralen Kontrazeptiva oder Schwangerschaften liegt vermutlich darin, dass sie die Reparationsphasen des ovariellen Oberflächenepithels *post ovulationem* reduzieren. Etwa 5-10 % der Ovarialkarzinome sind genetisch bedingt. Bei diesen Patientinnen findet man

Keimbahnmutationen in den Genen BRCA1 und BRCA2, deren Genprodukte an der DNA-Reparatur beteiligt sind [144]. Dadurch wird das Risiko, an einem Ovarial- oder Mammakarzinom zu erkranken, stark erhöht. Die Früherkennung des Ovarialkarzinoms beschränkt sich zurzeit auf regelmäßige gynäkologische Untersuchungen. Als weitere Methode ist das Screening des Tumormarkers CA125 hinzugekommen. Bei 25-50 % der Tumoren im Stadium I und 90 % der Tumoren im Stadium II ist der CA125-Wert im Blut erhöht [160]. Es gibt jedoch eine Reihe weiterer gynäkologischer und nicht-gynäkologischer Krankheitsbilder, bei denen der CA125-Wert ebenfalls erhöht ist, weswegen sowohl die Sensitivität als auch die Spezifität dieses Tests ihn nicht als verlässliches Screening qualifizieren.

Histologisch gliedert man das Ovarium in ein Oberflächenepithel (Müller-Epithel), das ovarielle Stroma und die Keimzellen. Aufgrund dieser drei Gewebekomponenten werden laut WHO-Klassifikation drei Hauptgruppen von Tumoren unterschieden: epitheliale, Keimstrang-Stroma- und Keimzelltumoren. 90 % der Ovarialmalignome sind Karzinome. Diese lassen sich in mehrere histologische Gruppen einteilen:

- serös: charakteristisches Merkmal sind tubenähnliche Entdifferenzierung
- muzinös: diese Tumoren sind durch ein hochprismatisches, schleimbildendes Epithel gekennzeichnet
- endometroid: aus konfluenten, atypischen endometroiden Drüsen aufgebaute Tumoren
- klarzellig: ein wichtiges Merkmal ist das wasserhelle Zytoplasma
- transitionalzellig: also dem Urothel ähnelnd
- nicht klassifizierbare Tumoren.

Das seröse Ovarialkarzinom ist der häufigste maligne Tumor des Ovars, gefolgt vom endometroiden und klarzelligen Ovarialkarzinom.

Zum Grading von Ovarialkarzinomen werden verschiedene Systeme verwendet. Oftmals gibt es jedoch Probleme in Bezug auf die Reproduzierbarkeit und die Konsistenz. Von Silverberg wurde ein universelles Grading-System erarbeitet [125], das praktikabel ist. Es gibt eine signifikante Korrelation zwischen dem histologischen Grad, dem Tumorstadium und dem Überleben. Schlecht differenzierte Karzinome sind häufig in einem fortgeschrittenen Stadium, während die meisten der Grad-1-Tumoren sich in einem frühen Krankheitsstadium befinden. Neben der Aszitesmenge ist das Grading der wichtigste Prognosefaktor für Patientinnen im Stadium I. Eine genaue Bestimmung des Tumorgrades ist gerade für diese Patientinnen außerordentlich wichtig, da sich daraus die weiteren therapeutischen Maßnahmen ableiten. Die

Stadieneinteilung erfolgt nach Vorgaben der *Fédération Internationale de Gynécologie et d'Obstétrique* (FIGO) und des *American Joint Committee on Cancer* (AJCC) [148].

Jeder klinisch nachgewiesene Tumor des Ovars muss operativ entfernt und histologisch beurteilt werden. Das Ausmaß des Eingriffs wird vor allem von der Art des Tumors und dessen Ausbreitung bestimmt, aber auch das Alter der Patientin und ihr Allgemeinzustand sind zu berücksichtigen. Während der Operation erfolgen die Exploration des gesamten Bauchraums und seiner Organe sowie eine Peritoneallavage des Beckens und die Bestimmung des Lymphknotenstatus. Die oberste Priorität hat die komplette Tumorentfernung während des Ersteingriffs. Der operativen Entfernung schließt sich außer bei Patientinnen mit Ovarialkarzinomen im FIGO Stadium IA eine adjuvante Chemotherapie an. Die momentane Standardtherapie besteht in einer Kombinationsbehandlung aus einem Platinanalogon und Paclitaxel. Nur etwa die Hälfte der Patientinnen mit fortgeschrittenem Ovarialkarzinom erfährt durch die Primärtherapie mit einer Platin-Taxan-Kombination eine Komplettremission. Bei den meisten Frauen tritt innerhalb der ersten 3 Jahre ein Rezidiv auf. Dabei unterscheidet man zwischen einem primären Progress, einem Früh- bzw. einem Spätrezidiv (innerhalb bzw. 6 Monate nach Primär-OP). Trotz der Fortschritte bei der chirurgischen und chemotherapeutischen Behandlung von Ovarialkarzinomen zählen daher nur etwa 20 % der Patientinnen zu den Langzeitüberlebenden. Aus diesem Grund gehen die Bestrebungen dahin, die Resistenzentwicklung zu beeinflussen bzw. zu prognostizieren, um auf diese Weise die Therapie zu individualisieren.

1.4 MDM2 und MDMX – Gemeinsamkeiten und Unterschiede

1.4.1 MDM2

Das MDM2-Gen wurde als eines von drei amplifizierten Genen „double minute" Chromosomen (dmin) in der spontan transformierten Mauszelllinie 3T3DM entdeckt [14]. Bei der Analyse der einzelnen Gene stellte sich heraus, dass nur das Produkt des murine double minute Gen 2 (MDM2) in der Lage ist, nach Transfektion in Fibroblasten die Zellen zu transformieren [32]. Dies äußerte sich in unkontrolliertem und tumorauslösendem Wachstum der Zellen. Das MDM2-Onkoprotein geriet anschließend sehr schnell in das Blickfeld des Interesses, weil entdeckt wurde, dass es an den Tumorsuppressor p53 binden [90] und diesen effizient inaktivieren kann [48].

Das humane MDM2-Gen ist auf dem Chromosomenarm 12q13.14 lokalisiert, einer Region, die in nahezu 1/3 aller Sarkome amplifiziert vorliegt [96] und in der Ovarialkarzinome häufig Zugewinne aufweisen [49]. Es kodiert für ein 90 kDa Protein [14,32]. Von MDM2 sind verschiedene Isoformen (Abb. 1-1) bekannt, die durch die Nutzung unterschiedlicher Promotoren oder durch alternatives Spleißen entstehen [45,98,124]. Die wichtigste und bei weitem am besten untersuchte Funktion von MDM2 ist die Bindung und Inaktivierung von p53 [38,90]. Mit der N-terminalen Domäne kann MDM2 an die α-Helix der transaktivierenden Domäne von p53 binden. Die am stärksten konservierte Domäne des MDM2-Proteins ist die C-terminale RING-Finger-Region, die eine Ubiquitin-Ligase-Aktivität gegenüber p53 besitzt, wodurch es p53 für den proteasomalen Abbau markiert. Somit kann MDM2 durch Bindung an p53 nicht nur dessen transaktivierende Domäne blockieren, sondern auch dessen Degradation einleiten [48,69,97]. Gleichzeitig wird durch wt-p53 die Expression des MDM2-Gens induziert. Dies ge-

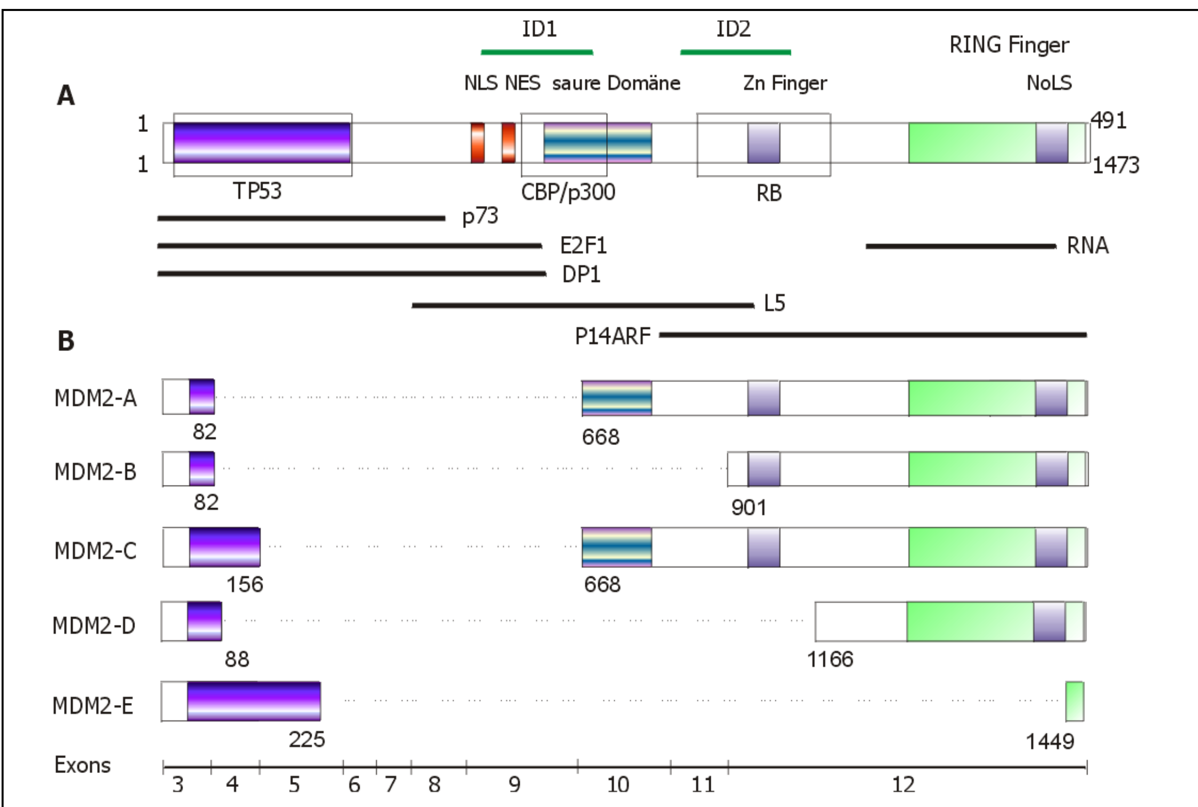

Abb. 1-1: Struktur des Onkogens MDM2 und verschiedener Isoformen

A. Das Produkt des Onkogens MDM2 besteht aus 491 Aminosäuren (die kodierende Region der cDNA aus 1473 Nukleotiden), die funktionellen Domänen sind durch farbige Boxen, die Bindungsstellen mit weiteren Proteinen durch horizontale Linien symbolisiert. ID1 und ID2 repräsentieren die beiden wachstumsinhibierende Domänen. **B**. Durch RT-PCR konnten verschiedene alternativ (A, B, C) und aberrant (D, E) gespleißte mRNA-Transkripte detektiert werden [124]. Diesen verkürzten Transkripten fehlen Teile der p53-bindenden Domäne, sowie der NLS und der NES-Sequenz. Die Zahlen repräsentieren jeweils das letzte und das erste in der cDNA der Spleißform enthaltene Nukleotid in Bezug auf die full-length MDM2-cDNA. NLS bzw. NoLS – Kernlokalisierungssequenz, NES – Kernexportsequenz.

schieht über den p53-spezifischen Promotor P2, der im Intron 1 des MDM2-Gens lokalisiert ist [158]. Das Produkt des Onkogens MDM2 und der Tumorsuppressor p53 sind somit durch einen so genannten autoregulatorischen „Feedback"-Loop (Abb. 1-2) miteinander verbunden [104]. In diesem Zusammenhang ist interessant, dass das Transkript, das vom P2-Promotor abgelesen wird, einen verkürzten 5'-untranslatierten Bereich aufweist und im Vergleich zum P1-Transkript wesentlich effektiver translatiert wird. Somit trägt die durch p53 induzierte MDM2-Expression zu einem beschleunigten Abbau von p53 bei [1]. Es konnte bereits für viele Tumorarten gezeigt werden, dass es zu Veränderungen des MDM2-Gens bzw. –Proteins im Sinne von Genamplifkation bzw. Überexpression kommt (Review in [99]). Sie stellen neben Genmutationen eine zusätzliche Möglichkeit zur Inaktivierung von p53 dar. Diese Mechanismen sind auch mit für die in der Klinik beobachteten Resistenzentwicklungen gegen Therapien mit Chemotherapeutika bzw. Bestrahlungen verantwortlich.

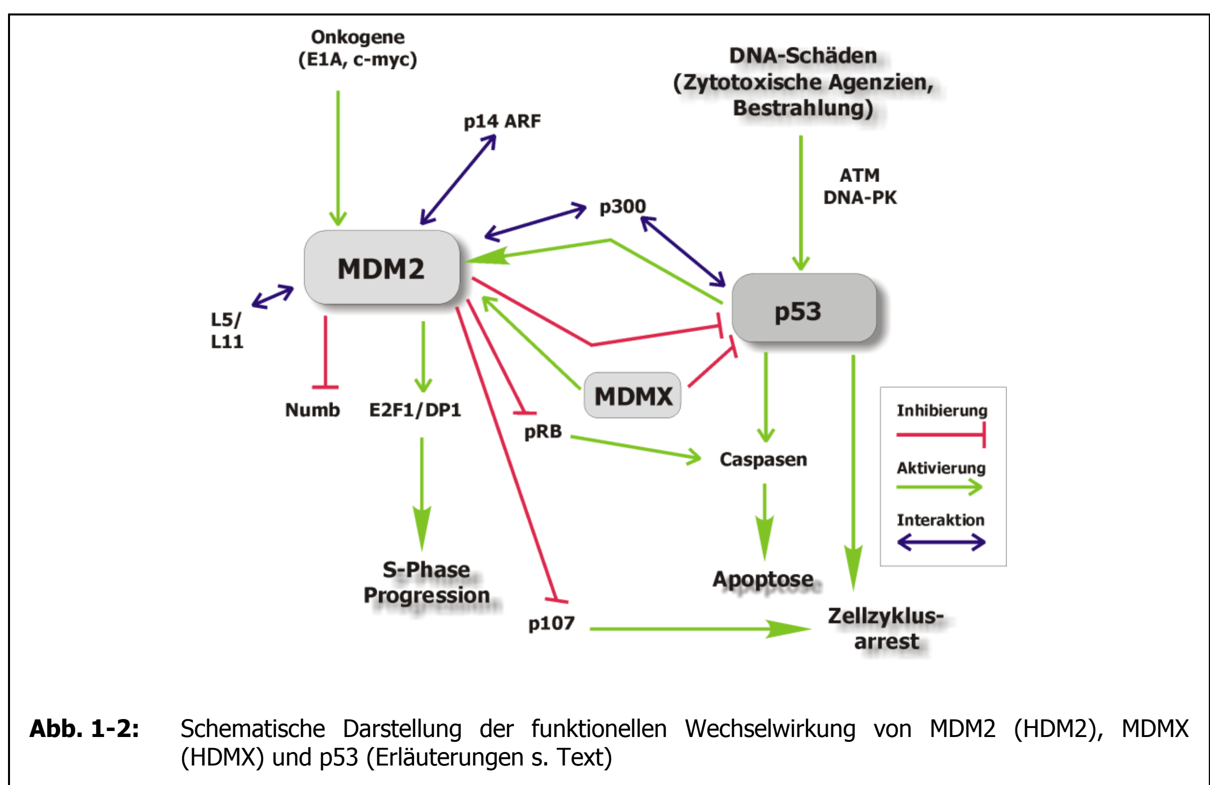

Abb. 1-2: Schematische Darstellung der funktionellen Wechselwirkung von MDM2 (HDM2), MDMX (HDMX) und p53 (Erläuterungen s. Text)

1.4.2 MDMX

MDMX ist ein Protein, das sehr große Ähnlichkeiten zu MDM2 aufweist [122,123]. Das Gen ist auf dem Chromosom 1 in der Region 1q32 lokalisiert, einer Region, die in Ovarialkarzinomen ebenfalls häufig Veränderungen aufweist [40,49]. Es konnte gezeigt werden, dass MDMX ebenfalls an p53 binden und so dessen Aktivität inhibieren kann. MDMX besitzt jedoch im

Gegensatz zu MDM2 keine Ligaseaktivität und kann daher p53 nicht für den proteasomalen Abbau markieren [57]. Daten aus *Knock-out*-Experimenten zeigten, dass MDMX-null-Mäuse sehr früh während der Embryonalentwicklung sterben. Dies kann durch die gleichzeitige Inaktivierung des p53-Gens verhindert werden. Somit konnte bewiesen werden, dass neben MDM2 auch MDMX eine wichtige Funktion bei der Regulierung der p53-Aktiviät spielt [100], aber auch, dass beide Proteine in voneinander unabhängigen Pathways integriert sind [87]. Bislang gibt es nur sehr wenige Untersuchungen zur Bedeutung von MDMX in Tumoren. Einige Daten weisen darauf hin, dass das MDMX-Gen in besonders aggressiven Glioblastomen amplifiziert vorliegt [117,118] und in vielen Tumorzelllinien (einschl. Ovarialkarzinomzelllinien) das Protein überexprimiert wird [112]. Aktuelle Untersuchungen an der Zelllinie MCF-7 (einer Mammakarzinomzelllinie) von Danovi *et al.* zeigen, dass das onkogene Potential von MDMX auf die Amplifikation des MDMX-Gens und die Überexpression des Proteins zurückzuführen ist [24]. Im Hinblick auf eine klinische und prognostische Relevanz von MDMX-Veränderungen im Ovarialkarzinom gibt es noch keine Daten aus der Literatur.

1.4.3 Spleißvarianten der MDM2- und MDMX-mRNA

Vor Beginn dieser Arbeit sind insgesamt fünf alternativ bzw. aberrant gespleißte mRNA-Transkripte in Blasen- und Ovarialkarzinomen beschrieben worden [124]. Diesen verkürzten Transkripten fehlen unterschiedlich große Teile der p53-bindenden und der sauren Domäne sowie die Kernlokalisierungs- und die Kernexportsequenz (NLS bzw. NES). In einem in vitro-Assay zeigte sich, dass alle Isoformen in der Lage sind, Fibroblastenzellen zu transformieren. Sie besitzen demnach tumorigene Eigenschaften. Bemerkenswert ist außerdem eine Korrelation zwischen dem Auftreten von Spleißformen und einem aggressiveren Tumorverhalten (höherer Tumorgrad, Tumorstadium, Invasivität) in verschiedenen Tumorarten [124]. Die Tatsache, dass die bisher beschriebenen Isoformen von MDM2 nicht mehr an p53 binden können, hat u.a. zur Folge, dass der autoregulatorische MDM2-p53-feedback-Loop aus dem Gleichgewicht gerät. Dadurch wird auf diese Weise unter Umständen die Tumorbildung gefördert. Es wird auch vermutet, dass bestimmte onkogene Eigenschaften von MDM2 in der C-terminalen Region (enthält den RING-Finger) lokalisiert sind und diese in den Isoformen verstärkt zur Geltung kommen.

Trotz oder gerade wegen der hier geschilderten Ergebnisse wirft die Existenz von MDM2-Isoformen zahlreiche bislang unbeantwortete Fragen auf, z.B.:

1. Ist die Expression von MDM2-Spleißformen auf Tumoren beschränkt oder findet man sie auch in Normalgewebe?

2. Wenn ja, haben die Produkte der alternativen Spleißformen eine normale physiologische Bedeutung, z.B. bei der Differenzierung?
3. Welche der Aktivitäten (Transformation, Apoptoseinduktion usw.) lassen sich dem „full-length"-MDM2 (FL-MDM2) bzw. den Spleißformen zuordnen?
4. Welchen Einfluss haben die Spleißformen auf die p53-Stabilität und –Aktivität?
5. Gibt es einen Zusammenhang zwischen der Amplifikation des MDM2-Gens und dem Auftreten von Spleißformen?
6. Hat die Expression von MDM2-Spleißvarianten einen Einfluss auf die individuelle Prognose eines Patienten und das Ansprechen auf bestimmte Therapieformen?

Die oben genannten Fragen zeigen, dass die Analyse der Expression und der Funktion von alternativen Spleißformen der MDM2-mRNA in Tumoren im Allgemeinen und bei WTS im Speziellen eine außerordentliche Bedeutung hat. Gleichzeitig wird auch die Notwendigkeit deutlich, Methoden zu entwickeln, die eine schnelle Charakterisierung von Spleißformen ermöglicht, da auch von vielen anderen Genen Spleißvarianten exprimiert werden [46].

1999 wurde auch für die MDMX-mRNA eine verkürzte Form identifiziert, welche als MDMX-S (short) bezeichnet wurde (Abb. 1-3). Diese Spleißvariante ist durch eine Deletion von Exon 6 (68 bp) charakterisiert. Diese Deletion führt nach Codon 114 zu einer Verschiebung des Leserahmens. Dadurch entsteht ein Stopcodon bei Aminosäurerest 127 im Maus-

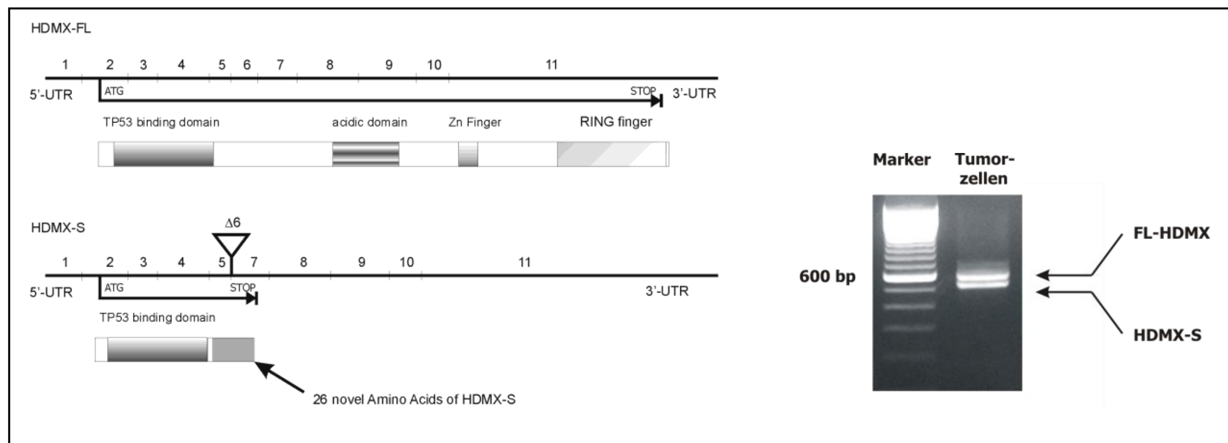

Abb. 1-3: Struktur der FL-MDMX und MDMX-S mRNA

Die Abbildung zeigt die Struktur der full-length MDMX-mRNA (oben links) und die Domänenstruktur des translatierten Proteins bzw. der MDMX-S Spleißvariante (unten links). Diese ist durch den Verlust von Exon 6 charakterisiert. Dadurch kommt es zu einer Verschiebung des Leserahmens und den Einbau eines vorzeitigen Stop-Codons nach 26 Aminosäuren. Deutlich erkennbar ist, dass MDMX-S praktisch nur aus der p53-bindenden Domäne besteht. Das Agarose-Gel zeigt beispielhaft die PCR-Produkte mit Primern, um die relative Expression von full-length MDMX (obere Bande) sowie MDMX-S (untere Bande) zu untersuchen. Abbildung entnommen aus Bartel *et al.,* 2005 [7]

protein bzw. ein Stopcodon bei Aminosäurerest 140 im humanen Gewebe [110]. Diese Isoform enthält die komplette p53-Bindedomäne und einige alternative C-terminale Aminosäuren (Abb. 1-2). Es konnte gezeigt werden, dass MDMX-S mit einer höheren Affinität als full-length MDMX an p53 bindet und effizienter in den Zellkern transportiert wird. MDMX-S unterdrückt die p53-vermittelte Transkription stärker als MDMX. Ursache für diese hohe Affinität sind die durch Verschiebung des Leserahmens neu entstandenen C-terminalen Aminosäuren. Weiterhin ist das MDMX-S Protein stabiler als MDMX, da es aufgrund der fehlenden RING-Fingerdomäne nicht mit HDM2 interagieren kann und so vor dem MDM2-vermittelten Abbau geschützt ist [111]. MDMX-S stellt somit eine weitere Möglichkeit zur Blockierung der p53-Aktivität dar.

1.5 Veränderungen des p53-MDM2-Pathways und deren Bedeutung als molekulare Prognosefaktoren

1.5.1 Weichteilsarkome

Das Onkogen MDM2 und das Tumorsuppressorgen p53 spielen auch in WTS eine Rolle. In einer Studie von Taubert *et al.* [131] wurde in WTS von insgesamt 146 Patienten der p53-Mutationsstatus bestimmt. Dabei konnte in 16 % der Fälle ein mutiertes p53-Gen detektiert werden [130,131]. Das MDM2-Gen ist in WTS in bis zu 30 % der Fälle amplifiziert [89], wobei die Amplifikationsfrequenz zwischen den einzelnen WTS-Entitäten stark variiert: während in Leiomyosarkomen kein bisher untersuchter Tumor ein amplifiziertes MDM2-Gen aufweist, lag in ca. 30 % der untersuchten Liposarkome das MDM2-Gen amplifiziert vor. Weiterhin wurde in zahlreichen WTS sowohl eine Überexpression der mRNA [37] als auch des Proteins beschrieben [74,107]. Von zwei Arbeitsgruppen wurde eine Korrelation zwischen der MDM2-Proteinüberexpression und einer schlechten Prognose nachgewiesen [23,150,152]. In Abhängigkeit vom eingesetzten Antikörper (N-terminales vs. C-terminales Epitop) erwies sich die alleinige MDM2-Überexpression als negativer Prognosemarker (RR=2,6; p=0,0035). Es zeigte sich, dass Patienten, die zusätzlich p53 überexprimieren, ein 4,6-fach erhöhtes Risiko besitzen (p=0,00001), am Tumor zu versterben. Für Patienten mit Extremitätentumoren war die prognostische Relevanz dabei am höchsten (RR=19; p=0,006) [151]. Diese Daten zeigen, dass die gemeinsame Überexpression von MDM2 und p53 ein unabhängiger negativer Prognosefaktor für WTS ist.

Ein anderes Bild ergibt sich, wenn man den Einfluss der MDM2-mRNA-Expression auf die Prognose betrachtet, denn in diesem Fall besitzen Patienten mit einer sehr niedrigen

(RR=13) bzw. einer sehr hohen mRNA-Menge eine schlechtere Prognose im Vergleich zu Patienten mit einer nur leicht erhöhten MDM2-mRNA-Expression [135]. Demgegenüber weisen Patienten mit einer stark erhöhten MDM2-mRNA-Menge ebenso eine schlechtere Prognose auf. Für diese Patienten ist das Risiko, am Tumor zu versterben, 3,2-fach erhöht [135]. Diese Befunde können dadurch erklärt werden, dass im Tumor die Menge an MDM2-mRNA-Transkripten, die vom p53-sensitiven Promotor P2 stammen, aufgrund einer p53-Fehlfunktion reduziert ist [88]. Eine leicht erhöhte MDM2-mRNA-Menge entspricht daher eher dem physiologischen Normalzustand und MDM2 kann so seine Funktionen, wie z.B. die Regulierung der p53-Menge, erfüllen. Demzufolge kann eine veränderte MDM2-mRNA-Expression (erhöht oder vermindert) als Ausdruck einer biologisch relevanten Fehlregulation aufgefasst werden.

1.5.2 Ovarialkarzinome

Trotz vielfältiger Bemühungen, molekulare Veränderungen als Prognosemarker zu etablieren, sind die Größe des Resttumors und die Aszites nachwievor in der multivariaten Analyse die einzigen signifikanten Marker mit prognostischer Bedeutung für Patientinnen mit einem fortgeschrittenen Ovarialkarzinom. Ein wichtiger Faktor der Zellantwort auf die Wirkung der platin-basierten Chemotherapie ist p53, dessen Funktionalität durch Mutationen der Keimbahn oder durch somatische Mutationen von p53 selbst oder durch Veränderung von regulatorischen Proteinen im p53-Pathway eingeschränkt ist. Ein Grund für die Chemoresistenz könnte demzufolge im Fehlen des funktionell aktiven p53-Proteins liegen. Die Mutationsrate des p53-Gens liegt bei Ovarialkarzinomen zwischen 40-80 % [33,34,50,116]. Laut vieler Studien ist eher die Überexpression von p53 [47,62,76,119,139] als die Mutation des p53-Gens [33,66,70,82] mit einer schlechten Prognose verbunden. Andere Autoren konnten jedoch keinen Zusammenhang zwischen der p53-Überexpression und der Prognose für Ovarialkarzinompatientinnen nachweisen [65,70,93,121,145]. Ein Problem bei der separaten Analyse von p53-Mutationen und der p53-Proteinexpression ist, dass bei immunhistochemischen Studien viele p53-Mutationen nicht erkannt werden; beispielsweise Non-sense-Mutationen oder Insertionen/Deletionen, wodurch ein verkürztes Protein entsteht. Andererseits ist eine Überexpression des p53-Proteins nicht notwendigerweise auf eine Mutation des p53-Gens zurückzuführen. Daher ist es wichtig, sowohl die Proteinexpression als auch den Genstatus von p53 zu analysieren. In einigen Studien wurde zwar neben der p53-Proteinexpression auch der p53-Mutationsstatus untersucht [50,116], aber in keiner dieser Arbeiten wurde der kombinierte Sta-

tus der p53-Alterationen mit dem Überleben oder dem Ansprechen auf die Chemotherapie korreliert.

1.6 Die MDM2-MDMX-p53-Interaktion als Ziel einer therapeutischen Intervention

Bei der Suche nach Angriffspunkten für eine Therapie, die auf der Beeinflussung kausaler Regulationsmechanismen basiert, deutet sich für das Onkoprotein MDM2 eine zentrale Rolle an. Da das MDM2-Protein den Abbau des Tumorsuppressors p53 beeinflusst, ist eine MDM2-Überexpression in seiner funktionellen Auswirkung mit einer Alteration des p53-Gens vergleichbar. Durch die Inhibierung der MDM2-Genexpression kann die Menge an MDM2-Protein in der Zelle reduziert werden, was meist zu einer Erhöhung der Menge an funktionellem p53-Protein führt. Hierdurch sollte Apoptose induzierbar und das Ansprechen eines Tumors auf eine Chemo- oder Strahlentherapie verbessert werden können.

In mehreren *in-vitro*-Studien konnte an Glioblastom- und Myelom-Zelllinien die Spezifität verschiedener MDM2-Antisense-Oligonukleotide (AS-ODNs) nachgewiesen werden [19,67]. Untersuchungen zeigten, dass nach Behandlung von Tumorzellen mit MDM2-AS-ODNs die MDM2-Proteinmenge signifikant reduziert werden konnte. Weitere Effekte der MDM2-AS-ODNs waren u.a. der Wachstumsarrest der Zellen in der G_1-Phase des Zellzyklus und eine deutlich verminderte Zellvitalität. Erste Versuche an WTS-Zelllinien, die von unserer Arbeitsgruppe durchgeführt wurden, ergaben, dass es in der MDM2-überexprimierenden Zelllinie US8-93 zur Reduzierung der Zellkoloniebildungsrate um bis zu 80 % kam [86].

Aus den bisherigen Studien in Bezug auf die Behandlung von Tumorzellen mit MDM2-AS-ODNs lassen sich die folgenden allgemeinen Schlussfolgerungen ziehen (Übersicht in Chen *et al.* [19]):

- MDM2-AS-ODNS stimulieren p53 über eine Reduktion der MDM2-Proteinmenge, der MDM2-p53-Proteinkomplexe und über einen MDM2-mRNA-Abbau,
- eine p53-Reaktivierung und Inhibierung von p53-unabhängigen Onkogen-Aktivitäten des MDM2-Genproduktes ist in Tumoren mit vorherrschender MDM2-Überexpression prinzipiell erreichbar,
- nichtmaligne Zellen sind toleranter gegenüber einer temporären MDM2-Inhibierung, was einer indirekten Selektivität der MDM2-AS-Therapie auf maligne Zellen gleichkommt.

Erste Resultate bestätigen die Wirkung von AS-ODNs als effektive Antitumor-Agenzien auch *in vivo* [138,141]. In Nacktmausmodellen wurde über eine signifikante Größenreduktion (>50 %) und ein im Vergleich zu Kontrollgruppen stark verzögertes Wachstum von Xenotransplantattumoren berichtet. Zusätzlich konnte ein kooperativer antiproliferativer Effekt durch Kombination mit Cisplatin, Taxol oder Topotecan beobachtet werden [138]. Ähnliche Effekte sind auch bei einer Kombinationstherapie von AS-ODNs und Irinotecan, einem Topoisomerase I-Inhibitor, in Mausmodellen für verschiedene Krebsarten (u.a. das Prostata- und Mammakarzinom) beobachtet worden [142]. Für WTS liegen bisher keine vergleichbaren Ergebnisse vor. Die an WTS-Zellkulturen erzielte Reduzierung des klonogenen Überlebens um bis zu 80 % durch eine MDM2-AS-Behandlung [86] zeigt jedoch deutlich das Ansprechen von WTS auf diese Therapie. Es schien daher dringend erforderlich, die gewonnenen Ergebnisse auf ein klinisch relevantes Tiermodell zu übertragen.

1.7 Zielstellung der Arbeit

Das Hauptziel dieser Arbeit bestand in der umfassenden Aufklärung von Alterationen des p53-MDM2-MDMX-Pathways in Tumoren mesenchymalen Ursprungs, den Weichteilsarkomen, sowie epithelialen Tumoren, hier am Beispiel der Ovarialkarzinome. Dabei stand die Analyse der bedeutsamen Tumorsuppressorproteine p53 und $p14^{ARF}$ sowie der Onkoproteine MDM2 und MDMX in Bezug auf Alterationen der Expression bzw. auf Genebene (Überexpression, Genamplifikation, Mutationen, Mikrosatelliteninstabilitäten, Einzelnukleotidpolymorphismen) im Vordergrund. Durch die Korrelation der molekularen Daten mit klinischen Parametern sollte anschließend ermittelt werden, ob bestimmte Veränderungen prognostische Relevanz besitzen (**Thematik 1** – WTS, **Thematik 2** - Ovarialkarzinome).

Die Detektion und Charakterisierung von alternativ gespleißten Transkripten der MDM2- und MDMX-mRNA in adulten WTS und in juvenilen RMS war ein weiterer Schwerpunkt der vorliegenden Arbeit. Darüberhinaus sollte untersucht werden, ob ein Zusammenhang zwischen dem Auftreten von Spleißformen und dem p53-Mutationsstatus bzw. einer p53-Proteinüberexpression besteht. (**Thematik 3**).

Aufbauend auf den Studien von Meye *et al.* [86], in denen in vitro durch Behandlung mit MDM2-AS-ODNs eine Wachstumsinhibierung von bis zu 80 % erzielt werden konnte, sollten Strategien für eine *in-vivo*-Applikation zur WTS-Therapie entwickelt werden. Das Ziel war zunächst die Etablierung eines Modells zur Behandlung von WTS-Rezidiven im Peritoneum in der Nacktratte, an dem die Wirksamkeit und p53-unabhängige Effekte der MDM2-AS-Konstrukte untersucht werden sollte. Diese Arbeiten sollen auch eine Grundlage für zukünftige Planungen von Kombinationstherapien von konventionellen Therapieformen (Zytostatika, Bestrahlung) mit einer Gentherapie für bestimmte WTS-Patienten sein (**Thematik 4**).

2. Material und Methoden

2.1. Methoden

Die in dieser Arbeit angewandten Methoden und Techniken sind ausführlich in den jeweiligen Publikationen beschrieben oder zitiert worden (s. Übersicht der eigenen Publikationen). Die Experimente wurden unter meiner Leitung bzw. unter meiner maßgeblichen Mitwirkung durchgeführt. Unter anderem handelt es sich um folgende Methoden:

- Isolieren von DNA, RNA und Protein aus Zellkulturen, Blut, Tumorfrischmaterial sowie aus fixiertem Tumormaterial
- reverse Transkription und PCR
- quantitative PCR („real-time PCR")
- Sequenzierung von DNA
- Klonieren von PCR-Produkten, Isolieren von Plasmid-DNA
- Western Blot zum Nachweis der Proteinexpression
- Immunhistochemie
- Kultivierung von Zelllinien
- Transfektion von Zellkulturen mit Antisense-Oligonukleotiden, Plasmiden sowie siRNA
- Durchlicht- und Fluoreszenzmikroskopie
- Xenotransplantation von humanen Tumorzellen auf Nacktratten
- Statistik (u.a. Kreuztabellen, Kaplan-Meier-Analysen, Cox-Regression, t-Test)

2.2. Tumormaterial und Patientenkollektive

2.2.1. Tumorzelllinien

Die verwendeten Zelllinien sind im Institut für Pathologie der Universität Halle-Wittenberg bzw. im Department of Molecular Pharmacology des St. Jude Children's Research Hospital (SJCRH, Memphis, TN, USA) kultiviert worden. Die kommerziell erhältlichen ATCC-Zelllinien (Manassas, VA, USA) wurden über WAK-Chemie (Bad Homburg) oder die „Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH" (Braunschweig) bezogen. Die Zelllinien Rh18, 28, 30, 36, 41 sind großzügiger weise von Prof. Peter Houghton vom SJCRH zur Verfügung gestellt worden. Die genannten Linien wurden im Department of Molecular Pharmacology des SJCRH aus Tumorproben als Primärkulturen etabliert.

2.2.2. Patientenkollektive

Weichteilsarkome

Die in dieser Arbeit verwendeten Tumorproben wurden freundlicherweise von der Chirurgischen Klinik des Universitätsklinikums Leipzig durch PD Dr. Peter Würl zur Verfügung gestellt, der die betreffenden Patienten operiert hat. Von sämtlichen Patienten wurden zusätzlich zum Tumormaterial auch Normalgewebe (Muskel) und Blutproben entnommen. Sowohl das Tumor- als auch das Normalgewebe sind bis zur weiteren Verwendung in flüssigem Stickstoff kryokonserviert worden. Die Verschlüsselung der Proben und die Anonymisierung der klinischen Daten erfolgten gemäß einer am Institut für Pathologie erstellten Datenbank und entsprechend ihres chronologischen Eingangs (LZ6 z.B. entspricht der 6. Tumorprobe aus der Chirurgischen Klinik des Universitätsklinikums Leipzig). Die Proben wurden durch erfahrene Pathologen histologisch charakterisiert und entsprechend der gültigen WHO-Klassifikation für WTS von 1993 eingeordnet [29].

Ovarialkarzinome

Paraffin-fixierte Proben von 107 invasiven Ovarialkarzinomen wurden nach Verfügbarkeit des Materials ausgewählt. Die Fälle wurden zwischen 1997 und 2005 am Institut für Pathologie der Martin-Luther-Universität Halle diagnostiziert. Die Studie wurde durch das lokale Ethikkomitee genehmigt. Alle histologischen Schnitte wurden durch zwei Pathologen (Elise Gradhand & Steffen Hauptmann) reevaluiert. Die Tumorproben und das Patientenkollektiv wurden in mehreren Publikationen ausführlich beschrieben [3,64]. Die histologische Klassifikation erfolgte nach den Richtlinien der WHO, das Grading nach Silverberg [125]. Klinische Daten wie Patientenalter, Resttumorgröße, FIGO Stadium, adjuvante Chemotherapie und Verlauf sind in Tabelle 2-1 zusammengestellt.

2.3. Versuchstiere

Alle Nacktratten (RH-nu/nu) stammten aus dem Zuchtbetrieb Harlan-Winkelmann (Borchen). Jeweils 3 - 4 Tiere wurden semisteril in Käfigen mit Einzelbelüftung im Tierstall der Veterinärmedizinischen Fakultät der Universität Leipzig mit einem 12/12 h Hell-Dunkel-Rhythmus gehalten. Den Tieren standen steriles handelsübliches Trockenfutter und Wasser ad libitum zur Verfügung. Es wurde darauf geachtet, dass die Tiere für eine Versuchsreihe jeweils aus der gleichen Lieferung stammten und etwa das gleiche Alter und Gewicht hatten (Alter bei Versuchsbeginn ca. 8-10 Wochen, Gewicht 80-110 g).

Tabelle 2-1: Zusammenfassung klinischer Daten der Ovarialkarzinompatientinnen

Klinische Parameter	Patientinnen (107)	
	No.	**%**
Tumortyp		
serös	62	57.9
endometroid	14	13.1
gemischt	11	10.3
klarzellig	9	8.4
transitionalzellig	1	0.9
undifferenziert	8	7.5
muzinös	2	1.9
Tumorstadium		
FIGO I	30	28.0
FIGO II	9	8.4
FIGO III	63	58.8
FIGO IV	5	4.7
Alter der Patientinnen		
Durchschnitt	63.5	
Median	64.0	
Therapie		
Cisplatin+Taxol	62	57.9
Platin-haltig, ohne Taxol	21	29.7
andere	2	1.9
keine (FIGO Ia)	5	4.7
abgelehnt, gestorben	13	12.1
keine Daten	4	3.7
Resttumor		
kein	39	41.0
< 1 cm	22	23.1
> 1 cm	34	35.8

3. Ergebnisse

3.1 Thematik 1 – Genetische Veränderungen von MDM2, MDMX, p53 bei WTS und Ovarialkarzinomen

3.1.1 Nachweis der Amplifikation des MDM2-Gens im WTS und Korrelation mit dem Überleben [4]

Von insgesamt 75 Tumorproben wurde der MDM2-Genstatus mittels einer semiquantitativen Multiplex-PCR [68] untersucht, von 63 Patienten stand auch Referenz-DNA von Lymphozyten aus dem peripheren Blut zur Verfügung. Insgesamt konnte in 28 % (21 von 75) der untersuchten WTS-Proben eine Amplifikation des MDM2-Gens detektiert werden [4]. Einzelne Proben wiesen ein bis zu 12-fach amplifiziertes MDM2-Gen auf (Abb. 3-1). Die MDM2-Genamplifikationen korrelierte mit einem geringeren Tumorstadium (p=0,022; χ^2-Test). Es war weiterhin von Interesse, ob die Amplifikation des MDM2-Gens eine erhöhte MDM2-mRNA-Expression zur Folge hat. Wir fanden heraus, dass die Amplifikation des MDM2-Gens signifikant mit einer erhöhten Expressionsrate der mRNA des MDM2-Gens korreliert (p=0,007, χ^2-Test). In allen Tumoren mit einer Amplifikation wurde gleichzeitig eine erhöhte mRNA-Expression detektiert, wohingegen keine Probe mit einer geringen Menge an MDM2-mRNA ein amplifiziertes MDM2-Gen aufwies. Mit Hilfe der Kaplan-Meier-Analyse wurde der Einfluss der Amplifikation des MDM2-Gens auf das Überleben von WTS-Patienten ermittelt (Tab. 3-1). Bemerkenswert war, dass die durchschnittliche Überlebenszeit für Patienten, in deren Tumor keine Amplifikation des MDM2-Gens nachweisbar war, 36 Monate betrug, während Patienten mit nachgewiesener MDM2-Genamplifikation im Durchschnitt 98 Monate überlebten (p=0,077, log-Rank-Test).

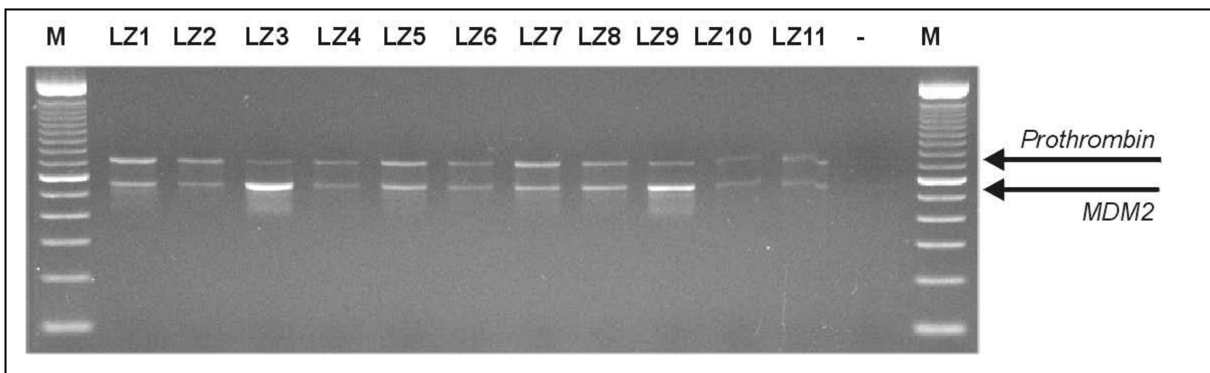

Abb. 3-1: Nachweis der Amplifikation des MDM2-Gens in WTS

Die Abbildung zeigt ein repräsentatives Agarosegel zum Nachweis von MDM2-Genamplifikationen. In einer Multiplex-PCR wird genomische DNA mit Primern für das MDM2 und das Prothrombin-Gen (Pro) zusammen amplifiziert. Anschließend erfolgten die Auftrennung der Produkte im Agarosegel und die densitometrische Analyse der Banden. Abbildung entnommen aus Bartel *et al.*, 2001 [4]

In der Cox-Regression zeigte sich, dass Patienten ohne MDM2-Amplifikation eine schlechtere Prognose im Vergleich zu WTS-Patienten haben, deren Tumor ein amplifiziertes MDM2-Gen trägt (p=0,04, relatives Risiko [RR] 4,5). Wird die Cox-Regression nach dem Tumorgrad adjustiert, ergibt sich für Patienten ohne Amplifikation ein vergleichbares Risiko am Tumor zu versterben (p=0,17, RR=4,4). Die Ergebnisse zeigen, dass der Nachweis einer Amplifikation des MDM2-Gens ein unabhängiger Prognosefaktor für WTS ist, und dass das Auftreten einer Amplifikation mit einer besseren Prognose für WTS-Patienten korreliert.

3.1.2 Mikrosatellitenanalyse für die MDM2-Marker D12S80b und D12S83a bei WTS [132]

Im Rahmen einer Analyse auf Mikrosatelliteninstabilitäten wurden WTS auch die beiden MDM2-Marker D12S80b (distal von kodierender Sequenz von MDM2) und D12S83a (proximal der kodierenden Sequenz von MDM2) auf Veränderungen hin untersucht. Von den 66 untersuchten Patientenproben wies nur ein geringer Anteil einen Verlust der Heterozygotie im MDM2-Marker D12S83a auf, d.h. in 8 % (5/66) der Tumoren konnte ein LOH („loss of heterozygosity") nachgewiesen werden. In einer dieser Proben wurde zusätzlich auch eine

Tab. 3-1 Kaplan-Meyer-Analyse des Einflusses molekularer und klinischer Faktoren auf die Überlebenszeit von WTS-Patienten. log-Rank – log-Rank-Test, WB – Western Blot.

Merkmal	Anzahl der Patienten	Überlebenszeit in Monaten (Median)	3-Jahres-Überlebensrate (%)	p (log-Rank)
MDM2 Spleißvarianten				
nein	34	68	60	
ja	43	57	56	0,39
MDM2 Amplifikation				
Nein	47	38	50	
ja	18	96	80	0,077
MDM2 Expression (WB)				
Keine	17	38	47	
Gering	17	35	49	
Erhöht	17	37	51	
Stark erhöht	8	68	71	0,94
p53 Expression (WB)				
Keine	15	68	85	
Gering	9	35	31	
Erhöht	11	25	36	
Stark erhöht	24	57	55	0,19
Tumorstadium				
1	22	96	94	
2	27	68	61	
3	25	24	34	
4	18	5	0	0,00001

Instabilität des Mikrosatellitenlocus festgestellt. Dem gegenüber wiesen 18 % (12/66) der WTS Veränderungen im MDM2-Marker D12S80b auf. Dabei wurde in jeweils 6 Tumoren ein LOH bzw. eine MSI (Mikrosatelliteninstabilität) detektiert.

Betrachtet man die Häufigkeiten des Auftretens von Veränderungen für die beiden MDM2-Mikrosatelliten D12S80b und D12S83a, so zeigt sich, dass der Verlust der Heterozygotie im Locus D12S80b signifikant mit der Amplifikation des MDM2-Gens korreliert (p = 0,003; χ^2-Test). Patienten, deren Tumoren für diesen Mikrosatellitenmarker einen LOH aufweisen, tragen in 4 von 5 Fällen (80 %) auch ein amplifiziertes MDM2-Gen. Demgegenüber wiesen 83 % der Tumorproben ohne Alteration auch keine Amplifikation des MDM2-Gens auf. Bei Patienten mit Mikrosatelliteninstabilitäten in den untersuchten Markern D12S80b und D12S83a konnte keine MDM2-Genamplifikation beobachtet werden.

3.1.3 MDMX-Genamplifikation in WTS [6,7]

Im Laufe der Arbeiten erschienen mehrere Publikationen, die auf eine Bedeutung von MDMX in Tumoren hindeuteten. Daher gingen wir der Frage nach, ob das MDMX-Gen ähnliche Alterationen aufweist wie MDM2. Die Amplifikation des MDMX-Gens wurde in insgesamt 66 WTS-Proben untersucht. Wir konnten nachweisen, dass das MDMX-Gen in 27 % (18 von 66) der untersuchten WTS amplifiziert vorlag. Die Bandenintensität der MDMX-Bande war in den einzelnen Tumorproben zwischen 3- bis 9-fach stärker als in den Lymphozyten der jeweiligen Patienten. Das Auftreten der MDMX-Genamplifikation korrelierte aber weder mit dem Tumorgrad (χ^2-Test, p=0,074) noch mit dem Tumorstadium (χ^2-Test, p=0,549). In der Kaplan-Meier-Analyse zeigte sich, dass Patienten, deren Tumor ein amplifiziertes MDMX-Gen aufweist, eine mittlere Überlebenszeit von 44 Monaten im Vergleich zu 66 Monaten für Patienten ohne MDMX-Genamplifikation (Abb. 3-2A). Dieser Unterschied war jedoch nicht signifikant (log-Rank-Test, p=0,27). In einer multivariaten Cox-Regressions-Analyse (Abb. 3-2B), adjustiert nach Tumorgrad und Art der Tumorresektion, konnten wir zeigen, dass das relative Risiko (RR), am Tumor zu versterben, für Patienten mit nachgewiesener MDMX-Genamplifikation signifikant erhöht war (RR=2,8; p=0,03). Zusammengefasst lässt sich sagen, dass das MDMX-Gen in einem Teil der WTS amplifiziert ist und dass dies mit einer schlechten Prognose für diese Patienten verbunden ist. Dies steht im Gegensatz zu dem Befund, dass das Vorhandensein einer MDM2-Genamplifikation ein positiver Prognosefaktor für WTS ist.

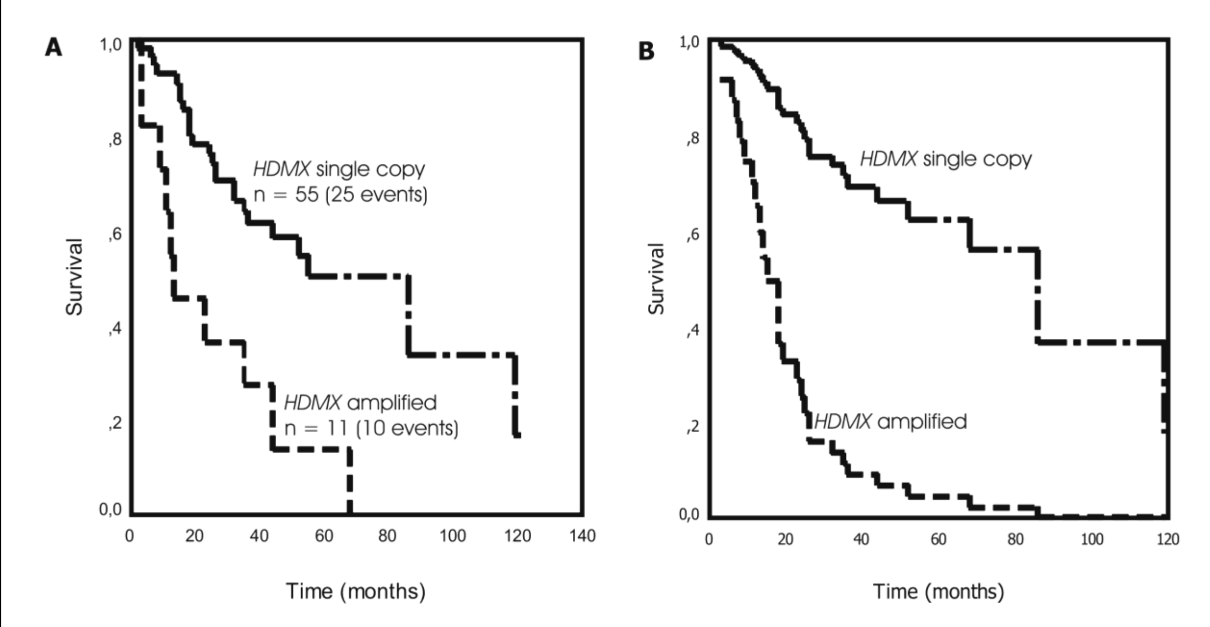

Abb. 3-2: Prognostische Bedeutung der MDMX-Amplifikation bei WTS

Die Amplifikation des MDMX-Gens war in WTS mit einem stark verkürzten Überleben (A – Kaplan-Meier-Kurve) und mit einem erhöhten Risiko, am Tumor zu versterben, assoziiert (B – multivariate Cox-Regression). Die Cox-Regression wurde nach dem Tumorgrad und dem p53-Mutationsstatus adjustiert. Beide Parameter sind unabhängige, prognostische Faktoren für WTS. Die Abbildung wurde aus Bartel *et al.,* 2005, [7] entnommen.

3.1.4 MDMX-mRNA-Expression in WTS [6,7]

Neben der MDMX-Genamplifikation wurde auch die MDMX-mRNA-Expression in 57 der 66 WTS-Proben untersucht. Insbesondere waren wir an der Analyse der MDMX-S-Spleißvariante interessiert. Diese Spleißvariante ist durch den Verlust von Exon 6 (68 bp) charakterisiert und wurde erstmals von Rallapalli *et al.* beschrieben [110]. Durch die Auswahl geeigneter PCR-Primer war es uns möglich, die PCR-Produkte, die von der cDNA der „full-length"-MDMX-mRNA (FL-MDMX) bzw. von der MDMX-S-Spleißvariante abstammen, zu unterscheiden. Wir konnten die FL-MDMX-mRNA in 54 der 57 (95 %) WTS-Proben nachweisen. In 12 von 54 Proben (22 %), die die MDMX-mRNA exprimierten, war nur die FL-MDMX-mRNA nachweisbar. In 42 von 54 WTS (78 %) wurden die Transkripte der MDMX-mRNA sowie der Spleißvariante MDMX-S exprimiert. Eine genauere Analyse ergab, dass in 48 % der Fälle (20 von 42) das FL-MDMX-Transkript und in 19 % (8 von 42) die MDMX-S-Spleißvariante wesentlich stärker im Vergleich zum FL-Transkript exprimiert wurde. In 33 % (14 von 42) der Fälle waren beide Transkripte nahezu gleich stark vorhanden. Als Vergleich haben wir die MDMX-mRNA-Expression in verschiedenen Normalgeweben untersucht und konnten feststellen, dass die MDMX-mRNA erst nach insgesamt 70 PCR-Zyklen nachweisbar war. Dabei dominerte in allen Normalgewebeproben das FL-MDMX-Transkript. In unserer

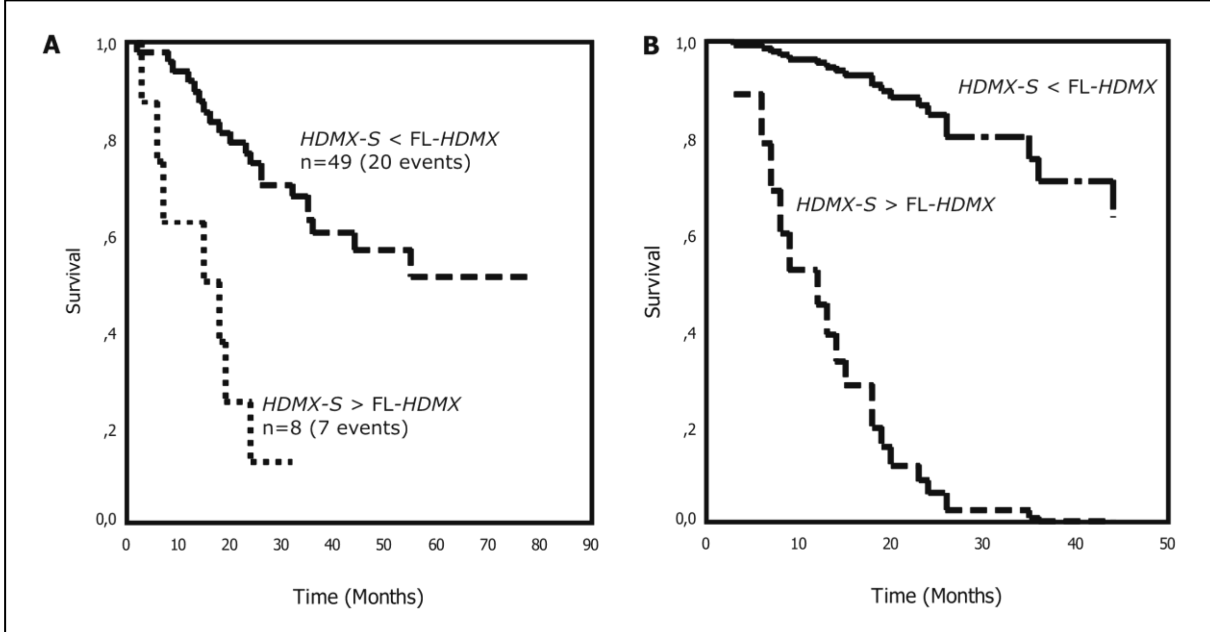

Abb. 3-3: Prognostische Bedeutung der MDMX-S-Überexpression bei WTS

Die Überexpression der MDMX-S-Spleißvariante war in WTS mit einem stark verkürzten Überleben (A – Kaplan-Meier-Kurve) und mit einem erhöhten Risiko, am Tumor zu versterben, assoziiert (B – multivariate Cox-Regression). Die Cox-Regression wurde nach der Tumorlokalisation und dem p53-Mutationsstatus adjustiert. Beide Parameter sind unabhängige, prognostische Faktoren für WTS. Die Abbildung wurde aus Bartel et al., 2005, [7] entnommen.

Studie gab es einen signifikanten Zusammenhang zwischen dem Tumorgrad und der Überexpression der MDMX-S-Spleißvariante (χ^2-Test, p=0,0005).

Berechnungen zur Überlebenszeit zeigten, dass Patienten mit einer deutlichen MDMX-S-Überexpression durchschnittlich 13 Monate nach Diagnosestellung verstarben, wohingegen Patienten, bei denen FL-MDMX-Menge größer oder gleich war im Verhältnis zu MDMX-S, durchschnittlich 53 Monate überlebten (p=0,0001). Die Cox-Regressionsanalyse, adjustiert nach Tumorgrad und Art der Tumorresektion, ergab zudem, dass Patienten mit MDMX-S-Überexpression eine deutlich schlechtere Prognose aufweisen als Patienten mit geringer MDMX-S-Expression (RR=9, p=0.0001). Zusammenfassend lässt sich festhalten, dass die Überexpression der MDMX-S-Spleißvariante ein unabhängiger prognostischer Faktor für WTS ist und mit einer signifikant kürzeren Überlebenszeit korreliert (Abb. 3-3).

3.1.5 Polymorphismen im MDM2-Gen [11,12,134]

Die Funktionalität von p53 spielt eine wichtige Rolle bei der Verhinderung der Entstehung von Tumoren. p53 selbst ist in mehr als 50 % der Tumoren durch Mutationen inaktiviert. Unsere Hypothese lautete, dass auch Sequenzvariationen von anderen Genen im p53-Pathway die Wahrscheinlichkeit einer Tumorentstehung erhöhen. Wir haben daraufhin

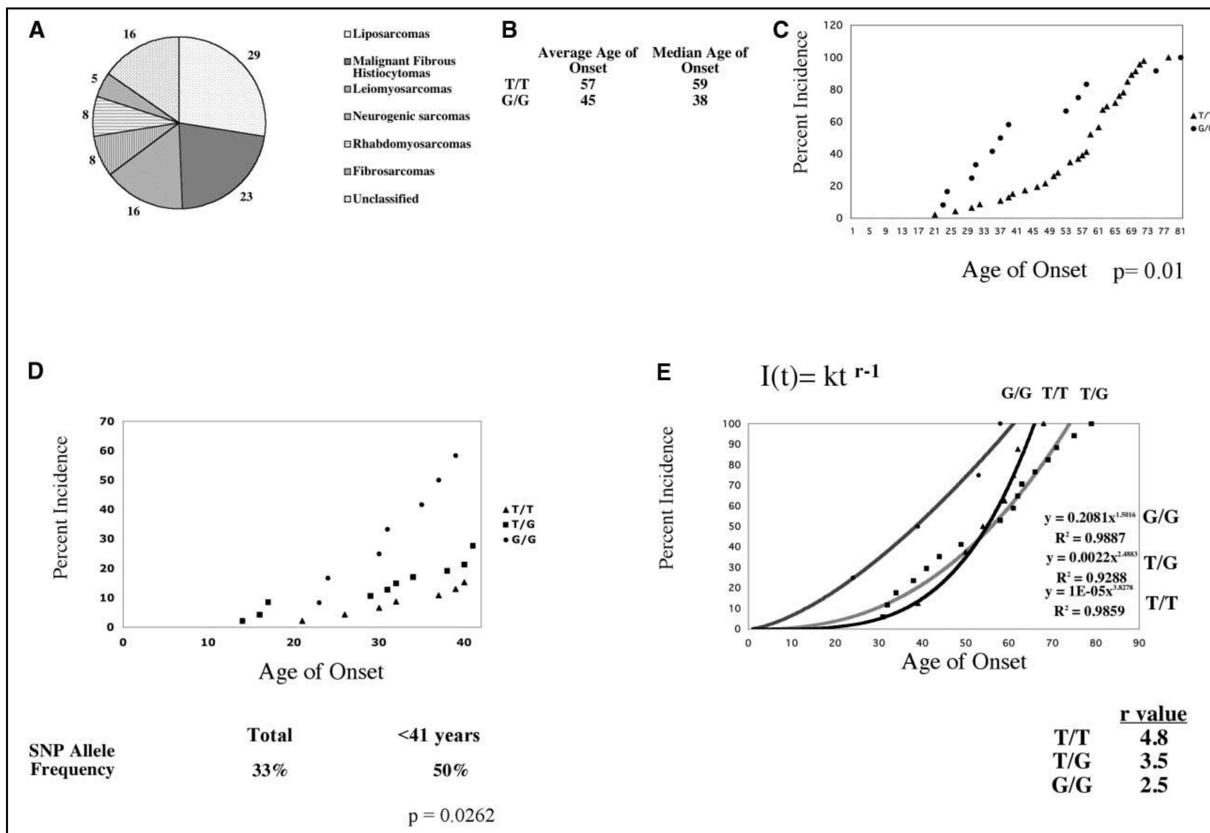

Abb. 3-4: SNP309 ist mit einem früheren Auftreten von sporadischen Weichteilsarkomen assoziiert

A – Das Diagramm zeigt die Verteilung der WTS-Subtypen in dieser Analyse. B – Patienten, die homozygot für den SNP309 (G/G) sind, wiesen ein 12 Jahre früheres Alter der Tumorentstehung auf. C – Im Diagramm ist die Anzahl der Individuen, die an einem WTS erkrankten, gegen das Alter der Diagnose aufgetragen. Es wird deutlich, dass Patienten mit einem G/G-Genotyp durchschnittlich früher an ihrem Tumor erkrankten als Patienten mit einem T/T-Genotyp (Wildtyp). D – Die Frequenz des G-Allels von SNP309 ist deutlich erhöht bei Patienten, die in einem Alter von unter 41 Jahren an ihrem Tumor erkrankten (p=0.0262). E – SNP309 ist möglicherweise ein „rate-limiting event“ bei Liposarkomen. Zur Entstehung einer Tumorzelle ist eine bestimmte Anzahl (r) von Ereignissen notwendig, die entsprechend der Formel $I(t)=kt^{r-1}$ berechnet. Der r-Wert, die Gleichung und der r^2-Wert für jeden Genotyp sind angegeben. Die Abbildung ist der Publikation Bond *et al.,* 2004, entnommen [11].

untersucht, ob in Tumoren Mutationen und/oder Polymorphismen des MDM2-Gens auftreten. Dazu wurde von 82 WTS-Patienten und 100 gesunden Probanden das Exons 12 des MDM2-Gens sequenziert. Es konnte weder in den Patienten noch in den gesunden Blutspendern eine Mutation detektiert werden. In den Untersuchungen fanden wir jedoch einen Einzelnukleotidpolymorphismus (SNP) im Kodon 354 (GAA > GAG), der in 8 % der Blutspender (8 von 100) und in 13 % der Tumorpatienten (11 von 82) auftrat. Der heterozygote Status des SNP korrelierte mit einer verminderten MDM2-mRNA-Expression (p=0.0032). Daraus lässt sich ableiten, dass das G-Allel möglicherweise zu einer verminderten mRNA-Stabilität führt. In einer Kaplan-Meier-Analyse konnten wir zeigen, dass Patienten, die einen homozygoten Wildtyp-Status aufwiesen, im Durchschnitt 88 Monate nach Diagnosestellung verstarben, während dies bei Patienten die heterozygot bzw. homozygot für den SNP waren, bereits nach durchschnittlich 45 Monaten der Fall war. Dies und der höhere Anteil an Tumorpatien-

ten mit dem SNP lassen den Schluss zu, dass dieser SNP mit einer erhöhten Tumorsuszeptibilität assoziiert ist.

Nach der Analyse der Zinkfinger-Region haben wir zusammen mit Arnold Levine (Institute of Advanced Studies, Princeton, USA) und Gareth Bond (Ludwig Institute for Cancer Research, Oxford, England) das Intron 1 des MDM2-Gens sequenziert. In dieser Region befindet sich der p53-sensitive P2-Promotor des MDM2-Gens [158]. Da die MDM2-Menge entscheidend für die p53-vermittelte Stressantwort ist, kann man davon ausgehen, dass natürlich vorkommende Sequenzvariationen (SNPs) die MDM2-Expression und die Funktionalität von p53 und somit die Fähigkeit von p53, die Entstehung von Tumoren zu verhindern, beeinflussen können. Es konnte an der 309. Position des Introns 1 ein T > G-Austausch identifiziert werden (SNP309). Dies führt zu einer verstärkten Bindung des Transkriptionsfaktors Sp1 an den Promotor und zu einer erhöhten MDM2-mRNA und –Proteinexpression [11]. Das Vorhandensein des G-Allels von SNP309 ist demnach mit einer Schwächung des p53-Pathways assoziiert. An unserem WTS-Patientenkollektiv konnten wir zeigen, dass der SNP309 mit einem früheren Auftreten von sporadischen WTS korreliert (Abb. 3-4). So entwickelten Patienten, die homozygot für SNP309 (G/G) waren, durchschnittlich 12 Jahre früher den Tumor als Patienten ohne SNP309 (T/T; p=0,01). Wir fanden weiterhin heraus, dass das G-Allel bei jüngeren Patienten deutlich häufiger auftrat. Die Allelfrequenz betrug bei Patienten unter 41 Jahren 50 % im Vergleich zu 33 % bei Patienten, die älter als 41 Jahre waren (p=0,026; Abb. 3-4D). Des Weiteren zeigen unsere Berechnungen, dass das G-Allel von SNP309 ein so genanntes „rate-limiting event" bei Liposarkomen ist (Abb. 3-4E). Das bedeutet, dass die Anzahl der notwendigen Ereignisse zur Tumorentstehung (z.B. Mutationen, Überexpression von Genen) herabgesetzt ist. Während bei Patienten, die homozygot für das T-Allel sind (Wildtyp), durchschnittlich 4,8 Ereignisse zur Tumorentstehung nötig sind, so sind es bei Patienten, die homozygot für das G-Allel sind, nur noch 2,5 Ereignisse [11]. Diese Ergebnisse zeigen eindeutig, dass das G-Allel von SNP309 mit einer Schwächung des p53-Pathways assoziiert ist, so dass die Entstehung von Tumoren in diesen Individuen beschleunigt wird.

Das untersuchte Patientenkollektiv besteht aus 58 Frauen und 47 Männern. Betrachtet man die geschlechtsspezifische Verteilung von SNP309, so fällt auf, dass nur 3 der 47 Männer einen G/G-Genotyp aufweisen. Das bedeutet, dass der Effekt einer frühen Tumorentstehung bei WTS nur bei Frauen mit einem G/G-Genotyp assoziiert ist. Bei weiblichen Patienten mit einem G/G-Genotyp trat der Tumor durchschnittlich 14 Jahre früher auf als bei Patientinnen mit einem T/T-Genotyp (T/T-Frauen: 59 Jahre; G/G-Frauen: 45 Jahre, p=0.028). Man kann daher vermuten, dass Östrogen eine wichtige Rolle in diesem Zusammenhang spielt.

Dies zeigt sich auch daran, dass bei 67 % der GG-Patientinnen der Tumor vor dem 51. Lebensjahr diagnostiziert wurde, bei den TT-Patientinnen war dies nur bei 27 % der Fall (p=0,05, exakter Test nach Fisher). Der Anteil der GG-Patientinnen betrug bei Frauen, deren Tumor vor dem 51. Lebensjahr auftrat, 27 %, bei Patientinnen über 51 Jahren nur 8% und bei männlichen Patienten 6 % (p=0,017, exakter Test nach Fisher). Diese Daten stützen die Hypothese, dass Geschlechtshormone, wie z.B. Östrogen, die Entstehung von Tumoren bei Individuen mit einem G/G-Genotyp weiter beschleunigen können.

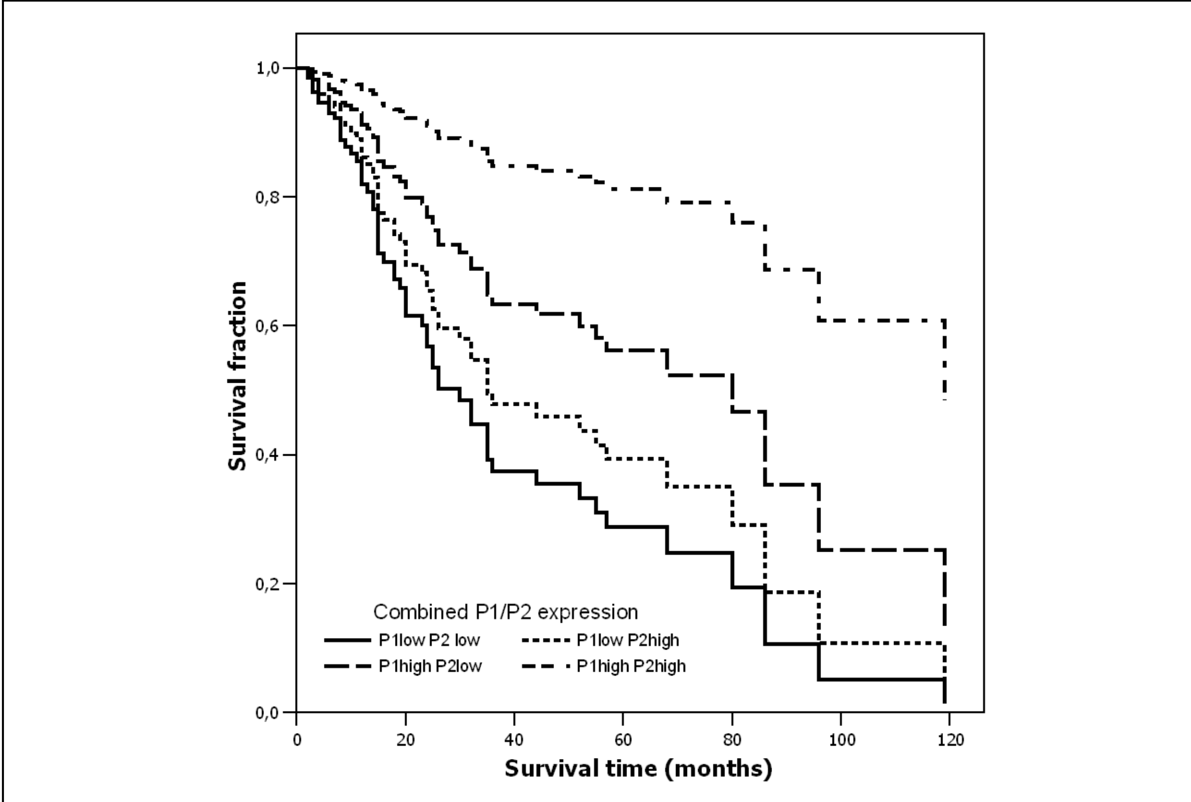

Abb. 3-5: Prognostische Bedeutung der MDM2-P1- und P2-Expression bei WTS

Die multivariate Cox-Regression (adjustiert nach Tumorstadium, Tumorlokalisation und Resektionsgrad) zeigt, dass die geringe Expression des P1- und P2-Transkripts mit einem stark erhöhten Risiko, am Tumor zu versterben, assoziiert ist. Die Abbildung ist der Publikation Taubert et al., 2008 entnommen [133].

3.1.6 MDM2-P1- und P2-Transkriptlevel bei WTS-Patienten [133]

Wir konnten in vorangegangenen Arbeiten nachweisen, dass sowohl die MDM2-Genamplifikation [4] als auch eine erhöhte MDM2-mRNA-Expression [135] mit einer guten Prognose für WTS-Patienten verbunden sind. Des Weiteren zeigen unsere Daten, dass Patienten, die homozygot für den SNP309 sind, im Durchschnitt 12 Jahre früher einen Tumor entwickeln [11] als Patienten mit dem Wildtyp. Daher war es naheliegend zu fragen, ob auch das

Expressionsniveau der mRNA-Transkripte, die von den beiden MDM2-Promotoren abgelesen werden, mit dem Alter bei Tumorentstehung bzw. der Prognose assoziiert sind. Die Transkriptmengen wurden mittels transkriptspezifischer Real-Time-PCR-Assays bestimmt. Wir nutzten den Median der Transkriptmengen, um die Werte in „geringe Expression“ bzw. „hohe Expression“ einzuteilen, wenn die Werte der Patienten unter bzw. über dem jeweiligen Median lagen.

Das durchschnittliche Alter bei Tumordiagnose betrug bei Patienten mit einer hohen P1-Expression 50,5 Jahre im Vergleich zu 61,5 Jahren bei Patienten mit einer geringen P1-Expression ($p<0,0001$; t-Test). Überraschenderweise konnten wir bei Patienten mit einer hohen bzw. geringen P2-Expression keinen Unterschied bezüglich des Alters bei Diagnosestellung ermitteln (56 vs. 55 Jahre; $p=0,72$; t-Test). Bei Patienten, die homozygot für den SNP309 waren (G/G) und geringe Mengen P2 expremierten, wurde der Tumor durchschnittlich 29 Jahre früher diagnostiziert als bei Patienten, die homozygot für das T-Allel des SNP309 waren (31,4 vs. 59,8 Jahre; $p<0,0001$, t-Test). Dies war nicht der Fall bei Patienten mit hohen P2-Mengen und unterschiedlichem SNP309-Status. Demgegenüber zeigte sich, dass Patienten mit geringer als auch mit hoher P1-Expression und dem G-Allel des SNP309 durchschnittlich früher an ihrem Tumor erkrankten. Der Altersunterschied war jedoch nur für Patienten mit geringer P1-Expression signifikant (GG + P1 niedrig: 45,1 Jahre vs. TT + P1 niedrig: 60,5 Jahre; $p=0,015$). Um den Effekt der MDM2-mRNA-Expression unabhängig vom Geschlecht und dem MDM2-Amplifikationsstatus zu untersuchen, haben wir das Alter der Diagnose bei Frauen berechnet, deren Tumor kein amplifiziertes MDM2-Gen aufwies. Es stellte sich heraus, dass der Tumor bei Frauen mit einem „single-copy“ MDM2-Gen und einer hohen P1-Expression durchschnittlich 10 Jahre früher im Vergleich zu Frauen mit einer geringen P1-Expression diagnostiziert wurde (51 Jahre vs. 61 Jahre; $p=0,086$). Diese Ergebnisse zeigen, dass die MDM2-Genamplifikation zwar mit einer erhöhten MDM2-Expression korreliert, jedoch nicht den Effekt der P1- und P2-Transkriptlevel auf das Alter der Tumorentstehung bei WTS-Patienten maskiert.

Während eine hohe P1-Expression mit einem früheren Auftreten eines Weichteilsarkoms assoziiert ist, so zeigte sich in der Kaplan-Meier-Analyse, dass Patienten mit einer hohen P1-Expression eine signifikant längere Überlebenszeit (Median: 119 Monate) als Patienten mit einer geringen P1-Expression (26 Monate, $p=0,0001$, log-Rank-Test) aufwiesen (Abb. 3-5). Bezüglich des Gesamtüberlebens zeigten sich auch deutliche Unterschiede zwischen Patienten mit geringer bzw. hoher P2-Expression (26 vs. 96 Monate, $p=0,0023$, log-Rank-Test). Der Effekt, dass eine hohe P1-Expression mit einer längeren Überlebenszeit assoziiert ist, war unabhängig vom Status der MDM2-Genamplifikation (MDM2 single-copy:

p=0,043; MDM2 amplifiziert: p=0,035; log-Rank-Test). In einer multivariaten Cox-Regressions-Analyse (adjustiert nach Tumorstadium, Lokalisation und Resektionsgrad) konnten wir geringe P1- und P2-Expressionslevel als unabhängige, negative Prognosefaktoren für Weichteilsarkompatienten identifizieren (relatives Risiko [RR] P1 gering = 3,7, p<0,0001; RR P2 gering = 2,5, p=0,001). Das Risiko, am Tumor zu versterben, war für Patienten, deren Tumor vor dem 51. Lebensjahr diagnostiziert wurde und durch eine geringe P1- bzw. P2-Expression charakterisiert war, um das 7,7-fache (P1; p=0,002) bzw. um das 13,1-fache (P2; p<0,0001) erhöht. Wir konnten diese Daten auch für Patientinnen mit einem „single-copy" MDM2-Gen bestätigen, um Einflüsse der MDM2-Amplifikation auszuschließen.

3.2 Thematik 2 - Genetische Veränderungen von p53, MDM2 und MDMX bei Ovarialkarzinomen [3]

3.2.1 p53-Mutationen und p53 Proteinexpression

Für die Analysen zu molekularen Veränderungen des p53-Pathways stand uns Material von 107 Ovarialkarzinomen zur Verfügung, das wir zunächst auf p53-Sequenzalterationen hin analysiert haben (Abb. 3-6). Wir konnten insgesamt 111 Sequenzveränderungen nachweisen. Dabei handelte es sich in 44 Fällen und Mutationen in den Exonen 4 – 8 (einschließlich 2 Mutationen in einem Intron), in den weiteren Fällen um bekannte Polymorphismen in Intron 3 bzw. Im Kodon 72. Die p53-Proteinexpression wurde von uns mittels Immunhistochemie an Paraffinschnitten untersucht (Abb. 3-6). In 51,4 % der Fälle (55 von 107) detektierten wir eine p53-Überexpression. 20 Fälle (18,7 %) wiesen nur eine geringe Färbung auf, und 32 Fälle (30,2 %) zeigten keine p53-Expression. Es zeigte sich, dass die p53-Proteinexpression nicht mit dem p53-Mutationsstatus korrelierte (p=0.59; χ^2-test). Der Anteil an Fällen mit einer p53-Überexpression und einem mutierten p53-Gen betrug 54 % (24 von 44). In 49 % der Fälle (31 von 63) konnte jedoch trotz eines p53-Wildtyp-Gens eine verstärkte p53-Expression detektiert werden. Wir haben daher die Tumoren entsprechend ihres kombinierten p53-Mutations/-Proteinexpressionsstatus in vier Gruppen eingeteilt. Fälle mit einem p53-Wildtyp-Gen und ohne immunhistochemisch nachweisbare p53-Expression wurden als „p53 normal" bezeichnet (29.9 %; 32 von 107). Hier gehen wir davon aus, dass p53 funktionell aktiv ist. Die anderen Gruppen enthielten Fälle mit einer p53-Alteration und setzten sich folgendermaßen zusammen: (i) p53-Überexpression, jedoch p53-Wildtyp-Gen (29 %; 31/107), (ii) p53-Mutation und p53-Überexpression (22.4 %; 24/107) sowie (iii) p53-Mutation, aber keine p53-Expression (18.7 %; 20/107).

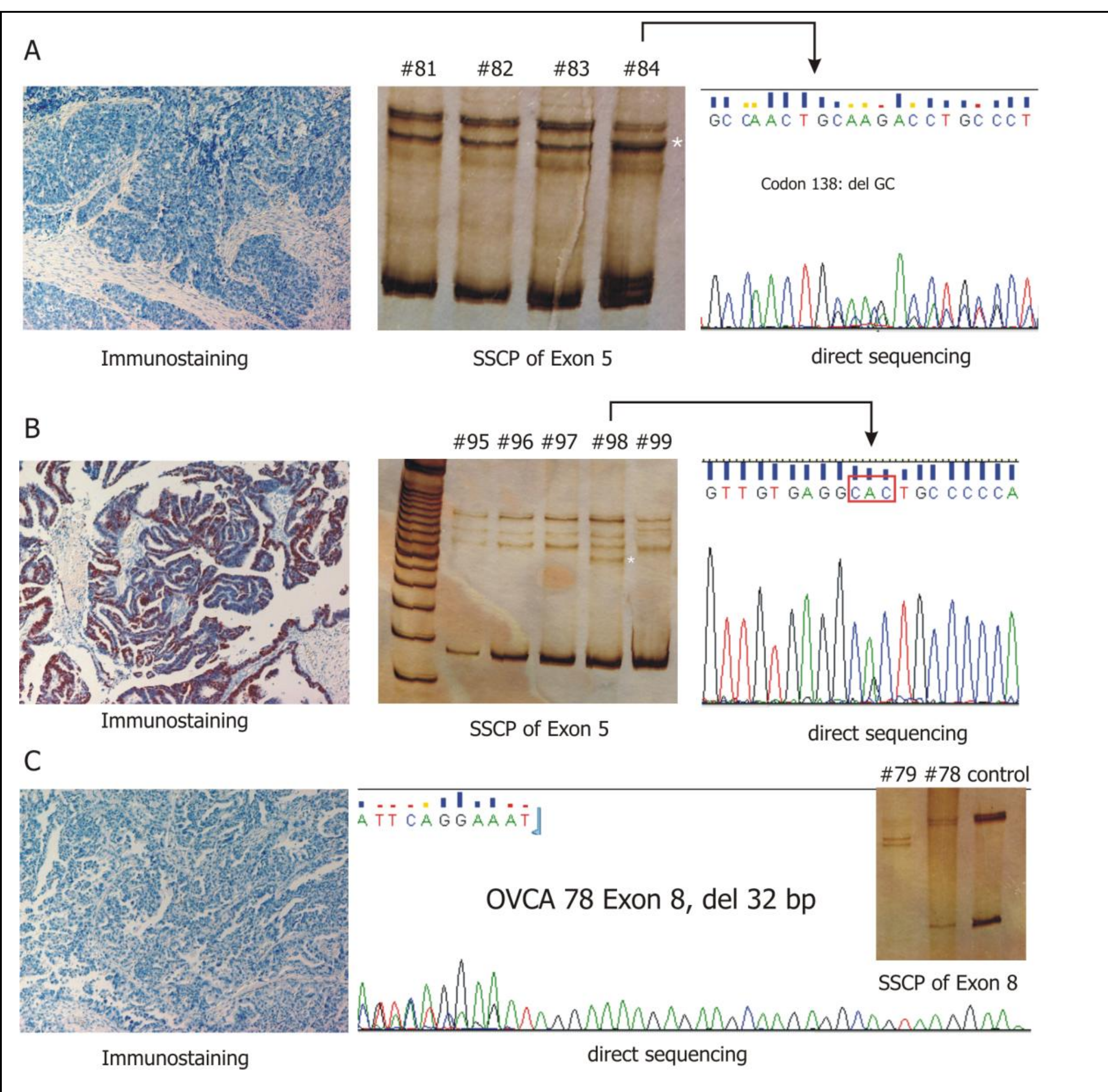

Abb. 3-6: SSCP, DNA-Sequenzierung und immunhistochemischer Nachweis der p53-Expression im Ovarialkarzinom

A – Die SSCP-Analyse von Exon 5 bei Fall Nr. 84 (seröses Ovarialkarzinom) zeigt eindeutig eine zusätzliche Bande (*). Die anschließende Sequenzierung bestätigte eine 2-bp-Deletion (GC) im Kodon 138, die zu einem frühzeitigen Stopp-Kodon führt. Daher war p53 bei diesem Fall immunhistochemisch nicht nachzuweisen. B – Durch SSCP und DNA-Sequenzierung von Exon 5 konnte im Fall Nr. 98 (seröses Ovarialkarzinom) eine Missense-Mutation im Kodon 175 (CGC>CAC; Arg>His) detektiert werden. Dieser Fall zeigte eine deutliche Überexpression von p53. C – SSCP, DNA-Sequenzierung und Immunhistochemie des Falls Nr. 78 (klarzelliges Ovarialkarzinom), der durch eine 32-bp-Deletion im Exon 8 charakterisiert ist. Die Abbildung ist der Publikation Bartel *et al.,* 2008, entnommen [3].

3.2.2 p53-Status und Ansprechen auf die Chemotherapie

Patientinnen, deren Tumor eine p53-Veränderung (Mutation und/oder Überexpression) aufwies, waren resistenter gegenüber einer Chemotherapie als Patientinnen mit normalem p53-Status (78 % vs. 52 %; p=0,027). Bei diesen Patientinnen war auch die Zeit bis zum Auftreten eines Rezidivs kürzer als bei Patientinnen ohne p53-Alteration (28 vs. 51 Monate,

p=0,075). In Bezug auf den Mutationsstatus wiesen Tumoren mit einem mutierten p53-Gen eine höhere Resistenz auf (79 %) als Tumoren mit einem p53-Wildtyp-Gen (64 %, p=0,148).

3.2.3 Status des SNP309 im MDM2-P2-Promotor bei Ovarialkarzinomen

Der Anteil an Patientinnen, die einen heterozygoten Genotyp (T/G) aufwiesen, war höher (52.4 %) und der Anteil mit einem homozygoten Genotyp (G/G) war etwas niedriger (7.8 %) als in gesunden Kontrollpersonen (T/G: 40 %; G/G: 12 %, [11]). Dieser Unterschied war jedoch nicht signifikant (p=0,53). Am Beispiel der Weichteilsarkome konnten wir zeigen, dass der SNP309 vor allem in Frauen mit einem aktiven Östrogen-Signalpathway einen Effekt hat [12]. Wir haben daher auch bei den Ovarialkarzinompatientinnen den Einfluss des Östrogenrezeptor (ER)-Status untersucht. In unserem Patientenkollektiv wiesen 40 % (43 von 107) eine sehr hohe und 17 % (18 von 107) eine geringe ER-Expression auf, während 43 % (46 von 107) keinen ER expremierten. In Patientinnen im FIGO III-Stadium mit ER-positiven Tumoren war das G-Allel von SNP309 mit einem 6 Jahre früheren (T/T: 70,6 Jahre; T/G+G/G: 64,4 Jahre, p=0,101) und bei Patientinnen mit einer sehr starken ER-Expression mit einem 8,5 Jahre früheren Auftreten des Tumors assoziiert (p=0,048). In ER-negativen Patientinnen fanden wir keine Unterschiede beim Alter der Tumorentstehung (p=0,44). Diese Daten unterstützen die Hypothese, dass das G-Allel von SNP309 einen intakten Östrogen-Signalpathway benötigt, um die Tumorentstehung zu beschleunigen [12].

3.2.4 p53- und SNP309-Status und der Einfluss auf das Gesamtüberleben.

Die kürzeste Überlebenszeit (42,8 Monate) wiesen Patientinnen auf, deren Tumor durch eine Überexpression von Wildtyp-p53 charakterisiert war. Besonders deutlich war der Unterschied im Vergleich zu Patientinnen mit einem p53-Wildtyp, jedoch ohne nachweisbare p53-Proteinexpression (p=0,019, log-Rank-Test). Zusammenfassend lässt sich sagen, dass Patientinnen mit einem „normalen“ p53 (Wildtyp-Gen, keine Proteinexpression) eine längere Überlebenszeit aufweisen als Patientinnen mit einer p53-Genmutation und/oder p53-Überexpression. Interessanterweise überlebten Patientinnen im FIGO III-Stadium und einem mutierten p53-Gen länger als Patientinnen mit einem p53-Wildtyp-Gen (59 vs. 41 Monate, p=0,058). Ein ähnliches Ergebnis - dass, ein geschwächter p53-Pathway mit einem längeren Überleben assoziiert war - fanden wir auch, als wir den Einfluss von SP309 auf das Gesamtüberleben der Ovarialkarzinompatientinnen untersuchten. Die durchschnittliche Überlebenszeit betrug für Patientinnen mit einem T/T-Genotyp (wt) 59 Monate und für Patientinnen mit dem G-Allel (T/G+G/G) 62 Monate (p=0,994). Im FIGO III-Stadium jedoch korrelierte das

G-Allel - also ein geschwächter p53-Pathway - mit einem längeren Überleben im Vergleich zum SNP309 Wildtyp-Status (57,5 Monate vs. 35,3 Monate, p=0.045, log-Rank-Test). Der Effekt des G-Allels auf das Gesamtüberleben war, wie schon beim Alter der Tumorentstehung, vom ER-Status des Tumors abhängig. Während bei Patientinnen ohne ER-Expression ein noch deutlicherer Unterschied in Bezug auf die Gesamtüberlebenszeit zu finden war (p=0,024, log-Rank-Test), so konnte bei Patientinnen mit ER-Expression kein signifikanter Unterschied der Überlebenszeiten detektiert werden (geringe ER-Expression: p=0,424; starke ER-Expression: p=0,828). Aus diesen Daten lässt sich ein Modell ableiten, nachdem ein geschwächter p53-Pathway—d.h. durch eine p53-Mutation oder das G-Allel von SNP309—mit einer besseren Prognose für Ovarialkarzinompatientinnen assoziiert ist.

3.2.5 MDMX-Alterationen in Ovarialkarzinomen (unveröffentlichte Daten)

Neben Veränderungen des MDM2-Gens haben wir auch den Status von MDMX in Ovarialkarzinomen untersucht. Während die klinische Bedeutung von MDM2-Veränderungen in Tumoren, u.a. in Ovarialkarzinomen [26], gut dokumentiert ist, existieren zum Einfluss von MDMX-Veränderungen auf den Tumorprozess nur sehr wenige Daten. Wir haben daher Sequenzveränderungen (Mutationen und SNPs) des MDMX-Gens in Ovarialkarzinomen untersucht. Dabei haben wir uns zunächst auf die RING-Finger-Domäne (Exon 11) konzentriert, da diese Region mit dem RING-Finger von MDM2 interagiert. Sequenzveränderungen in diesem Bereich könnten aus diesem Grund die Stabilität von MDMX und auch von MDM2 erhöhen und so den p53-Pathway weiter abschwächen. In nur zwei Fällen detektierten wir im Tumormaterial eine heterozygote Punktmutation im Kodon 421 (AGA → CGA). Dies führt zu einem Austausch von Arginin zu Glycin. Damit ist eine wesentliche Änderung der Proteinstruktur verbunden. Darüberhinaus konnten wir einen SNP im 3'-untranslatierten Bereich des MDMX-Gens identifizieren. Es handelt sich um einen A/C-Austausch an der Position 34091 des MDMX-Gens (SNP34091), 31 bp nach dem Stop-Kodon. Der Anteil an Individuen mit einem A/A-Genotyp von SNP34091 (wt) war bei „low-grade" Ovarialkarzinomen mit 48 % niedriger als bei gesunden Kontrollpersonen (55 %), bei „high-grade" Ovarialkarzinomen dagegen höher (62 %). Das A-Allel des SNP34091 war in den untersuchten Ovarialkarzinomen mit einer gesteigerten MDMX-mRNA- und Proteinexpression assoziiert (p=0,056; p=0,096). Dies könnte auf eine erhöhte mRNA-Stabilität zurückzuführen sein. Durch Datenbankrecherchen haben wir gefunden, dass der 3'-UTR bei Vorhandensein des C-Allels eine Bindungsstelle für die die microRNA miR-191 und miR191* darstellt. Durch die Bindung dieser microRNAs an die MDMX-mRNA werden möglicherweise deren Stabilität und Translation negativ beeinflusst. Im Falle des A-Allels entsteht keine Bindungsstelle für diese microRNAs.

Die mögliche Regulation der MDMX-Expression durch microRNA und deren Einfluss auf die Aktivität des p53-Pathways wird zurzeit in einem Kooperationsprojekt mit der Arbeitsgruppe um Jean-Christophe Marine vom IMB in Gent, Belgien, untersucht. In multivariaten Cox-Regressionsanalysen (adjustiert nach der Größe des Resttumors) konnten wir zeigen, dass das A-Allel des SNP34091 mit einem erhöhten Rezidivrisiko als auch mit einem erhöhten Risiko, am Tumor zu versterben, assoziiert war. Dieser Effekt war auch vom ER-Status der Patientinnen abhängig. Bei Patientinnen mit einem A/A-Genotyp und einem ER-negativen Karzinom war das Rezidivrisiko um das 8,2-fache(p=0,009) und das Risiko, am Tumor zu versterben, um das 5,5-fache (p=0,01) erhöht. Unsere Daten zeigen erstmalig, dass Veränderungen von MDMX, insbesondere der SNP34091, eine wichtige Rolle bei Ovarialkarzinomen spielen.

3.3 Thematik 3 - Spleißvarianten von MDM2 und MDMX - Auftreten, Prognose

3.3.1 Detektion verkürzter Transkripte der MDM2-mRNA in WTS [4,8]

Von Sigalas *et al.* konnten 1996 [124] u.a. in Blasentumoren insgesamt 5 verschiedene Transkripte der MDM2-mRNA nachgewiesen werden. Vorarbeiten aus unserer Arbeitsgruppe zeigten an ausgewählten WTS-Zelllinien, dass ebenfalls Transkripte verschiedener Größe im Northern Blot detektierbar waren [85]. Es sollte daher die Frage geklärt werden, ob auch in WTS-Tumorproben Spleißvarianten der MDM2-mRNA nachweisbar sind. Für die Analyse stand von 87 Patienten Tumorgewebe zur Verfügung.

Neben der Bande, die das FL-Transkript repräsentiert, waren in 51 von 85 Proben (60 %) zusätzlich kürzere Banden unterschiedlicher Länge nachweisbar. Die Anzahl der zusätzlichen Banden variierte zwischen den einzelnen Tumorproben. Während in einigen Proben nur eine weitere Bande mit stärkerer Intensität als die FL-Bande auftrat, war in anderen Proben eine Vielzahl von Banden mit geringerer Intensität detektierbar (Abb. 3-7). Es gelang, von 47 der 51 Proben einzelne Banden zu charakterisieren und eindeutig Spleißformen der MDM2-mRNA zuzuordnen, d.h. 55 % aller von uns untersuchten WTS-Proben exprimierten MDM2-mRNA-Spleißvarianten. In den untersuchten WTS-Proben konnten insgesamt 13 verschiedene Transkripte der MDM2-mRNA, einschließlich der FL-Form, nachgewiesen werden. Davon sind zwei Formen (MDM2-A und MDM2-B) bereits beschrieben worden [124]. Die anderen 10 Formen wurden im Rahmen dieser Studie erstmalig detektiert, charakterisiert und bezeichnet. Eine Übersicht über alle detektierten Spleißvarianten findet sich in Bartel *et al.* [8].

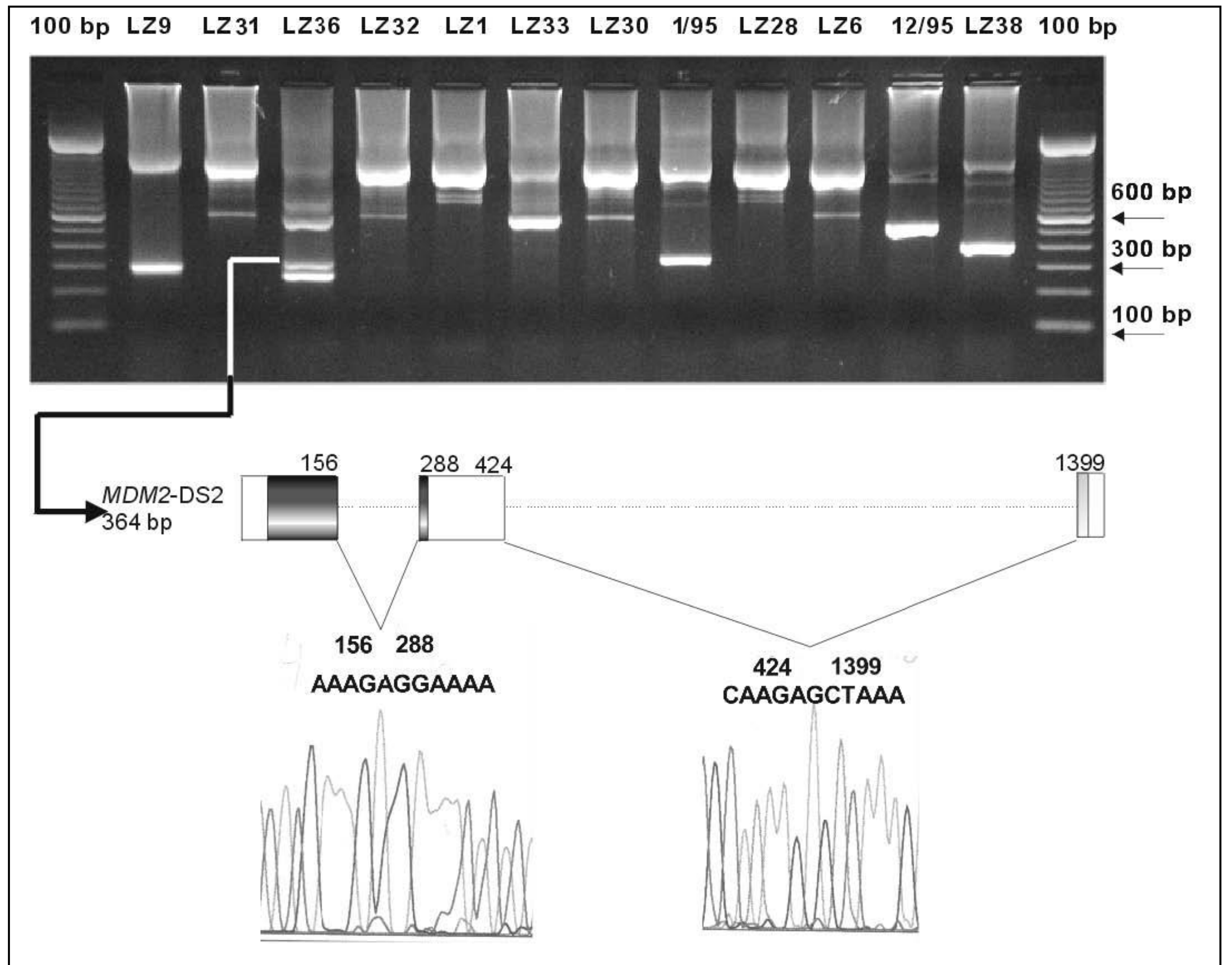

Abb. 3-7: Expression alternativ bzw. aberrant gespleißter MDM2-Transkripte in WTS

Die Abbildung zeigt ein repräsentatives Agarosegel einer nested-RT-PCR zum Nachweis der MDM2-mRNA-Expression in WTS (oben). Zusätzlich zur Bande des FL-Transkripts sind in allen hier gezeigten Tumorproben weitere Banden erkennbar, die MDM2-Spleißvarianten zugeordnet werden konnten. Im unteren Teil der Abbildung ist schematisch die Spleißvariante DS2 mit den Sequenzen der Spleißstellen dargestellt. Die Abbildung ist der Publikation Bartel et al., 2001, entnommen [4].

In Zusammenarbeit mit Linda Harris vom St. Jude Children's Research Hospital (Memphis, USA) wurden RMS-Zelllinien und –gewebeproben analysiert. Insgesamt wurden 11 verschiedene Spleißvarianten der MDM2-mRNA detektiert. Fünf dieser Spleißformen wurden bereits in anderen Tumoren beschrieben [4,124], während weitere sechs Spleißformen (MDM2-FB25 [Genbank-Eintrag: AF385322], -FB26 [AF385323], -FB28 [AF385324], -FB29 [AF385325], -FB30 [AF385326], -FB55 [AF385327]) von uns erstmals beschrieben wurden. Sieben der gefundenen Spleißformen waren alternativ gespleißt, d.h. an Exon/Intron-Grenzen. Der Leserahmen wurde dadurch nicht verschoben. Die Spleißvarianten FB25, FB26, F28 und FB29 dagegen sind Produkte eines aberranten Spleißprozesses. Durch die daraus resultierende Verschiebung des Leserahmens der mRNA weisen die abgeleiteten Proteinsequenzen eine veränderte Aminosäuresequenz am C-Terminus auf. Häufig sind die Proteine durch die Bildung eines frühen Stopp-Kodons weiter verkürzt. Mit der Ausnahme der Spleißform FB26 weisen alle hier gefundenen Varianten teilweise Verluste von Sequenzen der

p53-bindenden Domäne auf. Mit der Spleißvariante FB26 wurde von uns zum ersten Mal eine MDM2-Spleißvariante beschrieben, die eine vollständige, intakte p53-bindende Domäne aufweist. Es ist jedoch noch unklar, ob diese Isoform auch an p53 binden kann.

3.3.2 Detektion von MDM2-Spleißformen in Normalgewebe und Lymphozyten [5]

Von einigen Patienten konnte auch an den Tumor grenzendes Normalgewebe (Muskel) mit in die Untersuchungen einbezogen werden. Als weitere Kontrolle stand Gesamt-RNA aus Blutlymphozyten gesunder Spender sowie eine Knochenmarksprobe zur Verfügung. Von den 7 Normalgewebsproben wurde in 4 Fällen das FL-Transkript der MDM2-mRNA nachgewiesen. Davon wiesen 3 zusätzliche verkürzte Banden im Agarosegel auf. In nur einem Fall konnte lediglich die Expression des FL-Transkripts gezeigt werden. Überraschenderweise exprimierten 3 der untersuchten Normalgewebeproben kürzere Varianten der MDM2-mRNA, jedoch nicht das FL-Transkript. Einige der Banden konnten nach der Reinigung aus dem Gel reamplifiziert und anschließend sequenziert werden. Dabei konnte eine neue MDM2-Spleißform (MDM2-N) identifiziert werden.

In den untersuchten Blut- und Knochenmarkproben konnten zusätzlich zum FL-Transkript jeweils eine kürzere Bande detektiert werden. Die Sequenzanalyse ergab, dass es sich in beiden Fällen um bislang noch nicht beschriebene Spleißvarianten der MDM2-mRNA handelt. Wir konnten somit zeigen, dass die Expression von MDM2-Spleißvarianten nicht auf das Tumorgewebe beschränkt ist. Dies spricht für eine normale physiologische Funktion der MDM2-Isoformen. In weiterführenden Arbeiten wäre daher die Frage zu klären, ob es spezifische, tumorassoziierte MDM2-Isoformen gibt und wie das Spleißen der MDM2-mRNA reguliert wird.

3.3.3 Charakterisierung der Spleißvarianten der MDM2-mRNA

Die Banden, die vermutlich verkürzte Transkripte der MDM2-mRNA repräsentierten, wurden aus dem Agarosegel ausgeschnitten und gereinigt. Es gelang in nahezu allen Fällen, die gereinigten Fragmente mit dem Primerpaar MDM2-1/MDM2-8 zu reamplifizieren. Die so erhaltenen PCR-Produkte wurden direkt sequenziert. Die Sequenzen der PCR-Produkte sind mit der Sequenz der FL-MDM2-cDNA (Genbank-Eintrag NM002392) verglichen worden, um zu ermitteln, welche Domänen in den Spleißformen noch vorhanden sind. Einzelheiten zu den Varianten sind den Publikationen zu entnehmen, in denen die Transkripte ausführlich beschrieben wurden [2,4,5,8].

3.4 Thematik 4 - MDM2 als ein therapeutisches Target für WTS [2,149]

3.4.1 Etablierung eines WTS-Xenotransplantationsmodells in nu/nu-Ratten

Im Rahmen eines experimentellen *in-vivo*-Therapieansatzes sollten die Effekte einer MDM2-Antisense-Therapie studiert werden. Für die Etablierung eines Xenotransplantationsmodells für WTS in nu/nu-Ratten wurde die ATCC-Zelllinie RD verwendet. Zum Einen, weil diese Zelllinie nur ein mutiertes p53-Allel aufweist (das zweite Allel ist deletiert) und dadurch die Effekte einer MDM2-AS Therapie unabhängig von wt-p53-Protein untersucht werden konnten. Zum Anderen war durch Arbeiten aus unserer Arbeitsgruppe bekannt, dass diese Linie im Maus- und Rattenmodell reproduzierbar Tumoren bilden kann [85]. Diese Versuche wurden unter meiner maßgeblichen Mitarbeit an der Klinik für Allgemeinchirurgie der Universität Leipzig durchgeführt.

3.4.2 Behandlung von Xenotransplantattumoren mittels MDM2-AS Oligonukleotiden

In den Versuchsreihen wurde die Behandlung der Xenotransplantattumoren der STS-Zelllinie RD in den nachfolgenden Studien wie folgt durchgeführt: (i) es wurden verschiedene Konzentrationen des MDM2-AS2-ODNs eingesetzt und (ii) die Implantation der osmotischen Minipumpen erfolgte eine Woche nach der Xenotransplantation des Tumorgewebes. Es wurden insgesamt 24 Ratten behandelt, davon je 7 Tiere mit 100 µg bzw. 200 µg des MDM2-AS-ODN [67] und jeweils 5 Tiere als Kontrolle mit entweder 100 µg MDM2-SE-ODN oder physiologischer Kochsalzlösung. Die Behandlung der Tumoren mit MDM2-AS2-ODNs (100 bzw. 200 µg) führte zu einer signifikanten Reduzierung der Gesamttumormasse im Vergleich zu den mit Kochsalzlösung behandelten Tieren ($p = 0,016$ bzw. $p = 0,007$, Student t-Test). Nach 7 Tagen Behandlung mit 200 µg MDM2-AS-ODNs lag die durchschnittliche Tumormasse in dieser Gruppe bei 0,78 g ± 0,53 g (Abb. 3-8), dies entspricht etwa einer 71 %igen Verminderung im Vergleich zu der Kontrollgruppe, die mit physiologischer Kochsalzlösung behandelt worden ist (2,64 ± 1,33 g), und einer 56 %igen Reduzierung der Tumormasse im Vergleich zu der Gruppe, die mit MDM2-SE-ODNs behandelt wurde (1,74 ± 1,17 g). Die kontinuierliche intraperitoneale MDM2-AS-Behandlung inhibierte nicht nur die MDM2-Proteinexpression, sondern bewirkte auch einen Rückgang der mt-p53-Proteinmenge. Die erzielten Ergebnisse in dieser *in-vivo*-Studie zeigen somit das Potenzial einer Therapie mit MDM2-AS-ODNs unabhängig vom p53-Status des Tumors auf. Damit könnte MDM2 von allgemeiner Bedeutung für die Behandlung von Tumoren sein.

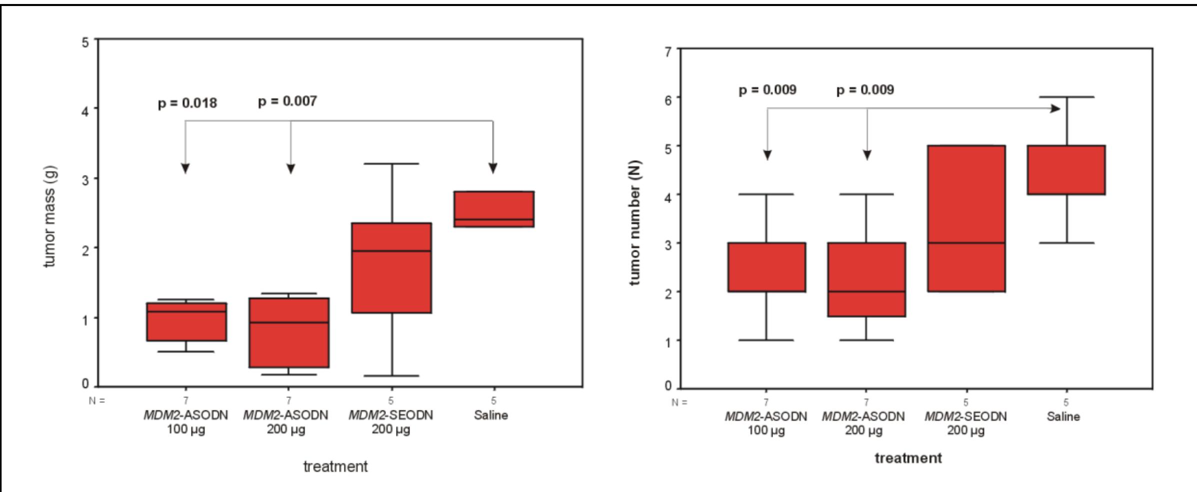

Abb. 3-8: Effekte der Behandlung von Xenografttumoren mit MDM2-AS-ODNs

Es ist deutlich die Reduzierung der Tumormasse (links) und der Tumorzahl (rechts) durch die MDM2-AS-Behandlung der Xenografttumoren erkennbar (Details s. Text). Die Abbildung ist der Publikation Würl *et al.*, 2002, [149] entnommen.

4. Diskussion

Das MDM2-Onkogen wurde ursprünglich in einer transformierten BALB/c-Mauszelllinie entdeckt, in der das Gen vielfach amplifiziert vorlag und das Protein überexprimiert wurde [14,32]. Das MDM2-Protein ist in der Lage, mit p53 Komplexe zu bilden und dadurch dessen Funktion als Transaktivator zu hemmen [56,90,97]. Andererseits wird die Expression von MDM2 durch p53 positiv reguliert. Damit sind das Onkogen MDM2 und der Tumorsuppressor p53 durch einen so genannten „autoregulatorischen" Feedback-Loop miteinander verbunden [105]. Ein weiterer negativer Regulator der p53-Aktivität ist das MDM2-Homologe MDMX [122,123]. MDMX kann sowohl an p53 als auch MDM2 binden. In einer Vielzahl von Tumorarten kommt es zu Störungen des Gleichgewichts zwischen MDM2, MDMX und p53 [137]. Eine Ursache können Mutationen des p53-Gens sein, wie dies in bis zu 50 % der Tumore der Fall ist [52]. Dies führt vielfach zur Expression eines funktionell inaktiven oder dominant negativen p53-Proteins. Andererseits ist das MDM2-Onkoprotein in vielen Tumorarten (u.a. in WTS) überexprimiert [38]. Dies ist bei einem Tumor mit wt-p53 einer Mutation des p53-Gens vergleichbar. Eine verstärkte Expression von MDM2 ist oftmals mit einer schlechten Prognose, insbesondere für WTS-Patienten, verbunden [23,150]. Die Ursache für die MDM2-Überexpression kann eine Amplifikation des MDM2-Gens sein. Oftmals kann dies aber auch auf verstärkte Transkription und/oder auf Transkripte mit einer unterschiedlichen Translationseffizienz zurückgeführt werden [1,72,73]. Ein weiterer Mechanismus, der zu einer veränderten MDM2-Expression in Tumoren beitragen kann, ist das Vorkommen von Spleißvarianten der MDM2-mRNA und verschiedener MDM2-Isoformen, wie es für verschiedene Tumorzelllinien aus Mausmodellen beschrieben worden ist [44,90,94,96]. Es war daher von Interesse zu prüfen, ob es in WTS (mesenchymalen Ursprungs) und Ovarialkarzinomen (epithelialen Ursprungs) zu Veränderungen im p53-MDM2-MDMX-Pathway kommt und ob diese eine prognostische Aussagekraft besitzen.

4.1 Molekulare Veränderungen des p53-MDM2-MDMX-Pathways und deren prognostische Bedeutung

Die Charakterisierung der Veränderungen des p53 –Pathways in WTS umfasste u.a. die Analyse des Amplifikationsstatus des MDM2- und des MDMX-Gens sowie die MDM2-Proteinexpression. Anschließend wurden die molekularen mit klinischen Daten korreliert, um zu prüfen, ob diese Einfluss auf den Krankheitsverlauf haben und eventuell als neue unabhängige Prognosefaktoren für WTS dienen können.

In unseren Untersuchungen konnte in 28 % (21 von 75) der untersuchten WTS-Proben eine Amplifikation des MDM2-Gens detektiert werden [4], wobei die meisten Proben eine 3 –

10-fache Amplifikation aufwiesen. Dieser Anteil ist etwas höher als die für WTS in einer MDM2-Amplifikationsdatenbank publizierten Werte [89]. Dort wird eine durchschnittliche Amplifikationsfrequenz von 20 % für WTS angegeben [23,37,71,91,92,96,101,102,107,113,140]. Cordon-Cardo *et al.*, die ein Kollektiv von 73 WTS-Patienten auf eine MDM2-Genamplifikation hin untersucht haben, fanden eine Rate von 15 % positiven Fällen [23], Florenes *et al.*, die insgesamt 97 WTS verschiedener Subtypen analysiert haben, berichten über eine Rate von 10 % [37]. Diese Unterschiede lassen sich möglicherweise auf die histologische Heterogenität und den differierenden Anteil der Tumoren in den einzelnen Malignitätsgraden der untersuchten WTS-Patientenkollektive zurückführen. Interessanterweise war die Amplifikation des MDM2-Gens mit einem geringen Tumorgrad verbunden. Während 58 % der Grad I Tumoren eine Amplifikation aufwiesen, so waren dies nur 15 % der WTS mit dem Tumorgrad II und III ($p = 0{,}001$, χ^2-Test). Ähnliche Befunde wurden Nakayama *et al.* [91] sowie von Nilbert *et al.* [92] beschrieben. Die beiden Arbeitsgruppen konnten in benignen Lipomen eine Amplifikationsfrequenz von 30 % nachweisen. In einer multivariaten Cox-Regression erwies sich die Amplifikation des MDM2-Gen als unabhängiger prognostischer Faktor für WTS (RR = 4,4, p = 0,17) [4].

Die Amplifikation des MDM2-Gens war in unseren Untersuchungen sehr stark mit einer hohen MDM2-mRNA-Expression verbunden ($p = 0{,}005$, χ^2-Test). In den von Florenes *et al.* untersuchten WTS wiesen alle Tumoren mit einem amplifizierten MDM2-Gen auch eine stark erhöhte MDM2-mRNA-Menge auf [37]. Da jedoch ebenfalls in Proben ohne Genamplifikation hohe mRNA-Level detektiert wurden, ist anzunehmen, dass noch weitere Mechanismen zu einer verstärkten MDM2-mRNA-Expression beitragen. In weiteren Arbeiten konnten wir zeigen, dass moderat erhöhte MDM2-mRNA-Mengen ebenfalls mit einer guten Prognose für WTS-Patienten korrelieren [135]. Der Befund, dass nachweisbare MDM2-mRNA-Mengen mit einer guten Prognose für die Patienten verbunden sind, konnte durch andere Arbeitsgruppen unabhängig voneinander an Ovarialkarzinomen [129] und Lungenkarzinomen [63] bestätigt werden. In diesen Arbeiten wurden jedoch keine Angaben zum Amplifikationsstatus des MDM2-Gens gemacht.

Im Gegensatz zu p53- und MDM2-Veränderungen gibt es bislang nur sehr wenige Daten in Bezug auf die MDMX-Genamplifikation und MDMX-mRNA-Expression in Tumoren [112,117,118]. Wir konnten erstmals zeigen, dass die *MDMX*-Genamplifikation und die MDMX-S-Überexpression unabhängige prognostische Bedeutung für WTS besitzen und mit einer schlechten Prognose für WTS-Patienten korrelieren. Unsere Daten deuten darauf hin, dass neben p53 und MDM2 auch MDMX einen Einfluss auf die Progression von WTS haben dürfte.

In dem von uns untersuchten WTS-Kollektiv war das MDMX-Gen in 27 % der Fälle amplifiziert. Riemenschneider *et al.* beschrieben, dass die MDMX-Genamplifikation und die

mRNA-Überexpression überwiegend in Glioblastomen gefunden wurde, die keine p53-Mutationen aufwiesen [117] und schlussfolgern daraus, dass die MDMX-Amplifikation ein neuer Mechanismus für die Entstehung maligner Gliome sein könnte, um sich der p53-kontrollierten Wachstumskontrolle zu entziehen. Dies konnte auch für unsere Patientenkollektive bestätigt werden: nur ein Tumor von 16 mit einer MDMX-Amplifikation wies auch eine p53-Mutation auf (eine intronische Mutation, F. Bartel, unveröffentlichte Daten). Daraus lässt sich schlussfolgern, dass die MDMX-Genamplifikation und/oder MDMX-Überexpression und p53-Mutationen unabhängige Mechanismen repräsentieren, um das p53-Protein in Tumoren zu inaktivieren.

Neben der MDMX-Genamplifikation haben wir auch die MDMX-mRNA-Expression in WTS untersucht. Wir konnten zeigen, dass ein Teil der Tumoren durch eine Überexpression der Spleißvariante MDMX-S charakterisiert ist, und dass dies mit einem 9-fach erhöhten Risiko, am Tumor zu versterben, korreliert. Weiterhin ist dies mit einer drastisch verkürzten Überlebenszeit für diese Patienten verbunden. Dies ist in Übereinstimmung mit Befunden von Riemenschneider und Mitarbeitern, die berichten, dass besonders hohe Mengen an MDMX-S im Vergleich zu FL-MDMX vor allem in Glioblastomen, jedoch nicht in anaplastischen Gliomen bzw. niedriggradigen Gliomen gefunden wurden [117]. Daten von Rallapalli *et al.* [110] und de Graaf *et al.* [25] zeigen, dass das verkürzte Protein MDMX-S sehr viel effektiver die p53-vermittelte Transaktivierung und die Apoptoseinduktion inhibieren kann als FL-MDMX. Es ist daher denkbar, dass die Aktivierung von *MDMX*-S zur Transformierung von Zellen beiträgt, indem es Wildtyp-p53 inaktiviert.

In Zusammenarbeit mit der Gruppe um Arnold J. Levine und Gareth Bond vom Institute of Advanced Studies, Princeton, USA, haben wir den Einfluss von SNPs im p53-Pathway auf die Tumorentstehung und den Krankheitsverlauf untersucht. Ein SNP im p53-sensitiven MDM2-P2-Promotor könnte das Gleichgewicht zwischen p53 und MDM2 stören und eine Schwächung der p53-Aktivität bewirken. Der identifizierte SNP309 (T>G) erhöht die Affinität des Transkriptionsfaktors Sp1 zum MDM2-Promotor, was eine erhöhte MDM2-Expression in Zellen mit einem G/G-Genotyp zur Folge hat [11]. Sowohl transformierte als auch nicht-transformierte Zellen, die homozygot für SNP309 (G/G) waren, zeigten eine deutlich abgeschwächte Antwort (Apoptose, Zellzyklusarrest) nach Behandlung mit DNA-schädigenden Agenzien im Vergleich zu SNP309-Wildtyp Zellen (T/T). Anschließend haben wir in unserem WTS-Kollektiv den Einfluss des SNP309 auf die Tumorentstehung und den Krankheitsverlauf untersucht. Es zeigte sich, dass Patienten mit dem G/G-Genotyp 12 Jahre früher an einem WTS erkrankten als Patienten mit dem T/T-Genotyp (Wildtyp). Die Allelverteilung in den Tumorproben entsprach der Verteilung wie sie auch in der Kontrollgruppe (gesunde Blutspender) gefunden wurde. Es scheint also keine Anreicherung des G-Allels in den Tumorproben zu geben. Neben der Korrelation mit einer früheren Tumorentstehung gab es

keine weitere Assoziation mit anderen klinischen Daten. Um die Frage zu klären, warum der SNP309 mit dem Alter bei Tumorentstehung korreliert, nicht jedoch mit einem generell erhöhten Risiko, am Tumor zu erkranken, sind vergleichende Studien mit höheren Fallzahlen notwendig. In Liposarkomen wiesen wir nach, dass das G-Allel ein so genanntes „rate-limiting event“ ist, d.h. dass die Zahl der zur Tumorentstehung notwendigen Alterationen (z.B. Mutationen) stark herabgesetzt ist [11]. Andererseits könnte es gewebespezifische Unterschiede in der Expression des Transkriptionsfaktors Sp1 geben, so dass der Effekt von SNP309 nicht in allen Geweben bzw. Tumorarten zum Tragen kommt.

Seit der erstmaligen Beschreibung des SNP309 konnte in zahlreichen anderen Tumorarten ein Zusammenhang mit einem früheren Auftreten des Tumors und auch mit einem erhöhten Risiko, am Tumor zu erkranken, gezeigt werden, u.a. bei B-Zell CLL [43], Mammakarzinom [156], Kolorektalkarzinom [84] und dem Magenkarzinom [95]. Demgegenüber konnte bei Basalzellkarzinomen [147], Lungenkarzinomen [108] sowie dem Prostatakarzinom [127] kein Einfluss des SNP309 auf die Tumorentstehung nachgewiesen werden. Die Ursache dafür könnte darin liegen, dass es neben dem SNP309 noch andere Polymorphismen gibt, die die Funktionalität von p53 beeinflussen. Ein weiterer Aspekt ist die Geschlechtsspezifität von SNP309. Bei WTS und dem Kolonkarzinom kommt es insbesondere bei Frauen, nicht jedoch bei Männern, zu einer beschleunigten Tumorentstehung bei Patienten mit dem G/G-Genotyp im Vergleich zum T/T-Genotyp [12]. Dies lässt den Schluss zu, dass das G-Allel bei einem intakten Östrogenrezeptor-Signalweg den p53-Pathway negativ beeinflusst.

In weiteren Arbeiten haben wir die Expression der promotorspezifischen Transkripte (P1, P2) der MDM2-mRNA und deren Einfluss auf die Tumorentstehung und Prognose von WTS-Patienten untersucht. Wir konnten erstmalig zeigen, dass eine hohe Expression des P1-Transkripts, nicht jedoch des P2-Transkripts, mit einem früheren Auftreten eines WTS verbunden ist, und dass eine geringe Expression von P1 und P2 mit einem stark verkürzten Gesamtüberleben assoziiert ist [133]. Diese Ergebnisse deuten darauf hin, dass MDM2 in Bezug auf Tumorenstehung und Krankheitsverlauf unterschiedlich wirken kann. Die Expression von p53 und MDM2 oszilliert bei der zellulären Stressantwort, wobei die der Anstieg bzw. Abfall der MDM2-Menge der von p53 zeitverzögert folgt [75]. Hu *et al.* fanden, dass geringste Änderungen der MDM2-Expression (erhöhte oder verminderte Expression) zu Störungen der Oszillation und somit zu einer Inaktivierung des p53-Pathways führen [54]. Des Weiteren gibt es Unterschiede in der Translationseffizienz der beiden Transkripte [13,20]. Laut Cheng und Cohen wird vom P2-Transkript hauptsächlich das $p90^{MDM2}$ translatiert, während vom P1-Transkript sowohl das $p90^{MDM2}$- als auch das verkürzte $p75^{MDM2}$-Protein gebildet wird. Die Autoren zeigten, dass $p90^{MDM2}$ am p53-MDM2-Feedback-Loop beteiligt ist, nicht jedoch $p75^{MDM2}$. Aktuelle Daten von Mendrysa *et al.* deuten darauf hin, dass bereits kleine Änderungen der MDM2-Expression einen starken Effekt auf die Bildung von Tumoren haben können.

Es wurde in $APC^{min/+}$-Mäusen (diese Mäuse weisen einen Defekt im *adenomus polyposis coli*-Gen auf und entwickeln spontan Adenome im Darm) untersucht, ob die erhöhte p53-Aktivität infolge einer verminderten MDM2-Expression die Bildung solider Tumoren verhindern kann. Während Mäuse mit einer normalen MDM2-Expression nach 5 Monaten 48 Adenome aufwiesen, so waren dies bei Mäusen mit einer um 20 % reduzierten MDM2-Expression nur 16 Adenome. Bei Mäusen mit ~30 % der normalen MDM2-Expression entwickelten noch weniger Tumoren [83]. Dies bestätigt die Daten aus unseren Studien, wonach eine erhöhte MDM2-Expression mit einer beschleunigten Tumorentstehung assoziiert ist [133].

Neben den Weichteilsarkomen untersuchten wir in unserer Arbeiten auch Veränderungen des p53-Pathways in Ovarialkarzinomen, u.a. den p53-Mutationsstatus, dessen Proteinexpression sowie den im p53-sensitiven MDM2-P2-Promotor liegenden SNP309 in einer Gruppe von 107 Ovarialkarzinomen. In 39 % (42 von 107) der Tumoren fanden wir p53-Mutationen, die eine Veränderung der Aminosäuresequenz bewirken. Dieser Prozentsatz ist nahe dem Bereich von 40-80 %, welcher in der Literatur für Ovarialkarzinome beschrieben wird [34]. Eine positive p53-Immunfärbung von 51 % liegt in dem publizierten Bereich von 29-62 % für FIGO I-IV Patientinnen [28,41,47,51,62,76,119,139].

Unsere Ergebnisse zeigen deutlich, dass die Immunhistochemie kein sicherer Marker für den p53-Genstatus ist. Wir fanden, dass die Hälfte der Fälle mit einem Wildtyp p53-Gen auch positiv für die p53-Immunfärbung waren, ein Prozentsatz der über dem liegt, was bisher von anderen Arbeitsgruppen veröffentlicht wurde [50,116]. Havrilesky *et al.* fanden in 28 % [50] und Reles *et al.* [116] in 38 % der von ihnen untersuchten Tumore eine Überexpression des p53-Proteins bei Vorhandenseins eines Wildtyp-Gens. Wildtyp-p53 ist normalerweise sehr instabil und ist in der Zelle mittels Immunhistochemie nicht nachweisbar [77]. Unter diesem Gesichtspunkt ist es überraschend, dass es sich bei 90% der Wildtyp-p53 überexpremierenden Tumore um high-grade Karzinome handelt. Die Ursache für diese unnormale Stabilität ist bisher unbekannt. Es ist möglich, dass es Veränderungen in der Funktionalität von p53-interagierenden Proteinen, wie MDM2 oder MDMX, gibt [155]. Weitere Studien sind nötig, um die Gründe für diesen Befund zu klären, besonders unter dem Aspekt, dass in unserer Studie 82 % dieser Tumoren ein Rezidiv bildeten, während dies nur 52 % der Patientinnen mit normalem p53 (Wildtyp-Gen, keine Expression) der Fall war.

In der Literatur gibt es Hinweise, dass eher die Überexpression von p53 [119,139] als Mutationen [33,70] mit einem verkürzten Gesamtüberleben korrelieren. Es muss jedoch hinzugefügt werden, dass in diesen Studien der p53-Mutations- und Proteinexpressionsstatus separat betrachtet wurden. Unsere Ergebnisse zeigen, dass eine Immunfärbung von >10 % der Tumorzellen mit einem verringertem Gesamtüberleben korreliert ($p = 0,0065$) und, dass das Auftreten von p53-Mutationen ($p = 0,86$) allein keinen Einfluss auf das Überleben hat. Wir

konnten dagegen zeigen, dass Patientinnen mit p53-Veränderungen generell eine schlechtere Prognose (p = 0,047) und eine verkürzte Gesamtüberlebenszeit (p = 0,05) hatten, im Vergleich zu Patientinnen mit normalem p53. Bei den Patientinnen mit verändertem p53 haben überraschender Weise die Frauen mit Wildtyp-p53-Überexpression die kürzeste Überlebenszeit. Da bisher nur wenige Studien [17,50,116] sowohl den p53-Genstatus als auch die Proteinexpression untersucht haben, wurde der prognostische Wert einer Überexpression von Wildtyp-p53 bisher unterschätzt. Weitere Studien sind notwendig um die Mechanismen der Wildtyp-p53-Überexpression zu klären, weil dies Einblicke in die Mechanismen der Resistenzentwicklung verspricht. Zusätzlich unterstreicht diese Studie die Bedeutung der p53-Funktionalität. Dies erscheint wichtiger als die getrennte Untersuchung von p53-Mutation oder Proteinexpression.

Zusätzlich zum p53-Status haben wir auch den SNP309 im p53-sensitiven P2-Promotor des MDM2-Gens untersucht und dabei gefunden, dass das G-Allel des SNP309 mit einem niedrigen Erkrankungsalter bei Frauen, aber nicht bei Männern, mit Weichteilsarkomen verbunden ist [12]. In unseren Studien an Ovarialkarzinomen konnten wir nachweisen, dass das G-Allel des SNP309 nur bei Tumoren mit einer sehr starken ER-Expression mit einem niedrigen Diagnosealter (8 Jahre früher; p = 0,048) verbunden ist [3]. Wenn das Niveau der ER-Expression in Ovarialkarzinomen nicht berücksichtigt wird, findet sich bei den verschiedenen SNP309-Genotypen kein Unterschied im Erkrankungsalter, wie es bereits vor kurzem publiziert wurde [15]. Hervorzuheben ist außerdem, dass das G-Allel des SNP309—das aufgrund zahlreicher Befunde mit einem geschwächten p53-Pathway assoziiert ist—ebenso wie ein mutiertes p53-Gen bei Patientinnen im FIGO III-Stadium mit einem längeren Gesamtüberleben korreliert. Dies zeigt die Komplexität der Wechselwirkung und den unterschiedlichen Einfluss von p53 und MDM2 auf die Entstehung und die Progression von Tumoren im Allgemeinen und Ovarialkarzinomen im Speziellen.

Alterationen des MDMX-Gens und deren Einfluss auf die Tumorentstehung und -progression sind in Ovarialkarzinomen bislang nicht untersucht worden. In einer kürzlich publizierten Studie von Reincke *et al.* über Sequenzveränderungen von MDMX in 43 Fällen von familiärem Brustkrebs zeigte deutlich, dass aktivierende Mutationen von MDMX sehr selten auftreten [115]. Nur 3 der 43 Patientinnen wiesen MDMX-Mutationen auf. Dies bestätigen auch erste Daten zum Mutationsstatus des MDMX-Gens bei Ovarialkarzinompatientinnen. Bei 3 von 102 untersuchen Patientinnen wurde eine Mutation detektiert (Bartel *et al.,* unveröffentlichte Daten). Wir konnten im Rahmen dieser Arbeit erstmals zeigen, dass der SNP im 3'-untranslatierten Bereich des MDMX-Gens (SNP34091) mit einem stark verkürzten Gesamtüberleben und einem kürzeren rezidivfreien Überleben korreliert. Das A-Allel von SNP34091 ist mit einer erhöhten MDMX-mRNA- und –Proteinexpression assoziiert, und dies führt in der Folge zu einer Inaktivierung des p53-Pathways. Der negative Effekt des A/A-

Genotyps auf das rezidivfreie und das Gesamtüberleben wurde von uns nur in den ER-negativen Fällen beobachtet. Der SNP im MDMX-Gen war jedoch nicht mit dem Alter der Tumorentstehung verknüpft. Daraus lässt sich ableiten, dass MDMX eher mit dem klinischen Verlauf und der Chemotherapieresistenz assoziiert ist als mit der Tumorentstehung. Im Gegensatz zum MDM2-Gen weist das MDMX-Gen keine Bindungsstellen für den Östrogen-Rezeptor auf. Daher kann die MDMX-Expression auch nicht durch den ER werden, wie dies für MDM2 in MCF-7-Zellen nachgewiesen werden konnte [103]. In unserem WTS-Kollektiv konnten wir eindrucksvoll zeigen, dass der Effekt des G-Allel von SNP309 durch einen intakten ER-Signalweg verstärkt wird [3,12]. Wir vermuten daher, dass der Effekt des SNP309 (also erhöhte MDM2-Mengen) vor allem in ER-positiven Tumoren (Geweben) eine Rolle spielt, und so den Effekt von SNP34091 (d.h. die Inaktivierung des p53-Pathways durch erhöhte MDMX-Mengen) in diesen Tumoren “überlagert“, während in ER-negativen Tumoren (Geweben) der Effekt des SNP34091 stärker ist.

Der ER-Status und das Risiko an einem Ovarialkarzinom zu erkranken ist auch im Zusammenhang mit der menopausalen Hormontherapie (MHT) von Bedeutung. Frauen in den Wechseljahren werden entweder mit Östrogen allein oder einer Kombination aus Östrogen und Progestin behandelt, um die Beschwerden wie beispielsweise Hitzewallungen zu mindern. Ob durch die MHT das Risiko steigt, an einem Ovarialkarzinom zu erkranken, ist bislang jedoch nur unzureichend untersucht worden. Eine aktuelle Meta-Analyse nach Auswertung von 42 Studien von Greiser *et al.* [42] kommt zu dem Schluss, dass das Risiko bei einer Behandlung mit Östrogen um das 1,28-fache ansteigt, wohingegen das Risiko, an einem Mammakarzinom infolge der MHT zu erkranken, nicht erhöht ist [55]. Unsere Daten belegen eindeutig einen Zusammenhang zwischen einer hohen ER-Expression, dem SNP309 und einem früheren Auftreten eines Ovarialkarzinoms. Es ist denkbar, dass bei Patientinnen, die eine MHT erhalten und heterozygot bzw. homozygot für den SNP309 sind (T/G bzw. G/G-Genotyp), ein höheres Risiko besitzen, infolge der MHT ein Ovarialkarzinom zu entwickeln. Es wäre daher interessant, in den im Review von Greiser *et al.* [42] beschriebenen Patientenkollektiven retrospektiv die Allelfrequenz des SNP309 zu bestimmen, um einen möglichen Zusammenhang zwischen der Gabe von Östrogen und dem SNP309-Status zu ermitteln.

Eine mögliche Erklärung für die erhöhten MDMX-mRNA und –Proteinmengen bei Patientinnen mit dem A/A-Genotyp könnte die unterschiedliche Bindung von Faktoren sein, die die mRNA-Stabilität und /oder die Translationseffizienz regulieren. Bei der Analyse des 3‘-UTR der MDMX-mRNA (http://mirna.imbb.forth.gr/microinspector/) stellte sich heraus, dass durch das C-Allel eine Bindungsstelle für die microRNAs hsa-miR191 und hsa-miR191* entsteht. Diese microRNAs binden jedoch nicht an das A-Allel von SNP34091. Daraus lässt sich ein Modell ableiten, in dem die MDMX-Expression in Individuen mit dem A/C und C/C-Genotyp von SNP34091 durch microRNAs beeinflusst wird. In aktuellen Arbeiten in Koope-

ration mit der Arbeitsgruppe um Jean-Christoph Marine vom IMB in Gent (Belgien) untersuchen wir den genauen Mechanismus der Regulation der MDMX-Expression durch hsa-miR191 und hsa-miR191* und den Einfluss auf die Funktionalität des p53-Pathways. Erste Ergebnisse zeigen, dass die Überexpression von hsa-miR191 in Zellen mit dem C/C-Genotyp (Efo-21) nicht jedoch in Zellen mit dem A/A-Genotyp (OAW-42) zu einer Reduzierung der MDMX-Proteinmenge kommt. Da die mRNA-Menge unverändert bleibt, beruht die Wirkung der microRNA hsa-miR191 auf einer Blockierung der Translation und nicht auf der Destabilisierung der mRNA.

Zusammenfassend lässt sich festhalten, dass der A/A-Genotyp von SNP34091 ein unabhängiger Prognosefaktor für Ovarialkarzinome ist und mit einem stark verkürzten rezidivfreien und Gesamtüberleben assoziiert ist. Das A-Allel korreliert mit einer erhöhten MDMX-Expression, was zu einer Inaktivierung des p53-Pathways in den betroffenen Tumoren führt. Obwohl der genaue Mechanismus noch nicht geklärt ist, deuten unsere Daten auf allelspezifische Unterschiede bei der Chemoresistenz bei Ovarialkarzinomen hin. Damit könnte MDMX ein vielversprechendes Zielprotein sein, um die Tumoren gegenüber einer Chemotherapie zu sensitivieren.

4.2 Expression von Spleißvarianten der MDM2-mRNA

Zu Beginn der Arbeiten wurden von Sigalas *et al.* insgesamt fünf verkürzte MDM2-mRNA-Transkripte in Blasen- und Ovarialkarzinomen nachgewiesen [124]. In 50 % der Ovarialkarzinomproben wurden alternativ gespleißte Transkripte detektiert. Für die von uns untersuchten WTS konnte ein ähnlich hoher Anteil ermittelt werden.

In unseren Studien korrelierte die Expression der Spleißformen mit dem Tumorgrad. Während nur 30 % der WTS mit Grad I verkürzte MDM2-Transkripte exprimierten, so betrug der Anteil bei den Malignitätsgrade II und III über 60 % [4]. Obwohl das Vorkommen der Spleißformen mit einem höheren Malignitätsgrad einhergeht, zeigte sich jedoch kein Einfluss auf das Überleben der Patienten. Vergleicht man unsere Ergebnisse mit Daten aus der Literatur, so bestätigt sich, dass MDM2-Spleißformen bevorzugt in Tumoren mit einem aggressiveren Phänotyp exprimiert werden. So wurden in 80 % der „high-grade“ Ovarialkarzinome verkürzte Transkripte gefunden, während nur 37 % der „low-grade“ Ovarialkarzinome davon betroffen waren [124]. Des Weiteren sind in 58 % der Tumoren im Stadium III und IV, aber nur in 22 % der Tumoren im Stadium I und II Spleißformen der MDM2-mRNA detektiert worden [124]. Von den ebenfalls von Sigalas *et al.* untersuchten Blasenkarzinomen exprimierten 57 % der invasiv wachsenden Tumoren, aber nur 6 % der oberflächlichen Tumoren Spleißformen. In der Untersuchung von Lukas *et al.* exprimierten von 38 Mammakarzinomen

13 Proben verkürzte Transkripte zusätzlich zur FL-Form [78]. Es zeigte sich kein Einfluss auf das Überleben der Patienten. Weitere Studien befassten sich mit Alterationen der MDM2-mRNA-Expression u.a. in Liposarkomen [128], Glioblastomen [81], nicht kleinzelligen Lungenkarzinomen [30] sowie Riesenzelltumoren des Knochens [31]. Übereinstimmend wurde ein Zusammenhang zwischen der Expression von MDM2-Spleißvarianten und einem erhöhten Malignitätsgrad gefunden [30,31,81,128]. Unsere Ergebnisse zeigen hingegen keinen eindeutigen Zusammenhang zwischen alternativem bzw. aberrantem Spleißen und einem aggressiverem Phänotyp. Sie deuten vielmehr darauf hin, dass auch die Expression von mRNA-Transkripten, die nicht an regulären Donor-/Akzeptorstellen gespleißt werden, der „normalen" physiologischen Situation entsprechen. Die von uns in Normalgewebe von Patienten und in peripheren Blutlymphozyten gesunder Spender nachgewiesenen Spleißvarianten MDM2-N sowie MDM2-BL sind beispielsweise an Sequenzen gespleißt worden, die innerhalb bekannter Exone lokalisiert sind [5].

Unsere Ergebnisse und die Analyse der Sequenzmotive zeigten, dass das aberrante Spleißen möglicherweise auf Alterationen des Spleißapparates zurückzuführen ist. Die von uns identifizierten Sequenzmotive (z.B. 5'-TG**C**CCAGTAT-3') beinhalten potenzielle Donor- und Akzeptorstellen. In einer Reihe von Genen (z.B. BRCA1, RB1, NF1) befinden sich in den Introns, aber auch in den Exons, eine Vielzahl von potenziellen Spleißstellen und Bindungsstellen für das heterogene nukleäre Ribonukleoprotein A1 (hnRNP A1), einer Komponente des Spleißapparates [10]. Es konnte gezeigt werden, dass schon geringe Änderungen der Affinität von hnRNP A1 zu den regulären Bindungsstellen zu einem fehlerhaften Ausschneiden von Exons führen können [10]. So kann beispielsweise durch Mutationen die Sekundärstruktur der mRNA beeinflusst werden, wodurch dann das hnRNP A1-Protein an alternative Sequenzen bindet. Die Folge könnte das alternative und/oder aberrante Spleißen der unreifen mRNA sein. Ein weiterer Aspekt ist der Einfluss von DNA-Schäden auf das Spleißverhalten von MDM2 (und MDMX). Chandler *et al.* zeigten, dass es nach Behandlung von Zellen mit ionisierender Strahlung nicht zu einer Änderung der Expression von MDM2-Spleißvarianten kommt. Werden die Zellen jedoch mit Cisplatin behandelt, so geht der Anteil des FL-MDM2 zurück, und es werden verstärkt verkürzte Transkripte der MDM2-mRNA expremiert [18]. Interessant sind in diesem Zusammenhang Arbeiten von Filipov *et al.*, wonach das Spleißverhalten der CD44-mRNA durch den ATM/ATR-Pathway reguliert wird [35]. Dies ließ sich auf die veränderte Expression von wichtigen Spleißfaktoren zurückführen. Aktuelle Arbeiten unserer Arbeitsgruppe beschäftigen sich u.a. mit der Fragestellung, wie das Spleißverhalten der MDM2/-X-mRNA in Ovarialkarzinomzelllinien reguliert wird und ob es einen Zusammenhang zwischen der Expression bestimmter Varianten (z.B. MDMX-S) und der Chemoresistenz gibt. In Vorversuchen unserer Arbeitsgruppe stelle sich heraus, dass es in den untersuchten Zelllinien (OAW-42, ES-2) unabhängig vom p53-Status zu einer Ände-

rung des Verhältnisses FL-MDMX/MDMX-S zugunsten von MDMX-S kommt (Wolf *et al.*, unveröffentlichte Daten). Da die MDMX-S-Isoform die p53-Aktivität wesentlich stärker inhibieren kann als MDMX selbst [110], könnte die in p53-wt-Zelllinien zu einem schlechteren Ansprechen der Zellen auf die Chemotherapie führen und somit zur Resistenz beitragen.

4.3 MDM2 als Ziel einer Gentherapie für WTS

In dieser Arbeit wurde erstmalig die Wirkung von MDM2-AS-ODNs auf WTS *in vivo* untersucht. Die Grundlage unseres Modells sind abdominale und intraperitoneale WTS, die durch konventionelle Therapieformen (chirurgische Eingriffe, Strahlen- und Chemotherapie) schwierig zu behandeln sind. Die peritoneale Tumoraussaat hat einen limitierenden Einfluss auf die Prognose. Daher ist es notwendig, alternative Therapiekonzepte, wie z.B. Antisense-Therapien gegen das MDM2-Onkogen, für diese WTS zu entwickeln. Unsere Ergebnisse

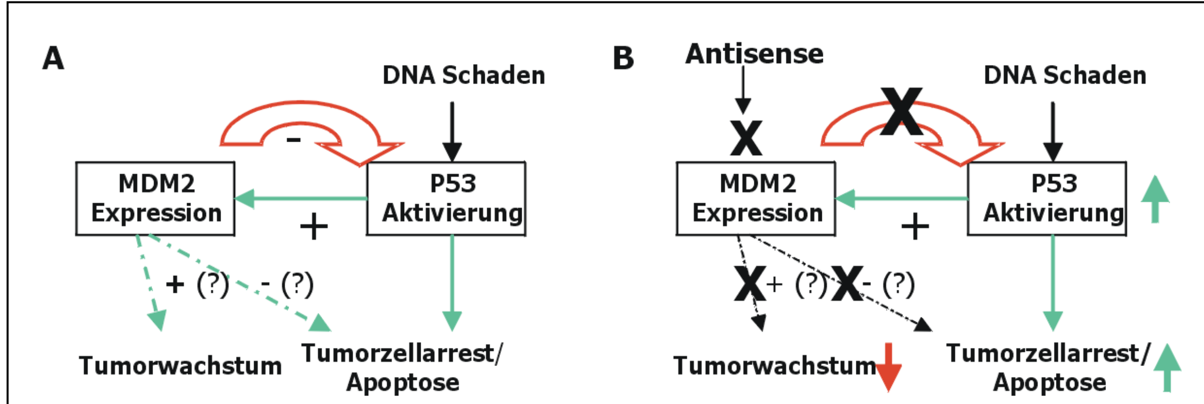

Abb. 4-1: Die MDM2-p53-Interaktion als Ziel einer therapeutischen Intervention

A. MDM2 ist ein negativer Regulator der p53-Funktion. p53 induziert die MDM2-Expression, z.B. nach einem DNA-Schaden. MDM2 wiederum inhibiert die Aktivitäten von p53 durch Komplexbildung und den Abbau von p53. Des Weiteren kann eine Überexpression von MDM2 unabhängig von p53 zur Progression des Zellzyklus, und damit zum Tumorwachstum, führen. **B.** Der postulierte Mechanismus einer MDM2-AS-Therapie. Die Inhibierung der MDM2-Expression führt zu einem Anstieg an freiem, und damit funktionellem p53 in der Zelle. Durch die p53-Aktivität kommt es zur Auslösung der Apoptose oder zum Zellzyklusarrest. Es wird weiterhin angenommen, dass durch die Reduzierung der MDM2-Proteinmenge in der Zelle die p53-unabhängigen Funktionen von MDM2 in Bezug auf das Tumorwachstum inhibiert werden.

zeigen die prinzipielle Eignung von MDM2 als Ziel einer molekular-basierten Therapie mittels Hemmung durch AS-ODNs auch in Tumoren mit mutiertem p53-Gen. Alle bisher in der Literatur beschriebenen *in vivo* Studien mit MDM2-AS-ODNs wurden mit Xenotransplantaten von Zelllinien durchgeführt, die ein wt-p53-Gen trugen. In unserer Arbeit ist erstmalig eine Zelllinie (RD) mit einem mutierten *p53*-Genstatus für die Untersuchungen verwendet worden.

Erste Studien zur Behandlung von WTS mittels MDM2-AS-ODNs wurden von Meye *et al.* an der MDM2-überexprimierenden Zelllinie US8-93 durchgeführt [86]. Es konnte gezeigt werden, dass die als Lipidkomplexe applizierten MDM2-AS-ODNs sowohl die MDM2-

Proteinexpression reduzieren als auch das klonogene Überleben der WTS-Zelllinie stark hemmen. In unseren Untersuchungen konnten wir in den Tieren, die mit verschiedenen Dosen an MDM2-AS-ONDs behandelt wurden, eine signifikante Reduzierung der Tumormasse und erstmals auch der Tumorzahl nachweisen. Dies stimmt mit Ergebnissen anderer Autoren überein, die ebenfalls über eine Verringerung der Tumorgröße, ein verzögertes Tumorwachstum und eine längere Lebensdauer von Mäusen berichten, die mit MDM2-AS-ONDs behandelt wurden [138,141].

Der Mechanismus für die antitumorale Wirkung der MDM2-AS-ODNs in Folge der Reduzierung der MDM2-Proteinmenge konnte ist in Abb. 4-1 dargestellt. In vielen Tumoren mit einem wt-p53-Gen führt die Verringerung von MDM2 zu einer Erhöhung der p53-Proteinmenge und anschließend zu Wachstumsarrest und Apoptose [19]. Die Annahme, dass MDM2-AS-ODNs nur in Zellen mit wt-p53 wirksam sind, konnte bislang nur für B-Zell-Lymphom-Zelllinien [16], jedoch nicht für andere Zellen [159], bestätigt werden. Studien von Chen *et al.* [19] an drei Zelllinien mit wt-p53-Genstatus zeigen wiederum, dass es nach Behandlung MDM2-AS-ODNs neben einer deutlichen, dosisabhängigen Reduzierung der MDM2-Proteinmenge zusätzlich zu einer 6-fachen Steigerung der p21-Expression kommt (jedoch nicht in einer als Kontrolle mitgeführten p53-defizienten Zelllinie). Dies deutet eindeutig auf eine durch die MDM2-AS-Behandlung hervorgerufene p53-Transaktivierung hin. Auf Grund der Reduzierung der MDM2-Proteinmenge steigt der Anteil an freiem (und somit funktionellem) p53-Protein, da dieses nicht mehr durch MDM2 komplexiert und zur Ubiquitinylierung markiert werden kann. Chen *et al.* [19] beobachteten in den behandelten Zellen auch eine Steigerung der Apoptoserate auf bis zu 80 % nach 24 h, die durch die Aktivierung von p53 erklärt werden kann.

Es ist jedoch auch denkbar, dass die Behandlung von Tumorzellen mit MDM2-AS-ONDs nicht nur den p53-MDM2-Feedback-Loop stört (Abb. 4-1), sondern auch die p53-unabhängigen, wachstumsfördernden Aktivitäten von MDM2 *in vivo* inhibiert. Aus der Literatur ist bekannt, dass MDM2 auch unabhängig vom p53-Genstatus onkogene Eigenschaften besitzt [23,27,59,79,80,114,124,143,150,154]. Die Ergebnisse dieser Arbeit zeigen, dass durch die Behandlung von WTS-Xenotransplantattumoren mit MDM2-AS-ODNs auch ohne funktionelles p53 die Tumormasse und die Anzahl der gebildeten Tumoren reduziert werden kann.

Überraschend war der Befund, dass es zu einer signifikanten Reduzierung der p53-Proteinexpression in den mit AS-ODNs behandelten Tieren kam. Es ist bekannt, dass MDM2 an wt- und mt-p53 binden kann [38], d.h. dass eine Reduzierung der MDM2-Proteinmenge in Folge einer Behandlung mit MDM2-AS-ODNs zu einer Stabilisierung von p53 führen könnte. Eine dieser beschriebenen Zelllinien trägt die gleiche Mutation im p53-Gen wie die Zelllinie RD, die in unseren *in vivo* Therapiestudien verwendet worden ist. In den von uns beschrie-

benen Experimenten wurde erstmalig eine Reduzierung der mt-p53-Proteinmenge auf Grund einer verringerten MDM2-Expression nachgewiesen [149]. Dies deutet darauf hin, dass mt-p53 durch MDM2 stabilisiert wird; d.h. MDM2 kann zwar an p53 binden, es jedoch nicht für den proteasomalen Abbau markieren. Für die Therapie von Tumorpatienten wäre die Reduzierung der mt-p53-Proteinmenge von großer Bedeutung, da etwa 50 % der Tumoren Mutationen im p53-Gen tragen [52], und Mutationen zu einer Stabilisierung von p53 beitragen können [157]. Unsere Ergebnisse zeigen in einem WTS-Xenotransplantatmodell, dass eine Reduzierung der Tumormasse und der Tumoranzahl durch MDM2-AS-ODNs unabhängig vom p53-Genstatus möglich ist. Dadurch wird die Hypothese unterstützt, dass eine MDM2-AS-ODN-Therapie in allen Tumoren, die durch eine MDM2-Überexpression charakterisiert sind, erfolgreich ist [159].

4.4 Zusammenfassung und Ausblick

Den Schwerpunkt der vorliegenden Arbeit bildete die Aufklärung der Mechanismen, die zur Fehlregulation des p53-MDM2-MDMX-Pathways in zwei völlig unterschiedlichen Tumorarten, den Weichteilsarkomen und den Ovarialkarzinomen, führen. Dies umfasste die Analyse von Alterationen wie z.B. Mutationen und Polymorphismen, Genamplifikationen, Überexpression der mRNA bzw. des Proteins sowie die Expression von Spleißvarianten und die Frage, ob diese Veränderungen können mit dem Krankheitsverlauf bzw. dem individuellen Todesrisiko betroffener Patienten korrelieren.

In 28 % der von uns untersuchten Weichteilsarkomproben konnte ein amplifiziertes MDM2-Gen nachgewiesen werden. Dies war mit einer erhöhten Expression der MDM2-mRNA assoziiert. Interessanterweise korrelierten sowohl die MDM2-Genamplifiation als auch eine leicht erhöhte MDM2-mRNA-Expression mit einer guten Prognose bei Weichteilsarkompatienten. Patienten ohne MDM2-Genamplifikation wiesen ein 4,5-fach erhöhtes Risiko auf, am Tumor zu versterben. Demgegenüber war jedoch für Patienten, deren Tumor durch eine MDMX-Genamplifikation charakterisiert war, das relative Risiko um das 2.8-Fache erhöht. Das MDMX-Gen war in 27 % der Weichteilsarkome amplifiziert. Neben dem MDMX-Genamplifikationsstatus wurde auch die Expression der „full-length“-MDMX-mRNA und der MDMX-S-Spleißvariante untersucht. Es stellte sich heraus, dass das Verhältnis „full-length“ MDMX/MDMX-S einen unabhängigen Prognosefaktor für Weichteilsarkome darstellt. Patienten, deren Tumor eine Überexpression der MDMX-S-Spleißvariante aufwiesen, verstarben durchschnittlich 13 Monate nach Diagnosestellung, während Patienten, bei denen das Verhältnis zu Gunsten der FL-MDMX-mRNA verschoben war, 53 Monate überlebten.

Bei der Sequenzanalyse des p53-sensitiven MDM2-P2-Promotors, die in Zusammenarbeit mit A.J. Levine und G.L. Bond (Princeton, USA) durchgeführt wurde, konnte ein Einzelnukleotidpolymophismus (T>G; SNP309) identifiziert werden. Das G-Allel war in Weichteilsarkompatienten mit einem 12 Jahre früheren Tumorauftreten assoziiert. Weitere Untersuchungen zeigten, dass das G-Allel die Tumorentwicklung vor allem in premenopausalen Frauen, nicht jedoch bei Männern, beschleunigen konnte. Bei den Ovarialkarzinomen konnte die Assoziation des G-Allels mit einem früheren Alter der Tumorentstehung nur bei einer Überexpression des Östrogen-Rezeptors beobachtet werden. Diese Daten stützen die Hypothese, dass Geschlechtshormone (hier: Östrogen), die Entwicklung von Tumoren bei Individuen mit einem G/G-Genotyp beschleunigen können. Bei einem SNP im 3'-UTR des MDMX-Gens zeigte sich ebenfalls eine Abhängigkeit von Östrogen-Rezeptor. Ausgehend von diesen Daten sollten sich prospektive Studien anschließen, in denen die hier beschriebenen Prognosefaktoren evaluiert werden. Dazu zählen der Status des SNP309, die Expression der MDMX-S-Spleißvariante sowie der Transkripte des MDM2-P1- bzw. –P2-Promotors.

Bei den von uns untersuchten Ovarialkarzinomen konnten wir ebenfalls zahlreiche Veränderungen des p53-MDM2-MDMX-Pathways detektieren, die einen Einfluss auf das rezidiv-freie und Gesamtüberleben der Patientinnen haben. Interessant war der Befund, dass Patientinnen, deren Tumor das Wildtyp-p53-Protein überexpremierte, die kürzeste Überlebenszeit aufwiesen. Dies zeigt deutlich, dass die isolierte Betrachtung des p53-Gen- bzw. Proteinexpressionsstatus nicht aussagekräftig ist. Somit lässt sich auch erklären, warum speziell beim Ovarialkarzinom sehr widersprüchliche Daten zur prognostischen Bedeutung des p53-Genstatus in der Literatur zu finden sind. Es sollten daher in Zukunft—wie in dieser Arbeit geschehen—der kombinierte p53-Gen- und Proteinexpressionsstatus ermittelt werden, um die Gruppe der Patientinnen zu identifizieren, die durch eine Überexpression von wt-p53 charakterisiert sind. Des Weiteren zeigen unsere Arbeiten die Bedeutung der Östrogenrezeptorexpression beim Ovarialkarzinom. Obwohl der ER-Status selbst keine prognostische Aussagekraft besitzt, jedoch war das G-Allel des SNP309 bei Patientinnen mit einem ER-positiven Tumor mit einem signifikant früheren Auftreten des Ovarialkarzinoms verbunden. Wie bereits für die WTS beschrieben, müssen sich auch beim Ovarialkarzinom prospektive Studien anschließen, um die Aussagekraft der genannten Prognosefaktoren zu untersuchen. Dabei sollten folgende Faktoren bestimmt werden: Genotyp des SNP309 im MDM2-Gens und des SNP34091 im MDMX-Gen, der p53-Gen- und Expressionstatus sowie der ER-Status der Ovarialkarzinome.

In weiterführenden Arbeiten werden wir die Regulation des Spleißmusters der MDM2/MDMX-mRNA nach Behandlung mit Carboplatin und einen möglichen Zusammenhang der Expression bestimmter Spleißvarianten mit der Chemoresistenz beim Ovarialkarzi-

nom untersuchen. Des Weiteren konnten wir in ersten Versuchen zeigen, dass das C-Allel des SNP34091 im MDMX-Gen eine Bindungsstelle für die microRNA hsa-miR191 bildet und dass die Überexpression von miR191 in Zellen mit einem C/C-Genotyp zu einer Verminderung der MDMX-Proteinmenge führt. Ziel der Versuche ist es daher nun zu untersuchen, welche Bedeutung microRNAs bei der Regulation des p53-MDM2-MDMX-Pathways spielen. Genaue Kenntnisse über die Alterationen und die Regulation dieses zentralen Pathways können in Zukunft dazu beitragen, die Chemoresistenz von Tumoren sowie den Verlauf von Tumorerkrankungen besser vorhersagen und somit die Resistenz überwinden zu können.

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Originalarbeiten – thematisch geordnet (Sonderdrucke im Anhang)

Thematik 1 – Genetische Veränderungen von MDM2, MDMX, p53 bei WTS und Ovarialkarzinomen

1. **Bartel F**, Meye A, Wurl P, Kappler M, Bache M, Lautenschlager C, Grunbaum U, Schmidt H, Taubert H. Amplification of the MDM2 gene, but not expression of splice variants of MDM2 MRNA, is associated with prognosis in soft tissue sarcoma. Int J Cancer 2001;95:168-75.
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Thematik 4 - MDM2 als ein therapeutisches Target für WTS

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Buchbeiträge

1. F. **Bartel**, J. Lasch. Virale und nichtvirale Gentherapie: Techniken und Ansätze (Kap. 4.1) in Diagnostik und Therapie der Weichteilsarkome - Stand und Perspektiven. Rath,F.W.; Schönfelder,M. (Hrsg.) Karger Verlag, Leipzig, 2002.
2. A. Meye und F. **Bartel** Bedeutung des Onkogens mdm2 für die Entwicklung neuer Therapiestrategien (Kap. 4.4) in Diagnostik und Therapie der Weichteilsarkome - Stand und Perspektiven. Rath,F.W.; Schönfelder,M. (Hrsg.) Karger Verlag, Leipzig, 2002.

Übersicht über Preise und Auszeichnungen

1. Bester Forschungsbeitrag 2004
   Jahrestagung der Deutschen Gesellschaft für Pathologie in Rostock

2. Bester Forschungsbeitrag 2007
   Jahrestagung der Deutschen Gesellschaft für Pathologie in Magdeburg

3. AACR Young Investigator Award 2007
   MDM2-Tagung in Woods Hole, USA

Übersicht über eingeworbene Drittmittel

1. Titel: „Untersuchungen zum Einfluss von HDMX und seiner Spleißvarianten auf die Funktionalität des p53-Tumorsuppressor-Pathways in Ovarialkarzinomen?"
   Dauer: 2 Jahre
   Projektträger: Land Sachsen-Anhalt
   Mittel: 18.000 €

2. Titel: „Untersuchung der klinischen Relevanz von Mdm2-Onkogen-Veränderungen in malignen Weichteilsarkomen: Angriffspunkt für die Entwicklung eines molekulargenetischen Therapiekonzeptes?"
   Dauer: 2 Jahre
   Projektträger: Deutsche Krebshilfe
   Mittel: 90.750 €

3. Titel: „Die Bedeutung des MDM2-Gens und seiner Produkte für die Diagnostik, Prognose und Therapie von Weichteilsarkomen" – Fortsetzungsantrag
   Dauer: 2 Jahre
   Projektträger: Deutsche Krebshilfe
   Mittel: 120.000 €

4. Titel: „Das Onkogen HDMX im humanen Ovarialkarzinom – Prognosefaktor und Ziel einer molekularen therapeutischen Intervention?“
   Dauer : 1 Jahr
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   Mittel: 36.491 €
5. Verlängerung der Juniorgruppe (Projekt 4)
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   Projektträger: Wilhelm-Roux-Programm – Juniorgruppe
   Mittel: 65.000 €

6. Verlängerung der Juniorgruppe (Projekt 4)
   Dauer : 1 Jahr
   Projektträger: Wilhelm-Roux-Programm – Juniorgruppe
   Mittel: 79.000 €

7. Titel: „Sind das Onkogen HDMX und seine Spleißvarianten im humanen Ovarialkarzinom?“
   Dauer : 2 Jahre
   Projektträger: Wilhelm-Sander-Stiftung
   Mittel: 69.620 €

8. Titel: „Untersuchungen zu Sequenzveränderungen des Onkogens HDMX in humanen Weichteilsarkomen und Ovarialkarzinomen“
   Dauer : 1 Jahre
   Projektträger: Wilhelm-Roux-Programm
   Mittel: 9.000 €

9. Titel: „Untersuchungen zur Regulation der MDMX-Expression durch die microRNA miR191“
   Dauer : 3 Monate (Reisestipendium)
   Projektträger: Fritz-Thyssen-Stiftung
   Mittel: 5.400 €

10. Titel: „Untersuchungen zum Zusammenhang der Expression von HDM2- und HDMX-Spleißvarianten nach DNA-Schäden und der Zytostatikaresistenz in Ovarialkarzinomen
    Dauer : 3 Jahre
    Projektträger: Deutsche Krebshilfe
    Mittel: 108.000 €

Lebenslauf

Persönliche Daten

geboren am 22. April 1972 in Ludwigslust

Eltern: Elisabeth und Manfred Bartel

Familienstand verheiratet, 2 Kinder

Ausbildung Studium der Biochemie an der Martin-Luther –University Halle-Wittenberg

Abschluss 1996: Diplom-Biochemiker Prädikat: „Gut"

Promotion zum Dr. rer. nat. am Fachbereich Biochemie der Martin-Luther-Universität Halle-Wittenberg

Abschluss 2002: magna cum laude

Wissenschaftlicher Werdegang

seit 1.9.2008 Leiter des Forschungslabors am Institut für Pathologie

2007 Verleihung des AACR Young Investigator Awards für herausragende Forschungsleistungen

seit 2005 Leiter einer Juniorgruppe am Institut für Pathologie, Universität Halle

2000 und 2002 mehrmonatige Arbeitsaufenthalte am St. Jude Children's Research Hospital, Memphis, TN, U.S.A.Schwerpunkt: Entwicklung adenoviraler Vektorsysteme zur Gentherapie von Weichteilsarkomen sowie die molekulare Charakterisierung kindlicher Rhabdomyosarkome

seit 1998 Universität Halle-Wittenberg, Medizinische Fakultät, Institut für Pathologie

Schwerpunkt: Charakterisierung des Onkogens MDM2 in Weichteilsarkomen (Genstatus, Amplifikation, Expressionsstudien, Analyse von Isoformen, Mutationen) sowie die Entwicklung und Testung von Gentherapiestrategien mit MDM2 als Target

1996 bis 1998 Universität Leipzig, Institut für Klinische Immunologie und Transfusionsmedizin, Abteilung Medizinische Biotechnologie, Leipzig - Schwerpunkt: Entwicklung von Assays zum patientenspezifischen Cytostatikascreening mit miniaturisierten Hohlfaserbioreaktoren

1995 Universität Halle-Wittenberg, Medizinische Fakultät, Institut für Medizinische Immunologie - Diplomarbeit zum Thema: Quantifizierung der Preproenkephalin-mRNA in Lymphozyten mittels kompetitiver RT-PCR

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Halle/Saale, 9. Dezember 2008 Dr. Frank Bartel

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AMPLIFICATION OF THE *MDM2* GENE, BUT NOT EXPRESSION OF SPLICE VARIANTS OF *MDM2* MRNA, IS ASSOCIATED WITH PROGNOSIS IN SOFT TISSUE SARCOMA

Frank BARTEL[1]*, Axel MEYE[2], Peter WÜRL[3], Matthias KAPPLER[1], Matthias BACHE[4], Christine LAUTENSCHLÄGER[5], Ulrich GRÜNBAUM[1], Hannelore SCHMIDT[1] and Helge TAUBERT[1]

[1]*Institute of Pathology, Department of Medicine, University of Halle-Wittenberg, Halle/Saale, Germany*
[2]*Clinic of Urology, Department of Medicine, Technical University Dresden, Dresden, Germany*
[3]*Surgical Clinic I, Faculty of Medicine, University of Leipzig, Leipzig, Germany*
[4]*Department of Radiotherapy, University of Halle-Wittenberg, Halle/Saale, Germany*
[5]*Institute of Medical Biometry and Computer Science, University of Halle-Wittenberg, Halle/Saale, Germany*

The *MDM2* gene encodes a 90-kDa oncoprotein that is overexpressed in several human carcinomas, osteosarcomas, gliomas and soft tissue sarcomas (STSs). This overexpression is the result of several mechanisms, for example, enhanced transcription or translation, gene amplification and alternative splicing. We found that 19 of 67 (28.4%) STS specimens contained an amplified *MDM2* gene. The amplification was more likely to be present in grade 1 tumors than in grade 2 or 3 tumors (58% of grade 1 tumors *vs.* 15% of grade 2 or 3 tumors, $p = 0.001$, χ^2 test). Furthermore, patients with tumors that contained an amplified *MDM2* gene had a survival estimate (87 months) that was longer than that of patients with tumors that lacked an amplified gene (40 months; $p = 0.02$, log-rank test). Alternatively and aberrantly spliced *MDM2* mRNAs were detected in human STSs by a highly sensitive reverse transcription-polymerase chain reaction method. Of 71 tumor samples, 38 (54%) showed evidence of the spliced forms, which included *MDM2-A*, *MDM2-B* and several variants exclusively expressed in STSs. A common feature of all forms was the absence of the MDM2 *N*-terminal region, which includes the TP53-binding region. Furthermore, the presence of the spliced forms was associated with elevated levels of TP53 ($p = 0.01$, χ^2 test). Although the presence of spliced forms was associated with late-stage tumor phenotypes ($p = 0.05$, χ^2 test), we observed no relationship between the presence of splice variants and patient outcome.
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Key words: *murine double minute 2 gene; alternative splicing; soft tissue sarcoma; mRNA; amplification*

The murine double minute 2 (*MDM2*) gene was originally isolated from the spontaneously transformed BALB/3T3 cell line by virtue of the gene's amplification.[1] Overexpression of the *MDM2* gene is correlated with tumorigenicity in nude mice.[2] Amplified *MDM2* genes have been detected in several human malignancies, especially in human soft tissue sarcomas (STSs; 30%).[3–9] The oncogenic potential of the *MDM2* gene product is mostly a result of its interaction with the tumor suppressor gene *TP53*.[10] MDM2 seems to play an important role in TP53 degradation: MDM2 binds to TP53 and acts as an ubiquitin ligase, and these actions lead to inhibition of TP53-mediated apoptosis and growth arrest.[11] This effect of MDM2 can be modulated by other proteins, for example, RB1 or p19^{ARF}.[12,13]

Overexpression of MDM2 is a characteristic feature that is often associated with a poor prognosis, particularly in STS and especially when TP53 is also overexpressed.[7,14] However, overexpression may occur independently of gene amplification and may be correlated with enhanced transcription or translation.[15–20] For patients with STS[21] or non–small-cell lung cancer,[22] strong *MDM2* mRNA expression is correlated with a better prognosis.

In addition to amplification, other alterations of *MDM2* include mutations[23] or alternative splicing.[24–26] The occurrence of different *MDM2* mRNAs has been well described, and recently investigators have evaluated the impact of spliced variant *MDM2* mRNAs on certain malignancies, for example, astrocytomas, bladder cancers, ovarian cancers and leukemias.[24,25] Less is known about the occurrence of truncated *MDM2* mRNAs in STS and their relation to *MDM2* amplification and protein expression and their impact on patient outcome. Therefore we comprehensively analyzed the *MDM2* status of STS samples with respect to the expression of splice variants, gene amplification status, and mRNA and protein levels. In addition, we determined whether amplification of the *MDM2* gene and the expression of *MDM2* splice variants are associated with particular tumor characteristics and patient outcome.

MATERIAL AND METHODS

Patients and tumor samples

Tumor tissue was collected from 71 patients with STS who underwent surgical procedures at the University of Leipzig. None of the patients underwent preoperative treatment for STS. Tissue specimens were taken intraoperatively, immediately frozen in liquid nitrogen and stored at −80°C. A surgeon (P.W.) examined the cryosections histologically. According to the classification standards of Enzinger and Weiss,[27] the 71 tumor samples comprised 23 liposarcomas, 15 malignant fibrous histiocytomas (MFH), 7 malignant neural tumors, 7 leiomyosarcomas, 6 fibrosarcomas, 5 rhabdomyosarcomas and 8 other sarcomas. Staging indicated that 19.9% were stage I, 35.5% were stage 2, 23.5% were stage 3 and 22.1% were stage 4.

Extraction methods

Total RNA from each tumor sample (10 30-μm-thick cryosections) was isolated with the RNeasy Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. DNA was extracted either from paraffin-embedded tissue or cryosections by following standard protocols that included the use of phenol-chloroform.[28] To extract protein, 10 30-μm-thick cryosections were incubated in Laemmli buffer and treated according to standard procedures.[28]

Abbreviations: amol, attomol; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; MDM2, murine double minute 2; MFH, malignant fibrous histiocytomas; RR, relative risk; STS, soft tissue sarcoma; zmol, zeptomol

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*Correspondence to: University of Halle-Wittenberg, Faculty of Medicine, Institute for Pathology, Magdeburger Strasse 14, D-06097 Halle/Saale, Germany. Fax: +49-345-557-1295.
E-mail: frank.bartel@medizin.uni-halle.de

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Reverse transcription-polymerase chain reaction (RT-PCR) for detection of MDM2 *mRNA splice variants*

Total RNA (250 ng) was reverse-transcribed by using the M-MLV reverse transcriptase Superscript RT (Life Technologies, Karlsruhe, Germany) at 42°C for 1 hr with random hexamer primers in a total volume of 20 μl. The integrity of RNA was tested by amplifying the cDNA with intron-spanning primers that were specific for the glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) gene (not shown).

The nested PCR method of detecting spliced products described previously[24] was modified. Briefly, 1/10th of the first-strand reaction was used in a 50-μl PCR mixture containing 1× PCR reaction buffer, 2.5 mM $MgCl_2$, 25 pmol of the external primer pair (mdm2–5 and mdm2–6, see Table I), 200 μM of each dNTP and 2.5 U of HotStarTaq DNA Polymerase (Qiagen). The first round of PCR was carried out in a PTC-2000 thermal cycler (MJ Research, Biozym, Hess. Oldendorf, Germany) for 25 cycles. Each cycle consisted of a 1-min period at 95°C, a 1-min period at 55°C and a 4.5-min period at 72°C. After the last cycle, an extension reaction was performed for 10 min at 72°C.

The second round of nested PCR was performed with 1/10th of the first-round PCR product and 2 different sets of internal primers (set 1, mdm2-1 and mdm2-8; set 2, mdm2-9 and mdm2-10). Each of the 35 cycles of PCR consisted of a 1-min period at 95°C, a 1-min period at 58°C and a 4.5-min period at 72°C. After the 35 cycles were complete, the final extension step (10 min at 72°C) was performed. The products of the second PCR reaction were analyzed on a 1.5% agarose gel and visualized under ultraviolet light after the gel had been stained with ethidium bromide. Spliced transcripts of the appropriate size were isolated from the gel by using a QIAEX kit (Qiagen), reamplified and directly sequenced (Thermosequenase TSII kit, Amersham Pharmacia, Freiburg, Germany) by an ABI 370 automatic sequencer (Perkin Elmer, Weiterstadt, Germany).

Semiquantitative PCR for the detection of amplified MDM2 *genes*

In semi-quantitative multiplex PCR,[29] 100 ng of genomic DNA was amplified with primers for the *MDM2* gene (mdm2–7 and mdm2–8) and with primers for the prothrombin gene (prot-1 and prot-2). The PCR mixture consisted of 1× PCR reaction buffer, 2.5 mM $MgCl_2$, 25 pmol of each primer pair, 200 μM of each dNTP and 2.5 U of HotStarTaq DNA Polymerase (Qiagen). PCR was performed under the conditions used in the first round of nested PCR. The PCR products were separated on a 1.5% agarose gel, and the gel was stained with ethidium bromide. The gel was scanned, and the band intensity was measured by densitometry (ImageMaster VDS 3.0 Software, Amersham Pharmacia). Finally, we calculated the ratio of the intensity of the MDM2 bands to the intensity of the prothrombin band. We considered any ratio greater than 3 to indicate amplification of the *MDM2* gene. Genomic DNA extracted from peripheral blood leukocytes of healthy volunteers served as a control.

Western blot analysis

Total protein (30 μg) was subjected to electrophoresis in a 10% polyacrylamide-sodium dodecyl sulfate (SDS) gel (Minigel system, Biometra Göttingen, Germany). Proteins were transferred to a PVDF Immobilon membrane (Millipore, Eschborn, Germany) at 200 mA for 90 min (Miniblotter, Biometra). After nonspecific binding sites were blocked by 0.1% Tween-20 containing 3% bovine serum albumin, the membrane was incubated for 1 hr with an anti-TP53 antibody (DO-7; 1:500 dilution; Dianova, Hamburg, Germany) or with an anti-MDM2 antibody (N20; 1:200 dilution; Santa Cruz Biotechnology, Heidelberg, Germany). The incubation with the primary antibody was followed by a 1-hr incubation with horseradish peroxidase-conjugated anti-rabbit IgG or anti-mouse IgG antibody (1:1,000 dilution; Dako, Denmark) at room temperature. For visualization of the bands, the membrane was placed into enhanced chemiluminescence substrate (Amersham, Braunschweig, Germany) for 1 min and then exposed to a Biomax film (Kodak, Stuttgart, Germany). Bound antibodies were removed from the membrane by incubation in washing buffer (2% SDS; 0.0625 M Tris-HCl, pH 6.7, 0.05 M β-mercaptoethanol) for 30 min at 50°C; the membrane was then washed twice with Tris-buffered saline with Tween-20 at room temperature. Blots were stored at 4°C until further use. The amounts of MDM2, TP53 and β-actin were compared with those of the same proteins isolated from a positive control cell line [RD cells (CCL 136, American Type Culture Collection, Manassas, VA), which overexpress a mutant form of TP53]. Afterward, densitometry (Imagemaster VDS 3.0 software, Pharmacia) was performed to determine the relative levels of MDM2 and TP53 protein. The relative amounts of MDM2 and TP53 were standardized to that of β-actin from the same sample. The resulting values were classified according to a semi-quantitative scale as no, moderate, marked, or strong expression.

Statistical analysis

We used SPSS 9.0 software (SPSS Inc., Chicago, IL) to perform statistical analyses, which included univariate Kaplan–Meier analysis, Cox regression analysis, the χ^2 test, and the log-rank test. A probability (p) of less than 0.05 was considered to indicate statistical significance.

RESULTS

MDM2 *gene amplification and mRNA expression*

We investigated *MDM2* gene amplification in 67 of 71 tumor samples. *MDM2* gene amplification was detected in 28.4% of the STS samples (19 of 67), and the intensity of the bands representing amplified *MDM2* genes were 3- to 12-fold greater than that of bands representing unamplified genes (Fig. 1). A more detailed analysis revealed that amplified *MDM2* genes were present in 53.8% (7 of 13) of stage 1 tumors, in 31.8% (7 of 22) of stage 2 tumors, in 15.4% (2 of 13) of stage 3 tumors and in 7.1% (1 of 14) of stage 4 tumors. The association that the amplified MDM2 gene was more likely to be present in stage 1 tumors than in stage 4 tumors was significant (χ^2 test; $p = 0.025$).

TABLE I – PRIMERS USED FOR RT-PCR, CYCLE SEQUENCING AND MULTIPLEX PCR

Target gene	Primer	Sequence	Reference
mdm2	mdm2-1	sense 5′-TCTGTACCTACTGATGGTGC3′	This study
	mdm2-5	sense 5′-CTGGGAGTCTTGAGGAAT-3′	Sigalas *et al.*[24]
	mdm2-6	antisense 5′-CAGGTTGTCTAAATTCCTAG-3′	Sigalas *et al.*[24]
	mdm2-7	sense 5′-GCTGACTATTGGAAATGCAC-3′	Schlott *et al.*[23]
	mdm2-8	antisense 5′-ATTGGTTGTCTACATACTGGGC-3′	Schlott *et al.*[23]
	mdm2-9	sense 5′-CGCGAAAACCCCGGGCAGGCAAATGTGCA-3′	Sigalas *et al.*[24]
	mdm2-10	antisense 5′-CTCTTATAGAC AGGTCAACTAG-3′	Sigalas *et al.*[24]
Prothrombin	prot-1	sense 5′-TCATCCTCAGTCCTAATGC-3′	This study
	prot-2	antisense 5′-AGACCCCAAGAAAGAAATGG-3′	This study

RT-PCR, reverse transcription-polymerase chain reaction.

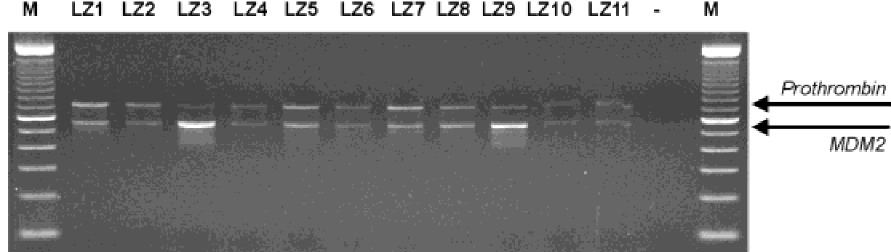

FIGURE 1 – Amplified *MDM2* gene in soft tissue sarcoma (STS) samples. A representative agarose gel showing the amplified *MDM2* gene in an STS sample. Note the amplified *MDM2* genes in samples LZ3 and LZ9: densitometry showed that the intensity of the *MDM2* bands in LZ3 is 12-fold greater than that in samples without amplified genes, and that of *MDM2* band in LZ9 is 9-fold greater than that in samples without amplified genes.

In our investigation, amplification was also associated with the grade of the tumor: in 58.8% of grade 1 tumors the *MDM2* gene was amplified, whereas only 15.2% of grade 2 and grade 3 tumors (7 of 46) showed evidence of amplification (χ^2 test; $p = 0.001$). Interestingly, amplified *MDM2* genes were present in 50% of the fibrosarcomas (3 of 6); this percentage was noticeably higher than that for other types of sarcomas. For example, only 7.1% of malignant fibrous histiocytomas (1 of 14, $p = 0.05$), 20% of neurogenic sarcomas (1 of 5) and 36.4% of liposarcomas (8 of 22, $p = 0.07$) contained amplified *MDM2* genes. All 4 rhabdomyosarcomas that were tested for *MDM2* gene amplification were negative.

We analyzed whether amplification of the *MDM2* gene was associated with increased expression of MDM2 mRNA or protein. The expression of *MDM2* mRNA and its relevance as a prognostic factor in STS has been described elsewhere.[21] We found a strong association between the amplification of the *MDM2* gene and the expression level of *MDM2* mRNA (χ^2 test, $p = 0.005$): 16 of the 17 tumors with an amplified *MDM2* gene showed an elevated amount of *MDM2* mRNA (>100 zmol *MDM2* mRNA per amol *GAPDH* mRNA), whereas only 1 tumor sample with an amplified *MDM2* gene had a low amount of *MDM2* mRNA (<100 zmol *MDM2* mRNA per amol *GAPDH* mRNA).

The survival estimates for patients with an STS that contained an amplified *MDM2* gene were significantly longer than those for patients with an STS that lacked an amplified *MDM2* gene (Table II; $p = 0.02$). Cox regression analysis showed that the patients whose tumors lacked the amplified *MDM2* gene had a worse prognosis ($p = 0.04$, RR = 4.5) than patients whose tumors contained the amplified gene. Multivariate Cox regression analysis (Fig. 2), when adjusted to tumor stage, revealed that the relative risk of tumor-related death was comparable for patients with and without amplified *MDM2* genes ($p = 0.17$, RR = 4.4). Adjusting the multivariate regression analysis for other factors, such as tumor type, was not meaningful, because of the heterogeneity of the STSs and the small number of representatives of some tumor types (data not shown).

Expression of alternatively and aberrantly spliced MDM2 *mRNA*

After nested PCR amplification of the complete coding region, we detected a pattern of specific PCR products of multiple sizes in 38 of the 71 tumor samples (53%; Fig. 3). Reamplification and direct sequencing of the different PCR products yielded evidence that confirmed the presence of spliced *MDM2* mRNAs. We detected the known variants *MDM2*-A (891 bp) and *MDM2*-B (657 bp) described by Sigalas *et al.*[24] and the newly discovered forms *MDM2*-DS2 (364 bp), *MDM2*-DS3 (294 bp), *MDM2*-KB2 (732 bp), *MDM2*-KB3 (219 bp), *MDM2*-PM2 (393 bp), *MDM2*-IS1 (456 bp), *MDM2*-EU2 (297 bp) and *MDM2*-JN1 (207 bp). The splice variant *MDM2*-A was detected in 7 tumor samples; the splice variant *MDM2*-B, in 17 tumor samples; and *MDM2*-PM2, in 2 tumor samples (Figs. 3–5, Table III). Each of the other forms was present only in single tumor samples. In the corresponding connective tissue of the patients, only the full-length *MDM2* transcript was present, or no *MDM2* transcripts were detected (data not shown).

TABLE II – UNIVARIATE ANALYSIS OF CLINICAL AND MOLECULAR CHARACTERISTICS AND THEIR IMPACT ON SURVIVAL OF PATIENTS WITH STS

Characteristic	No. of patients	Median survival (months)	3-year survival estimate (%)	p^1
MDM2 splice variants				
No	29	30	29	
Yes	35	64	56	0.19
MDM2 gene amplification				
No	44	40	31	
Yes	16	87	83	0.02
MDM2 expression				
No	16	45	51	
Weak	15	62	58	
Moderate	20	29	25	
Strong	8	96	n.d.	0.2
TP53 expression				
No	14	62	67	
Weak	9	13	57	
Moderate	11	27	18	
Strong	24	55	47	0.35
Tumor stage				
1	12	47	66	
2	23	81	76	
3	16	49	57	
4	13	15	0	0.0001

n.d., not determined.–[1]The log-rank test was used to determine statistical significance.

All splice forms of the *MDM2* mRNA contained at least part of the *N*-terminal region of the TP53-binding domain (Figs. 3–5). In addition, the splice variants lacked the nuclear export signal, the nuclear localization signal and the acidic domain. *MDM2*-A, *MDM2*-B and *MDM2*-KB2 are alternatively spliced transcripts, but the other 7 forms represent aberrantly spliced messages. The splice variant *MDM2*-KB2 is comparable to *MDM2*-B, except that exon 4 is still present in *MDM2*-KB2 (Fig. 4). The other aberrantly spliced transcripts exhibited a common pattern of splicing (Fig. 5). In brief, the *MDM2* mRNAs contained as many as 4 5- to 9-bp repeats (*e.g.* 5′-GAAAGAG-3′) that served as aberrant splice sites. For example, the splice variant *MDM2*-PM2 consisted of exons 1 and 3 and part of exon 4 to which exon 12 (the part of exon 12 that begins at nucleotide 1232 of the open reading frame) was spliced; this variant retained only 1 of the 4 GAAAGAG repeats.

Relation of splice variants to tumor type and stage

Only 25% (1 of 4) of rhabdomyosarcomas, 30% (7 of 23) of liposarcomas and 28% (2 of 7) of leiomyosarcomas contained spliced *MDM2* transcripts. The spliced transcripts were more frequently detected in fibrosarcomas (50%, 3 of 6) and in neurogenic

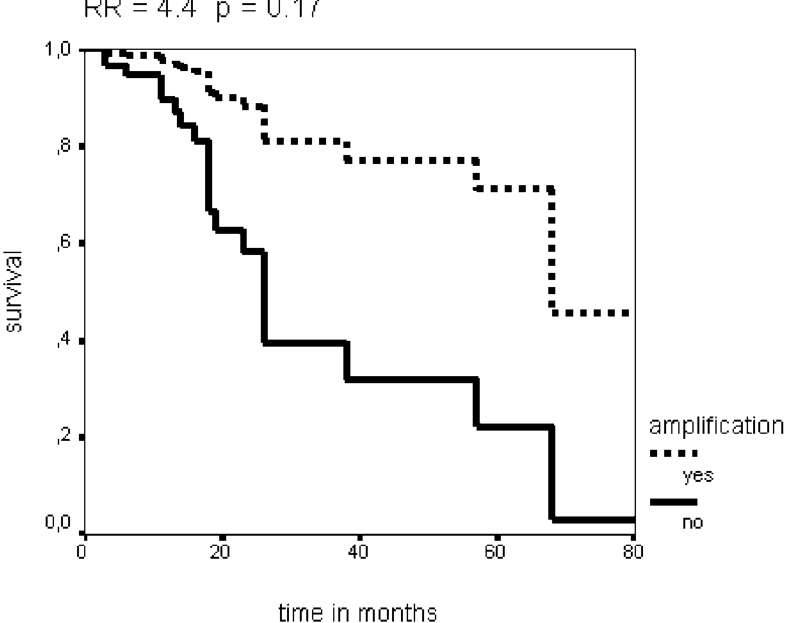

Figure 2 – Multivariate Cox model for analysis of amplification of the *MDM2* gene and survival estimates for patients with soft tissue sarcoma (all stages, $n = 67$, adjusted to tumor stage).

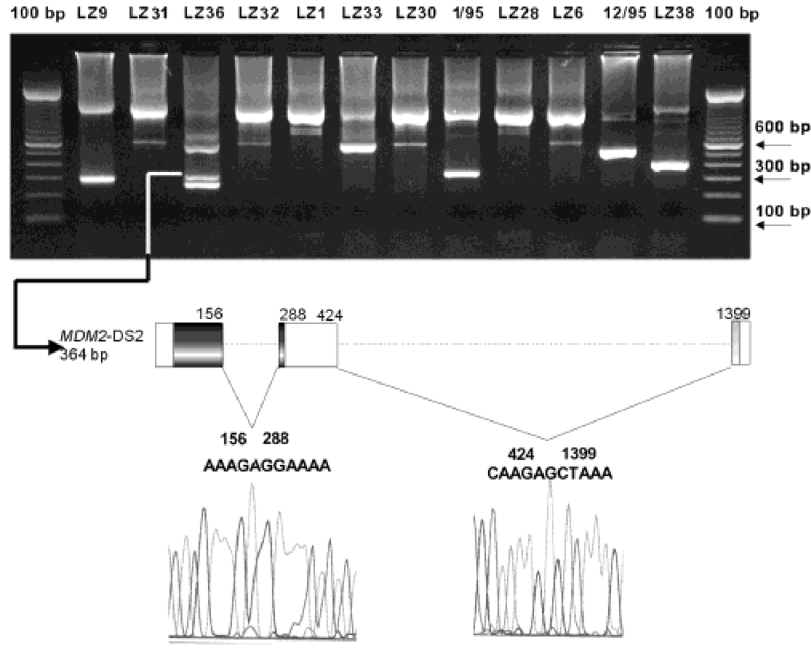

Figure 3 – Expression of alternatively or aberrantly spliced *MDM2* transcripts. (*Top*) Results of a nested reverse transcription-polymerase chain reaction. LZ9, LZ31 and LZ36 represent 3 different liposarcomas; LZ32, a fibrosarcoma; LZ1, 33 malignant fibrous histiocytomas; LZ30 and LZ1/95, 2 different neurogenic sarcomas; LZ28, a rhabdomyosarcoma; LZ6 and LZ12/95, 2 different leiomyosarcomas; and LZ38, an unclassified sarcoma. (*Bottom*) Schematic representation of the splice variant *MDM2*-DS2. The sequences of the splice sites are shown.

sarcomas (57%, 4 of 7). Nine of 14 (64%) malignant fibrous histiocytoma samples were positive for the spliced forms; this number of positive samples was significantly greater than the number of positive liposarcomas (χ^2 test; $p = 0.03$).

Next, we investigated whether the splicing of *MDM2* mRNA is related to a particular tumor stage. Spliced transcripts were detected in 31% (4 of 13) of stage 1 tumors and in 65% (26 of 40) of stage 2 or stage 3 tumors (χ^2 test; $p = 0.03$). These results indicate that splicing of the *MDM2* mRNA is associated with late-stage tumors.

The results of our Kaplan–Meier analysis showed that the median survival estimate for patients in whom truncated *MDM2* transcripts were expressed (64 months) was not significantly different from that for patients in whom only full-length *MDM2* transcripts were expressed (30 months; $p = 0.19$; Table III). After patients with stage 1 or stage 4 tumors were excluded from this analysis, the effect of expressing spliced forms of *MDM2* mRNA on outcome was even less significant ($p = 0.69$). Therefore, the expression of alternatively or aberrantly spliced

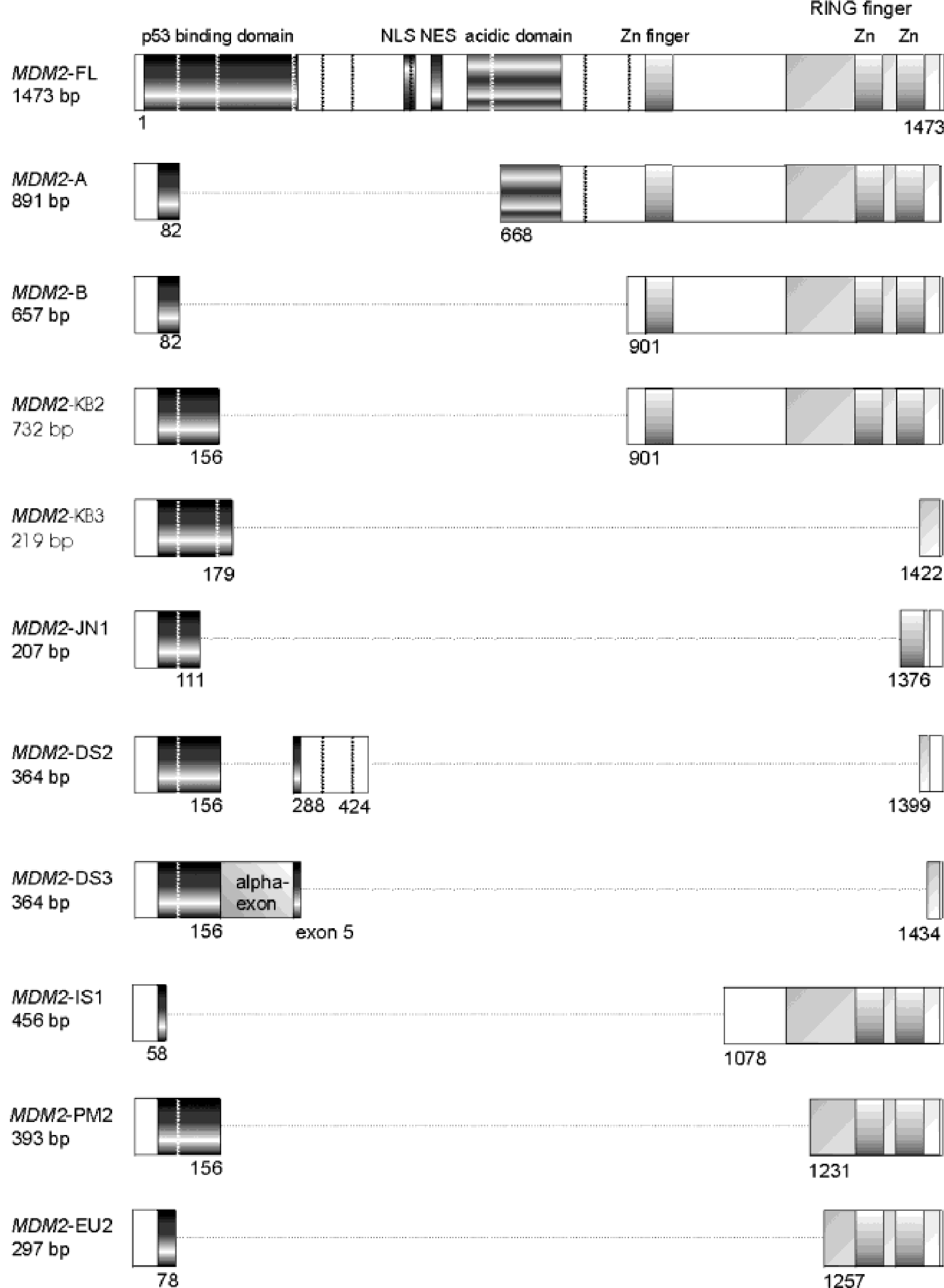

Figure 4 – Comparison of the *MDM2* splice variants. Schematic representation of the full-length transcript and the alternatively spliced forms of the *MDM2* mRNA in human soft tissue sarcoma samples. Most spliced forms lack the TP53-binding domain, the nuclear localization signal and the nuclear export signal sequences. The sequences of *MDM2*-KB2 (accession number AJ278975), *MDM2*-DS2 (accession number AJ276888), *MDM2*-IS1 (accession number AJ278976), *MDM2*-PM2 (accession number AJ278977), and *MDM2*-EU2 (accession number AJ278978) were submitted to GenBank. The dotted vertical lines indicate the borders between the exons starting with exon 3 of the *MDM2* gene.

MDM2 transcripts is not a predictor of outcome for patients with STS in our study.

Levels of MDM2 and TP53 protein in STSs

Levels of MDM2 and TP53 protein were detected by Western blot analysis and normalized to the amount of β-actin in the respective tumor samples (Fig. 6). The resulting levels were categorized as no, weak, moderate or strong expression (Table II). Most tumor samples showed either no (23%) or strong (39%) expression of TP53. This result could occur if the tumors lost 1 or both *TP53* alleles or if the tumors expressed TP53 protein abnormally. We found a strong association between the presence of alternatively and aberrantly spliced *MDM2* transcripts and an elevated level of TP53 (χ^2 test; $p = 0.01$). Eighty percent (20 of 25 samples) of the tumor samples with high

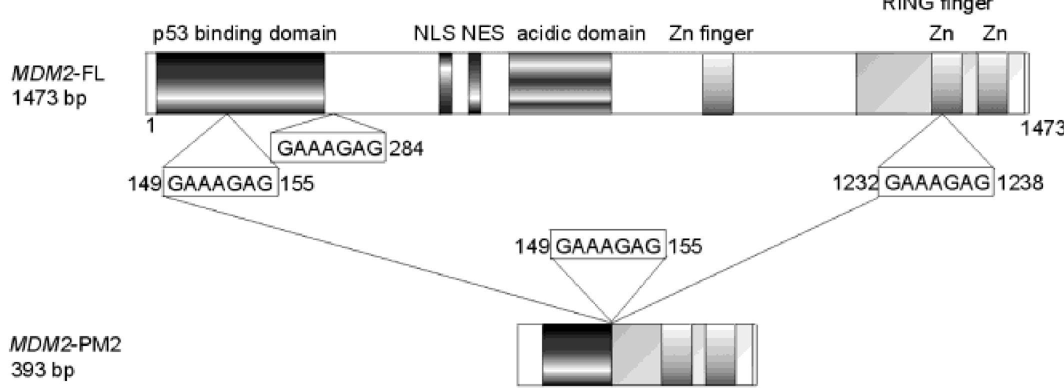

FIGURE 5 – Representation of the splicing pattern for *MDM2*-PM2. The regions encoding the different domains of the MDM2 protein are indicated.

TABLE III – SUMMARY OF CLINICAL AND MOLECULAR DATA FOR THE STS

	Liposarcomas	Fibrosarcomas	MFH	Neurogenic sarcomas	Rhabdomyosarcomas	Leiomyosarcomas	Other sarcomas
Total number	23	6	15	7	5	7	5
Tumors (*n*) per stage 1/2/3/4	10/7/2/4	2/3/1/0	0/4/8/3	0/5/2/0	0/0/5/0	1/2/1/3	0/3/0/2
Tumors (*n*) per grade 1/2/3	12/9/2	2/4/0	0/5/10	2/3/2	0/0/5	1/4/2	0/3/2
Amplification (yes/no)	8/14	3/3	1/13	1/5	0/4	3/4	1/5
Splice forms (yes/no)	7/16	3/3	10/5	4/3	3/2	5/2	5/0
MDM2 mRNA expression (w/m/ma/s)	2/5/9/6	2/1/2/1	3/1/10/1	1/0/3/3	4/0/0/1	3/0/3/1	0/1/3/1
MDM2 protein expression (n/w/m/s)	3/6/9/4	2/3/0/0	5/3/4/1	2/1/2/1	1/2/2/0	2/1/2/1	2/1/1/1
TP53 protein expression (n/w/m/s)	10/4/1/7	1/1/1/2	2/1/4/6	0/1/2/3	2/1/0/1	0/1/2/3	0/0/2/3

STS, soft tissue sarcoma; MFH, malignant fibrous histiocytoma; w, weak; m, moderate; ma, marked; s, strong; n, no.

levels of TP53 protein had detectable levels of truncated *MDM2* mRNAs, whereas only 33% of samples with no or moderate levels of TP53 protein expressed the splice variants. However, our Western blot analyses revealed no distinct association among the levels of *MDM2* mRNA, MDM2 protein and TP53 protein. Furthermore, we found that amplification of the *MDM2* gene resulted in an elevated level of *MDM2* mRNA but not necessarily in an increased level of MDM2 protein. Of the tumor samples with a high level of *MDM2* mRNA, 46% had an amplified *MDM2* gene but no detectable MDM2 protein (χ^2 test; $p < 0.05$). Western blot analysis of MDM2 expression showed no obvious relationship with prognosis (data not shown).

DISCUSSION

In this study, we analyzed in detail the relationship among *MDM2* gene amplification, mRNA and protein expression, and we evaluated the occurrence of alternatively and aberrantly spliced transcripts in 71 STS samples. Our results show that *MDM2* gene amplification is significantly related to a better prognosis for patients with STS ($p = 0.02$, log-rank test) and that alternative or aberrant splicing of *MDM2* mRNA occurs frequently in STSs and is associated with a more malignant phenotype. However, the presence of alternatively spliced *MDM2* transcripts is not valuable as an independent prognostic marker in STS.

MDM2 gene amplification was detected in 19 of 67 STS samples (28%) of different histologic origin. This rate is slightly higher than previously published values of *MDM2* amplification in STSs in general (20%).[9] The *MDM2* gene locus was amplified in 36% of the liposarcomas; the average of all published data for liposarcomas is 30%. The percentage of malignant fibrous histiocytomas with an *MDM2* amplification in our study (7%) is lower than the percentage (20%) calculated by Momand *et al.*[9] The finding that no *MDM2* amplification was present in 4 rhabdomyosarcomas is in agreement with the reports of Taylor *et al.*,[30] who found that only 2 of 20 samples (10%) contained amplified *MDM2* genes. Interestingly, in our study the amplification of the *MDM2* gene appeared to be significantly associated with tumor stage (χ^2 test; $p = 0.002$). Furthermore, our results indicate that *MDM2* gene amplification is a favorable prognostic factor in STS. Patients without the amplified gene have a 4.4-fold greater risk of tumor-related death than patients with an amplified *MDM2* gene. The median survival estimate for patients with an amplified *MDM2* gene was 87 months, and that for patients without an amplified *MDM2* gene was only 40 months ($p = 0.02$). The finding that an amplified *MDM2* gene rather than a single-copy

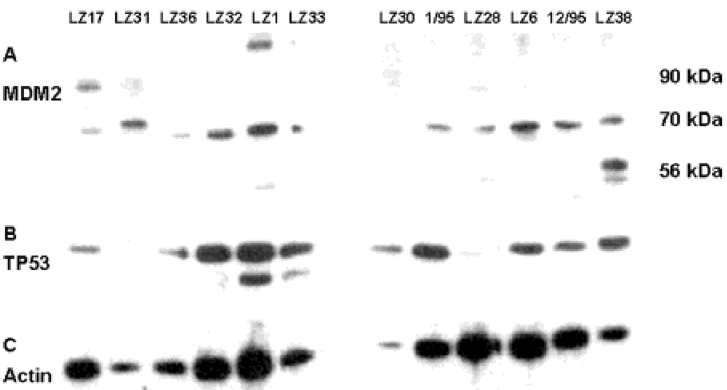

FIGURE 6 – Western blot analysis of MDM2 and TP53 protein expression in soft tissue sarcoma samples. The β-actin protein served as a control, and its expression level was used to standardize the MDM2 and TP53 protein expression levels (see Material and Methods). For a description of the tumor samples refer to Figure 2.

MDM2 gene is related to a better prognosis is somewhat surprising. Furthermore, one would expect that an amplified *MDM2* gene would coincide with overexpression of MDM2. However, there was no association between the amplification status and the level of MDM2 protein detected by Western blot methods.

It is accepted that many malignant tumors, especially sarcomas, possess elevated levels of MDM2, a feature that is mostly related to a poor prognosis.[7,14] However, these findings are based on immunohistochemical data. In the present study, we identified a strong association between an amplified *MDM2* gene and an elevated level of *MDM2* mRNA (χ^2 test; $p = 0.005$). In previous investigations, we found that an increased, not diminished, level of *MDM2* mRNA appeared to be a favorable independent prognostic marker in STS.[21] Our current findings indicate that amplification of the *MDM2* gene results in an increased mRNA level but is not necessarily associated with an increased accumulation of MDM2 protein. Several possible explanations could be given for this contrast. First, MDM2 overexpression might be the result of increased mRNA stabilization rather than enhanced transcription; similar findings have been observed for proteins such as heat-shock protein hsp70, insulin-like growth factor-binding protein 3, and waf-1.[31–33] Second, it is known that because of a promoter switch, tumor cells preferentially transcribe S-*MDM2* mRNA, which has a higher translation potential than the L-*MDM2* mRNA.[34] In our approach to quantifying the actual amount of *MDM2* mRNA, we could not distinguish between transcripts that arose from different promoters. Thus, it is possible that in some cells with increased levels of *MDM2* mRNA but no increase in MDM2 protein, the mRNA was of the L type. Third, it is noteworthy that there is generally little correlation between the abundance of mRNA species and the amount of protein that the mRNAs encode; this lack of correlation suggests that post-transcriptional regulation of gene expression is a frequent phenomenon.[35]

In our study, splice variants of *MDM2* mRNA were expressed in 53% of the STS samples and were present in all tumor types at almost the same frequency, but the percentage of MFH samples that contained these transcripts was significantly greater than that of liposarcomas (64% *vs.* 30%; χ^2 test; $p = 0.03$). Recent findings have shown that the presence of short splice variants of *MDM2* mRNA is correlated with a higher grade of ovarian cancers, bladder cancers, glioblastomas and astrocytomas.[24,25] A striking finding in our study was the association between the presence of short spliced forms and the overexpression of TP53 protein, which was detected by Western blotting methods. If one distinguishes between alternatively and aberrantly spliced transcripts, a highly significant correlation can be found between alternatively spliced transcripts and an overexpression of TP53 (χ^2 test; $p = 0.005$). Although *MDM2* mRNA splice variants seem to be associated with stabilized TP53, their function with respect to tumorigenesis remains unclear. However, the variants *MDM2*-A, *MDM2*-B, *MDM2*-C and *MDM2*-D, which lack the TP53-binding domain, fail to bind TP53 in an *in vitro* assay.[24] This failure to bind TP53 might contribute to stabilization of either mutant or wild-type TP53. The possibility that a stabilized wild-type or mutant TP53 could trigger the aberrant splicing of *MDM2* mRNA through an unknown mechanism has been proposed.[26] However, the disruption of the cell-cycle inhibitory domain, which occurs in most of the splice variants, may induce tumorigenesis. In most *MDM2* splice variants, including some aberrantly spliced transcripts (*MDM2*-D, -E,[24] -LN 229, -LN 18, -G 116, -G 150,[26] -IS1, -PM2, -EU2 [this study]), the C-terminal region, which contains the RING finger and 2 zinc finger domains, is retained, but whether the structure and the function of these domains change is unknown. We and others found that a shift in the original open reading frame may result in inactivation of the mRNA due to the introduction of a stop codon near the 5′ terminus of the mRNA or lead to a protein with an even further disrupted RING finger domain. A recently detected polymorphism in exon 12 of the *MDM2* gene[36] does not seem to affect either the frequency of splicing or the level of *MDM2* mRNA, but we observed a noticeable relationship between the occurrence of the polymorphism and a low level of MDM2 protein (data not shown). It must be emphasized that, to our knowledge, no one has determined whether splice variants detected by RT-PCR or Northern blot methods are translated into functional proteins. Investigations to elucidate this question are in progress.

One could postulate that splicing of distinct mRNAs is a common event during transformation, because of the differential expression of splicing regulatory factors.[37] Intronic mutations may also lead to an increased occurrence of splice variants. Alternative and aberrant splicing has been described for several mRNAs, such as CD44,[38] *bax*,[39] *ATM*[40] and *TP53*.[41] The expression of multiple CD44 isoforms may result in a higher risk of metastasis, multiple *TP53* isoforms may result in altered regulation of apoptosis and multiple *ATM* isoforms may cause an increased risk of microsatellite instability. Although the transforming ability of *MDM2* splice variants could be shown *in vitro*, the effect of *MDM2* isoforms on other target proteins *in vivo* remains unclear.

In summary, patients with STS that contains the amplified *MDM2* gene have a more favorable outcome than those whose tumors do not contain the amplified gene. *MDM2* amplification is also associated with an elevated level of *MDM2* mRNA but not with overexpression of MDM2 protein. Furthermore, the finding that the frequent occurrence of short *MDM2* splice variants is associated with an elevated level of TP53 suggests that in addition to *TP53* mutations and MDM2 overexpression, *MDM2* mRNA splice variants affect the tumor suppressor function of TP53 in STS.

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Significance of *HDMX*-S (or *MDM4*) mRNA splice variant overexpression and *HDMX* gene amplification on primary soft tissue sarcoma prognosis

Frank Bartel[1]*, Jördis Schulz[1], Anja Böhnke[1], Karen Blümke[1], Matthias Kappler[2], Matthias Bache[2], Hannelore Schmidt[1], Peter Würl[3], Helge Taubert[1] and Steffen Hauptmann[1]

[1]*Institute of Pathology, Faculty of Medicine, University of Halle-Wittenberg, Halle/Saale, Germany*
[2]*Department of Radiotherapy, Faculty of Medicine, University of Halle-Wittenberg, Halle/Saale, Germany*
[3]*Surgical Clinic I, Faculty of Medicine, University of Ulm, Ulm, Germany*

The product of the *HDMX* (or *MDM4*) gene is structurally related to the MDM2 oncoprotein and is also capable of interacting with the tumor suppressor protein p53. The aim of our study was to determine the amplification status of the *HDMX* gene and the expression of the *HDMX* mRNA (particularly that of the *HDMX*-S splice variant) in soft-tissue sarcomas (STS). Patients with STS were evaluated for the status of *HDMX* gene amplification ($n = 66$) and *HDMX*-S mRNA expression ($n = 57$) within their tumors. DNA, total RNA and protein were isolated from frozen tumor tissue. We determined that the *HDMX*-S splice variant transcript was predominant in a subset (14%) of tumor samples and that its expression was correlated with decreased patient survival (15 *vs.* 53 months, $p < 0.0001$, log-rank test) and with a 17-fold increased risk of a tumor-related death ($p < 0.0001$, multivariate Cox's regression model). The tumors from these patients also expressed elevated levels of HDMX-S protein. The *HDMX* gene was amplified in 17% of STSs, and the gene amplification was associated with poor prognosis (RR = 6.5, $p < 0.0001$). There was no correlation between the *HDMX* gene amplification and overexpression of the *HDMX*-S splice variant. In summary, our data indicate that both the overexpression of the *HDMX*-S transcript as well as *HDMX* gene amplification are important prognostic markers for STS.
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Key words: *HDMX*; amplification; overexpression; oncogene; prognosis; alternative splicing; cancer

Recently, a protein related to the product of the murine double minute gene 2 (MDM2), designated as HDMX, has been identified, and the *HDMX* gene was mapped to chromosome region 1q32.[1,2] It has been shown that HDMX (also referred to as MDM4 or MDMX in the mouse) is able to bind to p53, thereby inhibiting its activity. However, it fails to induce the nuclear export of p53 or its proteosomal degradation.[3] Although HDMX bears resemblance to MDM2, it has some distinct features. For example, whereas expression of MDM2 can be induced by DNA damaging agents, HDMX levels are not.[1] This suggests that the *HDMX* promoter, unlike the P2-promoter of the *MDM2* gene, is not transcriptionally transactivated by p53. Data from gene knockout experiments indicate that embryonic lethality of both $MDMX^{-/-}$ and $MDM2^{-/-}$ mice can be rescued by the loss of *p53*.[4,5] Therefore, it seems likely that MDM2 and MDMX act in nonoverlapping pathways to regulate p53.[5] However, data also show that HDMX actively regulates p53 activity through its interaction with MDM2,[6] suggesting that HDMX/MDMX is another important regulator of the p53-MDM2 network (reviewed in Michael and Oren[7]). These data raise the possibility that increased levels of HDMX result in the inactivation of p53 that may contribute to tumor development. In fact, several studies have revealed that the *HDMX* gene is a target of amplification at the 1q32 gene locus in malignant gliomas that have no *p53* mutations or *MDM2* amplification.[8,9] In addition, the *HDMX* gene has been shown to be amplified in breast cancer,[10] and the HDMX protein is aberrantly expressed in a variety of human tumor cell lines.[11] Furthermore, several splice variants of the *MDMX/HDMX* mRNA have been detected both in murine and human tumor cell lines.[12,13] For example, the *MDMX-S/HDMX*-S variant (also referred to as HDMX-E) is characterized by the loss of exon 6, which produces a shift of the reading frame, resulting in a premature stop codon and the incorporation of 13 and 26 unique amino acids in MDMX-S and HDMX-S, respectively (Fig. 1). The truncated proteins are much more effective than full-length MDMX in the inhibition of p53-mediated transcriptional activation and induction of apoptosis.[12,13]

To date, no information is available regarding the clinical significance of HDMX alterations in STS. Therefore, we were interested in evaluating gene amplification and mRNA expression of *HDMX* in STS. Here, we report that the *HDMX* gene is amplified in a subset of STSs and that *HDMX* gene amplification is associated with poor prognosis. Furthermore, higher levels of *HDMX*-S mRNA, when compared to the FL-*HDMX* transcript, predict a poor outcome and a 17-fold increased risk of a tumor-related death for STS patients.

Material and methods

STS patients and clinical data

For our study, tumor tissue samples from 66 adult patients with histologically verified STS were collected. These patients and tissue samples have been previously described.[14,15] The material included 18 liposarcomas (7 well differentiated, 8 myxoid, 1 round cell, 1 pleomorphic, 1 dedifferentiated), 14 malignant fibrous histiocytomas (MFH, 11 pleomorphic, 2 myxoid, 1 giant cell MFH), 14 rhabdomyosarcomas (9 embryonal, 5 alveolar), 4 leiomyosarcomas, 6 synovial sarcomas (4 biphasic, 2 monophasic), 5 neurogenic sarcomas (4 malignant schwannomas, 1 neuroepithelioma), 2 fibrosarcomas and 3 other sarcomas (2 malignant hemangiopericytomas, 1 malignant epithelial mesothelioma). The tissues were collected after surgical resection, snap-frozen in liquid nitrogen and stored at −80°C. The tumors were classified according to the van Unnik grading system[16] and the UICC guidelines.[17] None of the tumors had been exposed to any type of therapy prior to the samples being obtained.

RNA and DNA extraction, reverse transcription, PCR analysis and p53 sequence analysis

Total RNA was isolated using the RNeasy Mini Kit (Qiagen, Hilden, Germany), and genomic DNA was isolated using the QIAamp DNAMini Kit (Qiagen) according to the manufacturer's instructions. Subsequently, 250 ng RNA was reverse transcribed with random hexamer primers and the Superscript II™ reverse transcriptase (Invitrogen, Karlsruhe, Germany). The resulting cDNA was amplified by primers selected to allow the full-length *HDMX* transcript and the *HMDX*-S splice variant to be distinguished. The sequences of the primers used to amplify the *HDMX* cDNA were: MDMX-3 5′-GCAGTTTCTTCACTACCA-3′ and

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***Correspondence to:** Institute of Pathology, Faculty of Medicine, University of Halle-Wittenberg, Magdeburger Str. 14, D-06097 Halle/Saale, Germany. Fax: +49-345-557-1295.
E-mail: frank.bartel@medizin.uni-halle.de
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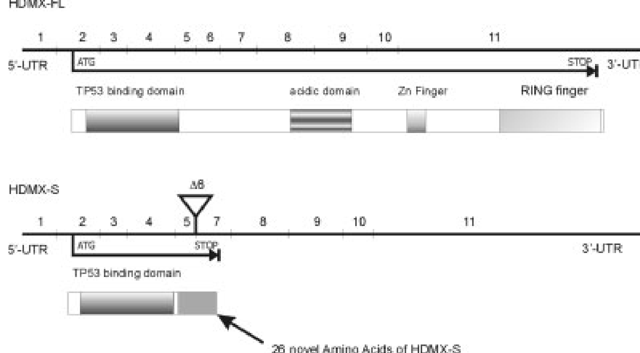


FIGURE 1 – Sequence comparison of FL-HDMX and HDMX-S. The alignment shows both the FL-HDMX (top) and the HDMX-S mRNA sequences (bottom) and the proteins that are translated from the respective transcripts. HDMX-S consists of only the p53-binding domain and 26 unique amino acids that are generated after shift in the open reading frame due to the loss of exon 6.

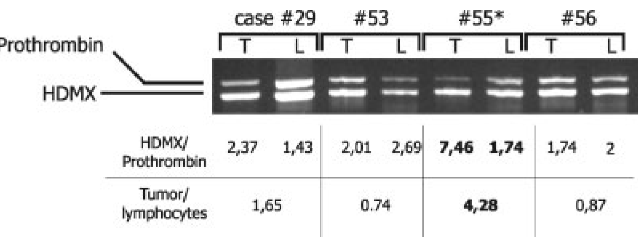


FIGURE 2 – *HDMX* gene amplification in soft-tissue sarcomas. Results of the semiquantitative multiplex PCR to determine *HDMX* gene amplification status in the tumor samples (T) and the lymphocytes (L) of the respective patients are shown. For example, in this representative agarose gel, the *HDMX* gene in sample #55 was considered to be amplified (marked with an asterisk).

MDMX-8 5′-AGCCTAGATGTTTCATCTTG-3′. Each PCR reaction was carried out in a total volume of 50 µL, which included 1/10 of the cDNA reaction, 1× reaction buffer, dNTP mix, 2.5 mM $MgCl_2$, 20 pmol of each primer, 1 U HotStarTaq (Qiagen), and 1× Q-solution (Qiagen). The PCR consisted of 35 cycles with 30 sec of denaturation at 95°C, 30 sec of primer annealing at 58°C and synthesis at 72°C for 60 sec. The PCR products were analyzed on a 1.5% agarose gel. The *HDMX*-S transcript is 68 bp smaller than the FL-*HDMX* transcript (Fig. 3).

Quantitative real-time RT-PCR was used to confirm the relative expression levels of the FL-*HDMX* and the *HDMX*-S mRNA. The reactions were carried out on a Rotor-Gene 3000 (Corbett Research, Sydney, Australia). We used a common forward primer for both FL-*HDMX* and *HDMX*-S; X quant fw 5′-CAGCAGGTGCGCAAGGTGAA-3′ and reverse primers specifically designed for amplification of either FL-*HDMX*, FL-AS 6 5′-CTGTGCGAGAGCGAGAGTCTG-3′ or *HDMX*-S, XS AS 5′-GCACTTTGCTGTAGTAGCAGTG-3′. Each reaction included 1/10 of the cDNA reaction, 10 µL of 2× Quantitect Sybr Green Master Mix (Qiagen) and 20 pmol of the respective primers in a total volume of 20 µL. The PCR consisted of 50 cycles with 30 sec of denaturation at 95°C, 30 sec of primer annealing at 58°C and synthesis at 72°C for 30 sec.

The p53 mutational status was assessed by sequencing exons 4 through 10. Primers and conditions are available on request.

Duplex-PCR analysis

Genomic DNA (20 ng) isolated from the tumor and peripheral blood lymphocytes of each patient was amplified with primers for *HDMX* and *Prothrombin*, as a reference for a single copy gene. The primer sequences were: MDMX-5 5′-CCTTGAGGAAGGATTGGTATT-3′, MDMX-2 5′-CTCTGACAGGTTGGAAATAA-3′, Prot-1 5′-TCATCCTCAGTCCTAATGC-3′, Prot-2 5′-AGACCCCAAGAAAGAAATGG-3′. The PCR reaction comprised of 1× reaction buffer, 2.5 mM $MgCl_2$, dNTP mix, 20 pmol of each primer, 1 U HotStarTaq (Qiagen) and 1× Q-solution in a total volume of 50 µL. The PCR was carried out for 30 cycles in a PCR machine (MJ Research, Waltham, MA). The PCR products were separated on a 2% agarose gel, and the band intensity was measured by densitometry. The *HDMX*/*Prothrombin* ratio was calculated. The *HDMX* gene was considered as amplified when the *HDMX*/*Prothrombin* ratio of the tumor sample was more than 3-fold greater than the ratio in the patient's corresponding lymphocytes.

In the samples where *HDMX* was found to be amplified, the amplification status of the following neighboring genes were also analyzed by duplex-PCR: *GAC1* (glioma amplified on chromosome 1), *REN* (renin), *ELK4* (Ets-domain protein 4), *PIK3C2B* (phosphatidylinositol-3-kinase class 2), *ELF3* (E74-like factor 3) as well as the anonymous gene *HDCMD38P*. The primers and PCR conditions for these genes have been previously described by Riemenschneider *et al.*[9]

Western blot hybridization

Protein extracts were electrophoresed by SDS-PAGE and the proteins were electrophoretically transferred onto nitrocellulose membranes. The membranes were then blocked with 10% nonfat dry milk in TBST buffer (10 mM Tris, pH 7.6, 150 mM NaCl, 0.05% Tween 20) for 1 hr at room temperature. The blots were then incubated with several antibodies independently; a rabbit polyclonal antiserum generated against HDMX-S (6658/2, 1:200 dilution), the respective preimmune serum, a rabbit polyclonal antiserum against FL-HDMX (p55, 1:200 dilution) and with an anti-actin antibody (1:2,000 dilution). The blots were then washed 3 times with TBST (5 min each wash) prior to incubation with a 1:2,000 dilution of the respective secondary peroxidase-conjugated antibody (anti-mouse or anti-rabbit) for 30 min at room temperature. Three washing steps (5 min each) were then followed by visualization of the bands by incubating the membranes at room temperature in ECL-substrate (Amersham, Freiburg, Germany) and exposure to BioMax film (Kodak, Stuttgart, Germany).

The p55 antiserum against FL-HDMX was kindly provided by A.G. Jochemsen from the Leiden University Medical Center.

Statistical evaluation

All statistics, including the Cox's proportional hazard model and the Kaplan-Meier survival estimates, were carried out using the SPSS 11.0 software (SPSS Science, Chicago, IL). A probability of $p < 0.05$ was considered as significant.

Results

HDMX *gene amplification in STS*

HDMX gene amplification in 66 STS samples was evaluated by multiplex PCR using *Prothrombin* as a single-copy reference gene. *HDMX* gene amplification was detected in 17% of the STS samples (11 of 66), with the intensities of the bands representing amplified *HDMX* genes measuring up to 9-fold greater when compared to the bands representing unamplified genes (Fig. 2). The highest frequency of *HDMX* gene amplification was present in leiomyosarcomas (4/14, 28%, Table I). Only 17% (3/18) of the liposarcomas and 7% (1/14) of MFH contained amplified *HDMX* genes, whereas all 6 synovial sarcomas and all 4 neurogenic sarcoma samples were negative with respect to *HDMX* gene amplification. The occurrence of an amplified *HDMX* gene correlated with the tumor's staging ($p = 0.015$, χ^2 test). However, there was no association between the tumor's grading and the presence of an amplified *HDMX* gene ($p = 0.92$, χ^2 test).

TABLE I – SUMMARY OF CLINICAL AND MOLECULAR DATA FOR THE STS

	HDMX amplification			*HDMX*-S expression		
	Total $n = 66$	Single copy[1] $n = 55$	Amplified[1] $n = 11$	Total $n = 57$	"Normal"[2] $n = 49$	Overexpression[2] $n = 8$
Men/women	29/37	24/31	5/6	28/29	27/22	1/7
Tumor type						
Liposarcoma	18	15	3	15	14	1
MFH	14	13	1	12	8	4
Fibrosarcoma	2	2	0	2	2	0
NS	4	4	0	5	5	0
RMS	4	3	1	4	3	1
LMS	14	10	4	11	10	1
Other STS	10[3]	8[3]	2	8[3]	7[3]	1
Tumor stage						
I	10	10	0	11	11	–
II	30	26	4	23	21	2
III	19	16	3	13	9	4
IV	7	3	4	10	8	2
Tumor localization						
Extremities	39	36	3	37	31	6
Thorax	4	3	1	3	1	2
Head	1	1	–	2	2	–
Abdomen	19	13	6	14	14	–
Other	3	2	1	1	1	–
Follow-up stage I–IV						
Alive	31	30	1	30	29	1
Expired	35	25	10	27	20	7

LMS, leiomyosarcomas; MFH, malignant fibrous histiocytomas; RMS, rhadomyosarcomas; STS, soft-tissue sarcomas.–[1]Single copy, ratio *HDMX/prothrombin* ≤ 3; amplified, ratio *HDMX/prothrombin* > 3.–[2]"Normal", *HDMX*-S/FL-*HDMX* ≤ 1; overexpression, *HDMX*-S/FL-*HDMX* > 1.–[3]One tumor could not be classified.

To investigate the size of the amplicon at the 1q32 locus, the amplification status of neighboring genes of *HDMX*, including *GAC1, REN, ELK4, PIK3C2B, ELF3* as well as *HDCMD38P*, were analyzed by duplex-PCR. The adjacent gene *GAC1* (distal to *HDMX*) was coamplified in 4 of 10 tumors (2 low-level and 2 high-level amplifications). The other distal located genes *HDCMD* and *ELF3* were amplified in 3 of 10 samples. *PIK3* and *REN* (which map proximal to *HDMX*) were not amplified in the 10 samples with *HDMX* amplification. However, the most proximal located gene, *ELK4*, showed high-level amplifications in 3 out of 10 samples. There was one example (LZ55) in which the largest amplicon included the genes *HDMX, GAC1, HDCMD38P* and *ELF3* (distal to *HDMX*). In another sample (LZ79), the most distal and most proximal located genes, *ELF3* and *ELK4*, respectively, were amplified, whereas other neighboring, more adjacent genes were not. In 5 tumor samples that showed no *HDMX* amplification, none of the neighboring genes were amplified (data not shown).

HDMX *mRNA expression in STS*

Expression of full-length *HDMX* and *HDMX*-S mRNA was evaluated in 57 STS samples. Primers were used that allowed the PCR products obtained from either FL-*HDMX* or *HDMX*-S cDNA to be distinguished, as described in Material and Methods. We detected *HDMX* mRNA in 54 of 57 (95%) samples (Fig. 3). In 1 of the 3 tumors that did not express *HDMX* mRNA, the *HDMX* gene was amplified. In 12 of 54 (22%) samples with detectable *HDMX* mRNA expression, only the full-length transcript could be detected (Fig. 3). In 42 of 54 (78%) of the samples, both transcripts were expressed, and of these the FL transcript was predominantly expressed in 48% (20 of 42), there was equivalent expression of both transcripts (FL-*HDMX* and *HDMX*-S) in 33% (14 of 42) of the samples, and the expression level of *HDMX*-S relative to FL-*HDMX* was elevated (referred to as *HDMX*-S overexpression) in 19% (8 of 42) of the samples. There was no association between *HDMX*-S overexpression and the p53 mutational status. Three of 8 patient samples contained a wild-type *p53* gene, and 5 of 8 were characterized by a mutated *p53* gene (Table II).

In the samples that showed *HDMX*-S overexpression, the expression ratio of *HDMX*-S compared to FL-*HDMX* was also analyzed by quantitative real-time RT-PCR. Samples ($n = 5$) with low *HDMX*-S expression were used as controls. We confirmed the densitometry results demonstrating that *HDMX*-S-mRNA was overexpressed in these samples. The ratio of *HDMX*-S/FL-*HDMX* in the samples that overexpressed *HDMX*-S ranged from 1.5-fold to 4.5-fold. In all of the control samples, the FL-*HDMX* transcript was predominantly expressed (data not shown).

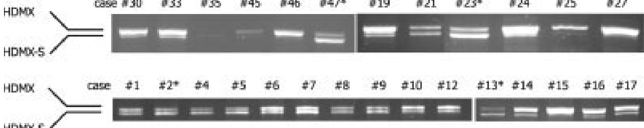


FIGURE 3 – Expression analysis of both full-length *HDMX* and *HDMX*-S mRNAs in soft-tissue sarcomas. The forward primer MDMX-3 was located in exon 2 just upstream of the translation start, and the reverse primer MDMX-8 was located in exon 8. Therefore, it was possible to distinguish between the full-length *HDMX*-mRNA (577 bp) and the *HDMX*-S mRNA (509 bp) RT-PCR products. The lanes marked with an asterisk represent samples in which the *HDMX*-S transcript was overexpressed compared to the full-length *HMDX*-mRNA.

The expression of *HDMX*-mRNA was also evaluated in normal tissues from several patients, including kidney, lung and spleen. The transcripts of FL-*HDMX* and *HDMX*-S were undetectable in normal tissues when analyzed under the same conditions as the tumor samples (data not shown). However, faint bands were visible after 2 rounds of PCR amplification (70 cycles). The FL-*HDMX* transcript was predominant in all normal tissues analyzed.

HDMX-*S and FL-HDMX protein expression*

To evaluate expression of proteins that are translated from the FL-*HDMX* and *HDMX*-S mRNA when *HDMX*-S mRNA is overexpressed, we generated a rabbit polyclonal serum (6658/2) against the 26 unique amino acids in HDMX-S (Fig. 1). For the

TABLE II – CHARACTERISTICS OF PATIENTS WITH OVEREXPRESSION OF THE *HDMX*-S SPLICE VARIANT AND *HDMX* GENE AMPLIFICATION

Case	Tumor subtype	Grade	Tumor localization	Status	*HDMX* amplified	*HDMX*-S overexpression	*HDM2* amplified[1]	p53 mutational status
HDMX-S overexpression								
LZ2	Pleo. MFH	III	Lower extr.	E	No	Yes	No	wt
LZ13	Emb. RMS	III	Thor.	E	Yes	Yes	No	mt
LZ23	Pleo. MFH	III	Lower extr.	E	No	Yes	No	mt
LZ47	Myx. LS	III	Thor.	E	No	Yes	No	mt (intr.)
LZ57	Pleo. MFH	III	Lower extr.	E	No	Yes	No	mt (intr.)
LZ94	Syn.	II	Lower extr.	E	No	Yes	No	wt
LZ98	LMS	III	Lower extr.	A	No	Yes	No	mt
LZ100	Pleo. MFH	III	Lower extr.	E	No	Yes	Yes	wt
HDMX gene amplified								
LZ3	Wd-LS	II	Retroperitoneal	E	Yes	No	Yes	wt
LZ5	LMS	III	Intraabdominal	E	Yes	No	No	wt
LZ10	Pleo. LS	II	Lower extr.	E	Yes	No	No	wt
LZ11	Myx. LS	II	Multi	E	Yes	No	No	wt
LZ13	Emb. RMS	III	Thor.	E	Yes	Yes	No	mt
LZ18	MPNST	II	Intraabdominal	E	Yes	No	No	wt
LZ20	Pleo. MFH	III	Pelvis	E	Yes	No	No	wt
LZ49	LMS	II	Intraabdominal	E	Yes	No	No	mt
LZ55	Fib. MT	II	Intraabdominal	E	Yes	No	No	mt (intr.)
LZ76	LMS	II	Lower extr.	A	Yes	No	Yes	n.d.
LZ79	Epith. LMS	III	Intraabdominal	E	Yes	No	No	wt

Pleo. MFH, pleomorphic malignant fibrous histiocytoma; MPNST, malignant peripheral nerve sheath tumors; fib. MT, fibrous mesothelioma; neu-epith, neuroepithelioma; emb. RMS, embryonic rhabdomyosarcoma; myx. LS, myxoid liposarcoma; wd-LS, well-differentiated liposarcomas; LMS, leiomyosarcoma; syn, synovial sarcoma; E, expired; A, alive, F, female; M, male; lower extr., lower extremities; upper extr., upper extremities; thor., thoraxwall.–[1]The results of the HDM2 amplification were published in Bartel *et al*.[18]

detection of the FL-HDMX protein, another rabbit antiserum p55 (kindly provided by A.G. Jochemsen, Leiden, NL) was used. The antiserum 6658/2 detected a band of approximately 22 kDA, which is barely visible in protein extracts from tumor cell lines when incubating the membrane with the preimmune serum (Fig. 4*a*). The calculated weight of the HDMX-S protein is 15 kDa, and the difference compared to the apparent weight observed after Western analysis suggests that HDMX-S undergoes posttranslational modification.[12] In our analysis, HDMX-S protein could be detected in cell lysates isolated from the samples that also showed elevated *HDMX*-S/FL-*HDMX* mRNA ratios. The band intensities have been analyzed by means of densitometry and were normalized against the actin expression levels within the same sample. We have found that the HDMX-S/FL-HDMX ratios were higher in these samples, at the protein level; however, the effect was not as pronounced as on the mRNA level. Since we have used different antisera with possibly different binding affinities to detect HDMX-S and FL-HDMX protein expression, it is not appropriate to draw conclusions about the exact HDMX-S/FL-HDMX protein ratio. Nonetheless, overexpression of the *HDMX*-S mRNA in STS is associated with but does not appear to correlate with elevated HDMX-S protein levels and averagely higher HDMX-S/FL-HDMX protein ratios.

Clinical significance of HDMX *gene amplification and* HDMX-*S overexpression*

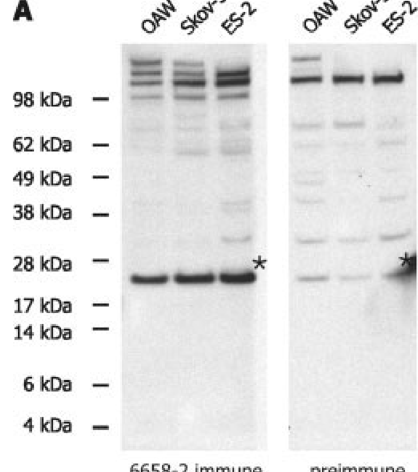
Kaplan-Meier analysis revealed that patients whose tumors do not contain *HDMX* gene amplification have a mean survival time of 68 months compared to 25 months for patients with an amplified *HDMX* gene (log-rank test, $p = 0.0004$, Fig. 5*a*). In a multivariate Cox's proportional-hazards regression model, which was adjusted to tumor grade and p53 mutational status, we demonstrated that the relative risk (RR) of tumor-related death for patients whose tumors carry an amplified *HDMX* gene was significantly increased (RR = 6.5; $p < 0.0001$, Fig. 5*b*). When the status of *MDM2* gene amplification, which predicts a favorable prognosis for STS patients,[18] was also included in the Cox's regression model, the relative risk for STS patients with an amplified *HDMX* gene alone was even greater compared to patients with *MDM2* gene amplification (data not shown). In summary, our data demonstrate that the *HDMX* gene is amplified in a subset of STS and that it predicts a poor prognosis for STS patients.

The clinical significance of *HDMX*-S overexpression in STS was also evaluated. Data demonstrated an association between tumor grade and overexpression of the *HDMX*-S splice form. Of the 8 tumors that overexpressed the *HDMX*-S variant, 1 was classified as a grade 2 tumor and 7 as grade 3 tumors (Table II). Furthermore, we analyzed whether *HDMX*-S overexpression was associated with a particular tumor type. It was determined that the frequency of overexpression was higher in MFHs (4 of 12, 33%) than in other tumor types (Table I). For example, in only 1 of 15 liposarcomas was the *HMDX*-S transcript predominantly expressed, and in fibrosarcomas and neurogenic sarcomas the levels of FL-*HMDX* mRNA were greater than that of the *HDMX*-S splice variant.

The survival estimates for patients whose tumors overexpressed the *HDMX*-S variant were significantly shorter than those for patients with lower or equal levels of *HDMX*-S compared to FL-*HDMX* (15 *vs.* 53 months, $p = 0.0001$, Fig. 6*a*). Remarkably, 7 of 8 patients with *HDMX*-S overexpression died within the observation time of 55 months. Cox-regression analysis, which was adjusted for the tumor localization and p53 mutational status (Fig. 6*b*), demonstrated that the patients whose STS overexpressed *HDMX*-S had a worse prognosis than patients with lower amounts of the *HDMX*-S splice form (RR = 17, $p < 0.0001$). A detailed analysis of MFHs and fibrosarcomas revealed that patients whose tumor overexpressed the *HDMX*-S variant had an 8.1-fold increased risk of tumor-related death (adjusted for tumor localization, $p = 0.017$, data not shown). In summary, our data demonstrate that overexpression of the splice variant *HDMX*-S is a prognostic factor that predicts a poor outcome and a significantly shortened survival time for STS patients.

Discussion

Alterations of the p53-MDM2-p14ARF-pathway, such as *p53* gene mutations and MDM2 overexpression, have been shown to be independent prognostic markers for the outcome of STS patients.[19–21] However, few studies have described gene amplification and overexpression of the homologue of the human *MDM2* gene, *HDMX*, in human tumors.[8,9,11] To our knowledge, our study is the first demonstration and analysis of the consequences of *HDMX* gene amplification and overexpression of the *HDMX*-S splice variant in human soft tissue sarcomas. Our results clearly



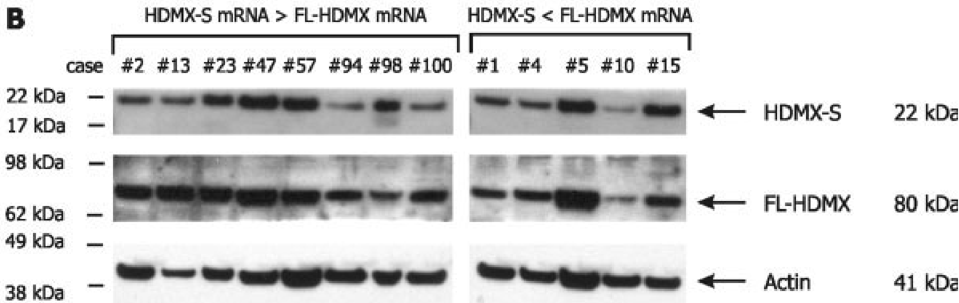


FIGURE 4 – Elevated *HDMX*-S transcript levels correlate with increased HDMX-S protein expression. (*a*) A rabbit polyclonal antiserum 6658/2 detected a band of about 22 kDa (asterisk) that is barely detectable when the blots are incubated with the preimmune serum. (*b*) The antiserum (6658/2) was used to evaluate HDMX-S protein expression in STS samples (upper panel). The rabbit polyclonal antiserum p55 was used to detect the expression of FL-HDMX in these tumors (middle panel). The band intensities were determined by densitometry and normalized against the corresponding actin band (lower panel). A comparison of the ratios of band intensities of HDMX-S/Actin and FL-HDMX/Actin revealed that the HDMX-S protein expression was generally slightly higher in the tumor samples with elevated HDMX-S mRNA levels than in the tumors with lower *HDMX*-S mRNA expression.

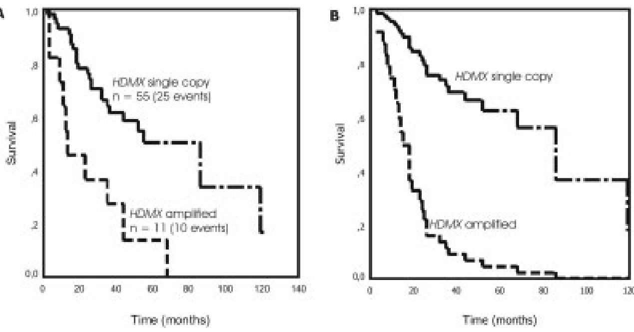


FIGURE 5 – *HDMX* gene amplification in the tumor samples was associated with decreased patient survival (*a*) and an increased risk of tumor-related death for STS patients (*b*). Cox's proportional-hazard regression model was adjusted to the tumor grading and p53 mutational status, both of which are independent prognostic factors in STS.

indicate that both *HDMX* gene amplification and *HDMX*-S overexpression have a significant impact on the prognosis of STS patients.

To study the role of *HDMX* amplification in STS, we analyzed its amplification status in primary STS. We found the *HDMX* gene to be amplified in 17% of the tumor samples. Riemenschneider *et al*. reported that amplification and overexpression of HDMX was exclusively found in glioblastomas without p53 mutations, more specifically in tumors without hot spot mutations, and they suggest that *HDMX* amplification represents a novel mechanism by which a small subset of malignant gliomas might escape p53-dependent growth control.[8] This is at least partially true for STS patients whose tumors carry *HDMX* gene amplifications because 7 of 11 STS tissue samples with an amplified *HDMX* gene contain a wild-type *p53* gene (Table II), 1 sample had a silent mutation (codon 213) and 1 had an intronic mutation, whereas only 1 STS sample had a mutation resulting in an amino acid change in codon 280 in 1 allele. Furthermore, findings from Ramos *et al*. support this hypothesis.[11] They have shown for a series of melanoma and ovarian cancer cell lines that there is an association between HDMX overexpression and the presence of a wild-type *p53* gene. It can be concluded from these data that *HDMX* amplification (and/or overexpression) and a mutated *p53* gene represent independent mechanisms resulting in the inactivation of p53 tumor suppressor protein.

In our study, we also observed that in well-differentiated liposarcomas and in a subset of MFHs either *HDMX* (located within 1q32) or *MDM2* (12q14-15) are co-amplified or neither of these 2 genes are amplified. In contrast, pleomorphic and myxoid liposarcomas contain only amplified *HDMX*, not *MDM2*. This has been confirmed by analyzing liposarcomas and MFHs by CGH and array-based CGH (H. Schmidt, data not shown). Previously, we demonstrated that the lack of *MDM2* gene amplification is correlated with a poor prognosis in STS.[18] When combining these data with the results of this study concerning *HDMX* gene amplification, it is apparent that patients whose tumor carries an amplified *MDM2* gene have the best outcome, whereas amplification of the *HDMX* gene is correlated with the worst prognosis. Patients with tumors in which either both of the genes or neither of the two is amplified demonstrate an intermediate phenotype. Furthermore, our studies show that *HDMX* is the main amplification target at the 1q32 locus and that other neighboring genes variably co-amplify.

We also evaluated our tumor samples for expression of *HDMX* mRNA, including the *HDMX*-S splice variant. Data demonstrate that *HDMX*-S is overexpressed in a subset of STS, and this amplification is associated with a 17-fold increased risk of tumor-related death for those patients. Furthermore, these patients are characterized by a strikingly decreased overall survival. Our data show that the *HDMX*-S transcript is predominantly expressed in a subgroup of MFHs with

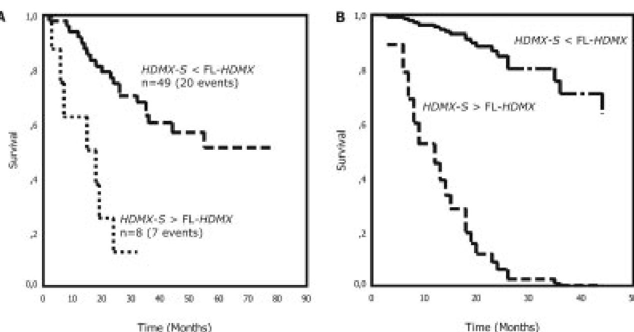


FIGURE 6 – Kaplan-Meier survival estimates revealed decreased survival for STS patients who overexpressed the *HDMX*-S splice variant in their tumors (*a*). The multivariate Cox's proportional-hazard regression model (adjusted to p53 mutational status and tumor localization) for *HDMX*-S expression in STS demonstrated that elevated *HDMX*-S mRNA expression is an independent prognostic factor and predicts a poor outcome for STS patients (*b*).

a particularly malignant phenotype. This is in accordance with data from Riemenschneider *et al.*,[8] who reported that expression of *HDMX*-S was elevated in glioblastomas compared to full-length *HDMX*. However, this association was not observed in anaplastic gliomas and low-grade gliomas.[8] Studies of Rallapalli *et al.*[12] and de Graaf *et al.*[13] revealed that the truncated proteins encoded by both *MDMX*-S and *HDMX*-S are more efficient at suppressing p53-mediated transactivation and induction of apoptosis than FL-MDMX or FL-HDMX, respectively. It is therefore conceivable that the upregulation of HDMX-S might contribute to the neoplastic transformation of cells by inactivating wt-p53. On the other hand, HDMX-S may possess other functions besides abrogating p53's activity. Regarding the correlation of HDMX-S overexpression and p53 mutational status, no data can be found in the literature. Riemenschneider *et al.*[8] report that the amount of HDMX-S is higher in relation to FL-HDMX in glioblastomas than in gliomas, and Rallapalli *et al.*[12] describe that HDMX-S is upregulated in transformed cells and downregulated in low passage cells. However, no information is given on the p53 mutational status of the tumors or cells described. We screened more than 40 human tumor cell lines with known p53 status. The screening revealed that FL-*HDMX* was the predominant transcript in wt-p53 cell lines such as the mamma carcinoma cell line MCF-7 and in the prostate cancer cell line LnCap. In these cell lines, the *HDMX*-S transcript was almost undetectable by RT-PCR. On the other hand, in cell lines that are p53-null, like Saos-2 (osteosarcoma cell line) and PC3 (prostate cancer), *HDMX*-S was predominantly expressed, and FL-*HDMX* was hardly detectable. In cell lines with p53 point mutations, it appears that the ratio *HDMX*-S/FL-*HDMX* is dependent on the type of the mutation (inactivating or silent, which domain, etc.; Bartel *et al.,* data not shown). These data suggest that there is a feedback mechanism between p53 and *HDMX*/*HDMX*-S expression. However, it has previously been shown that *HDMX* cannot be transcriptionally activated by p53, for example, after DNA damage.[1] Our data are preliminary and could not be confirmed in the tumor samples (Table II). We conclude that the expression, especially of *HDMX*-S, is independent of the p53 mutational status as it has been shown by the Cox's regressional hazard model.

In addition to mRNA analyses, we have also evaluated the expression of the proteins that are encoded by the *HDMX*-S and FL-*HDMX* transcripts. It is apparent from the Figures 3 and 4*b* that elevated mRNA levels do not always correlate with higher protein levels. It is generally accepted that there is not necessarily a correlation between the abundance of a given mRNA and the amount or protein it encodes,[22] suggesting that posttranscriptional regulation of gene expression is a frequent phenomenon. Reasons for this include the stability of the mRNA, the transcription and/or the translation efficacy and the stability of the protein, etc. Regarding HDMX-S and FL-HDMX, the ratio of the 2 proteins might be more important than their absolute expression levels because both proteins compete for p53 binding. It has been shown that HDMX-S has a greater affinity to p53 compared to FL-HDMX.[12] In our study, the average HDMX-S/FL-HDMX protein ratio was higher in those samples that were characterized by *HDMX*-S transcript overrepresentation. However, this was not as pronounced as on the mRNA level. Although the band intensities have been determined by densitometry and the values for the bands representing HDMX-S and FL-HDMX were normalized against the actin expression level within the same sample, the data should be used with caution. We have used different antisera for the detection of HDMX-S and FL-HDMX. The antisera could have different binding affinities for the respective protein. In future studies, the expression of FL-HDMX and HDMX-S should be analyzed with an antibody that recognizes an epitope that is present in both proteins. Nonetheless, we have clearly shown that the *HDMX*-S/FL-*HDMX* mRNA ratio is a strong, independent predictor of survival for STSs.

We demonstrated that *HDMX* mRNA is expressed at considerably higher levels in STS compared to corresponding normal tissues from several patients. The transcripts of both FL-*HDMX* and *HDMX*-S were undetectable after RT-PCR amplification of normal tissues when analyzed under comparable conditions as for tumor samples (data not shown). However, faint bands were visible after 2 rounds of PCR amplification (70 cycles), whereas in the tumor tissue detectable amounts were produced after only 35 amplification cycles. Riemenschneider *et al.* observed that *HDMX*-S mRNA is constitutively expressed in nonneoplastic brain tissue.[8] However, *HDMX* mRNA was expressed at higher levels in gliomas as shown by both RT-PCR and Northern blotting. This result was more pronounced in tumor samples that carried *HDMX* gene amplifications.[8] However, there was no correlation between *HDMX* gene amplification and *HDMX*-S overexpression in our STS samples. Therefore, it can be concluded that *(i)* both FL-*HDMX* and *HDMX*-S may be expressed in normal, untransformed tissues, albeit at very low, almost undetectable levels, and *(ii)* FL-*HDMX* compared to *HDMX*-S is the predominant transcript in normal tissues.

In summary, our data clearly indicate that *HDMX* gene amplification is a common event in STS and appears to be associated with poor prognosis. Furthermore, overexpression of the smaller *HDMX*-S transcript correlated with an unfavorable prognosis for STS patients. However, the mechanism by which *HDMX*-S expression is regulated must still be elucidated. Taken together, the data presented here suggest that the *HDMX* gene represents an important target of amplification in a subset of STS and that overexpression of HDMX-S could be another mechanism of inactivating p53 contributing to the process of immortalization and/or neoplastic transformation.

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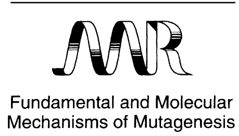

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A *Mbo*II polymorphism in exon 11 of the human *MDM2* gene occuring in normal blood donors and in soft tissue sarcoma patients: an indication for an increased cancer susceptibility?

Helge Taubert[a,*], Matthias Kappler[a], Axel Meye[a], Frank Bartel[a], Thilo Schlott[b], Christine Lautenschläger[c], Matthias Bache[d], Hannelore Schmidt[a], Peter Würl[e]

[a] *Institute of Pathology, Faculty of Medicine, Martin Luther University Halle-Wittenberg, Magdeburger Strasse 14, D-06097 Halle/Saale, Germany*
[b] *Department of Cytopathology, Georg-August-University, Robert-Koch-Strasse 40, D-37075 Göttingen, Germany*
[c] *Department of Radiotherapy, Martin Luther University Halle-Wittenberg, Dryanderstrasse 4-7, D-06097 Halle/Saale, Germany*
[d] *Institute of Medical Epidemiology, Biometry and Informatics, Martin Luther University Halle-Wittenberg, Magdeburger Strasse 27, D-06097 Halle/Saale, Germany*
[e] *Surgical Clinic 1, University of Leipzig, Liebigstrasse 20a, D-04301 Leipzig, Germany*

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Abstract

The human *MDM2* oncogene, well known as the tumor suppressor gene *p53*'s partner, plays an important role in tumorigenesis whether it is dependent on or independent of *TP53*. In this study, we investigated in a PCR-sequencing analysis the exon 11 of the human *MDM2* gene for gene alterations. A *Mbo*II polymorphism occurs in 8% of normal blood donors (8 out of 100 probands) and in 13% of the soft tissue sarcoma patients (11 out of 82 patients). Of note was that two STS patients carried the gene alteration only in the tumor specimens heterozygously but not in normal tissue. In a Kaplan–Meier analysis, patients without the polymorphism, indicated a median survival rate of 57 months, whereas, patients with the polymorphism survived on average only 38 months. We suggest that this polymorphism might be associated with an increased cancer susceptibility.
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Keywords: Murine double minute gene 2 (*MDM2*); DNA polymorphism; Soft tissue sarcoma; Cancer susceptibility

1. Introduction

The human *MDM2* oncogene, located on chromosome 12q13-14 is called the "big brother of *p53*" [1]. The reason behind this is that the MDM2 protein inhibits the tumor suppressor function of *TP53*. It promotes inactivation of *TP53* through its rapid degradation, inhibition of *TP53*-mediated apoptosis/growth arrest and by masking the transactivation domain of *TP53* thus impairing interaction with the transcriptional machinery (reviewed in [2]). Furthermore, *MDM2* can act independently of *TP53*, e.g. it interacts with the transcription factors of the E2F-family and the human TATA-binding protein [3,4], inhibits Rb growth regulatory function [5], contributes to tumorigenesis in *TP53*-/- mammary epithelial cells [6], mediates TGF-ß1 resistance [7] and inhibits the G_0/G_1-S-phase transition in normal human diploid

* Corresponding author. Tel.: +49-345-557-1293; fax: +49-345-557-1295.
E-mail address: helge.taubert@medizin.uni-halle.de (H. Taubert).

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cells [8]. This all suggests a general regulating role in the complex interaction of tumor-associated genes. What is especially well described is: a disturbance of normal *MDM2* function in tumors and tumor cell lines by gene amplification, different mRNA levels or mRNA splice products, and protein overexpression including soft tissue sarcomas [2,9–19]. However, to date, except for three studies, mutational analysis and investigations concerning polymorphisms in the human *MDM2* gene have not been comprehensively done. The above mentioned studies dealt with: one polymorphism [20], some mutations in different tumors [21] and the absence of mutations in codon 17 [22]. Data concerning gene alterations and their potential effects are yet to be collected and investigated.

2. Material and methods

2.1. Tissue specimens and patients

Blood from 100 healthy donors and frozen tissue from 82 soft tissue sarcoma patients were collected from the Institute of Human Genetics and Medical Biology and from the Institute of Pathology at the Martin-Luther University Halle-Wittenberg, Germany between 1995–1999.

2.2. DNA preparation, PCR and sequencing

The DNA was isolated from 10 to 15 cryo-sections (30 μm thick in diameter) as previously described [23,24]. In a PCR reaction, a 548 bp fragment was amplified starting at base four of exon 11 of the human *MDM2* gene (GenBank AJ251943). The 20 oligomer primers, OL7 in sense direction: 5′ GCTGACTATTGGAAATGCAC 3′ OL8 in antisense direction: 5′ ATTGGTTGTCTACATACTGG 3′ were applied as described by Schlott et al. [21]. In brief, PCR conditions are described as the following: pre-denaturation at 95°C for 5 min and 40 cycles of denaturation at 95°C for 45 s, annealing and DNA synthesis for 1 min at 55°C. Cycle sequencing was done using the same primers and conditions as for the PCR amplification products (TMII sequenase kit/Amersham Pharmacia, Freiburg, Germany). Products were sequenced on an ABI373 (Perkin Elmer, Weiterstadt, Germany). All tumor samples were sequenced in both directions and samples from blood donors were sequenced in sense direction. Striking cases were double checked to exclude errors from the Taq polymerase and further confirmed by a restriction endonuclease digest.

2.3. Restriction endonuclease digest

The 548 bp PCR product was first digested with *Hin*dIII (Promega, Germany) and the 254 bp band was cut out and the DNA was regained by a cleaning kit (QIAquick Gel extraction kit/Qiagen, Hilden, Germany). The DNA was digested further by *Mbo*II (Amersham Pharmacia, Freiburg, Germany) overnight and separated in a 2.5% agarose gel.

3. Results

A 548 bp fragment located in exon 11 of the human *MDM*2 gene was amplified with PCR from samples taken from 100 normal blood donors and samples from 82 soft tissue sarcoma patients (108 samples) as previously described [21]. An A → G (GAA → GAG) polymorphism located in codon 354 was identified by sequencing (Fig. 1) (GeneBank/EMBL accession no. AJ251943). This polymorphism does not change the coded amino acid; it remains a glutamine. It was observed in 8% of the normal blood donors (8 out of 100 probands) and in 13% of soft tissue sarcoma patients (11 out of 82 patients).

This polymorphism, located 162 bp downstream of the 5′-end of exon 11, is part of a *Mbo*II recognition site (Fig. 2). Since the restriction enzyme *Mbo*II usually performs incomplete digestion, the 548 bp PCR fragment was cut with *Hin*dIII first, resulting in a 254 bp and a 294 bp fragment, each with a reduced number of *Mbo*II sites. Following further digestion of the 254 bp *Hin*dIII fragment with *Mbo*II 109 + 79 + 66 bp fragments could be identified in the DNA without a polymorphism and 109 + 145 bp fragments were detected in the DNA with a polymorphism (Fig. 2). The allelic frequency estimated in 100 blood donors was 4.5% and it was 8.5% in the 82 unrelated soft tissue sarcoma patients, which is remarkable but not significant ($P = 0.2$; Fisher's exact test) in comparison to the blood donors. But this is rather related to the still small number of patients and blood donors investigated than to a too small difference of the allelic frequencies.

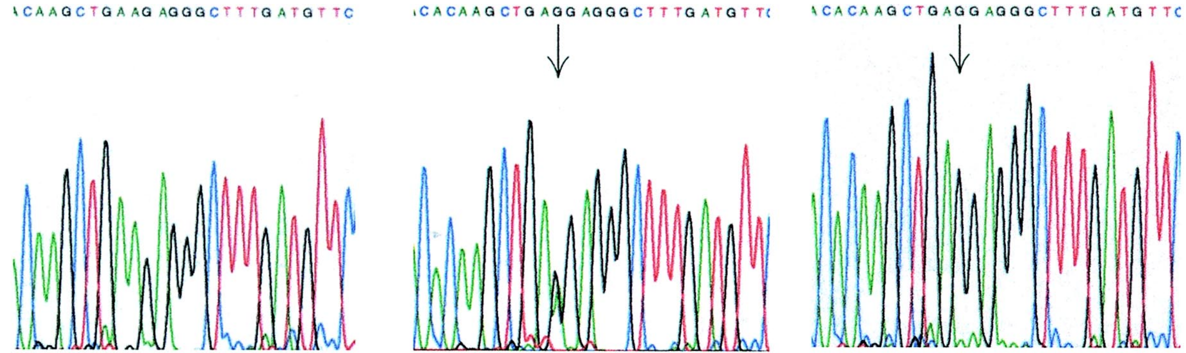

Fig. 1. Sequence polymorphism in codon 354 of exon 11 of the *MDM2* gene. The normal sequence (left part), the polymorphism heterozygously (middle part) and the polymorphism homozygously (right part) is shown.

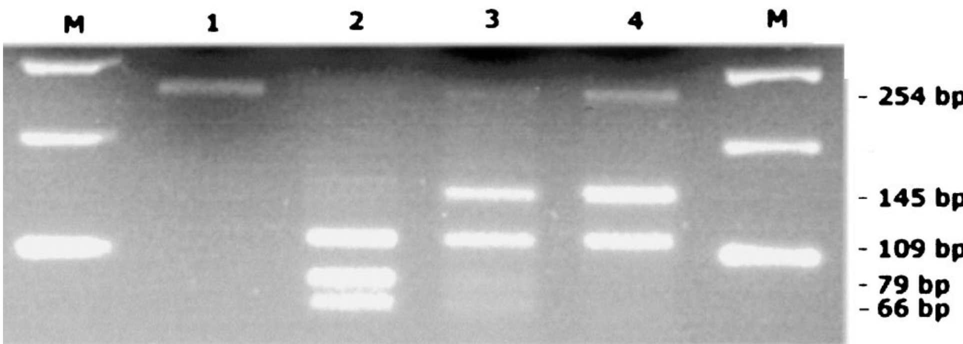

Fig. 2. An *Mbo*II digest recognizes the normal sequence but not the polymorphism in codon 354. An ethidium bromide-stained agarose gel of amplified genomic DNA digested with *Mbo*II is shown. A 254 bp *Hin*dIII fragment from exon 11 of human *MDM* gene results after digestion with *Mbo*II in 109 + 66 + 79 bp fragments for samples without a polymorphism (lane 2) and an additional band at 145 bp in the presence of a heterozygous polymorphism (lane 3, note the weaker 99 and 66 bp bands). Only 109 + 145 bp fragments are seen for the sample with a homozygous polymorphism (lane 4). For comparison an undigested sample (lane 1) and a 100 bp ladder (M) (Amersham Pharmacia, Freiburg, Germany) were used.

One blood donor carried the polymorphism homozygously. Out of the STS patients one carried this gene alteration in normal tissue heterozygously but homo(hemi)zygously in the tumor sample. What we found striking was that two STS patients carried the gene alteration only in the tumor specimens heterozygously but not in normal tissue.

A Kaplan–Meier analysis was performed to test if the occurrence of this gene alteration could have an effect on the prognosis of the STS patients. Patients without the polymorphism showed an average survival time of 38 months (median survival; 95% confidence interval 14–62 months), whereas, patients without the polymorphism survived on the average for 57 months (median survival; 95% confidence interval 14–100 months).

4. Discussion

Despite the important role the *MDM2* oncogene plays in malignant tumorigenesis, especially in soft tissue sarcomas, [1] only a few mutations [21] and just one polymorphism in the transcribed but untranslated 5′-region of the human *MDM2* gene [20] have been previously described in other tumors. The gene

alteration we found occurred in about 8% of healthy blood donors and in 13% of STS patients. Therefore, this study describes a second polymorphism found in the *MDM2* gene.

Surprisingly, we have found that the identified gene alteration was observed in two cases in soft tissue sarcoma specimens but not in normal tissue of the same patients. Furthermore, while investigating five AML patients, in one case, a polymorphism was found, but not in normal lymphocytes from the same patient (Dr. T. Schlott, unpublished results). These findings suggest that gene alteration can also occur somatically. Furthermore, what is remarkable is the result that in a Kaplan–Meier analysis, STS patients with the gene alteration had in average a median survival of 38 months while patients without the gene alteration had in contrast an average median survival of 57 months. This finding indicates that gene alteration plays a possible role in cancer susceptibility.

It has been hypothesized that the inheritance of common polymorphic variants on some genes carry a covert, or more subtle cancer risk [25,26]. In view of this, an interesting example for hypothesis is the TP53 protein that closely interacts with the *MDM2* gene/protein. It has been controversially discussed if there is an increased tumor susceptibility with a TP53 polymorphism. On the other hand, several authors describe the overpresence of special haplotypes of the codon 72 polymorphism as an indicator for increased susceptibility for lung cancer [27–29], colon cancer [30], breast cancer [31] and ovarian cancer [32]. In contrast, this was not found by others for lung cancer [33], breast cancer [34] and cervical cancer [35,36]. Furthermore, occurrence of a polymorphism in the gluthatione S-transferase locus (GSTM3), is suggested to be a risk factor for the basal cell carcinomas of the skin [37]. Altogether in a study for 18 genes investigating the correlation between polymorphisms and the risk of breast cancer three genes possessed a significant correlation (*CYP19*, *GSTP-1*, *TP53*) [38]. Recently, Kong and associates [39] could show that a polymorphism in the *CyclinD1* gene is associated with an increased expression of mRNA splice variants. The proteins translated from these splice variants had an increased half life and patients with these splice variants had a 11 year earlier onset of tumor development. The question if the polymorphism identified by us correlates with the occurrence of *MDM2* splice variants in STS (Bartel et al. unpublished results) is currently under investigation.

Summing up, a polymorphism in exon 11 of the human *MDM2* gene occurring in normal blood donors and soft tissue sarcoma patients has been described for the first time in our investigations. Its sporadic occurrence in STS but not in normal tissue of the same patients and its association with a decreased average survival rate suggests a role in cancer susceptibility.

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A Single Nucleotide Polymorphism in the *MDM2* Promoter Attenuates the p53 Tumor Suppressor Pathway and Accelerates Tumor Formation in Humans

Gareth L. Bond,[2,8] Wenwei Hu,[2,8]
Elisabeth E. Bond,[2] Harlan Robins,[1]
Stuart G. Lutzker,[2] Nicoleta C. Arva,[7]
Jill Bargonetti,[7] Frank Bartel,[4] Helge Taubert,[4]
Peter Wuerl,[5] Kenan Onel,[6] Linwah Yip,[3]
Shih-Jen Hwang,[3] Louise C. Strong,[3]
Guillermina Lozano,[3] and Arnold J. Levine[1,2,*]
[1]School of Natural Sciences
Institute for Advanced Study
Princeton, New Jersey 08540
[2]Cancer Institute of New Jersey
University of Medicine and Dentistry of New Jersey
New Brunswick, New Jersey 08903
[3]Section of Cancer Genetics
M.D. Anderson Cancer Center
University of Texas
Houston, Texas 77030
[4]Institute of Pathology
Faculty of Medicine
Martin-Luther-University
Halle/Saale 06097
Germany
[5]Surgical Clinic
University of Ulm
Ulm 89075
Germany
[6]Department of Medicine
Weill College of Medicine
Cornell University
New York, New York 10021
[7]Department of Biological Sciences
Hunter College and Graduate School
The City University of New York
695 Park Avenue
New York, New York 10021

Summary

The tumor suppressor p53 gene is mutated in minimally half of all cancers. It is therefore reasonable to assume that naturally occurring polymorphic genetic variants in the p53 stress response pathway might determine an individual's susceptibility to cancer. A central node in the p53 pathway is the MDM2 protein, a direct negative regulator of p53. In this report, a single nucleotide polymorphism (SNP309) is found in the *MDM2* promoter and is shown to increase the affinity of the transcriptional activator Sp1, resulting in higher levels of MDM2 RNA and protein and the subsequent attenuation of the p53 pathway. In humans, SNP309 is shown to associate with accelerated tumor formation in both hereditary and sporadic cancers. A model is proposed whereby SNP309 serves as a rate-limiting event in carcinogenesis.

*Correspondence: alevine@ias.edu
[8]These authors contributed equally to this work.

Introduction

The tumor suppressor protein, p53, is activated upon cellular stresses such as DNA damage and oncogene activation and initiates a transcriptional program which leads to DNA repair, cell cycle arrest, and in some cases, apoptosis (Jin and Levine, 2001). The p53 stress response pathway has been shown to be crucial for the prevention of tumor formation. For example, both mice and humans harboring a germline inactivating mutation in one allele of the p53 gene develop tumors very early in life and at dramatically high frequencies (Donehower et al., 1992; Garber et al., 1992; Li et al., 1990). Somatic inactivating mutations of the p53 gene are also found in over 50% of all human tumors (Lain and Lane, 2003). Together, these observations and many others support the importance of the p53 pathway in tumor suppression. It is therefore reasonable to assume that naturally occurring polymorphic genetic variants in critical nodes of the p53 pathway might underlie the variation seen between individuals in their susceptibility to cancer and the progression of their disease.

The search for genetic variation in the p53 pathway was begun by looking in the *MDM2* gene, which encodes an important negative regulator of p53. MDM2 directly binds to and inhibits p53 by regulating its location, stability, and activity as a transcriptional activator (Michael and Oren, 2003). *MDM2* is an essential gene in murine development, as a knockout embryo dies before implantation in the uterus. This lethal phenotype is rescued by knocking out the p53 gene, clearly demonstrating an important genetic interaction between these two genes in murine development (Jones et al., 1995; Montes de Oca Luna et al., 1995). Mendrysa et al. (2003) demonstrated the importance of this interaction in the adult mouse by genetically altering mice to express reduced levels of Mdm2. These mice are small, lymphopenic, and radiosensitive, with increased apoptosis in both lymphocytes and epithelial cells. These phenotypes were all shown to be p53 dependent, thereby further demonstrating that Mdm2 is a key negative regulator of p53 in both the developing and mature mouse. In humans, a subset of tumors overexpress MDM2 mRNA and protein; this overexpression is associated with accelerated cancer progression and lack of response to therapy (Freedman and Levine, 1999). In a subset of these tumors, overexpression of MDM2 was mutually exclusive to p53 mutation, which could suggest that overexpression of MDM2 can substitute for inactivating p53 by mutation (Leach et al., 1993; Oliner et al., 1992).

As MDM2 expression levels seem to be vital to a well-regulated p53 response, naturally occurring sequence variations in the *MDM2* promoter may result in altered expression of the MDM2 protein, thereby impacting p53 tumor suppression and potentially cancer in humans. In this report, data are presented which support this hypothesis.

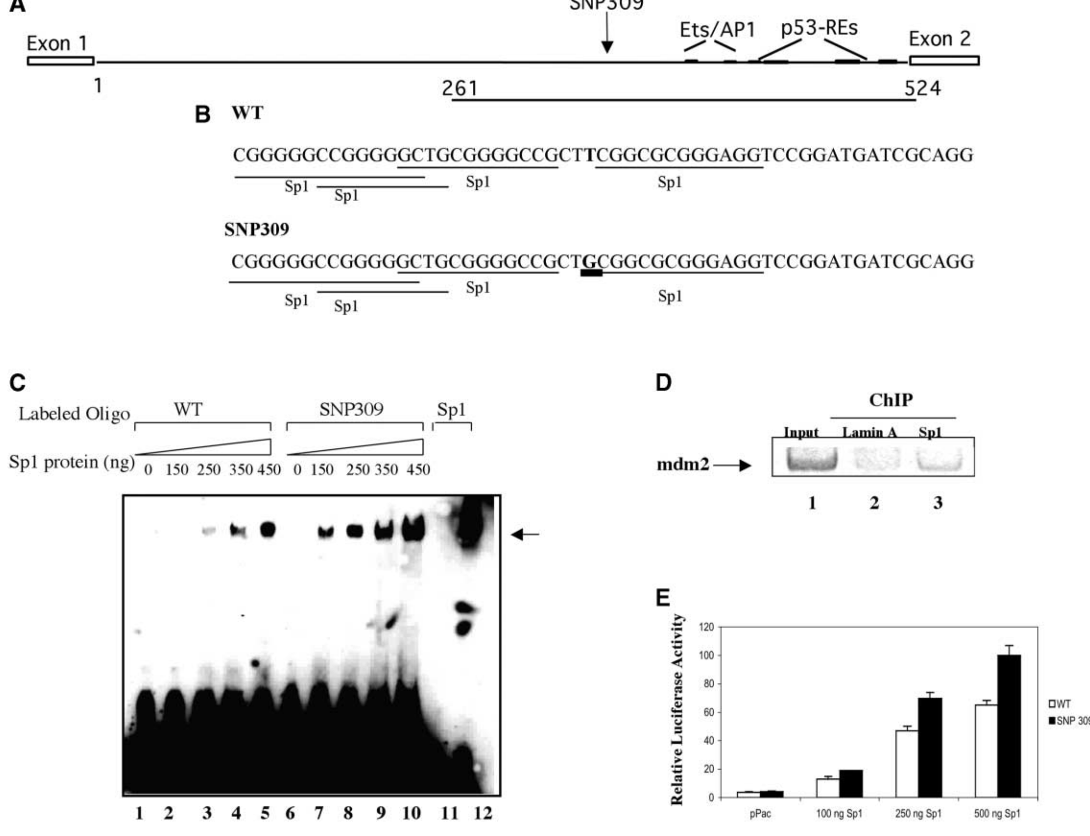


Figure 1. The *MDM2* Promoter Contains a Single Nucleotide Polymorphism Which Alters the Affinity of the Transcriptional Activator Sp1

(A) A schematic diagram depicts the intronic promoter of the *MDM2* gene. The position of SNP309 is indicated in relation to the exon/intron boundaries and the transcription factor binding sites for p53 and Ets/AP-1. The region analyzed for sequence variation is marked by the bar drawn below the diagram.

(B) The analysis of transcription factor binding sites in the region containing SNP309 is depicted. Potential Sp1 sites are underlined.

(C) An autoradiograph of an electrophoretic mobility shift assay is depicted where varying concentrations of purified Sp1 protein were incubated with biotin-labeled oligonucleotides containing either SNP309 (G/G; lanes 6–10), wild-type sequence (T/T; lanes 1–5), or an Sp1 optimal binding site (lane 11, oligo alone, and lane 12, oligo and Sp1). The arrow indicates the specific Sp1-DNA complex.

(D) Chromatin immunoprecipitations (ChIP) were preformed using antibodies against either Sp1 or lamin A. The presence of the *MDM2* promoter was assayed for using PCR. The PCR products are depicted here after electrophoreses on an 8% nondenaturing polyacrylamide gel and subsequent staining with EtBr.

(E) The relative measured luciferase levels are depicted in a bar graph, whereby pGL2 luciferase reporter plasmids containing either SNP309 sequence or wild-type sequence were cotransfected with pPac (control) or pPac-Sp1 expression plasmids. Each value is the average of at least three independent experiments and the error bars represent the standard deviations.

Results

The search for genetic variation in the *MDM2* promoter was focused to a well-characterized region in the first intron of the intronic promoter, which is utilized by both the p53 and ras pathways to activate *MDM2* transcription (Ries et al., 2000; Zauberman et al., 1995). To look for sequence variation in this region, 300 base pairs from genomic DNAs isolated from 50 healthy volunteers were amplified by PCR and sequenced (Figure 1A). Two single nucleotide polymorphisms (SNPs) were found in this region. One of these, SNP309 (a T to G change at the 309th nucleotide in the first intron), was found at relatively high frequency both in the heterozygous state (T/G, 40%) and in the homozygous state (G/G, 12%). The other SNP, SNP344 (a T to A change at the 344th nucleotide in the first intron), is rare and is not further studied (found only in the heterozygous state in 8% of people).

Analysis of this region of the *MDM2* promoter using a computer algorithm (AliBaba) revealed several putative binding sites for the transcription factor Sp1 (Figure 1B). Interestingly, the presence of SNP309, a T to G change, extended the length of one of the putative Sp1 DNA binding sites, suggesting that the presence of SNP309 could increase the affinity of Sp1 to this region of the *MDM2* promoter.

In order to characterize the potential Sp1 binding sites identified by computer analysis and to investigate the functional consequences of SNP309, electro-mobility shift assays (EMSAs) were performed. EMSAs were carried out with purified recombinant human Sp1 protein and labeled double-stranded oligonucleotides containing either the wild-type sequence (T/T) or the SNP309 sequence (G/G). Interestingly, as predicted by the computer analysis, the binding affinity of oligonucleotides containing SNP309 to a range of concentrations (150 ng–450 ng) of purified Sp1 is much higher (2–4-fold) than

that of the wild-type sequence (Figure 1C, lanes 2–5 versus lanes 7–10). The same assays were carried out using HeLa cell nuclear extracts as a source of protein instead of purified Sp1, and similar results were obtained (data not shown). These data suggest that Sp1 can bind to its putative consensus site in the *MDM2* promoter and that SNP309 can greatly enhance its binding affinity of this site.

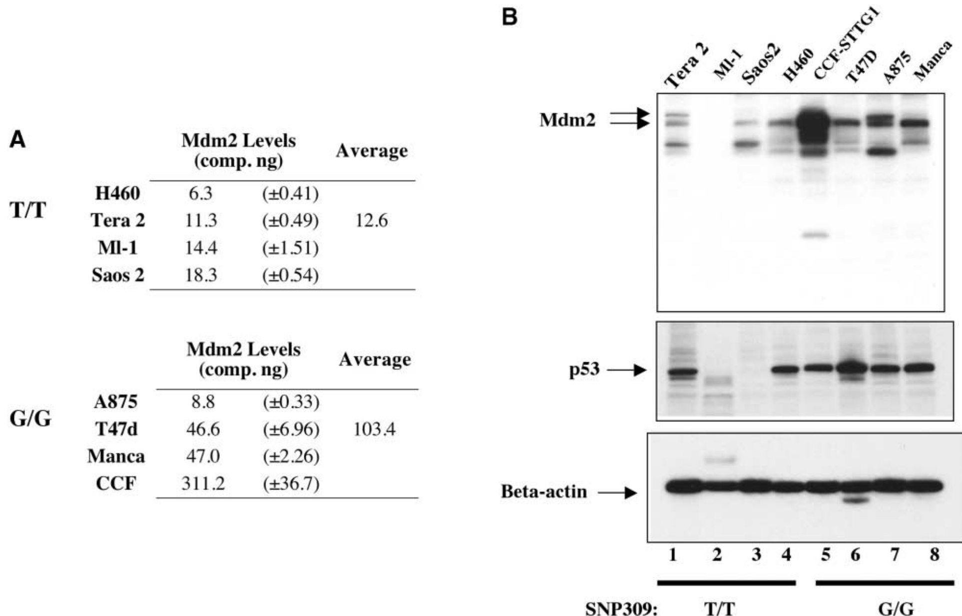
To verify the presence of Sp1 on the *MDM2* promoter in vivo, a chromatin immunoprecipitation (ChIP) was performed. Lysates were prepared from growing Manca cells, homozygous for SNP309 (G/G), and immunoprecipitations were carried out using antibodies against either Sp1 or lamin A. After extensive washing and elution, the DNA was purified and the presence of the *MDM2* promoter was assayed for using PCR. As seen in Figure 1D, the *MDM2* promoter was detected in the ChIP using the Sp1 antibody (lane 3) but not using the lamin A antibody (lane 2). These data suggest that Sp1 binds this region of the *MDM2* promoter in vivo.

To investigate a possible role of Sp1 in the transactivation of the *MDM2* promoter and the influence of SNP309, *Drosophila* SL2 cells, which are deficient in Sp-related proteins, were utilized. SL2 cells were transiently transfected with an Sp1 expression vector (pPac-Sp1) and a luciferase reporter plasmid driven by the *MDM2* promoter either wild-type (T/T) or homozygous (G/G) for SNP309. As shown in Figure 1E, cotransfection of Sp1 strongly stimulated luciferase expression of the reporter plasmid driven by the *MDM2* promoter, as measured by luciferase activity, suggesting that Sp1 can bind to this region of the *MDM2* promoter and activate transcription. Interestingly, the presence of SNP309 in the reporter plasmid consistently showed higher Sp1-induced luciferase expression ($\sim$50%) over the presence of wild-type sequence in the reporter plasmid, as the increased binding affinity of Sp1 to SNP309-containing oligonucleotides would have predicted. Similar results were obtained when both reporter plasmids were transfected into the mammalian HeLa cell line which has an abundance of Sp1. The reporter plasmid containing SNP309 (G) yielded significantly higher luciferase levels (60%) than the plasmid containing the wild-type sequence (T) (data not shown).

Together, these data suggest that Sp1 can bind to the *MDM2* promoter and activate transcription and that the presence of SNP309 further stimulates the activity of Sp1 by enhancing its DNA binding affinity to the *MDM2* promoter. If true, individuals homozygous for SNP309 (G/G) should show heightened levels of MDM2 when compared to individuals wild-type for SNP309 (T/T). To address this, cells in culture were employed. Forty-three tumor-derived cell lines were genotyped for SNP309. SNP309 was present at a frequency similar to that found in the normal volunteers. MDM2 RNA and protein levels were compared in four cell lines homozygous for SNP309 (G/G) to the levels found in four cell lines wild-type for SNP309 (T/T). To compare MDM2 RNA levels, total RNA was isolated from growing cells. MDM2 RNA levels were measured by real-time PCR (TaqMan). The presence of SNP309 correlated with high expression of the MDM2 transcript (on average 8-fold) when compared to the levels seen in cells wild-type for SNP309 (T/T) (Figure 2A). MDM2 protein levels were also found to be significantly higher (on average 4-fold) in cell lines homozygous for SNP309 (G/G), as seen in the Western blot analysis of total cell lysates (Figure 2B). Interestingly, three of the four cell lines homozygous for SNP309 (A875, CCF-STTG1, and T47D) have been previously reported to overexpress MDM2 when compared to cell lines derived from similar tumor types (Landers et al., 1997; Lu et al., 2002; Phelps et al., 2003). MDM2 protein levels were found to be intermediate in four heterozygous (T/G) cell lines (MDA-231, MCF-7, WM-9, and CACL-7336): on average 1.9-fold higher than T/T cells (data not shown). Thus, together these data support an association of SNP309 with the increased levels of MDM2.

To test if the Sp1 transcription factor is indeed responsible for the heightened levels of MDM2 in cells homozygous for SNP309, endogenous Sp1 was inhibited, and the resulting effect on endogenous MDM2 levels was analyzed. Sp1 was inhibited using two different approaches. First, Sp1 levels were reduced by transfecting siRNAs specific to Sp1 RNA. Second, Sp1 activity was inhibited by treating cells with mithramycin A, an aureolic antibiotic that has been shown to selectively inhibit Sp transcription factor-mediated transcriptional activation (Blume et al., 1991).

Sp1 siRNA reduced the protein levels of Sp1 over 2-fold in all three cell lines tested, as shown in Figures 3A and 3B, when compared to Sp1 levels in cells transfected with either a nonspecific siRNA (NS, Figure 3A, lanes 3, 6, and 9) or siRNAs targeted against lamin A/B transcripts, although lamin A/B protein levels were dramatically reduced (Figure 3A, lanes 1, 4, and 7). Sp1 siRNA had no effect on another Sp family member, Sp3 (Figure 3A). As expected, reduction of Sp1 levels lead to the reduction of the protein levels of one of its known target genes, cyclin D1, in all three cell lines tested (Figure 3A; Grinstein et al., 2002). As predicted, reduction of Sp1 levels dramatically decreased MDM2 levels, up to 3-fold in cells homozygous for SNP309 (A875 and T47D), while in cells wild-type for SNP309 (HeLa), reduction of Sp1 levels had no significant effect on MDM2 levels (Figures 3A and 3B). Inhibition of Sp1 activity by treating cells with mithramycin A showed similar effects on MDM2 levels. A cell line homozygous for SNP309 (T47D) and a cell line wild-type for SNP309 (HL60) were treated with various concentrations of mithramycin A for 24 hr, and MDM2 protein levels were analyzed as shown in Figure 3C. In cells homozygous for SNP309 (T47D), mithramycin A treatment significantly decreased MDM2 levels. Specifically, 200 nM mithramycin A reduced MDM2 levels more than 5-fold in T47D, while no significant effect was seen in cells wild-type for SNP309 (HL 60). Similar effects were seen in other cell lines as shown in Figure 3D. In two more cell lines homozygous for SNP309 (Manca and A875), mithramycin A treatment significantly decreased MDM2 levels. However, mithramycin A only slightly reduced the MDM2 levels in two more cell lines wild-type for SNP309 (HeLa and ML-1). In summary, both methods of Sp1 inhibition can preferentially reduce the heightened levels of MDM2 in cells homozygous for SNP309, thereby supporting the hypothesis that the Sp1 transcription factor is indeed re-



A

		Mdm2 Levels (comp. ng)		Average
T/T	H460	6.3	(±0.41)	
	Tera 2	11.3	(±0.49)	12.6
	Ml-1	14.4	(±1.51)	
	Saos 2	18.3	(±0.54)	

		Mdm2 Levels (comp. ng)		Average
G/G	A875	8.8	(±0.33)	
	T47d	46.6	(±6.96)	103.4
	Manca	47.0	(±2.26)	
	CCF	311.2	(±36.7)	

Figure 2. SNP309 Associates with the Overexpression of MDM2

(A) The levels of *MDM2* RNA from tumor-derived cell lines are shown as measured by real-time PCR (TaqMan). All values have been normalized to the level of GAPDH and are the averages of three independent readings. The standard deviation is given in the parenthesis next to each value.
(B) A Western blot analysis of MDM2 levels found in total cell lysates is shown. The upper panel is a Western blot using a monoclonal MDM2 antibody (SMP-14). Full-length MDM2 protein runs as a doublet and is indicated by arrows. The middle panel is a Western blot of the same filter using a monoclonal antibody to p53 (Do-1) and the lower panel is a Western blot of the same filter using a monoclonal antibody to actin.

sponsible for the heightened levels of MDM2 in cells homozygous for SNP309.

Overexpression of MDM2 can lead to the inactivation of the p53 pathway (Lain and Lane, 2003). We therefore reasoned that the SNP309-homozygous cell lines, with higher levels of MDM2, should have an attenuated p53 response. To test the p53 response, these cells were treated with the chemotherapeutic drug etoposide (VP16) to induce DNA damage. This DNA damage activates the p53 pathway, leading to DNA repair, cell cycle arrest, and apoptosis. Significant death (20%–35% of the total cell population) was observed in cells that are wild-type at p53 and wild-type for SNP309 (T/T) (H460, ML-1, Tera-2: Figure 4A, lanes 1–3). Interestingly, cells with wild-type p53 that are homozygous for SNP309 (G/G) (Manca, CCF-STTG1, and A875: Figure 4A, lanes 6–8) showed much lower death rates (on average 2%–3% of the total cell population). In fact, the percentage of cells dying after treatment was similar to that seen in cells either mutant (T47D) or null (Saos2) for the p53 gene itself (compare lanes 5 and 4 with 1–3). Four cell lines heterozygous for SNP309 (T/G) and wild-type for p53 were also assayed for their response to etoposide and found to be intermediate in their response: on average 5%–7% of the total cell population (data not shown). Together, these data demonstrate that the DNA damage response pathway is attenuated in the SNP309 homozygous cell lines.

These data support the model that the heightened MDM2 levels in SNP309 cells attenuate the p53 pathway. If true, reducing the MDM2 levels should allow for the p53 pathway to be activated in the SNP309 homozygous cells lines. To test this, MDM2 levels were reduced by blocking Sp1-mediated transcription by mithramycin A treatment, and the activity of the p53 pathway was assessed by measuring the apoptotic response after DNA damage (etoposide treatment). Specifically, Manca (homozygous for SNP309) and ML-1 (wild-type for SNP309) were treated with various concentrations of mithramycin A and etoposide for 48 hr, after which the percentage of cells undergoing apoptosis was measured. Interestingly, as shown in Figure 4B, the percentage of etoposide-induced cell death increased significantly (2–3 fold) in Manca (SNP309 homozygous cell line) with mithramycin A treatment. However, in ML-1 (SNP 309 wild-type cell line), mithramycin A treatment had no obvious effect on etoposide-induced cell death. Similar results were obtained using various incubation times (data not shown). These data demonstrate that reduction of MDM2 levels by inhibiting Sp1 activity can reverse the attenuated p53 response in the SNP309 homozygous cell lines, thereby supporting the model that the heightened MDM2 levels due to increased Sp1 activity on the *MDM2* promoter in SNP309 cells result in the attenuation of the p53 pathway.

p53 responds to DNA damage by activating a transcriptional program (Jin and Levine, 2001). If the low death rates, seen after etoposide treatment in the homozygous SNP309 cell lines, is due to inhibition of p53, the p53 transcriptional program should be weakened in these cells. To test this, cells with wild-type p53 were treated with etoposide, and then the induction of 27 known p53-responsive genes was compared in the cells either homozygous for SNP309 or wild-type for SNP309. RNA was isolated from cells before and after etoposide treatment, and gene expression was analyzed using the

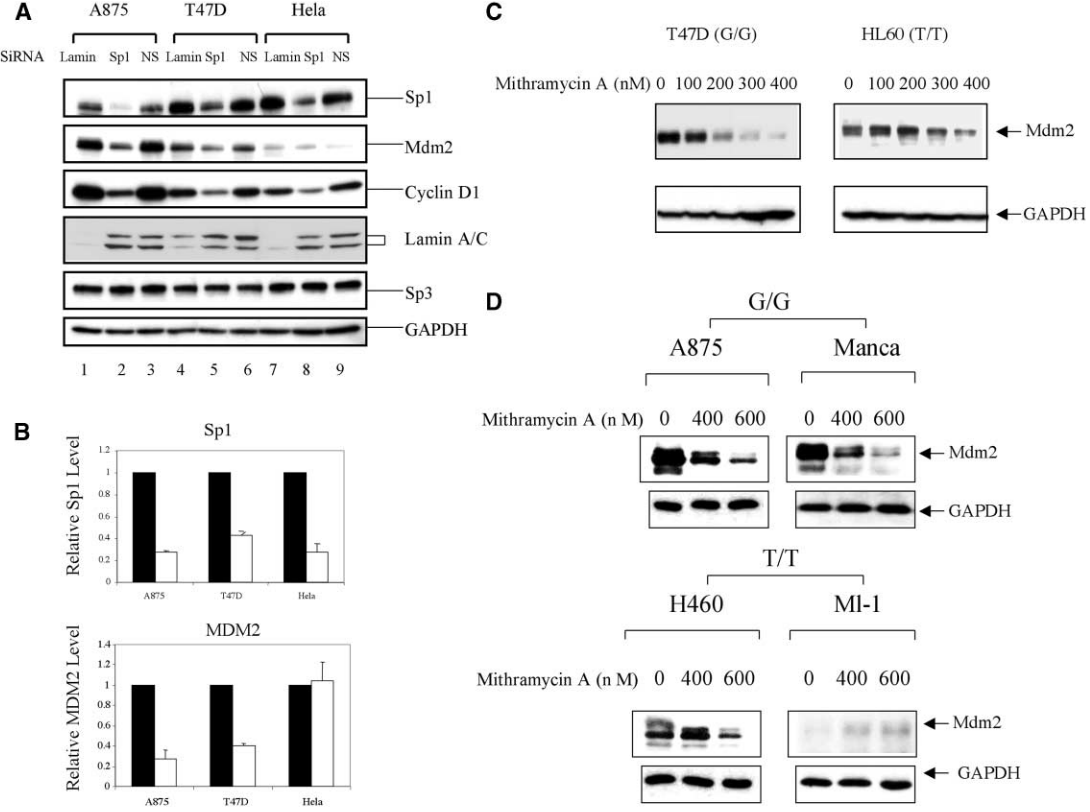


Figure 3. Inhibition of Endogenous Sp1 Can Reduce the Heightened Levels of Endogenous MDM2 in Cells Homozygous for SNP309

(A) Cell lines homozygous for SNP309 (G/G; A875 and T47D) and wild-type for SNP309 (T/T; HeLa) were transfected with siRNA specific for Sp1, for lamin A/B, or a nonspecific siRNA (NS). Depicted here are autoradiographs of Western blots using antibodies specific for Sp1, Sp3, MDM2, cyclin D1, lamin A/B, and Gapdh. Arrows indicate the positions of the proteins.

(B) The bar graphs represent the quantification of Western blot analysis in (A). The relative expression levels in cells transfected with siRNA for Sp1 was determined as the percentage of either Sp1 or MDM2 in the cells transfected with Sp1-siRNA to those transfected with nonspecific siRNA. Each value is the average of at least three independent experiments and the error bars represent the standard deviations.

(C and D) Cell lines either homozygous for SNP309 (G/G; T47D, A875, and Manca) or wild-type for SNP309 (T/T; HL60, H460, and Ml-1) were treated with various concentrations of mithramycin A for 24 hr. Autoradiograms of Western blots using either MDM2 or Gapdh antibodies are shown. Arrows indicate the positions of the proteins.

AffyMetrix GeneChip array (Human Genome U95A Array). Cells wild-type for SNP309 induced multiple known p53-responsive genes above 5-fold (Figure 4C). Specifically, nine genes in the lung carcinoma cell line, H460 (Figure 4C, lane 1), six genes in the myeloid leukemic cell line, ML-1 (Figure 4C, lane 2), and four genes in the testicular teratoma cell line, Tera-2 (Figure 4C, lane 3), were induced. In contrast, cells homozygous for SNP309 showed a much-weakened p53-dependent transcriptional response. The melanoma cell line, A875 (Figure 4C, lane 4), did not induce any known p53-responsive gene above 5-fold and both the astrocytoma cell line, CCF-STTG1 (Figure 4C, lane 6), and the Burkitt's lymphoma cell line, Manca (Figure 4C, lane 5), only induced one gene above 5-fold. These data demonstrate that the ability of p53 to act as a transcriptional activator for known target genes is attenuated in cells homozygous for SNP309.

Taken together, these data support a model that cells homozygous for SNP309 (G/G) express higher levels of MDM2, thereby attenuating the p53 pathway. MDM2 has been shown to inhibit p53 minimally by three different mechanisms. One well-studied mechanism is its ability to serve as an E3 ubiquitin ligase, targeting p53 for proteosamal degredation (Haupt et al., 1997; Honda et al., 1997; Kubbutat et al., 1997). Upon certain cellular stresses, such as DNA damage, p53 levels rise, as the half-life of p53 dramatically increases. The increase of the half-life of p53 has been attributed to the inability of MDM2 to target p53 for degradation after DNA damage. Specifically, immediately after DNA damage, MDM2 levels decrease dramatically as does its affinity for p53 binding (Michael and Oren, 2003).

To address what effect the elevated MDM2 levels in G/G cells have on p53 levels, the levels of wt p53 in cells before and after stress (DNA damage) were monitored. As seen in Figure 2B there are no significant differences in basal wild-type p53 levels between cells wild-type or homozygous for SNP309 (compare p53 levels in lanes 1, 2, and 3 to those in lanes 5, 7, and 8). To rule out the possibility that MDM2 cannot target p53 for degradation in G/G cells, MDM2 levels were artificially reduced in nonstressed cells, and p53 levels were monitored. As seen in Figure 4D, reduction of MDM2 levels, using siRNAs, in the two G/G cell lines tested led to the significant stabilization of p53. The stabilized p53 is active, as p21 levels also rise, and p21 is a well-known

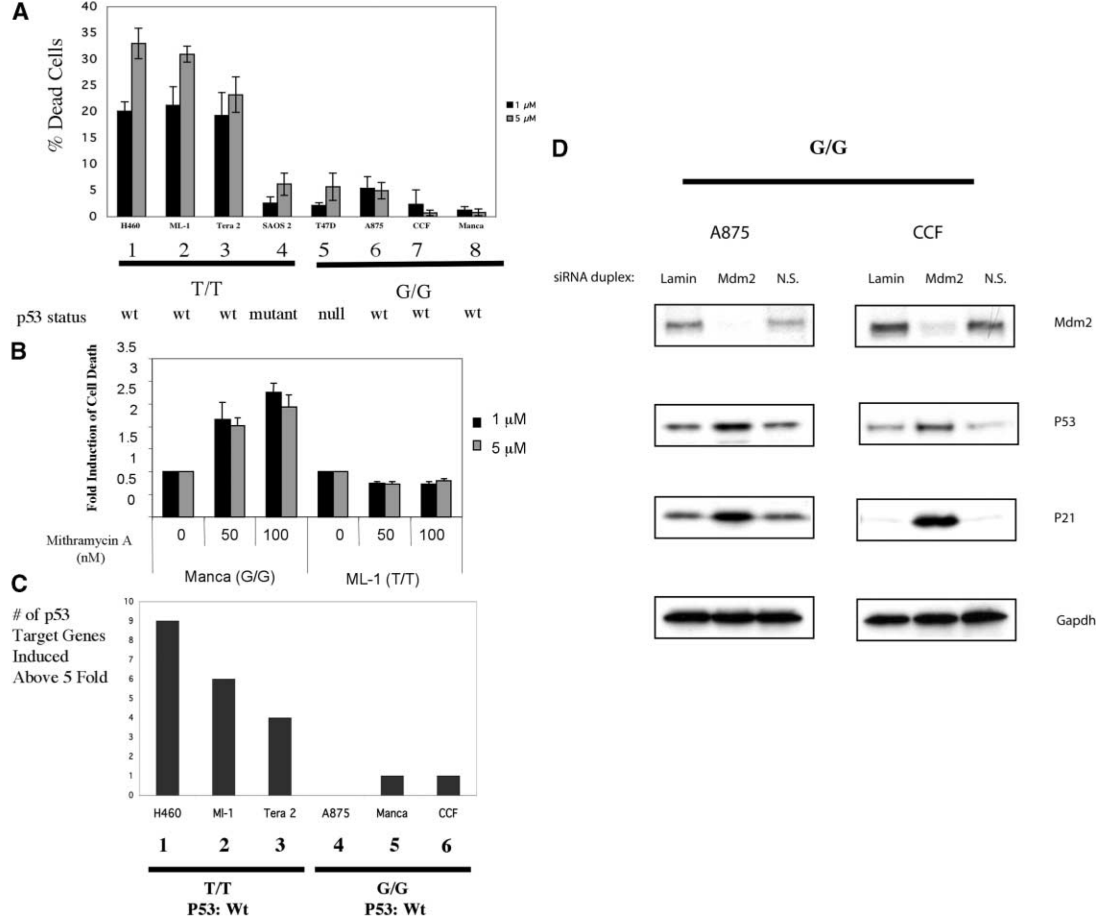


Figure 4. p53 Activity Is Attenuated in Cells Homozygous for SNP309

(A) In the bar graph, the percent of the population of cells which are dead after 24 hr of either 1 μM (black) or 5 μM (gray) etoposide treatment is plotted for each cell line. Each value is the average of three independent experiments and the error bars represent the standard deviations.

(B) Inhibition of Sp1 activity by mithramycin A treatment increases DNA damage-induced cell death in cells homozygous for SNP309. Manca (homozygous for SNP309) and ML-1 (wild-type for SNP309) were treated with either 1 μM (black) or 5 μM (gray) etoposide and various concentrations of mithramycin A for 48 hr. The percentage of the dead cell induced by etoposide and mithramycin A treatment was measured and subtracted with any death induced by mithramycin A treatment alone. Fold induction of cell death is depicted in the bar graph. Each value is the average of at least three independent experiments and the error bars represent the standard deviation.

(C) In the bar graph, the number of known p53 target genes induced after DNA damage (etoposide treatment) above 5-fold from levels found in untreated cells is plotted for each cell line.

(D) MDM2 can inhibit p53 in cell lines homozygous for SNP309 (G/G; A875 and CCF). Cells were transfected with siRNA specific for *MDM2*, for lamin A/B or a nonspecific siRNA (NS). Depicted here are autoradiographs of Western blots using antibodies specific for MDM2, p53, p21, and Gapdh.

p53 transcriptional target. These data suggest that MDM2 can target p53 for degradation in G/G cells, but that, in nonstressed cells, heightened levels of MDM2 in G/G cells do not further reduce the levels of wt p53.

As mentioned above, the heightened MDM2 levels could also inhibit the proper stabilization of p53 in response to stress, thereby attenuating the p53 pathway. To address this possibility, the stabilization of p53 after etoposide treatment was monitored (Figures 5A and 5B). Cells were treated with etoposide and harvested after 1, 2, and 3 hr of treatment. The p53 levels were measured by Western blotting and the fold induction of p53 levels after etoposide treatment was calculated for each cell line and each time point. The values are depicted in the bar graph in Figure 5B. As expected, the p53 levels in cells wild-type for SNP309 (T/T) increased 4-fold or above after only 1 hr of etoposide treatment. Interestingly, there was no significant stabilization of p53 in cells homozygous for SNP309 (G/G) in the times tested. These data support the hypothesis that the heightened levels of MDM2 in SNP309 cells result in the inability to properly stabilize p53 in response to cellular stresses like DNA damage but that the heightened levels of MDM2 do not further reduce the levels of wt p53 in nonstressed cells. The inability to properly stabilize p53 in response to DNA damage provides one possible mechanism for the observed attenuation of p53 pathway in cells homozygous for SNP309 (G/G).

The data thus far support a model that cells homozygous for SNP309 express higher levels of MDM2, thereby weakening the p53 pathway. Humans who carry a germline p53 mutation in one allele (Li-Fraumeni Syn-

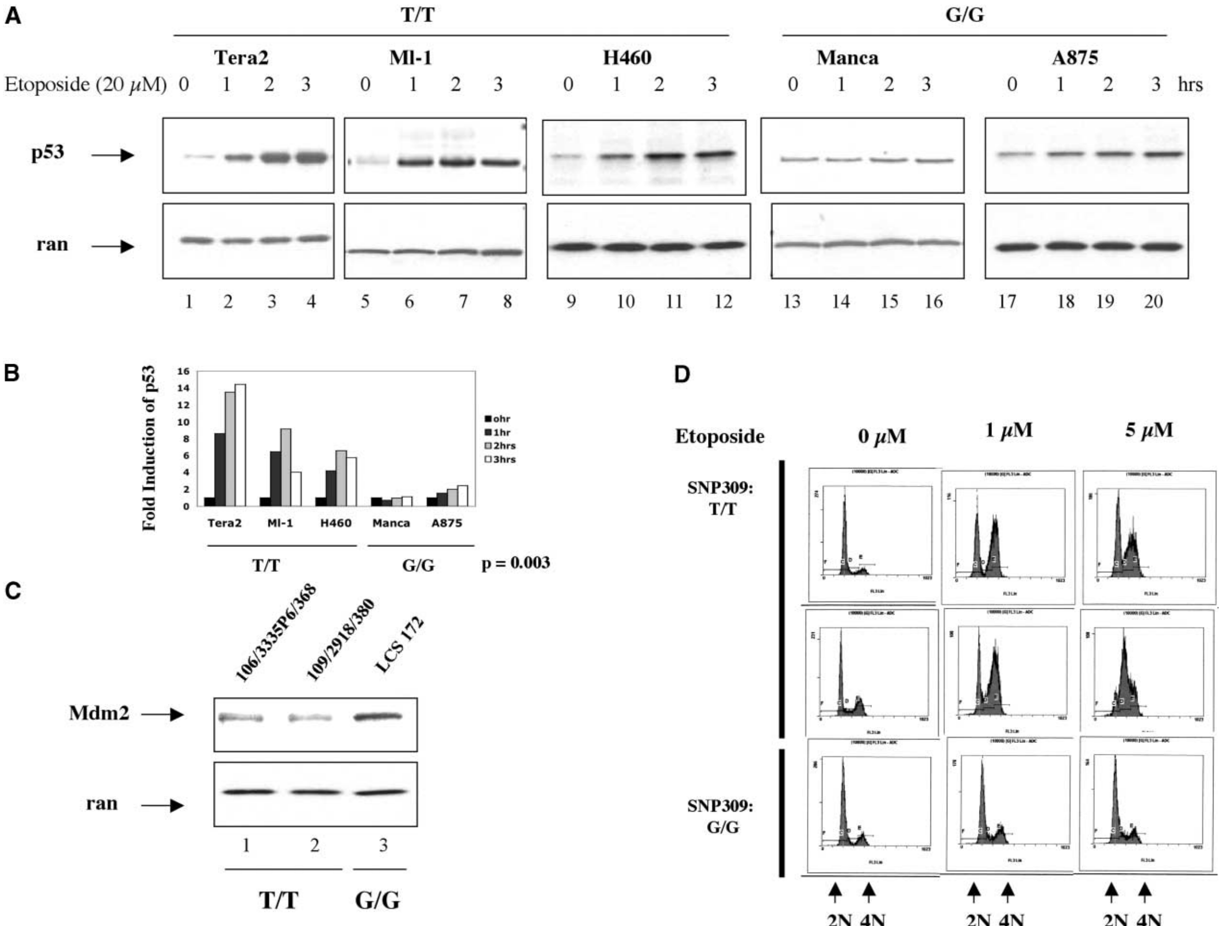


Figure 5. The Stabilization of p53 after DNA Damage Is Impaired in Cells Homozygous for SNP309, and the p53 DNA Damage Response Is Attenuated in Li-Fraumeni Fibroblasts Homozygous for SNP309 with High MDM2 Levels

(A) Cell lines either wild-type (T/T) or homozygous (G/G) for SNP309 were treated with etoposide and harvested after 1, 2, and 3 hr of treatment. Depicted here are autoradiographs of Western blots using antibodies specific for p53 and ran. The autoradiograms are depicted, and exposures were chosen to highlight the induction of p53 after etoposide treatment for each cell line. The p53 levels were measured and normalized to ran levels from the autoradiograms in (A), and the fold induction of p53 after etoposide treatment from basal levels was calculated for each cell line and each time point. The values are depicted in the bar graph in (B).

(C) A Western blot analysis of MDM2 levels found in total cell lysates is shown. The upper panel is a Western blot using a monoclonal MDM2 antibody. Full-length MDM2 protein is indicated an arrow. The lower panel is a Western blot of the same filter using a monoclonal antibody to ran.

(D) The fibroblastoid cell line derived from a germline p53 mutation carrier (Li-Fraumeni) and homozygous for SNP309 has an altered DNA damage response when compared to Li-Fraumeni fibroblastoid cell lines wild-type for SNP309. Histograms are depicted of the DNA content in cells after 24 hr of varying concentrations of etoposide treatment (0, 1, and 5 μM, indicated above the panels).

drome) develop tumors at very high frequencies (Garber et al., 1992; Li et al., 1990). These individuals develop tumors on average at very young ages and can develop multiple primary tumors throughout a lifetime. We hypothesized that increased levels of MDM2 by SNP309 could further weaken the p53 pathway in Li-Fraumeni individuals and further impact tumorigenesis. To test this, the first approach was to analyze MDM2 levels and the DNA damage response (etoposide treatment) in fibroblasts derived from Li-Fraumeni individuals who were either wild-type for SNP309 (T/T) or homozygous for SNP309 (G/G). MDM2 levels were analyzed by Western blotting for two fibroblast cell lines wild-type for SNP309 (T/T) and one fibroblast cell line derived from the Li-Fraumeni individual homozygous for SNP309 (G/G). As predicted by the analysis of tumor-derived cell lines, the G/G fibroblasts showed significantly higher MDM2 levels (above 3-fold) when compared to those found in both T/T fibroblast cell lines (Figure 5C). Upon etoposide treatment, the two fibroblast cell lines wild-type for SNP309 (T/T) showed a characteristic p53-mediated G2 arrest as noted by the accumulation of cells with tetraploid DNA content (Figure 5D). In contrast, the fibroblast cell line homozygous for SNP309 (G/G) did not show such a response. In fact, no significant difference in the DNA content of the SNP309 homozygous (G/G) cells was seen. These data suggested that both MDM2 levels and the p53 pathway in Li-Fraumeni individuals, as measured by the DNA damage response in fibroblasts, could be greatly affected by the presence of SNP309.

If SNP309 further debilitates the p53 pathway in Li-

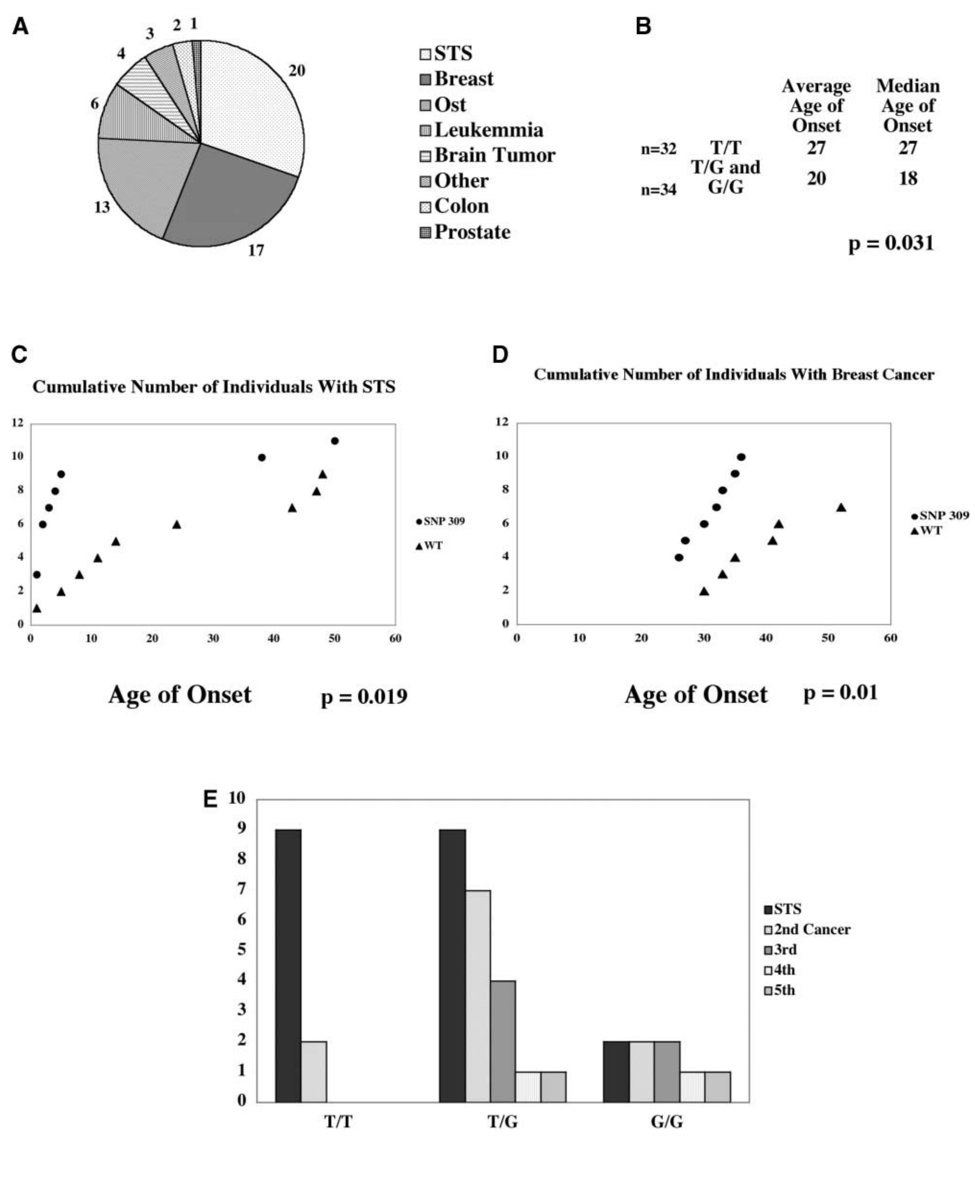


Figure 6. SNP309 Associates with an Accelerated Age of Onset of Tumors and the Occurrence of Multiple Subsequent Tumors in Li-Fraumeni Individuals

(A) Of the 88 individuals in the Li-Fraumeni cohort, 66 were diagnosed with at least one cancer at a median age of 22 years old. The first cancers the 66 individuals were diagnosed with are shown in the pie chart. Individuals either T/G or G/G are diagnosed on average 7 years earlier than wild-type (T/T) individuals as depicted in (B). The cumulative number of individuals either with SNP309 (heterozygous or homozygous) (circles) or wild-type for SNP309 (triangles) is plotted against the age of onset of STS (C) and breast cancer (D).

(E) The bar graph depicts the number of individuals who were first diagnosed with STS (black) and then with subsequent cancers (second, third, fourth, and fifth) for individuals either wild-type (T/T), heterozygous (T/G), or homozygous (G/G) for SNP309.

Fraumeni individuals, tumor development might also be affected. To address this possibility, 88 individuals who are members of Li-Fraumeni families and have germline mutations in one allele of p53 were studied, and the frequency of SNP309 was found to be similar to the frequencies found in the 50 normal volunteers (Hwang et al., 2003). Sixty-six have been diagnosed with at least one cancer so far. Soft tissue sarcomas (STS) (20 individuals), breast cancer (17), and osteosarcomas (13) were the most prevalent first cancers (Figure 6A). Interestingly, individuals who carried SNP309, in either the heterozygous or homozygous state, showed a significantly earlier age of onset for all tumor types (Figure 6B). For individuals with SNP309, the median age of tumor onset was 18 years old, while in wild-type individuals the median age of tumor onset was 27 years old ($p = 0.031$). Specifically, the median age of onset of STS in wild-type individuals was fourteen years old, while in SNP309 individuals the median age of onset was two years old ($p = 0.019$, Figure 6C). In those who developed breast

cancer, the median age of onset in wild-type individuals was 39 years old, and in SNP309 individuals the median age of onset was 29 years old ($p = 0.01$, Figure 6D). Taken together, those carrying SNP309 developed all tumor types 9 years earlier, STS on average 12 years earlier and breast cancer on average 10 years earlier than those who do not carry SNP309. These data support the hypothesis that the presence of SNP309 further debilitates the p53 pathway in Li-Fraumeni individuals, resulting in an even earlier tumor onset.

As mentioned above, another hallmark of the Li-Fraumeni syndrome is the occurrence of multiple primary tumors in a lifetime. Interestingly, the presence of SNP309 also correlated with the occurrence of multiple tumors in those individuals first diagnosed with STS ($p = 0.0014$, Fisher's exact test, Figure 6E). Specifically, both individuals homozygous for SNP309 (G/G) developed a second and a third cancer, and one developed a fourth and a fifth cancer. Of the nine people heterozygous for SNP309 (T/G), seven developed a second cancer, four a third, and one a fourth and a fifth cancer. In contrast, of the nine people wild-type for SNP309 (T/T), only two developed a second cancer and none a third, a fourth, or a fifth cancer. These data indicate an association with the presence of SNP309 and the occurrence of independent subsequent cancers. Together, these data support a model whereby the presence of SNP309 in the promoter of the *MDM2* gene leads to overexpression of the MDM2 protein, which then inhibits p53 and impacts two major hallmarks of the Li-Fraumeni Syndrome, namely the early age of onset of tumors and the occurrence of multiple primary tumors in a lifetime.

The next question raised was if SNP309 caused a weakened p53 pathway, did this SNP act upon sporadic cancers as well as genetically altered individuals with a p53 defect. To address this question, a group of patients who develop sporadic adult STS and had no known hereditary cancer predisposition and no known germline p53 mutation was studied. As seen in the pie chart in Figure 7A, the sarcomas come from a variety of tissues. Interestingly, as in the Li-Fraumeni individuals, SNP309 associates with an earlier age of onset of STS (Figure 7B). Specifically, those individuals homozygous for SNP309 were diagnosed on average 12 years earlier than those individuals without SNP309 ($p = 0.01$, Figure 7C). As seen in Figure 7D, the frequency of the SNP309 G allele was greatly increased in those individuals who developed STS at a young age. Those who developed STS below the age of 41 years old had an allele frequency for SNP309 of 50%, while the allele frequency is only 33% for the whole group ($p = 0.0262$, Figure 7D). These data demonstrate that SNP309 does not require the presence of an inactivating germline p53 mutation to associate with earlier STS tumor formation.

Discussion

Experimental data have been presented in this report which support the hypothesis that single nucleotide polymorphisms in the tumor suppressor p53 pathway can be a part of the genetic variation which underlies the phenotypic variation seen in individuals' susceptibility to cancer. To summarize, the results in biochemical assays in cell culture systems demonstrate that SNP309 in the promoter of the *MDM2* gene increases the binding affinity of the transcriptional activator Sp1, which results in high levels of MDM2 RNA and protein. The heightened MDM2 levels were shown to lead to the attenuation of the p53 DNA damage response, in concordance with the observations that MDM2 is as a key negative regulator of p53. The results of clinical epidemiological studies demonstrated that SNP309 associates with minimally a 9-year earlier onset of tumors in both hereditary and sporadic cancers. Together, these data support a model whereby SNP309 enhances the affinity of the transcriptional activator Sp1 to the promoter of the *MDM2* gene, resulting in heightened transcription. Heightened levels of MDM2 lead to the direct inhibition of p53, which releases the cell from p53 tumor suppression. Inhibition of endogenous Sp1 using either siRNA or mithramycin A can significantly reduce SNP309-related overexpression of MDM2. The experiments presented here demonstrate that reduction of SNP309-related overexpression of MDM2 by mithramycin A treatment can reverse the attenuated p53 response. Mithramycin A is an aureolic antibiotic that has been used in humans to treat several types of cancer and hypercalcemia associated with cancers (Hurtado and Esbrit, 2002). The data presented here suggest that treating cancer patients homozygous for SNP309 with mithramycin A may enhance their response to chemotherapeutic drugs by reducing MDM2 levels to allow for a p53-dependent apoptotic response induced by most chemotheraputic agents.

The model proposed here states that the heightened levels of MDM2, due to SNP309, result in accelerated tumor formation in humans. There is much evidence in the literature to support the claim that high MDM2 levels can positively impact tumorigenesis. For example, Jones et al. (1998) created mice which overexpress Mdm2 by using the entire *MDM2* gene as a *trans*-gene. These mice expressed an average of 4-fold more Mdm2 in various tissues relative to nontransgenic mice. Interestingly, 100% of the Mdm2-overexpressing mice developed spontaneous tumors in a lifetime. Lundgren et al. (1997) also showed that targeted overexpression of Mdm2 in the murine mammary epithelium results in tumors, albeit with a lower penitence (16%). These two studies, together with numerous accounts of MDM2 overexpression or amplification in a variety of human cancers, support the idea that heightened levels of MDM2, by SNP309, could positively impact tumor formation (Cordon-Cardo et al., 1994; Momand et al., 1998; Oliner et al., 1992; Taubert et al., 2000).

MDM2 is a key negative regulator of p53. MDM2 can regulate the degradation of p53 as an E3 ubiquitin ligase, targeting p53 for proteosamal degredation (Michael and Oren, 2003). Observations in this report support the hypothesis that the heightened levels of MDM2 in SNP309 cells (G/G) result in the inability to properly stabilize p53 in response to cellular stresses like DNA damage; this hypothesis offers one possible mechanism of p53 attenuation by MDM2 in SNP309 (G/G) cells. Interestingly, the heightened levels of MDM2 do not further reduce the levels of wild-type p53 in nonstressed cells. One explanation for these observations is that the ability of MDM2 to regulate p53 levels may not be limiting in nonstressed cells, so heightened levels of MDM2 would

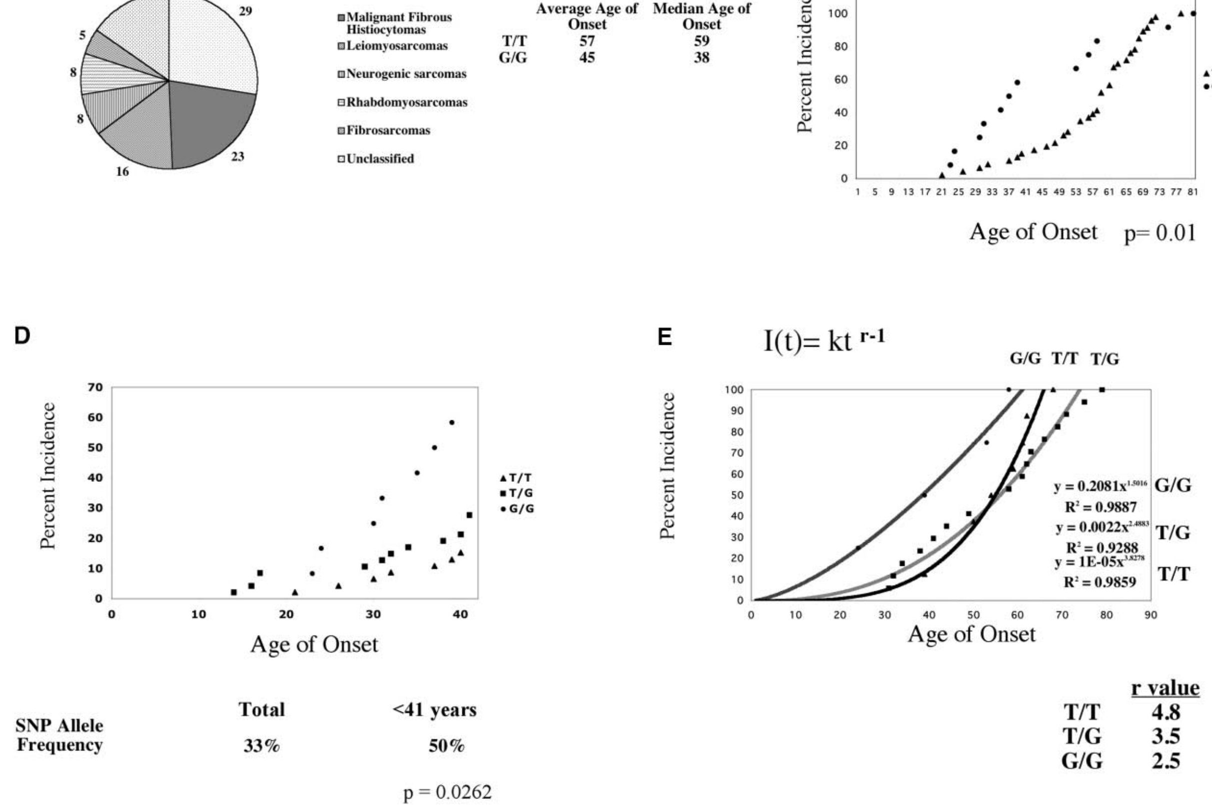


Figure 7. SNP309 Associates with an Accelerated Age of Onset of Tumors in Sporadic Soft Tissue Sarcoma

(A) The subtypes of STS are depicted in the pie chart. Individuals homozygous for SNP309 showed a significantly earlier age of onset of STS, on average 12 years earlier (B). The cumulative number of individuals either homozygous for SNP309 (circles) or wild-type for SNP309 (triangles) is plotted against the age of onset of STS (C).

(D) SNP309 is at a higher frequency in the population with early onset STS. The cumulative number of individuals either homozygous for SNP309 (circles), heterozygous for SNP309 (squares), or wild-type for SNP309 (triangles) is plotted against the age of onset of STS in those who were diagnosed below the age of 41 years old (one standard deviation from the median age of onset).

(E) SNP309 could serve as a rate-limiting event in liposarcoma. The number of rate-limiting events required to create a cancer cell and subsequent tumor was calculated using $I(t) = kt^{r-1}$, whereby r is the number of rate-limiting events. The R value was calculated for each genotype by plotting the cumulative number of individuals either homozygous for SNP309 (circles), heterozygous for SNP309 (squares), or wild-type for SNP309 (triangles) against the age of onset of liposarcoma and performing a nonlinear regression as a power function to obtain the exponent for each curve. The equations for the trend-line, R^2 value, and R value are displayed for each genotype.

have no further impact on p53 levels. Only when MDM2 activity is reduced after cellular stress (or artificially, e.g., siRNA in nonstressed cells, Figure 4D) does MDM2 become limiting, where heightened MDM2 levels would result in less induced p53 (Figures 5A and 5B).

The results presented in this report suggest that SNP309 can impact tumorigenesis in humans who carry a germline inactivating mutation in one p53 allele (Li-Fraumeni Syndrome). The data suggest that SNP309 can further lower the age of onset of tumors in these individuals on average 9 years and increase the occurrence of multiple primary tumors in a lifetime. One possible scenario to explain these observations is that high levels of MDM2 and just one wild-type p53 allele in those Li-Fraumeni individuals with SNP309 produce a severely weakened p53 tumor suppressor pathway resulting in a higher mutation rate, poorer DNA repair processes, and reduced apoptosis leading to faster and more frequent tumor formation.

Fifty years ago it was noted that age-specific incidence of many tumors increases with the power of age, and it was proposed that the age-specific increase could be correlated to the number of rate-limiting steps involved in the formation of a cancer cell and subsequent tumor. Specifically, the function $I(t) = kt^{r-1}$ was used to describe the incidence of cancer (I) observed at a given age (t), whereby r is the number of rate-limiting events which have to occur in cells at the constant rate (k). This equation or derivatives thereof have been successfully used to describe many cancers (Knudson, 2001), most notably in Alfred G. Knudson's description of hereditary and nonhereditary forms of retinoblastoma, which later became known as the two hit theory. As shown in Figures 6 and 7, the age-incidence curves of those patients with SNP309 (G/G) vary greatly from those individuals with a genotype of T/T or even T/G. Interestingly, when the number of rate-limiting events (R value) is calculated for each genotype at the SNP309 locus in sporadic liposarcomas, T/T gives a value of 4.8, T/G 3.5, and G/G 2.5 (Figure 7E). The fact that the heterozygote reduces

the R value by one and the homozygote by two suggests that the G allele can serve as a rate-limiting event in the formation of a sarcoma.

It is not difficult to propose a model to explain why SNP309 could be a rate-limiting event in carcinogenesis. Over the past 50 years, it has become clear that age-specific incidence of cancer is dependent minimally on three factors: the number of rate-limiting mutations required for a given cancer, the mutation rate per mitosis, and the net proliferation rate of the effected cells (cell division rate minus cell death rate; Knudson, 2001). Inhibition of the p53 pathway by SNP309 could potentially affect all three of these factors (Jin and Levine, 2001; Lain and Lane, 2003). The p53 gene itself is thought of as a rate-limiting mutation in many cancer types, as it is found mutated in over 50% of all human tumors, and humans who carry a germline p53 mutation develop cancer with increased incidence and on average early in life. The wild-type p53 pathway is also thought to reduce mutation rates per mitosis, as loss of p53 leads to defective centrosome replication and numerous chromosomal abnormalities. Finally, the p53 pathway also impacts the net proliferation rate of cells, as it functions to arrest the cell cycle and induce apoptosis upon stress signals like DNA damage and oncogene activation. Therefore, inhibition of the p53 pathway by SNP309 could affect all three factors, which have been shown to influence the age-specific incidence of cancer to accelerate carcinogenesis in an individual.

Experimental Procedures

Sequence Analysis

The *MDM2* promoter was analyzed for sequence variation by PCR amplification and subsequent sequencing, primer 1: CGGGAGTT CAGGGTAAAGGT and primer 2: AGCAAGTCGGTGCTTACCTG.

Electrophoretic Mobility Shift Assays (EMSA)

EMSAs were performed with a LightShiftTM Chemiluminescent EMSA Kit (PIERCE, Rockford, Illinois). The binding reactions were performed for 20 min at room temperature in 10 mM Tris-HCl ([pH 7.5] at 25°C), 1 mM $MgCl_2$, 50 mM NaCl, and 0.5 mM DTT, 4% glycerol, 50 μg/ml poly (dI-dC) (dI-dC), 10 fmol biotin 3′-end-labeled double-stranded oligonucleotides, and purified recombinant Sp1 protein. After incubation, samples were separated on a native 4% polyacrylamide gel and then transferred to a nylon membrane. The positions of the biotin end-labeled oligonucleotides were detected by a chemiluminescent reaction with streptavidin-horseradish peroxidase according to the manufacturer's instruction and visualized by autoradiography. The nucleotide sequence of the double-stranded oligonucleotides with either wild-type sequence or the SNP is as follows: 5′-CCGGGGGCTGCGGGGCCGCTT/GCGGCGC GGGAGGTCCGGATG-3′.

Chromatin Immunoprecipitation

Proteins were crosslinked to DNA in 1% formaldehyde. After washing, cells were lysed in detergent lysis buffer. Lysates were washed and sonicated. Two micrograms of antibodies were added and incubated overnight. Protein A/G Plus beads (Santa Cruz) were used, and after extensive washing, crosslinks were removed at 65°C overnight in an elution buffer (1% SDS, 0.1 M $NaHCO_3$). DNA was isolated using the QIAquick PCR purification kit (Qiagen). Ten percent of purified DNA was analyzed by PCR. The entire PCR reaction was analyzed by gel electrophoreses on an 8% nondenaturing polyacrylimide gel and subsequent ethidium bromide staining.

Luciferase Reporter Assays

The *MDM2* promoter-luciferase reporter plasmids containing either the wild-type sequence or the SNP309 sequence were constructed by tandemly inserting two copies of double-stranded oligonucleotides containing the same sequence as used for the EMSAs into pGL2 luciferase reporter plasmid (Promega). The clones were confirmed by DNA sequencing.

Drosophila Schneider's SL2 cells were seeded at 1.5×10^6 cells per well in a six-well plate 24 hr before transfection. Transient transfections were preformed using CellfectAMINE (Invitrogen) according to the manufacturer's instructions. The DNA transfection mixture contained 250 ng reporter plasmid, varying amounts of pPac-Sp1 plasmid, and empty pPac vector to normalize DNA concentrations. The expression plasmids pPac and pPac-Sp1 were kindly provided by Dr. Richard D. Kolodoner (La Jolla, California). Cells were harvested 48 hr after transfection in reporter lysis buffer (Promega) and assayed for luciferase activity. Each extract was analyzed in duplicate, and at least three independent experiments were performed. Luciferase activities were normalized to cellular protein, measured by the Bio-Rad protein assay system.

RNA Analysis

To analyze *MDM2* RNA levels, total RNA was isolated from cell pellets using RNeasy (Qiagen). cDNAs were made using TaqMan reverse transcription reagents from Applied Biosystems. Real-time PCR was carried out using the ABI Prism 7000 sequence detection system. Probe and primer sets for *MDM2* and Gapdh were purchased as predeveloped assays from Applied Biosystems.

Protein Analysis

To analyze protein levels, total cell extracts were made by using a detergent lysis buffer (50 mM Tris [pH 7.5] 150 mM NaCl, 1% NP40, 0.1% SDS, 0.5% deoxycholic acid, 5mM EDTA, and a protease inhibitor cocktail [Complete Mini, Roche]). Thirty micrograms of total protein was run on a 4%–20% tris-glycine gel (Invitrogen) and transferred to a PVDF membrane. MDM2 was detected using the mouse monoclonal antibody SMP-14; Sp1 and Sp3 were detected using the rabbit polyclonal antibody PEP2 and D-20, respectively. Lamin A/C, cyclin D1, p53, and actin were detected using the mouse monoclonal antibody 346, DCS-6, Do-1, and C-2, respectively. Gapdh was detected using the goat polyclonal antibody V-18. All antibodies were purchased from Santa Cruz.

Gene Silencing with siRNA

Sp1 siRNA targeted to AATGAGAACAGCAACAACTCC was used to lower Sp1 expression. Two hundred picomoles siRNA duplex was transfected into cells at 30%–50% confluencey using oligofectamine reagent (Invitrogen) according to the manufacturer's instructions. Control siRNA duplex has no known target in mammalian genomes and was used as follows: sense UUCUCCGAACGUGUCACGUdTdT, antisense ACGUGACACGUUCGGAGAAdTdT. Lamin A/C siRNAs were purchased from Qiagen and *MDM2* siRNAs from Dharmacom. Cells were lysed 48 hr after transfection, and protein levels of Sp1, Sp3, and MDM2 were analyzed as described above.

Mithramycin A Treatment

Logarithmically growing cells were treated with various concentrations of mithramycin A (Sigma) for 24 hr. After treatment, cells were lysed and protein levels of MDM2 were analyzed.

Cell Viability Analysis

To analyze induction of cell death after etoposide treatment, growing cells were treated with either 1 μM or 5 μM etoposide for 24 hr. To analyze etoposide-induced cell death after inhibition of Sp1, growing cells were treated with various concentrations of mithramycin A and etoposide for 48 hr. After treatment, cells were harvested and viability was measured using the Guava ViaCount assay (Guava).

Cell Cycle Analysis

To analyze the DNA damage response in fibroblasts, all cells were treated with two concentrations of etoposide (1 μM and 5 μM) for 24 hr. Cells were harvested and fixed with methanol and kept at −20°C for 40 min or overnight. After washing and equlibration in PBS, the cell pellet was resuspended in 1 ml of staining solution containing 50 μg of RNase A and 0.5 μg of propidium iodide per ml in PBS and kept at room temperature for over 30 min. The cells

were then applied to the fluorescence-activated cell sorter (FACSCalibur; Becton Dickson). The FACSCalibur program was used to sort and count the cells.

Statistical Analysis

A randomization test is employed to determine the statistical significance of the age of onset of cancer between the groups with and without SNP309. The two groups are compared pair-wise, and each instance of an element from the second group greater than an element of the first group adds one to the distance. This total distance is the cutoff. The lists are then randomly permuted, holding fixed the number of elements of each list. The calculated p value is the percent of randomized groups that have a distance less than the cutoff as determined by a large Monte Carlo simulation.

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MDM2 SNP309 Accelerates Tumor Formation in a Gender-Specific and Hormone-Dependent Manner

Gareth L. Bond,[1,2] Kim M. Hirshfield,[2] Tomas Kirchhoff,[3] Gabriella Alexe,[1] Elisabeth E. Bond,[2] Harlan Robins,[1] Frank Bartel,[4] Helge Taubert,[4] Peter Wuerl,[5] William Hait,[2] Deborah Toppmeyer,[2] Kenneth Offit,[3] and Arnold J. Levine[1,2]

[1]The Institute for Advanced Study, Princeton, New Jersey; [2]The Cancer Institute of New Jersey, New Brunswick, New Jersey; [3]Memorial Sloan Kettering Cancer Center, New York, New York; [4]Martin-Luther-University, Halle/Salle, Germany; and [5]University of Ulm, Ulm, Germany

Abstract

The importance of the p53 stress response pathway in the suppression of tumor formation is well documented. In a previous report, a single nucleotide polymorphism (SNP309 T/G) was found in the promoter of the *MDM2* gene resulting in higher levels of MDM2 RNA and protein and, consequently, in the attenuation of the p53 pathway both *in vitro* and *in vivo*. As the SNP309 locus is found in a region of the *MDM2* promoter, which is regulated by hormonal signaling pathways, and the G-allele of SNP309 increases the affinity of a well-described cotranscriptional activator of nuclear hormone receptors (i.e., Sp1), the hypothesis that the SNP309 locus could alter the effects of hormones on tumorigenesis was tested *in vivo* in humans. Data obtained from patients with three different sporadic cancers, from four independent case studies, support this hypothesis, providing an example for the genetic basis of gender differences in cancer and showing that the genotype at a specific locus can affect how hormones, like estrogen, affect tumorigenesis in humans. (Cancer Res 2006; 66(10): 5104-10)

Introduction

On exposure to cellular stresses, the p53 protein is stabilized, increases its concentration, and becomes active as a transcription factor initiating a transcriptional program, which leads to DNA repair, cell cycle arrest, cellular senescence, or apoptosis. The p53 stress response pathway functions as a critical tumor suppressor pathway, which is underlined by the observation that the p53 gene is one of the most commonly mutated genes in human tumors (1). Furthermore, both mice and humans harboring a germ-line inactivating mutation in just one allele of the p53 gene develop tumors early in life and at very high frequencies (2–5). A previous report provided evidence that naturally occurring polymorphic genetic variant in the p53 stress response pathway influences an individual's susceptibility to cancer (6).

Both genetic and biochemical studies have shown that the *MDM2* oncogene is a key negative regulator of the p53 protein (7). Since the initial publication (6), multiple studies have shown that the G-allele of the single nucleotide polymorphism (SNP309, T/G) in the promoter of the *MDM2* gene was associated with the attenuation of the p53 pathway and an enhanced early onset of, and increased risk for, tumorigenesis (6, 8–14). In various reports, results were presented that support the model that the G-allele of SNP309 increases the DNA binding affinity of the transcriptional activator Sp1, which results in high levels of MDM2 mRNA and protein in human cells and tissue with this allele (10, 15, 16). The heightened MDM2 levels were shown to lead to the attenuation of the p53 DNA damage response induced by various chemotherapeutic drugs (6, 15), in concordance with the well-described role of MDM2 in the negative regulation of the p53 protein. In humans, two independent reports have shown that germ-line p53 mutation carriers who possessed the G-allele of SNP309 were diagnosed with cancer, on average, 7 or 10 years earlier than those who were homozygous for the T-allele (6, 9). It was proposed that high levels of MDM2, resulting from the G-allele of SNP309 and just one wild-type p53 allele, produce a severely weakened p53 tumor suppressor pathway resulting in a higher mutation rate, poorer DNA repair processes, and reduced apoptosis leading to faster and more frequent tumor formation (6, 16).

Estrogen signaling has been shown to regulate MDM2 expression levels. Many reports have shown that MDM2 mRNA and protein levels are heightened in breast tumors which express the estrogen receptor (ER), a critical component of the estrogen signaling pathway (17–21). In fact, the expression of ERα was shown to induce transcription of MDM2 (22). The regulation of MDM2 expression by estrogen, as well as by thyroid hormone, is mediated, at least in part, through a well-characterized region of the MDM2 promoter (20, 22–24). Both the ER and the thyroid hormone receptor are known to bind to this region of the promoter and activate transcription of the MDM2 gene (23, 24). Interestingly, SNP309 is found in this region of the promoter. The SNP309 locus potentially could affect the transcriptional regulation of MDM2 by hormone receptors as the G-allele of SNP309 increases the affinity of the MDM2 promoter for Sp1, which is a well-characterized cotranscriptional activator for multiple hormone receptors, including the ER (25–27). These observations suggest the possibility that the SNP309 locus could alter the effects of hormones, like estrogen, on tumorigenesis, and therefore contribute to the gender differences observed in many different types of cancer. In this report, data are presented from four independent studies that support this hypothesis.

Materials and Methods

Statistical analysis. A randomization test is employed to determine the statistical significance of the age of onset of cancer between the different genotypes. The groups are compared pairwise and each instance of an element from the second group adds one to the distance. This total distance is the cutoff. The lists are then randomly permuted, holding fixed the number of elements of each list. The calculated P value is the percent of

Note: G.L. Bond and K.M. Hirshfield contributed equally to this manuscript.

Requests for reprints: Arnold Levine, The Institute for Advanced Study, Einstein Drive, Princeton, NJ 08540-0631. Phone: 609-734-8005; Fax: 609-924-7592; E-mail: alevine@ias.edu.

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randomized groups that have a distance less than the cutoff as determined by a large Monte Carlo simulation.

Ashkenazi lymphoma and breast cancer cases. Lymphoma cases were derived from 678 cases of non-Hodgkin's lymphoma ascertained at a single center in the New York metropolitan area during the period of April 2000 to March 2005. Of these, 162 were classified as diffuse large B-cell lymphoma (DLBCL). The breast cancer cases were derived from 658 individuals with invasive ductal carcinoma (IDC). The patients were Caucasians and self-identified as of Ashkenazi Jewish ethnicity. Pathology reports, and in the case of breast tumors, ER status, of all cases were reviewed and age of diagnosis was recorded. Germ-line DNA from cases was collected and permanently stripped of identifiers before genotyping, in accord with an Institutional Review Board–approved protocol.

Controls were drawn from DNA of 976 healthy men and women, all of Ashkenazi ancestry by self-report of religion and country of origin of parents and grandparents. Age range was 18 to 65 years. Control DNA samples were available from the New York Cancer Project, an ongoing cohort study of over 17,000 volunteers of varying ethnic backgrounds who live in the New York metropolitan area (28).

Soft-tissue sarcoma cases. Samples originated from 105 sporadic soft-tissue sarcoma (STS) cases diagnosed from 1991 to 2001 at the Surgical Clinic 1, University of Leipzig and at the Institute of Pathology of the Martin-Luther-University Halle, Germany. All patients had a R0-resection of a their primary tumor done by the same team. The patients were of Caucasian ethnicity with an age range of 14 to 84 years (average, 55 years). The STS samples consisted of 29 liposarcomas, 23 malignant fibrous histiocytomas, 16 leiomyosarcomas, 12 neurogenic sarcomas, 8 rhabdomyosarcomas, 8 synovial sarcomas, 5 fibrosarcomas, and 4 other types. One hundred four healthy blood donors of Caucasian ethnicity (non-Jewish) from Germany served as controls.

Breast cancer cases. Between April 2004 and October 2005, 258 Caucasian women previously diagnosed with infiltrating ductal carcinoma of the breast were accrued to this study at The Cancer Institute of New Jersey (New Brunswick, NJ). Pathology reports were reviewed on all cases. Venipuncture was done and genomic DNA was prepared from whole blood. Age and menopausal status at diagnosis, ER status, and degree of positivity were recorded. All information was devoid of identifiers and kept in a database. Data collection was in accord with an approval by the Institutional Review Board at the Cancer Institute of New Jersey.

Sequence analysis. The status of the SNP309 locus was determined for the p53 germ-line mutation carriers, the unaffected non-Ashkenazi Jewish individuals, the STS patients, and the second group of Caucasian IDC

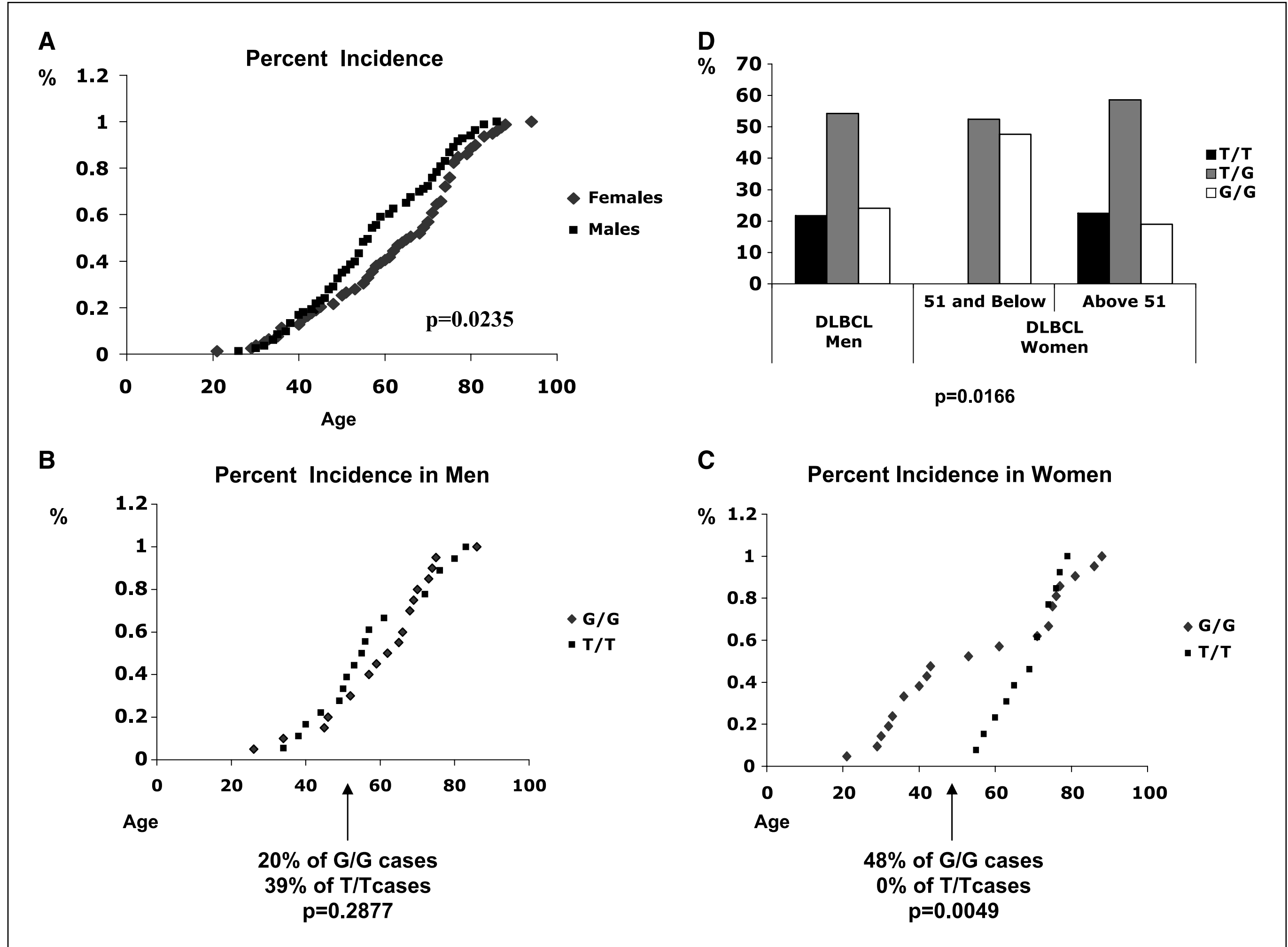

Figure 1. The G-allele of SNP309 associates with an accelerated age of onset of DLBCL in younger females but not in males. DLBCL has a well-documented gender difference in tumor incidence. *A,* the cumulative incidence of cancer for both men (*black squares*) and women (*gray diamonds*) is plotted as a function of age. The cumulative incidence of DLBCL for both the individuals T/T in genotype (*black squares*) and G/G in genotype (*gray diamonds*) is plotted as a function of age for males (*B*) and females (*C*). Female DLBCL patients diagnosed below the average age of menopause (51 years) are enriched for the G-allele of SNP309. *D,* relative ratios of the three genotypes at the SNP309 locus for the male DLBCL patients, the female DLBCL patients diagnosed at ≤51 years of age, and females diagnosed >51 years of age.

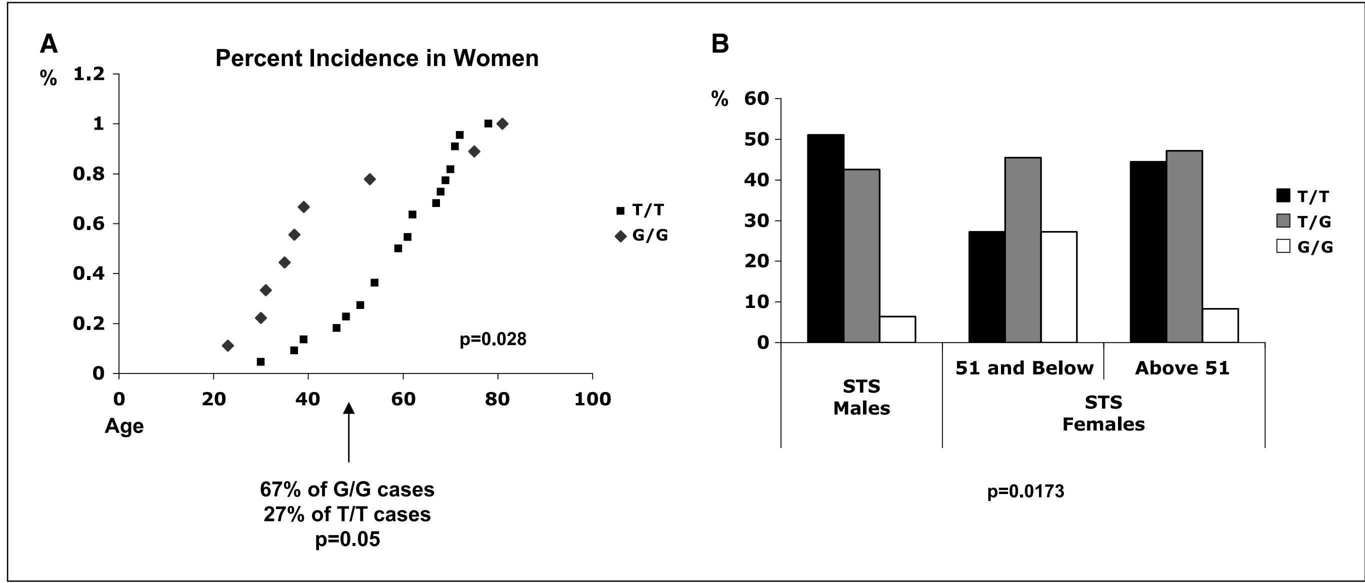

Figure 2. The G-allele of SNP309 associates with an accelerated age of onset of STS in younger females. *A,* the cumulative incidence of STS for both the women T/T in genotype (*black squares*) and G/G in genotype (*gray diamonds*) is plotted as a function of age. Female STS patients diagnosed below the average age of menopause (51 years) are enriched for the G-allele of SNP309. *B,* the relative ratios of the three genotypes at the SNP309 locus for the male STS patients, the female STS patients diagnosed at ≤51 years of age, and females diagnosed >51 years of age.

patients by PCR amplification and subsequent sequencing (primer 1, CGGGAGTTCAGGGTAAAGGT; primer 2, AGCAAGTCGGTGCTTACCTG).

The status of the SNP309 locus was determined for the unaffected Ashkenazi Jewish individuals, DLBCL patients, and the first group of Caucasian Ashkenazi Jewish IDC patients by using the combination of *Msp*IA1 RFLP analysis and 5′ allelic discrimination assay (TaqMan). For the *Msp*A1 RFLP analysis, primers 5′-CGGGAGTTCAGGGTAAAGGT-3′ and 5′-AGCAAGTCGGTGCTTACCTG-3′ were used. PCR was done under standard conditions using 20 ng of genomic DNA and annealing temperature of 66°C. The resulting PCR product (351 bp) was digested by *Msp*IA1. *Msp*IA1 cleaves final PCR product on two sites, one is constitutive that served as an internal control of enzymatic digestion and allele G of SNP309 generates specific *Msp*IA1 restriction site.

The TaqMan 5′ allelic discrimination PCR assay was done in 384-well plates. Each well contained 5.0 ng DNA, 2.5 μL TaqMan Universal PCR Master Mix (Applied Biosystems, Foster City, CA), 0.0625 μL of probe and primer solution (Assays-on-Demand, Applied Biosystems), and 2.4375 μL distilled water. The PCR reaction was initiated at 95°C for 10 minutes, followed by 47 cycles of 92°C for 30 seconds and 60°C for 60 seconds. Following PCR, fluorescence was measured in an ABI 7900 HT Sequence Detector (Applied Biosystems) and the genotype clusters were manually scored using Sequence Detection Software 2.0 (Applied Biosystems).

Results

Diffuse large B-cell lymphoma. To test if SNP309 can contribute to the gender differences observed in cancer, a type of cancer was studied with a well-documented gender difference in tumor incidence (i.e., non-Hodgkin's lymphoma). Non-Hodgkin's lymphoma is the fifth most common cancer in both men and women but men have an increased risk for developing non-Hodgkin's lymphoma worldwide, as well as an earlier average age of tumor diagnosis (29, 30). In this study, Caucasians of Ashkenazi Jewish ethnicity with DLBCL were analyzed. It is interesting to note that Ashkenazi Jewish individuals have a higher relative frequency of the G-allele compared with Caucasians not of Ashkenazi descent. Specifically, 976 Ashkenazi Jewish individuals who had never been diagnosed with cancer, on sample ascertainment, were found to have the following relative frequency of three different genotypes at the SNP309 locus: T/T, 27%; T/G, 49%; G/G, 24%. One hundred four Caucasians, not of Ashkenazi decent and who had never been diagnosed with cancer, on sample ascertainment, were found to have the following relative frequencies: T/T, 38%; T/G, 51%; G/G, 10%.

As previously observed (29, 30), in 162 Caucasians of Ashkenazi Jewish ethnicity diagnosed with DLBCL, the 83 male patients were diagnosed, on average, 5 years earlier than the 79 female patients (Fig. 1*A*). Specifically, the male patients were diagnosed, on average, at 57 years of age (range, 25-85 years) and females at 62 years of age (range, 21-93 years). The male DLBCL patients showed no large differences in the age of tumor diagnosis when separated into the different genotypes of the SNP309 locus (T/T males, 58 years of age versus G/G males, 60 years of age; Fig. 1*B*). In contrast, G/G women showed a 13-year earlier tumor diagnosis than T/T women [T/T women, 68 years of age (range, 55-78 years) versus G/G women, 55 years of age (range, 21-87 years); Fig. 1*C*].

Estrogen has been proposed to play a role in the gender-specific differences in DLBCL incidence, as women exposed to exogenous estrogens have been observed to have altered risk for DLBCL (31–33). Indeed, ER, a critical component of the estrogen-signaling pathway, is expressed in multiple B-cell lymphomas (34–36). If estrogen signaling is working to allow the G-allele of SNP309 to accelerate tumor formation in women, then differences in the age-dependent incidence of DLBCL between G/G women and T/T women should be the largest in women below the average age of menopause (51 years) when female specific hormones like estrogen are at their highest levels. As seen in the Fig. 1*C*, where the cumulative incidence of DLBCL for each genotype is plotted as a function of age, the greatest differences in the age-dependent incidence between the two genotypes are seen in women <51 years of age. Indeed, there are no women with a T/T genotype in these cases diagnosed with DLBCL under the age of 55 years. By contrast, over half of the G/G women had already been diagnosed with DLBCL by this age (P = 0.0027, two-tailed Fisher's exact test). The

G/G women make up 48% of the female DLBCL cases diagnosed by the age of 51 years and only 19% of the female cases >51 years of age and only 24% of the male DLBCL cases (Fig. 1*D*; *P* = 0.0166, two-tailed Fisher's exact test). Together, these data support the hypothesis that estrogen could play a role in the ability of the G-allele of SNP309 to accelerate DLBCL formation in women as the greatest differences in the age-dependent incidence between G/G women and T/T women are the greatest below the average age of menopause when gender-specific hormones, like estrogen, are at the highest levels.

Soft-tissue sarcoma. The G-allele of SNP309 in its homozygous state (G/G) has been previously shown to associate with a 12-year earlier age of onset of sporadic STS compared with T/T individuals (6). The STS patients in this study were made up of 58 Caucasian women and 47 Caucasian men. When separated by gender, it became clear that the significant earlier age of onset of STS observed in these cases is only associated with the G/G women, as only 3 of 47 male patients had the G/G genotype. Specifically, G/G women were diagnosed on average 14 years earlier than T/T women [T/T women, 59 years of age (range, 30-78 years) versus G/G women, 45 years of age (range, 23-81 years); Fig. 2*A*; *P* = 0.028, randomization test]. As the presence of ER in STS is well documented (37–40), the possibility that estrogen might be playing a role in the ability of the G-allele of SNP309 to accelerate STS formation in women was tested. Indeed, the largest differences in the age-dependent STS incidence between G/G women and T/T women were also seen in women below the average age of menopause (51 years) when gender-specific hormones, such as estrogen, are at their highest levels. Specifically, 67% of G/G women were diagnosed with STS by the age of 51 years, but only 27% of T/T women (*P* = 0.05, one-tailed Fisher's exact test). In fact, the overall frequency of the G/G genotype is 27% in female STS cases diagnosed by the age of 51 years and only 8% in female cases diagnosed >51 years of age and 6% in male STS cases (Fig. 2*B*; *P* = 0.0173, one-tailed Fisher's exact test). Together these data further support the hypothesis that gender-specific hormones, like estrogen, could be playing a role in the ability of the G-allele of SNP309 to accelerate both sporadic DLBCL formation and sporadic STS tumor formation.

Invasive ductal breast carcinoma. To test if the G-allele of SNP309 can indeed accelerate tumorigenesis only in the presence of an active estrogen-signaling pathway *in vivo*, women with the same tumor type were studied. The tumor type studied was sporadic IDC of the breast, which accounts for the vast majority of all breast cancers (41). Some women developed IDC with an intact estrogen pathway whereas others did not. DNA was isolated from lymphocytes from women with IDC of the breast and the status of SNP309 was determined. All patients were Caucasians of self-identified Ashkenazi Jewish ethnicity. Individuals with the most common breast cancer susceptibility alleles of the *BRCA1* and *BRCA2* genes were excluded from this study. In each case, pathology reports, including analysis of ER (when available), and age at diagnosis were recorded. At diagnosis, the levels of the ER, a critical component of the estrogen-signaling pathway, are measured in the tumor to determine the most appropriate course of therapy. ER is detectable in 60% to 70% of all breast cancers and its expression level correlates with the tumor dependence on estrogen for growth (42, 43).

Of the 658 IDC patients, 136 patients were noted to have no significant levels of ER expression (<10% of tumor cells stained positive for ER) whereas 472 were noted to have significant levels of ER staining (≥10% of tumors cells stained positive for ER) and the expression level of ER was not known for 50 patients. Of the 472 patients noted to have significant levels of ER staining, 127 patients were known to have high levels of ER expression (≥50% of the tumor cells stained positive for ER).

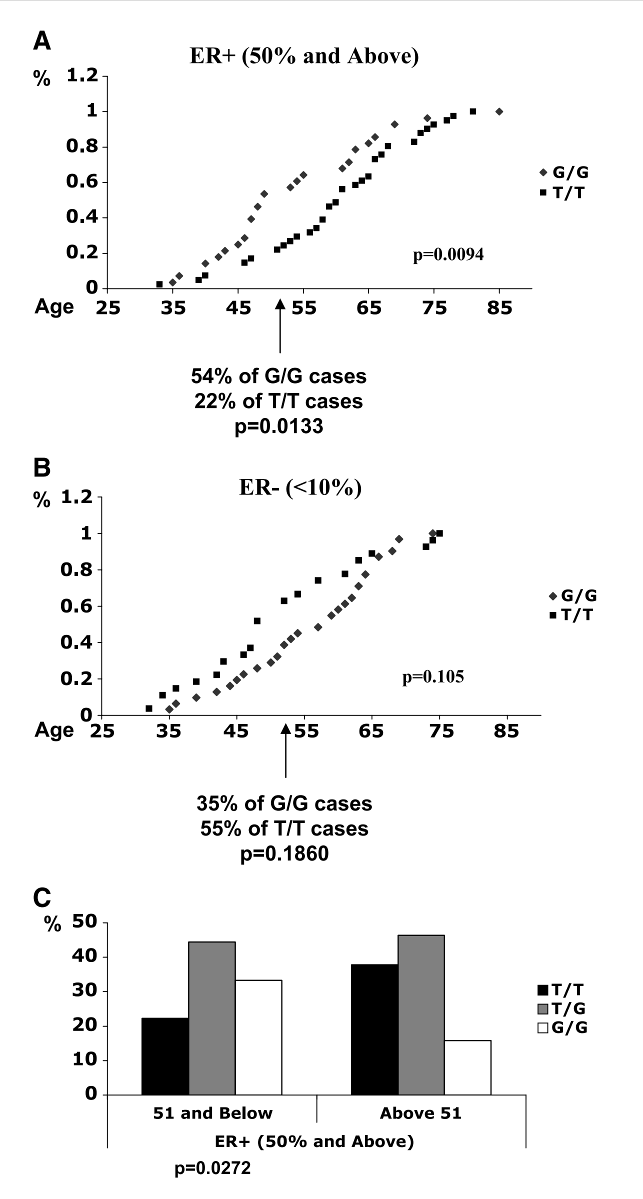

Figure 3. The G-allele of SNP309 associates with an accelerated age of onset in high ER-positive, but not in ER-negative, IDC of the breast in Caucasians (Ashkenazi Jewish). The cumulative incidence of IDC for both the individuals T/T in genotype (*black squares*) and G/G in genotype (*gray diamonds*) is plotted as a function of age for high ER-positive tumors (>50% of the tumor cells stained positive for ER; *A*) and ER-negative tumors (<10%; *B*). Female patients with high ER-positive IDC (>50% of the tumor cells stained positive for ER) diagnosed below the average age of menopause (51 years) are enriched for the G-allele of SNP309. *C,* relative ratios of the three genotypes at the SNP309 locus for the female high ER-positive IDC patients diagnosed at ≤51 years of age and females diagnosed >51 years of age.

If the G-allele of SNP309 can accelerate tumorigenesis only in the presence of an active estrogen-signaling pathway, then an earlier age of tumor onset for G/G women versus T/T women should be greatest in the formation of tumors that express high levels of ER, as the percent of tumor cells which stain positive for ER has been shown to correlate with the tumor dependence on estrogen for growth (42, 43). Indeed, in those 100 women whose tumors

expressed high levels of ER (≥50%), G/G women showed a 7-year average earlier age of onset of IDC compared with T/T women and an 11-year median earlier onset [T/T women, 60 years of age (range, 33-81 years) versus G/G women, 53 years of age (range, 35-84 years); $P = 0.0094$, randomization test; Fig. 3*A*]. No significant differences were observed in those women whose tumors expressed low levels of ER [<10% (Fig. 3*B*) or <50% (data not shown)]. Specifically, the ER-negative T/T patients were diagnosed on average at 51 years of age and G/G patients at 56 years of age. Interestingly, the acceleration of high ER-positive (≥50%) IDC formation in G/G women compared with T/T women was also greatest below the average age of menopause (51 years) when estrogen levels are at their highest. Fifty-four percent of the patients with the G/G genotype had been diagnosed with high ER-positive (≥50%) IDC by the age of 51 years, in contrast to only 22% of the T/T patients (Fig. 3*A*; $P = 0.0133$, one-tailed Fisher's exact test). In fact, women with the G/G genotype make up 33% of high ER-positive (≥50%) IDC cases diagnosed by the age of 51 years, as opposed to 16% of high ER-positive (≥50%) IDC cases diagnosed >51 years of age (Fig. 3*C*; $P = 0.0272$, two-tailed Fisher's exact test). These data further support the hypothesis that an active estrogen-signaling pathway allows for the G-allele of SNP309 to accelerate tumor formation in women.

To confirm these observations with an independent study, the hypothesis was tested again in a second group of Caucasian IDC patients not selected for Ashkenazi Jewish ethnicity. In the 258 IDC patients in this study, 71 patients were noted to have no significant levels of ER expression (<10% of tumor cells stained positive for ER) whereas 184 were noted to have significant levels of ER staining (≥10% of tumor cells stained positive for ER) and the expression level of ER was not known for 3 patients. Of the 184 patients noted to have significant levels of ER staining, 117 patients were known to have high levels of ER expression (≥50% of the tumor cells stained positive for ER).

As previously observed, in the 117 women in this second group whose tumors expressed high levels of ER (>50%), G/G women again showed a 7-year earlier onset of IDC compared with T/T women [T/T women, 54 years of age (range, 29-76 years) versus

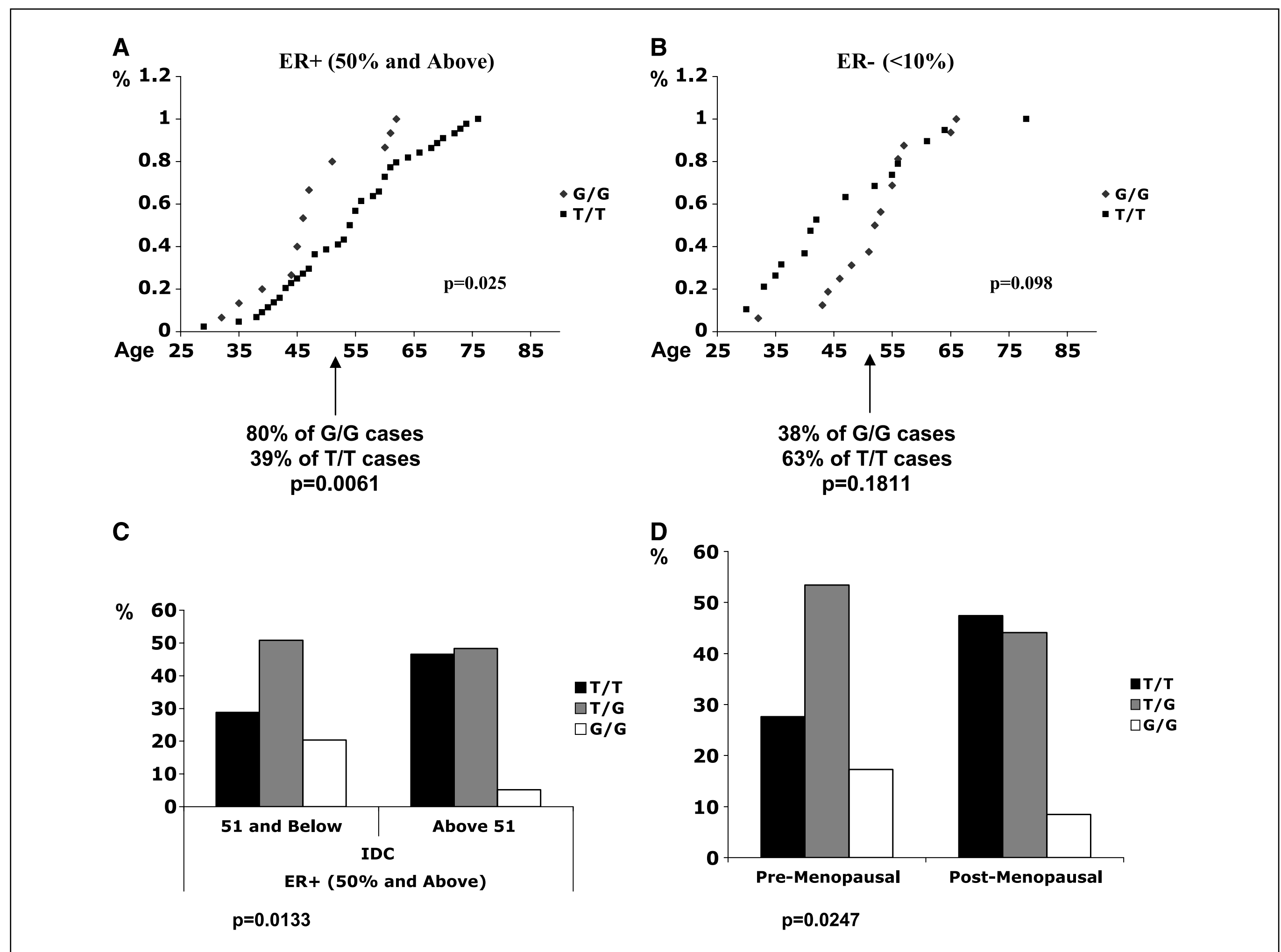

Figure 4. The G-allele of SNP309 associates with an accelerated age of onset in high ER-positive but not ER-negative IDC of the breast in a second independent group of Caucasians. The cumulative incidence of IDC for both the individuals T/T in genotype (*black squares*) and G/G in genotype (*gray diamonds*) is plotted as a function of age for high ER-positive tumors (>50% of the tumor cells stained positive for ER; *A*) and ER-negative tumors (<10%; *B*). Female patients with high ER-positive IDC (>50% of the tumor cells stained positive for ER) diagnosed below the average age of menopause (51 years; *C*) or premenopausal (*D*) are enriched for the G-allele of SNP309. *C* and *D,* relative ratios of the three genotypes at the SNP309 locus for the female high ER-positive IDC patients diagnosed at ≤51 years of age or premenopausal and females diagnosed >51 years of age or postmenopausal.

G/G women, 47 years of age (range, 32-62 years); $P = 0.025$, randomization test; Fig. 4*A*]. Again, no significant differences were observed in the 71 women whose tumors expressed lower levels of ER [<10% (Fig. 4*B*) or <50% (data not shown)]. The acceleration of high ER-positive (≥50%) IDC formation in G/G women compared with T/T women was also greatest below the average age of menopause (51 years) when estrogen levels are at their highest. Eighty percent of the G/G patients had been diagnosed with ER-positive IDC by the age of 51 years, but only 39% of the T/T patients ($P = 0.0061$, one-sided Fisher's exact test). In fact, G/G women make up 20% of high ER-positive (≥50%) IDC cases diagnosed by the age of 51 years, as opposed to 5% of high ER-positive (≥50%) IDC cases diagnosed >51 years of age (Fig. 4*C*; $P = 0.0133$, one-sided Fisher's exact test). In this study, the menopause status of the patients at diagnosis was available and, as predicted, individuals with the G-allele of SNP309 were enriched in the premenopausal high ER-positive (≥50%) IDC cases compared with the postmenopausal cases. Specifically, individuals with the G-allele of SNP309 made up 72% of the premenopausal cases and only 53% of the postmenopausal cases (Fig. 4*D*; $P = 0.0247$, one-sided Fisher's exact test).

Discussion

In summary, the results of four independent studies of three different sporadic cancers (DLBCL, STS, and IDC) support the model that an active estrogen-signaling pathway, either directly or indirectly, allows for the G-allele of SNP309 to accelerate tumor formation in women. Specifically, in DLBCL patients (Fig. 1), the G-allele of SNP309 only associated with an earlier age of diagnosis in female patients and not in male patients, similar to what was previously observed in a study of male and female colorectal cancer patients (8). That this gender-specific difference could be due to gender-specific hormones was supported by the observations that both in female DLBCL patients (Fig. 1) and in female STS patients (Fig. 2), the differences in age-specific cancer incidence between G/G and T/T women were the largest below the average age of menopause (51 years) when gender-specific hormones, like estrogen, are at their highest levels. That the estrogen-signaling pathway could be either directly or indirectly involved was supported by the observations that the G-allele of SNP309 only associated with an earlier age of onset in high ER-positive (≥50%), but not in ER-negative, IDC formation, which was seen in two independent case studies (Figs. 3 and 4). Further support came from the observation that the differences in age-specific cancer incidence between G/G and T/T women with high ER-positive (≥50%) IDC were the largest below the average age of menopause (51 years) when estrogen levels are at their highest (Figs. 3*A* and 4*A*). This model, in which an active estrogen-signaling pathway allows for the G-allele of SNP309 to accelerate tumor formation in women, may help explain the genetic basis for the frequently observed gender differences in cancer and shows that the genotype at a specific locus can affect how hormones, like estrogen, will affect tumorigenesis in humans.

The estrogen-signaling pathway can be regulated in humans for a variety of reasons (e.g., contraception, relief of menopausal symptoms, as well as cancer prevention and treatment). Estrogen signaling manipulation has been shown to alter both cancer incidence and progression (42–44). This study suggests that women with a G/G genotype for SNP309 could be affected differently by estrogen signaling manipulation than women with a T/T genotype. Specifically, in the case studies of DLBCL, STS, and high ER-positive (≥50%) IDC of the breast, a total of 147 female cancer patients diagnosed below the average age of menopause, when estrogen levels are at their highest, were greatly and significantly enriched for women with a G/G genotype (29.3%) when compared with the 234 female patients diagnosed after the average age of menopause (12.8% G/G; $P < 0.0001$, two-sided Fisher's exact test) when estrogen levels in women measurably decrease (Figs. 1*D*, 2*B*, 3*C*, and 4*C*; Table 1). These observations suggest that increasing estrogen levels in postmenopausal women with a G/G genotype, to alleviate menopausal symptoms, could significantly increase their risk to develop these cancers. Indeed, studies have shown the association of exogenous estrogen use by menopausal women lends to an increased risk of developing ER-positive IDC and other cancers (44–46). In contrast, these observations imply that women with a G/G genotype and these cancers (DLBCL, STS, and high ER-positive IDC) would benefit from decreasing estrogen levels and this could significantly retard the progression of their disease. Indeed, many studies have shown that reducing estrogen signaling in patients with high ER-positive IDC significantly retards tumor

Table 1. Distribution of MDM2 SNP309 in patients from four independent case studies of three different tumor types

	DLBCL, *n* (%)			STS, *n* (%)			IDC, *n* (%)		IDC, *n* (%)		Total, *n* (%)	
	Males	Females		Males	Females		ER+ (≥50%)		ER+ (≥50%)		Females	
		≤51 y	>51 y		≤51 y	>51 y	≤51 y	>51 y	≤51 y	>51 y	≤51 y	>51 y
T/T	18 (22%)	0 (0%)	13 (22%)	24 (51%)	6 (27%)	16 (44%)	10 (22%)	31 (38%)	17 (29%)	27 (47%)	33 (22.4%)	87 (37.2%)
T/G	45 (54%)	11 (52%)	34 (59%)	20 (43%)	10 (45%)	17 (47%)	20 (44%)	38 (46%)	30 (51%)	28 (48%)	71 (48.3%)	117 (50.0%)
G/G	20 (24%)	10 (48%)	11 (19%)	3 (6%)	6 (27%)	3 (8%)	15 (33%)	13 (16%)	12 (20%)	3 (5%)	43 (29.3%)	30 (12.8%)
Total	83	21	58	47	22	36	45	82	59	58	147	234
P value for enrichment of G/G		$P = 0.0166$			$P = 0.0173$		$P = 0.0272$		$P = 0.0133$		$P < 0.0001$	
Fisher's exact test		two-tailed			one-tailed		two-tailed		one-tailed		two-tailed	
Race		Caucasian			Caucasian		Caucasian		Caucasian		Caucasian	
Ethnicity		Ashkenazi Jewish					Ashkenazi Jewish					

growth and results in longer overall survival rates (43, 47). It will be interesting to determine if reduction of estrogen signaling will also prove to be an effective treatment for G/G women with DLBCL and STS, as the results presented here predict.

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Q2 # Association of HDM2 Transcript Levels with Age of Onset and Prognosis in Soft Tissue Sarcomas

Helge Taubert,[1] Frank Bartel,[1,4] Thomas Greither,[1] Matthias Bache,[2] Matthias Kappler,[2] Thomas Köhler,[5] Anja Böhnke,[1,4] Christine Lautenschläger,[3] Hannelore Schmidt,[1] Hans-Jürgen Holzhausen,[1] Steffen Hauptmann,[1] and Peter Würl[6]

[1]Institute of Pathology and [2]Department of Radiotherapy, [3]Institute of Medical Biometry and Informatics, and [4]Junior Research Group, Institute of Pathology, Martin-Luther-University Halle-Wittenberg, Halle, Germany; [5]AJ Roboscreen, Leipzig, Germany; and [6]Clinic of General and Transplantation Surgery, University of Ulm, Ulm, Germany

Abstract

Q3 **The p53 stress response is crucial for the prevention of tumor formation. The oncogene HDM2 is one of the key negative regulators of p53 and is a central node in the p53 pathway. P53 and HDM2 form an oscillating feedback loop. HDM2 expression is regulated by different promoters. To evaluate its clinical relevance, we determined the levels of HDM2 transcripts originating from the constitutive P1 and p53-sensitive P2 promoter in 133 soft tissue sarcomas and correlated the results with the age of diagnosis and the patients' outcome. We show that only high levels of the HDM2-P1 transcript but not the P2 transcript are associated with an 11-year earlier age of onset (50.5 years) compared with low P1 levels (61.5 years; $P < 0.0001$, *t* test). In addition, low P1 and P2 mRNA expression levels were independent predictors of poor outcome for patients with soft tissue sarcomas (low P1: relative risk, 3.7; $P < 0.0001$; low P2: relative risk, 2.5; $P = 0.001$). A change in the expression levels of the HDM2 transcripts originating from the two HDM2 promoters could disrupt the oscillating P53-HDM2 feedback loop in a way that elevated levels of HDM2-P1 transcript are associated with an earlier age of tumor onset and that reduced levels of HDM2-P1 or HDM2-P2 transcripts are correlated with poor prognosis of patients with soft tissue sarcomas. (Mol Cancer Res 2008;6(10):1–7)**

Introduction

HDM2, the human orthologue of the mouse MDM2 oncogene is well known as an antagonist of the tumor suppressor p53 (1, 2). Transcription of HDM2 is accomplished by two promoters, the p53-independent P1 promoter and the p53-sensitive P2 promoter (3). A third promoter, designated P3, has been identified in intron 3 of the *HDM2* gene (4). In most cells, the transcription from the P1 promoter occurs at low levels (5). The P2 promoter is highly induced by p53 due to the presence of p53-responsive elements (6). Because translation starts in exon 3, the proteins that are translated from the two respective transcripts are identical, but the difference in the 5′-untranslated region is responsible for the higher translation efficiency of the P2 transcript (5, 7). A polymorphism in the HDM2-P2 promoter (SNP309) adjacent to an Sp1 binding site can enhance the binding of the Sp1 transcription factor and subsequently increase the expression of HDM2-P2 transcript and protein (8). Interestingly, the homozygosity of SNP309 is associated with an accelerated age of onset of tumors, e.g., by 12 years in soft tissue sarcomas (STS; ref. 8). Furthermore, we have shown that HDM2 SNP309 accelerates tumor formation in a gender-specific and hormone-dependent manner (9). In this study, we were interested if the transcript levels from either the HDM2-P1 or the HDM2-P2 promoter have a different clinical relevance. We measured HDM2 mRNA expression originating from the HDM2-P1 or HDM2-P2 promoters and correlated the results with onset and survival of patients with sporadic STS.

Results

HDM2-P1 and HDM2-P2 Transcript Levels of Patients with STS

The levels of the HDM2 transcript variants arising from the P1 and P2 promoters were analyzed by quantitative real-time reverse transcription-PCR. The median expression levels of the P1-HDM2 and P2 HDM2 transcripts were 5.7 and 7.2 zmol per amol *GAPDH*, respectively. We classified tumor samples with an expression below the median of the respective mRNA as "low expression" and tumor samples with expression levels above the median as "high expression." A high P1 expression was significantly correlated with a high P2 expression, and vice versa ($P < 0.0001$, χ^2 test; Table 1). Q4

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H. Taubert and F. Bartel contributed equally to this study.
Requests for reprints: Helge Taubert, Institute of Pathology, Faculty of Medicine, University Halle-Wittenberg, Magdeburger Strasse 14, D-06097 Halle/Saale, Germany. Phone: 49-345-557-1293; Fax: 49-345-557-1295. E-mail: helge.taubert@medizin.uni-halle.de
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Correlation of HDM2-P1 and HDM2-P2 Transcript Levels with Age of Onset of STS

Q5 In our previous work, we have shown that *HDM2* gene amplification (10) and elevated HDM2 mRNA levels (10) were correlated with a favorable prognosis for patients with STS. Furthermore, we have recently published that a single nucleotide polymorphism in the HDM2-P2 promoter (SNP309) is associated with a 12-year earlier age of onset for those individuals who are homozygous for SNP309 (8). Therefore, we asked whether the expression of the two different HDM2 transcript variants derived from either the constitutive P1 or the p53-sensitive P2 promoter is also associated with the age of onset and prognosis in STS and if other mechanisms besides gene amplification might influence HDM2 mRNA expression in STS.

We found that the average age of onset for STS patients with high expression of the P1 transcript was 50.5 years compared with 61.5 years for low P1 expression ($P < 0.0001$; *t* test). Surprisingly, there was no difference regarding the average age of onset for patients with low or high P2 transcript levels (56 versus 55 years; $P = 0.72$; *t* test).

Effect of HDM2-P1 and HDM2-P2 Transcript Levels on Overall Survival and Prognosis in STS

Low P1 transcript levels were correlated with a significantly decreased overall survival (median, 26 months) compared with high P1 expression levels (median, 119 months; log rank test; $P = 0.0001$). There was also a significant difference in the mean overall survival for patients whose tumors expressed low or high levels of the HDM2-P2 transcript (26 versus 96 months; $P = 0.0023$).

In the multivariate Cox's regression hazard analysis that was adjusted to tumor stage, localization, and extent of surgical therapy, we identified low P1 and low P2 levels (relative risk, 3.7; $P < 0.0001$; relative risk, 2.5; $P = 0.001$, respectively), as well as combined low amounts of P1 mRNA and P2 mRNA (relative risk, 5.9; $P < 0.0001$) as independent risk factors that were correlated with poor prognosis for patients with STS (Fig. 1, Table 2).

Correlation of HDM2-P1 and HDM2-P2 Transcript Levels with SNP309 Status

Previously, we could show that SNP309 status was correlated with an early onset of tumor formation in female patients with STS (9). Therefore, we investigated if P1 or P2 promoter transcript expression was correlated with SNP309 status. In a bivariate correlation, we did not detect an association between SNP309 and P1/P2 promoter transcript levels in all patients with STS. However, when we considered only female patients, a correlation between SNP309 and P2 promoter transcript expression ($P = 0.036$), and as a trend, an association with P1 promoter transcript expression ($P = 0.062$)

Table 1. Correlation of HDM2-P1 and HDM2-P2 Transcript Levels with Clinical Variables of Patients with STS

Characteristics	Cases	HDM2-P1 expression		HDM2-P2 expression	
		Low (n = 59)	High (n = 74)	Low (n = 58)	High (n = 75)
Sex					
Male	57	23	34	24	33
Female	76	36	40	34	42
Histologic subtype					
LS	32	8	24	12	20
MFH	32	23	9	16	16
FS	9	3	6	2	7
RMS	9	4	5	6	3
LMS	21	9	12	12	9
NS	13	6	7	4	9
Syn	9	2	7	4	5
Other	8	4	4	2	6
Tumor grade					
1	21	5	16	7	14
2	70	28	42	27	43
3	41	26	15	24	17
Tumor stage					
I	20	5	15	7	13
II	55	22	33	20	35
III	45	24	21	26	19
IV	13	8	5	5	8
Complete resection					
Radical (R_0)	95	40	55	40	55
Not radical (R_1)	38	19	19	18	20
Localization					
Extremities	87	42	45	38	49
Trunc wall	11	6	5	7	4
Head/neck	4	2	2	2	2
Abdomen/retroperitoneum	26	8	18	9	17
Patients follow-up					
Alive	71	21	50	23	48
Dead	62	38	24	35	27

Abbreviations: LS, liposarcomas; MFH, malignant fibrous histiocytoma; FS, fibrosarcoma; RMS, rhabdomyosarcoma; LMS, leiomyosarcoma; NS, neurogenic sarcoma; Syn, synovial sarcoma.

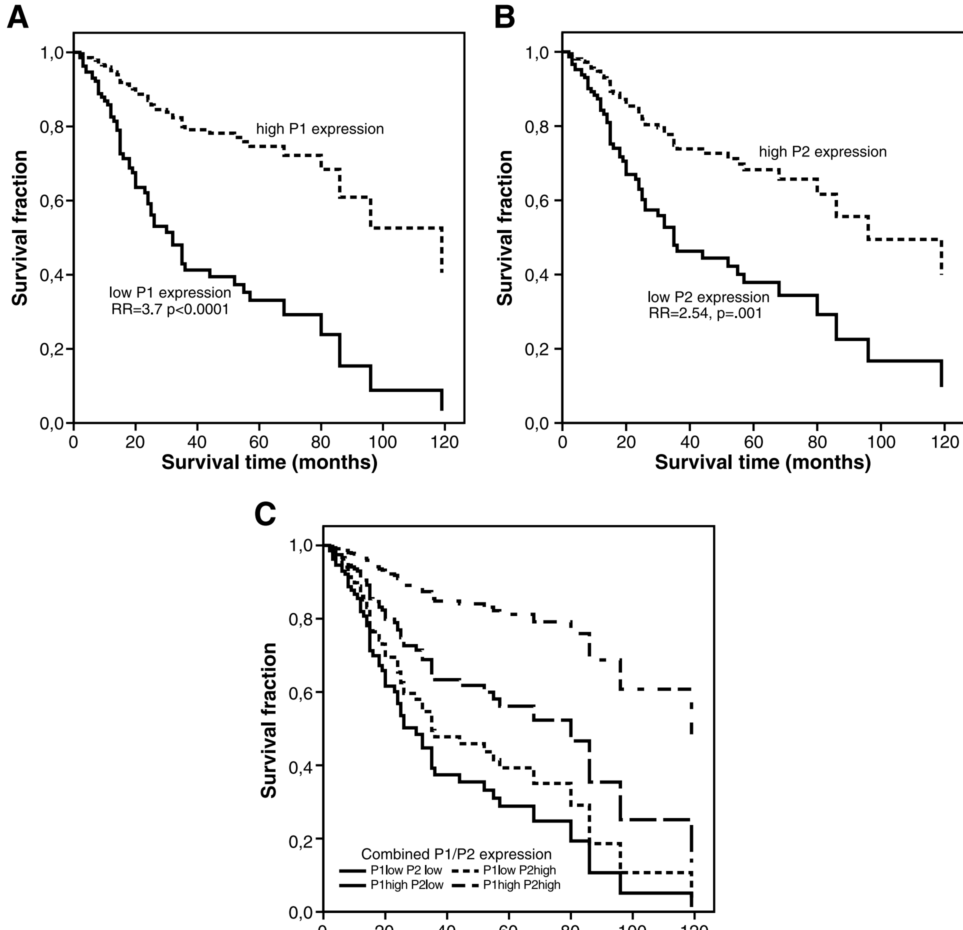

FIGURE 1. The multivariate Cox's proportional-hazard regression model (adjusted to tumor's stage, localization, and status of surgical resection) showed that (**A**) decreased HDM2-P1 and (**B**) decreased HDM2-P2 mRNA expression as well as (**C**) combined decreased P1 and P2 levels are independent prognostic factors that predict poor outcome for patients with STS.

could be found. In a grouped analysis, in which P1 promoter and P2 promoter expression was separated into low or high expression, a correlation between heterozygous/homozygous SNP309 and P1 promoter transcript expression (P = 0.015, Fisher's exact test), but not for the P2 promoter expression, could be seen in female patients. Twenty-one out of 32 female patients (66%) with heterozygous/homozygous SNP309 have a higher transcript of P1 promoter compared with 7 out of 22 females (32%) with wild-type SNP309, whose tumors possessed a higher P1 promoter transcript level. There was no correlation between SNP309 status and P1/P2 promoter expression in male patients.

Correlation of HDM2-P1 and HDM2-P2 Transcript Levels with HDM2 and p53 Protein Expression

We studied protein expression of HDM2 and p53 by immunohistochemistry. Because HDM2 and P53 have short half-lives in normal tissue, both proteins are not detectable in most normal tissues. The detection of HDM2 or p53 protein is considered an alteration in their protein expression. However, there was no association between P1 or P2 promoter transcription and HDM2 protein or p53 protein expression in male or in female patients (independent if premenopausal or postmenopausal female patients).

Correlation of SNP309 with HDM2 and p53 Protein Expression

We did not see a correlation between SNP309 status and HDM2 protein expression, either in male patients or in all female patients. However, if we separate female patients into premenopausal and postmenopausal groups, all 20 premenopausal patients showed either expression of HDM2 and/or heterozygous/homozygous SNP309 in their tumors (P = 0.035; Fisher's exact test). Furthermore, we found a correlation of SNP309 status with p53 protein expression in female patients (P = 0.019; Fisher's exact test) but not in male patients. Eighteen out of 21 female patients (87%) with wild-type SNP309 were p53-positive, whereas 19 out of 34 patients (56%) with heterozygous/homozygous SNP309 were p53-positive. There seems to be a complementing effect of p53 positivity and heterozygous/homozygous SNP309 in female patients with STS. After separating female patients into premenopausal and postmenopausal groups, we still see an association between SNP309 and p53 protein expression only in premenopausal women. All 5 female patients with wild-type SNP309 were p53-positive, whereas 7 out of 15 patients with heterozygous/homozygous SNP309 were p53-positive. In other words, all of the premenopausal STS female patients either showed p53 positivity or were heterozygous/homozygous for SNP309. Q6

Table 2. Prognostic Value of HDM2-P1 and HDM2-P2 Transcript Levels in STS

Variables	Univariate analysis		Multivariate analysis	
	RR (95% CI)*	*P*	RR (95% CI)*	*P*
Tumor stage				
I	1.00		1.00	
II	2.82 (0.84-9.53)		2.43 (0.7-8.2)	
III	6.76 (2.03-22.4)		6.93 (2.07-23.2)	
IV	31.4 (8.71-113.7)	<0.001	20.04 (5.1-78.7)	<0.001
Complete resection				
Radical (R_0)	1.00		1.00	
Not radical (R_1)	2.31 (1.4-3.82)	0.001	1.64 (0.91-2.97)	0.09
Localization				
Extremities	1.00		1.00	
Trunc wall	1.11 (0.39-3.15)		1.22 (0.42-3.57)	
Head and neck	0.84-9.27		1.06 (0.29-3.83)	
Abdomen/retroperitoneum	2.08 (1.15-3.77)	0.015	1.48 (0.75-2.94)	0.259
HDM2-P1 expression				
Low (<5.6 zmol)	2.82 (1.66-4.79)	<0.001	3.77 (2.05-6.94)	<0.001
Elevated (≥5.6 zmol)	1.00		1.00	
HDM2-P2 expression				
Low (<7.1 zmol)	2.16 (1.3-3.6)	0.003	2.54 (1.44-4.48)	0.001
Elevated (≥7.1 zmol)	1.00		1.00	
Combined P1-P2 expression				
P1 low, P2 low	4.14 (2.1-8.2)	<0.001	5.95 (2.76-12.8)	<0.001
P1 high. P2 low	1.36 (0.69-2.68)		1.33 (0.63-2.78)	
P1 low, P2 high	1.92 (0.91-4.05)		2.15 (0.94-4.9)	
P1 high, P2 high	1.00		1.00	

NOTE: Multivariate Cox's proportional-hazard regression model (adjusted to tumor's stage, localization, and status of surgical resection).
For all results, please refer to Supplementary Fig. S1.
*RR, relative risk of tumor-related death.

Discussion

This is the first report about the effect of transcript levels from two different HDM2 promoters, P1 and P2, on the age of onset and outcome of STS. The key results of our study were that (*a*) high levels of the P1 transcript, but not of the P2 transcript, are significantly associated with an earlier age of onset for STS, and (*b*) with respect to overall survival, low levels of the HDM2-P1 mRNA and P2 mRNA are correlated with an unfavorable prognosis (Fig. 2). These results seem to be contradictory. The group of Moshe Oren could show that expression of p53 and HDM2 is oscillating in a feedback loop (11). Based on this finding, Hu et al. recently showed that changes, i.e., either an increase or a decrease in the expression levels of p53 or HDM2, disturb this oscillation (12). They found that higher levels of HDM2 in cells homozygous for SNP309 do not permit coordinated p53-HDM2 oscillation after stress. This might decrease the efficiency of the p53 pathway and correlate with the development of cancers at an earlier age of onset in females (12). Previously, we could show that SNP309 (G/G) accelerates STS formation by 12 years (9), and in this study, we show that P1 transcript expression results in an early tumor onset by 11 years. In line with these results, an increased expression of P1 promoter transcript was significantly correlated with heterozygous/homozygous SNP309. This finding is surprising but it still fits in our previous results that SNP309 is correlated with increased HDM2 transcript levels (8). Altogether, we see a strong correlation between P1 and P2 promoter expression which suggests that their regulation is coordinated and/or that their expression levels might affect each other. However, it is difficult to explain how a polymorphism in the P2 promoter could affect the expression of P1 promoter. Recently, Liang and Lunec (4) have described the existence of a third promoter (P3) in intron 3 of the *hdm2* gene. It has been shown that wild-type p53 specifically binds to this region, and surprisingly, this leads to a decreased activity of the P3 promoter. Furthermore, the P3 promoter had a suppressive effect on P2, especially at high levels of p53 (4), thereby adding a new level to the autoregulatory p53-MDM2 feedback loop. It would be of interest to investigate if the P3 or P2 promoters could affect the P1 promoter. Furthermore, in cell lines homozygous for SNP309, an increased resistance against different chemotherapies and radiation has been shown (8, 13). From our results, it is tempting to speculate that this resistance is rather associated with P1 promoter transcript than with P2 promoter transcript level, which might be relevant for future STS therapies.

Several studies describe a relationship between SNP309 and p53 mutational status in colorectal, bladder, ovarian, and breast cancer (14-17). Patients with homozygous SNP309 (G/G) retain wild-type p53 and patients with wild-type SNP309 (T/T) carry p53 mutations which can effect either early onset of tumor development and/or impair prognosis. In STS, the p53 mutational rate is rather low (~18% in this study); therefore, we investigated P53 protein expression by immunohistochemistry. P53 protein expression is normally not detectable in tissues because of its short half-life of ~20 minutes. Detection of P53 protein expression can be associated with mutational status and/or with conformational changes of P53 and has a prognostic effect for patients with STS (18). In our study group, we found a significant correlation between wild-type SNP309 and the detection of P53 protein in female patients but not in male patients. We suggest that there is a

complementing effect between p53 positivity and heterozygous/homozygous SNP309 both affecting the p53/hdm2 pathway in female patients with STS.

Recently, Cheng and Cohen (19) showed that HDM2-P2 promoter preferentially produce $p90^{HDM2}$ protein, whereas HDM2-P1 promoter transcripts contribute equally to $p90^{HDM2}$ and $p75^{HDM2}$ protein products. They suggest that $p90^{HDM2}$ participates in the p53/HDM2 feedback loop, whereas $p75^{HDM2}$ does not (19). In our study, P1 and P2 transcript levels did not correlate with HDM2 protein levels detected by Western analysis (75 and 90 kDa protein products) or detected by immunohistochemistry (data not shown). However, several studies provided evidence that there can be little correlation between the mRNA and protein levels in yeast, in human liver, and in lung adenocarcinomas (20-22).

The finding that low levels of HDM2-P1 and HDM2-P2 transcripts were correlated with poor overall survival is in agreement with our previous result that, in general, HDM2 transcript levels are significantly associated with tumor-related death for STS (23). Although a higher mdm2-mRNA expression was associated with a better prognosis in patients with ovarian carcinomas (24), several publications describe a correlation between a high HDM2 transcript level and poor outcome for different cancer patients (reviewed in ref. 23). Further studies on the expression profiles of different HDM2 transcripts are necessary to resolve this contradiction.

Recent work from Mendrysa et al. (25) showed that a subtle decrease in the levels of MDM2 has a profound effect on tumor formation. After 5 months of age, mice with wild-type levels of MDM2 had 48 intestinal adenomas on average, whereas mice expressing ~80% of the wild-type MDM2 levels had only 16 tumors, and mice with ~30% of wild-type MDM2 expression had a further reduced number of tumors. These results are confirmed by data from this study, in which an attenuated p53-pathway (high HDM2 expression) associates with an accelerated onset of STS.

In conclusion, our results show that high levels of the HDM2-P1 transcript accelerate the onset of STS. Additionally, we have identified a group of patients whose tumors express low levels of either the P1 or P2 transcript, which are independent prognostic markers predicting poor outcome. Our data supports the finding that changes in the HDM2 transcript level can disturb the oscillating p53-HDM2 feedback loop. In this study, we showed that up-regulation or down-regulation of HDM2 transcripts could affect tumor onset or prognosis in patients with STS.

Materials and Methods

Study Subjects

Tumor tissue samples from 133 adult patients with histologically verified primary STS were analyzed (Table 1; Supplementary Fig. S1). The patient and tissue samples have been previously described (26, 27). The tissue was collected following surgical resection and snap-frozen in liquid nitrogen. The tumors were classified according to the van Unnik grading system (28) and the International Union Against Cancer guidelines (29). All patients gave their written consent. The study was approved by the local ethics committee.

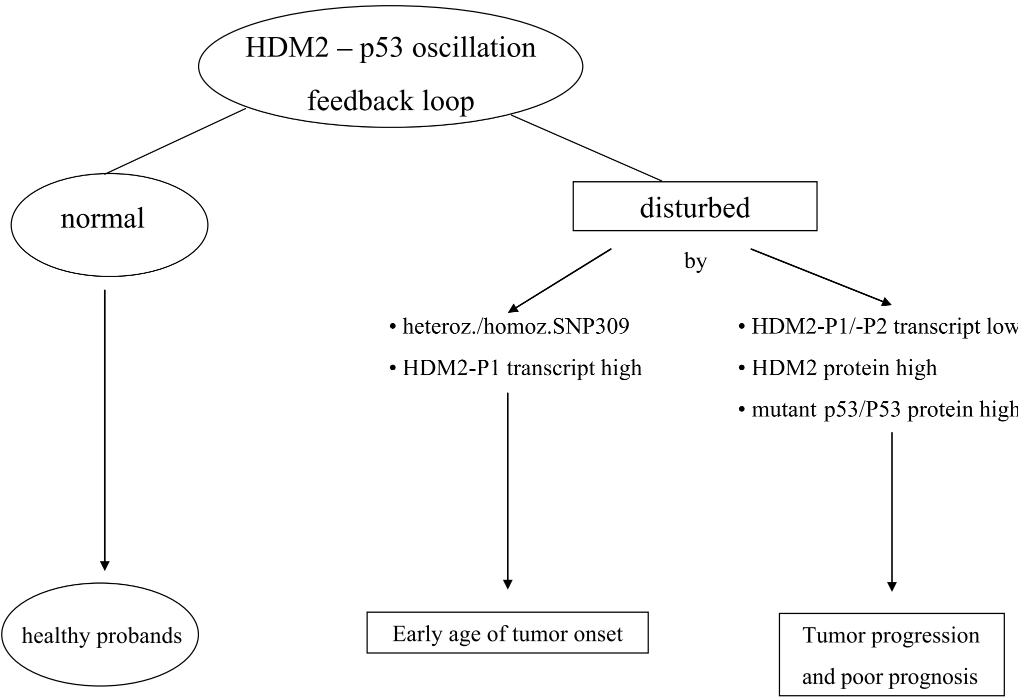

FIGURE 2. Model of disturbance of HDM2-p53 oscillation feedback loop describes which changes in this oscillating equilibrium can affect the age of tumor onset or prognosis of patients with STS. Model is based on the results of refs. (8-10, 17, 18, 25, 31).

Measurement of HDM2-P1 and HDM2-P2 Transcript Levels

Total RNA was isolated using the RNeasy Mini Kit (Qiagen) according to the manufacturer's instructions. Subsequently, 250 ng of RNA was reverse-transcribed with random hexamer primers and the Superscript II reverse transcriptase (Invitrogen). We used two custom-designed assays to measure the levels of the *HDM2* transcripts from either the constitutive P1 or the p53-sensitive P2 promoter (AJ Roboscreen AG). PCR primers were modified after Phelps et al. (ref. 30); P1-fw (GACTCCAAGCGCGAAAACC), P1-bw (CCATCAGTAGGTACAGACATGTTGGT), P2-fw (CGGACGCACGCCACTT), and P2-bw (CCATCAGTAGGTACAGACATGTTGGT). The *GAPDH*-mRNA expression values were determined with a commercially available assay according the manufacturer's instructions (AJ Roboscreen AG). The cDNA amount in each patient sample was calculated according to a simultaneously amplified reference DNA. The P1 and P2 transcript levels in each patient sample are given as zmol P1 (P2)-*HDM2* mRNA (zeptomole, 10^{-21} mol) per amol *GAPDH*-mRNA (attomole, 10^{-18} mol). The p53 gene status of our patient cohort has been described previously (ref. 31; Supplementary Fig. S1).

Immunohistochemical and Western Blot Detection of HDM2 and P53 Protein

Immunohistochemical detection of HDM2 and p53 proteins was done as previously reported (18, 32). We applied 19E3 antibody (Medac) for HDM2 and DO-1 antibody (Oncogene Science) for P53 staining. Western immunoblot was carried out as previously described (33) with the HDM2 antibody N20 (Santa Cruz Biotechnology).

Statistical Analyses

All statistics, including Cox's proportional hazard model and the Kaplan-Meier survival estimates, were carried out using the SPSS 12.0G software (SPSS Science). $P < 0.05$ was considered to be significant. Multivariate Cox's regression models were adjusted to the tumor's stage, localization, and type of resection unless otherwise stated.

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Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Einfluss von Mutationen und Polymorphismen im p53-Tumorsuppressorpathway auf die Entstehung und Prognose von Ovarialkarzinomen

Both somatic and germline genetics of the p53-pathway influence ovarian cancer incidence and survival

Anja Böhnke[1], Juliane Jung[1], Helge Taubert[2], Steffen Hauptmann[2] und Frank Bartel[1]

Abstract

Purpose: Although TP53 is one of the most studied genes/proteins in ovarian carcinomas, the predictive value of TP53 alterations is still ambiguous. *Experimental design:* We performed analyses of the *TP53* mutational status and its protein expression by immunohistochemistry. Moreover, the single nucleotide polymorphism SNP309 in the P2-promotor of the *HDM2* gene was investigated. We correlated the results with the age of onset and the outcome of 107 ovarian carcinoma patients. *Results*: In our study, we identified a large group of patients with TP53 overexpression despite having a wild-type gene (49 % of all patients with wild-type TP53). This was associated with a significantly shortened overall survival time ($p = 0.019$). Patients with TP53 alterations (especially those with overexpression of wild-type TP53) were also more refractory to chemotherapy than patients with normal TP53 ($p = 0.027$). The G-allele of the SNP309 is associated with an earlier age of onset in estrogen receptor expressing FIGO stage III patients ($p = 0.048$). In contrast, in FIGO III patients, a weakened TP53 pathway (either G-allele of SNP309 or a TP53 mutation) is correlated with an increased overall survival compared with patients whose tumors are wild-type for TP53 and SNP309 ($p = 0.0035$). *Conclusion:* Our study provides evidence that both germ line and somatic alterations of the TP53 pathway influence incidence and survival of ovarian carcinoma, and it underscores the importance of assessing the functionality of TP53 in order to predict sensitivity of platin-based chemotherapies and patient outcome.

Einleitung

Das Ovarialkarzinom weist in der westlichen Welt unter den gynäkologischen Tumoren die höchste Sterblichkeitsrate auf (20). Die meisten Patientinnen mit Ovarialkarzinomen befinden sich zum Zeitpunkt der Diagnosestellung in fortgeschrittenen Krankheitsstadien (22). Die 5-Jahresüberlebensrate beträgt für die FIGO-Stadien III und IV nur noch 15 – 25 % (6,20). Trotz großer Bemühungen, molekulare Veränderungen als prognostische Marker zu identifizieren, bleiben in multivariaten Analysen die Resttumorgröße und der Aszites die einzigen signifikanten Parameter für Patientinnen in fortgeschrittenen Stadien. Die Standardtherapie für Ovarialkarzinompatientinnen in diesen Stadien ist die Tumorentfernung, gefolgt von einer Chemotherapie mit Carboplatin und Taxol. Leider tritt aufgrund einer erworbenen Chemoresistenz bei fast allen Patientinnen ein Rezidiv auf. Daher sind Faktoren, die die Resistenzentwicklung von Ovarialkarzinompatientinnen beeinflussen von großem Interesse. Ein Schlüsselprotein für die zelluläre Antwort auf Platin-haltige Chemotherapeutika ist TP53, dessen Funktionalität durch Mutationen oder Polymorphismen im TP53-Signalweg verringert werden kann. Daher könnte eine Ursache für die Chemoresistenz im Fehlen von funktionsfähigem TP53 liegen.

Für das Ovarialkarzinom liegt die TP53-Mutationsfrequenzen zwischen 40 – 80 % (9, 10, 13, 30). In der Literatur zeigt sich der Trend, dass eher eine TP53-Überexpression (12, 16, 23, 31, 34) als TP53-Mutationen (9, 19, 21, 25) mit einem verkürzten Gesamtüberleben korrelieren. In anderen Studien wurde wiederum kein signifikanter Zusammenhang zwischen Überexpression und Prognose gefunden (18, 21, 27, 32, 35). Problematisch an diesen Studien ist, dass einerseits in immunhistochemischen Untersuchungen keine verkürzten Proteine erkannt werden, welche durch nonsense Mutationen oder Insertionen/Deletionen

[1] Juniorgruppe, Institut für Pathologie, Universitätsklinikum Halle-Wittenberg
[2] Institut für Pathologie, Universitätsklinikum Halle-Wittenberg, Halle/Saale

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entstehen können. Andererseits ist eine TP53 Überexpression nicht zwangsläufig mit Mutationen verbunden. Deshalb ist der Gesamtstatus von p53-Veränderungen (Genmutationen und/oder Überexpression) informativer als Sequenzierung oder Immunhistochemie allein. Obwohl einige Studien sowohl den TP53-Gen- als auch den Proteinexpressionsstatus bestimmt haben (13, 30), wurde in keiner dieser Untersuchungen der Gesamtstatus von TP53 Veränderungen mit dem Überleben oder dem Ansprechen auf Chemotherapie korreliert.

Das HDM2-Onkogen ist eines der wichtigsten negativen Regulatoren des TP53-Proteins (4). Es konnte gezeigt werden, dass das G-Allel des Einzelnukleotid-Polymorphismus SNP309 im TP53-sensitiven P2-Promotors des HDM2-Onkogens mit einer Abschwächung des TP53-Tumorsuppressorsignalweges assoziiert ist (2). Verschiedene Publikationen liefern Beweise, dass das G-Allel des SNP309 mit einem erhöhten Risiko für Tumorentstehung und einem früheren Auftreten der Erkrankung korreliert (1, 5, 15, 26). Die HDM2-Expression wird auch durch den Östrogenrezeptor-Signalweg beeinflusst, da die Transkription von HDM2-P2 durch ERα induzierbar ist (29). Interessanterweise ist die ERα-Bindestelle in der Region lokalisiert, welche den SNP309 enthält. In einer vorherigen Publikation konnten wir zeigen, dass der SNP309 den Effekt von Hormonen, wie Östrogen, auf die Tumorgenese verändert, wodurch es zu geschlechtsspezifischen Unterschieden in verschiedenen Karzinomen kommt (3).

Ziel dieser Studie war es, den Status von SNP309, *TP53* Sequenzveränderungen und TP53-Proteinexpression einer Gruppe von 107 Ovarialkarzinompatientinnen (mit kompletten klinischen Daten, gesammelt am Institut für Pathologie der Universität Halle) zu bestimmen und zu untersuchen ob Polymorphismen oder Mutationen im TP53-Signalweg als signifikante Marker für die Prognose von Ovarialkarzinompatientinnen genutzt werden können.

Material und Methoden

Patientenkollektiv und klinische Daten. Paraffin-fixierte Proben von 107 invasiven Ovarialkarzinomen wurden nach Verfügbarkeit des Materials ausgewählt. Die Fälle wurden zwischen 1997 und 2005 am Institut für Pathologie der Martin-Luther-Universität Halle diagnostiziert. Die Studie wurde durch das lokale Ethikkomitee genehmigt. Alle histologischen Schnitte wurden durch 2 Pathologen (EG & SH) reevaluiert. Die Tumorproben und das Patientenkollektiv wurden bereits in anderen Arbeiten ausführlich beschrieben (17). Die histologische Klassifikation erfolgte nach den Richtlinien der WHO, das Grading nach Silverberg (33). Klinische Daten wie, Patientenalter, Resttumorgröße, FIGO Stadium, adjuvante Chemotherapie und Verlauf sind in Tabelle 1 zusammengestellt.

Immunhistochemie. Die Analyse der TP53 Proteinexpression erfolgte unter Verwendung des monoklonalen Maus-Antikörpers DO-7 (DAKO, Hamburg) und die Analyse der Östrogenrezeptorexpression unter Verwendung des anti-ER-Antikörpers SP1 (Labvision, Deutschland). Die Immunreaktivität für TP53 und ER wurde als Prozentzahl von gefärbten Zellen bestimmt, durch das Auszählen von durchschnittlich 150 Tumorzellen. Tumore mit mehr als 10 % gefärbten Zellen wurden als TP53 oder ER-positiv eingestuft.

Single Strand Conformation Polymorphism (SSCP) Analyse von TP53. Die DNA-Isolation aus Paraffin-fixierten Proben erfolgte nach Standardprotokoll (30). Ein Schnitt jeder Tumorprobe wurde zur Feststellung des Tumorzellgehalts H&E gefärbt, um sicherzustellen, dass die Mehrheit des verwendeten Materials aus Tumorzellen besteht.

Mittels PCR wurden die Exone 3–9 des TP53-Gens amplifiziert. Die Primer für jedes Exon waren im Intron lokalisiert, wodurch auch die Möglichkeit bestand, Spleißstellen mit zu untersuchen. Die Primersequenzen und die PCR-Bedingungen wurden bereits veröffentlicht (30). 10–20 µL jedes PCR-Poduktes wurden über Nacht bei –20 °C gefällt und Konformationsveränderungen durch SSCP auf einem denaturierenden Polyacrylamid-Gel bestimmt. Als Kontrolle diente DNA von gesunden Blutspendern.

TP53 Sequenzanalyse. Die Exone 5–8 wurden bei allen Proben sequenziert. War eine

Tabelle 1: Zusammenfassung klinischer Daten der Ovarialkarzinompatientinnen.

Klinische Parameter	Patientinnen (107)	
	No.	%
Tumortyp		
serös	62	57.9
endometroid	14	13.1
gemischt	11	10.3
klarzellig	9	8.4
transitionalzellig	1	0.9
undifferenziert	8	7.5
muzinös	2	1.9
Tumorstadium		
FIGO I	30	28.0
FIGO II	9	8.4
FIGO III	63	58.8
FIGO IV	5	4.7
Alter der Patientinnen		
Durchschnitt	63.5	
Median	64.0	
Therapie		
Cisplatin+Taxol	62	57.9
Platin-haltig, ohne Taxol	21	29.7
andere	2	1.9
keine (FIGO Ia)	5	4.7
abgelehnt, gestorben	13	12.1
keine Daten	4	3.7
Resttumor		
kein	39	41.0
<1 cm	22	23.1
>1 cm	34	35.8

Veränderung im SSCP-Gel nachweisbar, wurden auch die Exone 3, 4 sowie 9 sequenziert. Die Sequenzierung in sense und antisense Richtung erfolgte unter Verwendung des BigDye Terminator Cycle Sequencing 3.1 Kits (Applied Biosystems, Darmstadt). Die Sequenzierreaktion erfolgte nach Angaben des Herstellers.

Bestimmung des SNP309 Status. Der SNP309-Status von 103 Ovarialkarzinomen wurde durch PCR und anschließende direkte Sequenzierung bestimmt (2).

Statistische Auswertung. Alle statistischen Berechnungen, einschließlich Cox-Regression und Kaplan-Meier-Analyse, wurden mit der SPSS 11.0 Software durchgeführt (SPSS-Science, Chicago, USA). Eine Wahrscheinlichkeit von $p<0,05$ wurde als signifikant eingestuft.

Ergebnisse

TP53-Mutationen und TP53-Proteinexpression. In 107 Patientenproben konnten wir insgesamt 111 Sequenzveränderungen in exonischen sowie intronischen Bereichen des TP53-Gens nachweisen, davon lagen 44 Mutationen im Bereich von Exon 4–8, einschließlich zweier Mutationen im Intron. Bei 22 der Sequenzveränderungen handelte es um eine 16bp-Insertion im Intron 3 und weitere 45 Veränderungen wurden im Codon 72 detektiert, beides bereits bekannte Polymorphismen. 93 % (39/42) der Mutationen, welche die TP53-Proteinsequenz beeinflussen, wurden in Exon 5-8 gefunden. Dabei handelte es sich in 63 % (27/42) um Missense-Mutationen und 9,3 % (4/42) waren Nonsense-Mutationen, bei denen der Basenaustausch zu einem vor-

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zeitigen Stopcodon führt, 19 % (8/42) waren Deletionen und bei 7 % (3/42) der Mutationen handelte es sich um Insertionen. Am häufigsten von Veränderungen betroffen waren dabei mit jeweils 3 Fällen Codon 175 und Codon 245. 8 der 42 Mutationen im Exonbereich konnten in dieser Arbeit das erste Mal im Ovarialkarzinom nachgewiesen werden, weitere 4 Mutationen wurden in dieser Studie erstmalig beschrieben (28).

Wir konnten zeigen, dass 51,4 % (55/107) der Fälle eine Überexpression des TP53-Proteins aufwiesen. 20 Fälle (18,7 %) zeigten eine Färbung von 10% der Tumorzellen oder weniger und in 32 (30,2%) Fällen war die Färbung negativ. Normales ovarielles Oberflächenepithel diente als Kontrolle und zeigte keine TP53-Expression.

Verhältnis von TP53-Expression und Genstatus. In unserer Studie korrelierte eine TP53-Überexpression nicht mit dem Mutationsstatus ($p = 0,59$; χ^2-Test). 54 % (24/44) der Fälle mit mutiertem TP53 zeigten auch eine Überexpression des TP53-Proteins; jedoch lag der Prozentsatz der Fälle mit Wildtyp-Gen und überexpremierten TP53 bei 49 % (31/63).
Für eine bessere Auswertbarkeit unterteilten wir die Fälle in 4 Gruppen. Proben mit Wildtyp TP53-Gen und nicht nachweisbarem Protein wurden als „TP53 normal" klassifiziert, da hier vermutlich funktionsfähiges TP53-Protein vorlag (32/107, 29,9 %). Die weiteren Gruppen enthielten Fälle mit TP53-Veränderungen, (i) Fälle die Wildtyp-TP53 überexpremieren (31/107, 29 %), (ii) Fälle mit TP53-Mutation und Protein-überexpression (24/107, 22,4 %) und (iii) Fälle mit TP53-Mutation, jedoch nicht detektierbarem TP53-Protein (20/107, 18,75 %).

TP53-Mutationen und TP53-Überexpression konnten häufiger in fortgeschrittenen Stadien des Ovarialkarzinoms nachgewiesen werden (Tabelle 2)

TP53 Status und Ansprechen auf Chemotherapie. Wir konnten zeigen, dass Patientinnen mit TP53-Veränderungen signifikant resistenter (78 %) gegenüber Chemotherapeutika waren, als Patientinnen mit „normalem" TP53 (52 %, $p = 0,027$). Dies galt auch für die Proteinexpression beim Vergleich von Tumoren mit TP53-Überexpression (83 % $p = 0,02$) mit Tumoren ohne TP53 (58 %). Patientinnen mit TP53-Mutationen waren ebenfalls resistenter gegenüber einer Chemotherapie (79 %) im Vergleich zu Patientinnen mit Wildtyp TP53-Gen (64 %). Jedoch war diese Assoziation nicht signifikant ($p = 0,148$). Patientinnen mit verändertem TP53 (Mutation und/oder Überexpression) entwickelten schneller ein Rezidiv als Patientinnen mit normalem TP53 (28 vs. 51 Monate, $p = 0,075$).

SNP309 Status im *HDM2* P2-Promotor. Der SNP309-tatus wurde in 103 der 107 Ovarialkarzinompatientinnen untersucht. Wir konnten eine verhältnismäßig hohe Frequenz des heterozygoten Zustandes T/G (52,4 %) und eine geringe Prozentzahl an homozygoten G/G (7,8 %) Veränderungen im Vergleich zu gesunden Blutspendern (T/G: 40 %, G/G: 12 %, (21)) nachweisen. Wie wir bereits vor kurzem zeigen konnten, spielt das G-Allel hauptsächlich bei Patientinnen mit aktivem Östrogen-Signalweg eine Rolle. Deshalb untersuchten wir, ob die Assoziation von SNP309 und dem Alter

Tabelle 2: Zusammenhang von TP53-Status und FIGO-Stadium beim Ovarialkarzinom.

FIGO Stadium	*TP53*-Mutationsstatus		TP53-Expression	
	Wildtyp	mutiert	negativ	positiv
I	22	8 (26 %)	22	8 (27 %)
II	7	2 (22 %)	5	4 (44 %)
III	32	31 (49.2 %)	23	40 (63 %)
IV	2	3 (60 %)	2	3 (66 %)
Signifikanz (p)	p = 0.096		p = 0.01	
Zusammenfassung	63	44 (41.1 %)	53	55 (51.4 %)

Abbildung 1 A

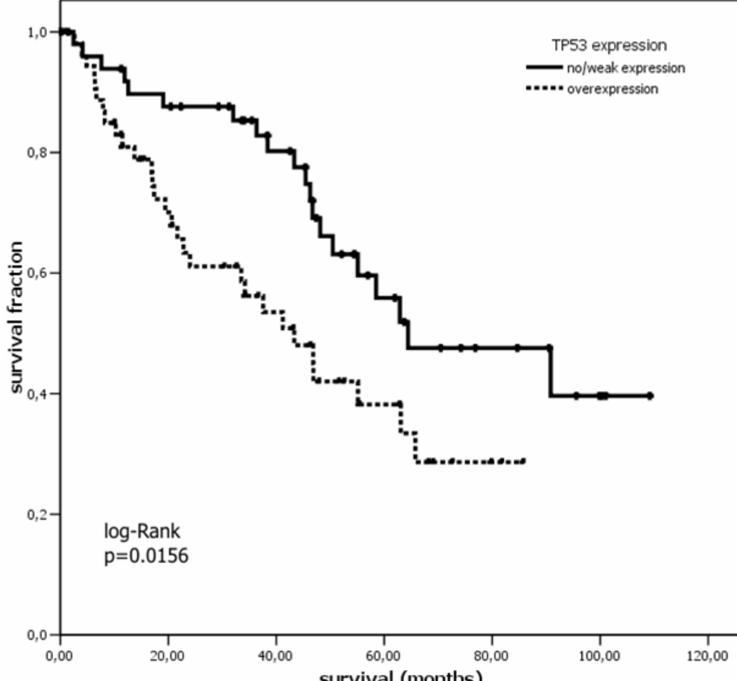

Abb. 1:
A Gesamtüberleben von Ovarialkarzinompatientinnen mit unterschiedlichem TP53-Expressionsstatus.

Abbildung 1 B

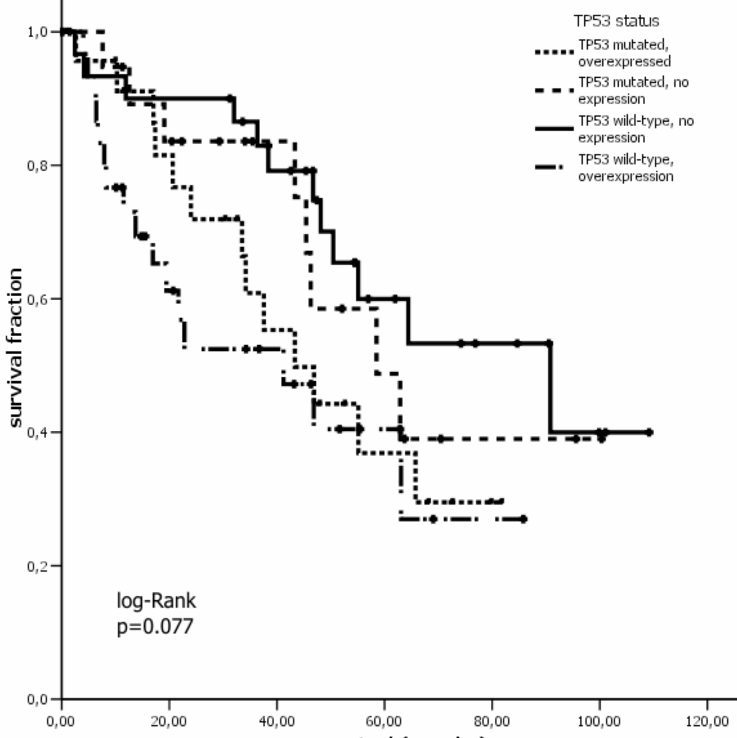

Abb. 1:
B Gesamtüberleben von Ovarialkarzinompatientinnen mit kombinierten TP53 Protein und Mutationsstatus (siehe Text für weitere Details).

bei Diagnosestellung durch die Expression des ER auch bei Ovarialkarzinompatientinnen beeinflusst wird. 43 % (46/107) der Tumoren waren ER-negativ, 17 % (18/107) zeigten eine geringe und 40 % (43/107) eine hohe ER-Expression (Daten nicht gezeigt). In FIGO III-Patientinnen ist die Existenz des SNP309 mit einem fast 6 Jahre früheren Auftreten des Tumors für Patientinnen mit nachweisbarer ER-Expression verbunden (T/T: 70,6 Jahre; T/G+G/G: 64,4 Jahre), obwohl der Unterschied nicht statistisch signifikant ist (p=0,101). Für Patientinnen mit stark erhöhter ER-Expression beträgt der Unterschied sogar 8,5 Jahre (p = 0,048). In ER-negativen Patientinnen fand sich kein Unterschied hinsichtlich des Alters bei Diagnosestellung (p = 0,44). Diese Ergebnisse unterstützen die Hypothese, dass das G-Allel des SNP309 einen intakten Östrogen-Signalweg benötigt, um die Tumorentstehung zu beschleunigen.

TP53, SNP309 und Gesamtüberleben. Bei Kombination des TP53 Mutations- und Proteinexpressionsstatus haben Patientinnen mit einer Überexpression des Wildtyp-Proteins das kürzeste Gesamtüberleben (42,8 Monate) im Vergleich zu Patientinnen mit Expression des mutierten Gens (48,2 Monate) und Patientinnen ohne nachweisbare TP53-Expression (TP53 Wildtyp: 72,4 Monate, TP53 mutiert: 64,4 Monate). Allerdings ist der Unterschied zwischen allen Gruppen nur geringfügig signifikant (p = 0,077, log-Rank-Test, Abb. 1 B). Die Überlebenszeiten erreichten jedoch statistische Signifikanz bei Vergleich der TP53-Expression bei Patientinnen mit Wildtyp TP53-Gen (p = 0,019, log-Rank-Test). Zusammenfassend lässt sich sagen, dass Patientinnen mit normalem TP53 (Wildtyp-Gen und kein nachweisbares Protein) die längste Überlebenszeit hatten im Vergleich zu Patientinnen, deren Tumore eine Überexpression von Wildtyp oder mutiertem TP53 zeigten.

Patientinnen mit TP53-Überexpression hatten im Vergleich zu Patientinnen mit keiner oder schwacher Expression eine signifikant verkürzte Gesamtüberlebenszeit (45,8 vs. 71,7 Monate; p = 0,016; log-Rank-Test; Abb. 1 A). Wir fanden jedoch keinen Unterschied im Gesamtüberleben für Patientinnen mit Wildtyp oder mutiertem TP53-Gen (p=0,86; log-Rank-Test). Es ist jedoch überraschend, dass Patientinnen mit Tumoren im FIGO III-Stadium und Wildtyp-P53-Gen ein verkürztes Gesamtüberleben gegenüber Patientinnen mit mutiertem TP53-Gen hatten. Dieser Unterschied erreicht nahezu statistische Signifikanz (41 vs. 59 Monate; p = 0,058). Wir machten ähnliche Beobachtungen bei der Untersuchung der Bedeutung des SNP309 auf das Gesamtüberleben. Die durchschnittliche Überlebenszeit für Frauen mit T/T Status betrug 59 Monate, für Patientinnen mit G-Allel jedoch 62 Monate (p = 0,994). Im FIGO III-Stadium ist das G-Allel mit einem signifikant verlängertem Gesamtüberleben verbunden (57,5 Monate) im Vergleich zu Frauen mit dem T/T Genotyp (35,3 Monate; p = 0,045; log-Rank-Test). Bei weiterer Unterteilung der FIGO III-Patientinnen entsprechend des ER-Status (Abb. 2 A, B) konnten wir zeigen, dass Patientinnen mit ER-negativen Tumoren und mit T/G- bzw. G/G-Status eine signifikant verlängerte Überlebenszeit (p = 0,024, log-Rank-Test) hatten im Vergleich zu Frauen mit T/T-Genotyp des SNP309 (69 vs. 34 Monate). Im Gegensatz dazu beeinflusst das G-Allel nicht das Gesamtüberleben von FIGO III-Patientinnen mit ER-positiven Tumoren (geringe Expression: p = 0,424; starke Expression: p = 0,828). Aus diesen Ergebnissen kann man ableiten, dass ein geschwächter TP53-Signalweg, sei es durch mutiertes TP53 oder dem G-Allel des SNP309, mit einer besseren Prognose für Ovarialkarzinompatientinnen verbunden ist.

In der univariaten Cox-Regression konnten wir zeigen, dass die Überexpression von Wildtyp-TP53 (p = 0,016) und ein immunhistochemischer Proteinnachweis (p=0,018) prognostische Faktoren sind, während die Existenz von TP53-Mutationen allein das Risiko, am Tumor zu versterben, nicht erhöht (p = 0,864). Andere prognostische Faktoren waren eine undifferenzierte Histologie (p = 0,032), das Auftreten von Aszites (p = 0,01), die Resttumorgröße (p < 0,001), das FIGO-Stadium (p < 0,001) sowie das Fortschreiten der Erkrankung (relatives Risiko 22,3; p = 0,002). In der multivariaten Cox-Regression waren jedoch nur das FIGO-Stadium (p = 0,008) und die Resttumorgröße (p = 0,008) unabhängige prognostische Marker für das Gesamtüberleben. Interessanterweise

Abbildung 2 A

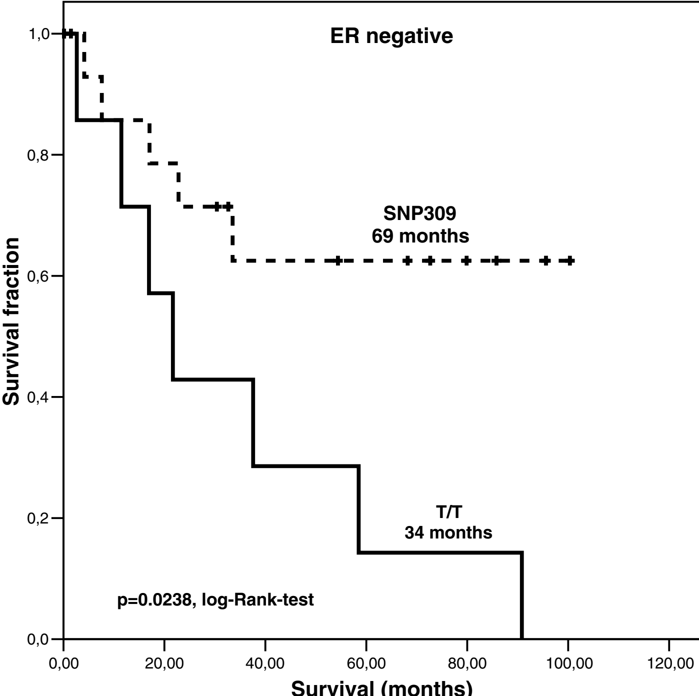

Abbildung 2 B

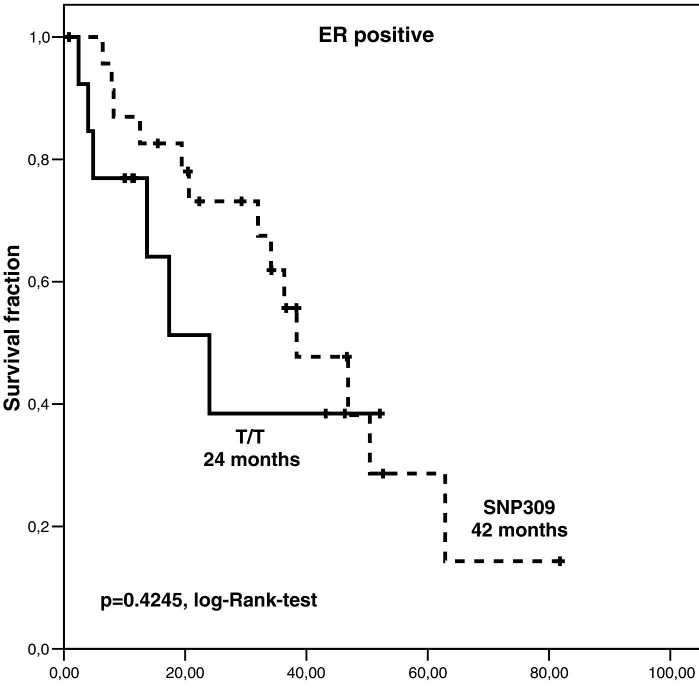

Abb. 2:
A, B Gesamtüberleben von Ovarialkarzinompatientinnen mit unterschiedlichem SNP309-Genotyp und Östrogenrezeptor-Expression.

korrelierte das Pro/Pro-Allel des Arg72Pro SNP in der multivariaten Cox-Regression (adjustiert nach FIGO Stadium und Resttumor) mit einem 6,4-fach erhöhten Risiko, am Tumor zu versterben.

Diskussion

In unserer Studie untersuchten wir das TP53-Gen, dessen Proteinexpression sowie den Status des im TP53-sensitiven HDM2-P2-Promotor liegenden SNP309 in einer Gruppe von 107 Ovarialkarzinomen. In 39 % (42 von 107) der Tumore fanden wir TP53-Mutationen, die eine Veränderung der Aminosäuresequenz bewirken. Dieser Prozentsatz ist nahe dem Bereich von 40–80 %, welcher in der Literatur für Ovarialkarzinome beschrieben wird (10). Eine positive TP53-Immunfärbung von 51 % liegt in dem publizierten Bereich von 29–62 % für FIGO I-IV Patientinnen (8, 11, 12, 14, 16, 23, 31, 34).

Unsere Ergebnisse zeigen deutlich, dass die Immunhistochemie kein sicherer Marker für den TP53-Genstatus ist. Wir fanden, dass 49,2% der Fälle mit einem Wildtyp TP53-Gen auch positiv für die TP53-Immunfärbung waren, ein Prozentsatz der über dem liegt, was bisher von anderen Arbeitsgruppen veröffentlicht wurde (13,30). Havrilesky *et al.* fanden in 28 % (13) und Reles *et al.* (30) in 38 % der von ihnen untersuchten Tumore eine Überexpression des TP53-Proteins bei Vorhandenseins eines Wildtyp-Gens. Wildtyp-TP53 ist normalerweise sehr instabil und wird in der Zelle nur in geringem Maße expremiert, da TP53 ein Schlüsselregulator für Zellwachstum und Apoptose ist (24). Unter diesem Gesichtspunkt ist es überraschend, dass es sich bei 90 % der Wildtyp-TP53 überexpremierenden Tumore um high-grade Karzinome handelt. Die Ursache für diese unnormale Stabilität ist bisher unbekannt. Es ist möglich, dass es Veränderungen in der Funktionalität von TP53 interagierenden Proteinen gibt, wie HDM2 (36) oder HDMX (36). Weitere Studien sind nötig, um die Gründe für diesen Befund zu klären, besonders unter dem Aspekt, dass in unserer Studie 82 % dieser Tumoren ein Rezidiv bekamen. Wohingegen nur 52 % der Patientinnen mit normalem TP53 (Wildtyp-Gen, keine Expression) ein Rezidiv bekamen.

In der Literatur gibt es Hinweise, dass eher die Überexpression von TP53 (31,34) als Mutationen (9,21) mit einem verkürzten Gesamtüberleben korrelieren. Unsere Ergebnisse zeigen, dass eine Immunfärbung von >10 % der Tumorzellen mit einem verringertem Gesamtüberleben korreliert (p = 0,0065) und, dass das Auftreten von TP53-Mutationen (p = 0,86) allein keinen Einfluss auf das Überleben hat. Wir konnten auch zeigen, dass Patientinnen mit TP53-Veränderungen generell eine schlechtere Prognose (p = 0,047) und eine verkürzte Gesamtüberlebenszeit (p = 0,05) hatten, im Vergleich zu Patientinnen mit normalem TP53. Bei den Patientinnen mit verändertem TP53 haben überraschender Weise die Frauen mit Wildtyp-TP53 Überexpression die kürzeste Überlebenszeit. Da bisher nur wenige Studien (7, 13, 30) sowohl den TP53-Genstatus als auch die Proteinexpression untersucht haben, wurde der prognostische Wert einer Überexpression von Wildtyp-TP53 bisher unterschätzt.

Zusätzlich zum TP53-Status haben wir auch den SNP309 im TP53-sensitiven P2-Promotor des HDM2-Gens untersucht. Wie wir vor kurzem zeigen konnten, ist das G-Allel des SNP309 mit einem jüngeren Erkrankungsalter für Frauen, aber nicht für Männer, mit B-Zell-Lymphomen und Weichteilsarkomen verbunden (3). In dieser Studie konnte nachgewiesen werden, dass das G-Allel des SNP309 nur bei Tumoren mit einer sehr starken ER-Expression mit einem früheren Diagnosealter (8 Jahre früher; p = 0,048) verbunden ist, aber nicht für ER negative Ovarialkarzinome (1 Jahr früher; p = 0,77). Wenn das Niveau der ER-Expression in Ovarialkarzinomen nicht berücksichtigt wird, findet sich bei den verschiedenen SNP309-Genotypen kein Unterschied im Erkrankungsalter, wie es bereits vor kurzem publiziert wurde (45).

Die beste Prognose konnten wir bei Patientinnen mit normalem TP53-Status finden im Gegensatz zu Patientinnen mit TP53-Überexpression unabhängig von der Existenz von Mutationen. Wichtig ist, dass es eine große Gruppe von Patientinnen gibt, deren Tumoren Wildtyp-TP53 überexpremieren. Bei diesen Frauen kommt es bei einer hohen Prozentzahl zu einer frühen Rezidivbildung und sie zeigten,

die kürzeste Gesamtüberlebenszeit. Weitere Studien sind notwendig um die Mechanismen der Wildtyp-TP53-Überexpression zu klären, weil dies Einblicke in die Mechanismen der Resistenzentwicklung verspricht. Zusätzlich unterstreicht diese Studie die Bedeutung der TP53-Funktionalität. Dies erscheint wichtiger als die alleinige Untersuchung von TP53-Mutation oder Proteinexpression. Nur so wird es möglich sein, das Ansprechen auf eine Platinhaltige Chemotherapie und die Prognose von Patientinnen verlässlich vorherzusagen.

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Wir danken Gareth L. Bond vom Institute of Advanced Studies, Princeton, NJ, für seine kritische Durchsicht des Manuskripts sowie die vielen hilfreichen Diskussionen. Des Weiteren möchten wir uns für die Unterstützung bei der Durchführung dieser Arbeit bei den Mitarbeitern unserer Arbeitsgruppe bedanken: Birgit Wypior, Ilona Wiederhold, Ute Rolle, Jana Beer, Tina Große und Kathrin Spröte.

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Both Germ Line and Somatic Genetics of the p53 Pathway Affect Ovarian Cancer Incidence and Survival

Frank Bartel,[1] Juliane Jung,[1] Anja Böhnke,[1] Elise Gradhand,[2] Katharina Zeng,[2] Christoph Thomssen,[3] and Steffen Hauptmann[2]

Abstract **Purpose:** Although p53 is one of the most studied genes/proteins in ovarian carcinomas, the predictive value of p53 alterations is still ambiguous.

Experimental Design: We performed analyses of the *TP53* mutational status and its protein expression using immunohistochemistry. Moreover, the single nucleotide polymorphism SNP309 in the P2 promoter of the *MDM2* gene was investigated. We correlated the results with age of onset and outcome from 107 patients with ovarian carcinoma.

Results: In our study, we identified a large group of patients with p53 overexpression despite having a wild-type gene (49% of all patients with wild-type TP53). This was associated with a significantly shortened overall survival time ($P = 0.019$). Patients with p53 alterations (especially those with overexpression of wild-type TP53) were also more refractory to chemotherapy compared with patients with normal p53 ($P = 0.027$). The *G*-allele of SNP309 is associated with an earlier age of onset in patients with estrogen receptor – overexpressing FIGO stage III disease ($P = 0.048$). In contrast, in patients with FIGO stage III disease, a weakened p53 pathway (either the *G*-allele of SNP309 or a *TP53* mutation) was correlated with increased overall survival compared with patients whose tumors were wild-type for both *TP53* and SNP309 ($P = 0.0035$).

Conclusion: Our study provides evidence that both germ line and somatic alterations of the p53 pathway influence the incidence and survival of ovarian carcinoma, and it underscores the importance of assessing the functionality of p53 in order to predict the sensitivity of platinum-based chemotherapies and patient outcome.

Ovarian cancer is the leading cause of death among patients with gynecological cancer in the Western world (1). Most women with ovarian cancer present with advanced stages of disease at the time of diagnosis (2). The 5-year overall survival rates are only 15% to 25% for advanced stages (International Federation of Gynecology and Obstetrics, FIGO stages III and IV) of ovarian cancer (1, 3). Despite many efforts to establish molecular alterations as prognostic markers, residual disease and ascites are still the only significant variables in multivariate analyses in patients with advanced disease. The standard treatment for patients with advanced ovarian carcinoma is tumor debulking, followed by chemotherapy with carboplatin and taxol. Unfortunately, the disease will recur in nearly all patients due to acquired chemoresistance. Therefore, factors modulating chemoresistance are of outmost importance in the treatment of patients with ovarian carcinomas. An important factor for a cellular response to platinum-based chemotherapeutic agents is p53 whose functionality is attenuated by both germ line and somatic genetic events of TP53 itself or of regulators located upstream and downstream in the p53 tumor suppressor pathway. Therefore, resistance to these drugs may be due to the lack of functional p53.

In ovarian carcinomas, mutation frequencies, determined by direct sequencing, range from 40% to 80% (4 – 7). There seems to be a trend in which overexpression of TP53 (8 – 12) rather than mutation of *TP53* (4, 13 – 15), is correlated with shortened overall survival. In other studies, no significant association between p53 overexpression and a poorer outcome for patients with ovarian cancer was found (15 – 19). A problem related to studies separately analyzing *TP53* mutations and p53 protein is that immunohistochemistry will miss cases with truncating mutations, such as nonsense mutations and deletions/insertions, and on the other hand, overexpression of TP53 is not necessarily associated with mutations. Therefore, the status of p53 alterations (gene mutations and/or overexpression) will be more informative than the mutational and protein expression status alone. Although a few studies have evaluated both the *TP53* gene and protein expression status (i.e., refs. 5, 6), in

Authors' Affiliations: [1]Junior Research Group and [2]Institute of Pathology, Faculty of Medicine, University of Halle-Wittenberg, Halle/Saale, Germany, and [3]Department of Gynecology, Martin-Luther-University Halle-Wittenberg, Halle, Germany

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Note: F. Bartel and J. Jung contributed equally to the results of this study.

Requests for reprints: Frank Bartel, Institute of Pathology, Faculty of Medicine, University of Halle-Wittenberg, Magdeburger Str. 14, D-06097 Halle/Saale, Germany. Phone: 49-345-557-4272; Fax: 49-345-557-1295; E-mail: frank.bartel@medizin.uni-halle.de.

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none of them have the authors correlated the combined status of p53 alterations with survival or response to chemotherapy.

The MDM2 oncogene is the key negative regulator of the p53 protein (20). It has been shown that the *G*-allele of a single nucleotide polymorphism (SNP309) in the p53-sensitive P2 promoter of the *MDM2* gene is associated with the attenuation of the p53 tumor suppressor pathway (21). Numerous reports provide evidence that the *G*-allele of SNP309 is correlated with an earlier age of onset and an increased risk of tumorigenesis (22 – 25). MDM2 expression is also regulated by estrogen receptor (ER) signaling with transcription of the MDM2-P2 transcript induced in ERα-positive cell lines (26). Interestingly, the ERα-binding site is located within the region that contains SNP309. In a previous report, we showed that SNP309 alters the effect of hormones, such as estrogen, on tumorigenesis, and contributes to the gender differences observed in many cancers (27).

The aim of this study was therefore to investigate the status of SNP309, *TP53* sequence alterations, and p53 protein expression in a group of 107 ovarian cancer patients with complete clinical data collected at the Institute of Pathology, University of Halle (Germany) and to test whether both germ line and somatic p53 alterations were associated with tumor characteristics that could be used as reproducible markers for clinical outcome.

Materials and Methods

Patient population and clinical data. Paraffin-embedded tissue samples from 107 invasive ovarian carcinomas which were diagnosed at the Institute of Pathology, Martin-Luther-University Halle-Wittenberg between 1997 and 2005 were selected based on the availability of tissue. The study was approved by the local ethical committee. All histologic slides were re-evaluated by two pathologists (E. Gradhand and S. Hauptmann) using a multihead microscope. Tumor patients and tissue samples were, in part, described elsewhere (28). Histology was classified according to the WHO, and grading was assessed according to Silverberg (29). Data retrieved from clinical files included the patient's age, amount of residual tumor, FIGO stage, adjuvant chemotherapy, and follow-up (Table 1).

Immunohistochemistry. The analysis of the p53 protein expression was carried out using the DO-7 mouse monoclonal antibody (DAKO), and analysis of the ER used the anti-ER antibody SP1 (Labvision, Germany). The immunoreactivity for p53 and ER was scored as the percentage of stained cells by counting 150 tumor cells on average. Tumors with >10% stained cells were considered to be either p53 or ER-positive.

***Single-strand conformation polymorphism analysis for* TP53.** DNA from paraffin-embedded tissue sections were isolated according to standard procedures (30). One section of each tumor sample was stained with H&E in order to confirm that the majority of the tissue was comprised of tumor cells.

We used PCR to amplify exons 3 to 9 of the *TP53* gene. The primers for each exon were located in intronic sequences; therefore, flanking splice sites could be analyzed. The sequences of the primers and the condition for the PCR amplification have been published previously (6). Ten to 20 μL of each PCR product was precipitated overnight at -20°C and the conformational changes of the PCR products were subsequently analyzed by single-strand conformational polymorphism (SSCP) on a denaturing polyacrylamide gel. DNA from healthy volunteers served as wild-type controls.

***Sequence analysis for* TP53.** All samples identified as having conformational changes, and in addition, all samples from exons 5 to 8, were analyzed by direct sequencing in both sense and antisense directions using the BigDye Terminator Cycle Sequencing 3.1 Kit (Applied Biosystems). The sequencing reactions were carried out according to the manufacturer's instructions.

Determination of SNP309 status. The SNP309 status of 103 ovarian carcinoma samples was determined by PCR and subsequent direct sequencing of the P2-promoter region of the *MDM2* gene as described elsewhere (21).

Statistical evaluation. All statistics, including Cox's proportional regression hazard model and the Kaplan-Meier survival estimates, were carried out using the SPSS 12.0G software (SPSS Science). $P < 0.05$ was considered to be significant.

Results

TP53 *mutations and p53 protein expression.* In 107 patient samples, we detected a total of 111 sequence alterations in exons and introns of the *TP53* gene (Table 2; Fig. 1), of which 44 were mutations in exons 4 to 8, including two mutations in introns. Twenty-two of the sequence alterations were a 16-bp insertion in intron 3, and 45 sequence alterations were found in codon 72, which are known as polymorphisms.

Of the mutations that affected the amino acid sequence of p53, 93% (39 of 42) were found in exons 5 to 8 (Table 2). Sixty-three percent (27 of 42) of the mutations were missense mutations, 9.3% (4 of 42) were nonsense mutations in which a single nucleotide exchange resulted in a premature stop codon. Furthermore, we detected small frameshifting mutations of which seven were deletions and three were insertions of 1 or 2 bp, respectively. In addition, there was one large deletion of 32 bp in exon 8 (Table 2). The most frequently affected were

Table 1. Summary of clinicopathologic data of patients with ovarian cancer

Characteristics	Patients (107) No. (%)
Tumor cell type	
Serous	62 (57.9)
Endometrioid	14 (13.1)
Mixed	11 (10.3)
Clear cell	9 (8.4)
TCC	1 (0.9)
UC	8 (7.5)
MC	2 (1.9)
FIGO tumor stage	
I	30 (28.0)
II	9 (8.4)
III	63 (58.8)
IV	5 (4.7)
Patient age (y)	
Mean	63.5
Median	64.0
SD	11.5
Type of therapy	
Cisplatin + taxol	62 (57.9)
Platinum-based chemotherapy without taxol	21 (29.7)
Other	2 (1.9)
None (FIGO Ia)	5 (4.7)
Refused/dead	13 (12.1)
Missing	4 (3.7)
Residual tumor	
None	39 (41.0)
<1 cm	22 (23.1)
>1 cm	34 (35.8)

Abbreviations: TCC, transitional cell carcinoma of the ovary; UC, undifferentiated carcinoma of the ovary; MC, mucinous carcinoma of the ovary.

Table 2. *TP53* mutations in ovarian cancer

Case no.	Histology	Exon	Codon	Conserved region	Mutation type	Change*	Wild-type	Mutated	Wild-type AA	Mutated AA
9	Mixed	4	91	NC	nonsense	G>A	TGG	TAG	Trp	STOP
37	ser	4	91	NC	nonsense	G>A	TGG	TAG	Trp	STOP
64	ser	4	98	NC	Missense	C>T	CCT	CCT	Pro	Leu
84	ser	5	138	Co	Deletion	-GC (*)				
115	mixed	5	141	Co	Missense	G>A	TGC	TAC	Cys	Tyr
116	ser	5	141	Co	Missense	G>A	TGC	TAC	Cys	Tyr
8	endo	5	149	NC	Insertion	+T	TCC	TTC	Ser	Phe
109	ser	5	150	NC	Missense	C>T	ACA	ATA	Thr	Ile
99	ser	5	152	NC	Deletion	C	CCG		STOP	
68	ser	5	153	NC	Insertion	+T				
34	un	5	175	Co	Missense	G>A	CGC	CAC	Arg	His
39	ser	5	175	Co	Missense	G>A	CGC	CAC	Arg	His
98	ser	5	175	Co	Missense	G>A	CGC	CAC	Arg	His
67	ser	5	178	Co	Missense	C>T	CAC	TAC	His	Tyr
111	ser	5	179	Co	Missense	A>G	CAT	CGT	His	Arg
31	un	Int5								
3	ser	6	195	NC	Missense	T>C	ATC	ACC	Ile	Thr
24	TCC	6	195	NC	Missense	T>C	ATC	ACC	Ile	Thr
20	EC	6	200	NC	Deletion	T				
61	ser	6	201	NC	nonsense	T>A	TTG	TAG	Leu	STOP
91	ser	6	206	NC	Deletion	TT (*)				
60	un	6	216	NC	Missense	G>A	GTG	ATG	Val	Met
32	ser	6	220	NC	Missense	A>G	TAT	TGT	Tyr	Cys
15	ser	7	225	NC	Missense	G>T	GTT	TTT	Val	Phe
106	endo	7	229	NC	Insertion	+T (*)	GAC	TGA	Asp	STOP
79	ser	7	239	Co	Missense	A>G	AAC	AGC	Asn	Ser
45	ser	7	245	Co	Missense	G>A	GGC	AGC	Gly	Ser
47	un	7	245	Co	Missense	G>T	GGC	TGC	Gly	Cys
59	ser	7	245	Co	Missense	G>A	GGC	GAC	Gly	Asp
4	ser	7	248	Co	Missense	G>A	CGG	CAG	Arg	Glu
69	ser	7	248	Co	Missense	G>A	CGG	CAG	Arg	Gln
40	clear	7	257	Co	Missense	T>A	CTG	CAG	Leu	Gln
33	clear	7	259	NC	Missense	G>T	GAC	TAC	Asp	Tyr
118	ser	Int7	Splice							
81	ser	8	267	NC	Deletion	-G				
76	mixed	8	272	Co	Missense	G>A	GTG	ATG	Val	Met
42	mixed	8	273	Co	Missense	G>A	CGT	CAT	Arg	His
41	ser	8	274	Co	Missense	G>T	GTT	TTT	Val	Phe
27	ser	8	275	Co	Missense	G>T	TGT	TTT	Cys	Phe
80	mixed	8	280	Co	Missense	G>C	AGA	ACA	Arg	Thr
78	clear	8	285	Co	Deletion	-32 (*)				
7	mixed	8	291	NC	Deletion	-G				
43	ser	8	294	NC	nonsense	G>T	GAG	TAG	Glu	STOP
86	ser	8	306	NC	Deletion	-C	CGA			

Abbreviations: Ser, serous ovarian carcinoma; Endo, endometroid ovarian carcinoma; TCC, transitional cell carcinoma of the ovary; UC, undifferentiated carcinoma of the ovary; MC, mucinous carcinoma of the ovary; NC, non-conserved region of the *TP53* gene; Co, highly conserved region of the *TP53* gene.
*Mutations which have not been described as of October 2006 according to Petitjean et al. (31).

codons 175 and 245, with three cases each. To our knowledge, 12 of the 42 exonic mutations have been described here for the first time to occur in ovarian cancer, including 4 that have not been published thus far (31).

We found that 51.4% (55 of 107) cases showed overexpression of p53. Twenty cases (18.7%) showed immunostaining of 10% or less of the cells and 32 cases (30.2%) were negative. Benign ovarian control tissue was negative for TP53.

Relationship of p53 protein expression and* TP53 *gene status. In our study, overexpression of p53, as detected by immunohistochemistry, was not correlated with the *TP53* mutational status (P = 0.59; χ^2 test). Fifty-four percent (24 of 44) of cases with a mutated *TP53* gene also showed overexpression of p53 protein; however, the percentage of cases with a wild-type *TP53* gene that overexpress p53 was 49% (31 of 63).

We divided the cases according to their combined p53 mutational/protein expression status into four groups. Cases with a wild-type *TP53* gene and undetectable p53 protein were designated as "p53 normal" and considered to have functional p53 (32 of 107 cases, 29.9%); other groups included cases with an alteration of p53, i.e. (*a*) cases that overexpressed wild-type p53 (31 of 107, 29%), (*b*) cases with *TP53* mutations and overexpression of the protein (24 of 107, 22.4%), and (*c*) cases with *TP53* mutations but undetectable p53 protein (20 of 107, 18.7%).

TP53 mutations and p53 overexpression were more frequent in advanced stage ovarian carcinomas; however, only the latter

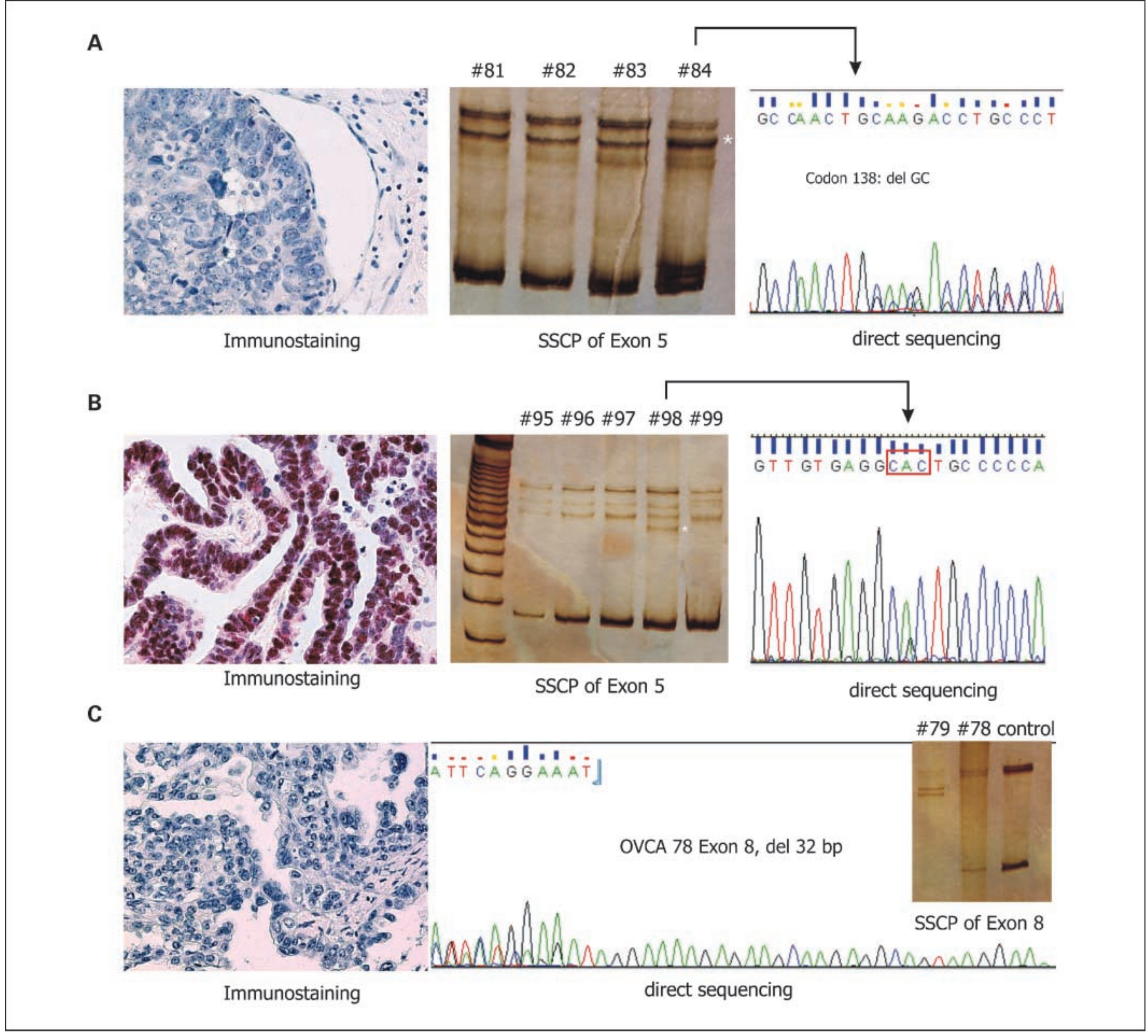

Fig. 1. SSCP, DNA sequencing, and immunohistochemical detection of p53 expression in ovarian cancer. *A,* the SSCP analysis of exon 5 from case no. 84 (serous ovarian carcinoma) clearly shows the appearance of an additional band (*). The subsequent DNA sequencing confirmed a 2-bp deletion (GC) in codon 138 that results in a premature stop codon. P53 was not detectable by immunohistochemistry in case no. 84. *B,* SSCP and DNA sequencing of exon 5 from case no. 98 (serous ovarian carcinoma) revealed a missense mutation in codon 175 (CGC to CAC) resulting in an Arg to His substitution. P53 immunostaining showed overexpression of the TP53 protein. *C,* SSCP, DNA sequencing, and immunohistochemical detection of TP53 expression in case no. 78 (clear cell ovarian carcinoma) which is characterized by a 32-bp deletion in exon 8 of the *TP53* gene and the absence of p53 immunostaining.

correlation was significant ($P = 0.096$ and $P = 0.01$, respectively; Table 3).

P53 status and response to chemotherapy. We found that patients whose tumors have p53 alterations are significantly more resistant (78%) than patients whose tumors harbor normal p53 (52%; $P = 0.027$). This was also true for protein expression status when comparing tumors either with overexpression of p53 (83%; $P = 0.02$) or no p53 (58%). Patients with *TP53* mutations were also more resistant to chemotherapy (79%) compared with patients with wild-type *TP53* (64%). However, this association was not significant ($P = 0.148$). Patients with altered TP53 (mutation and/or overexpression)

Table 3. Association of FIGO stage and p53 status in ovarian cancer

FIGO stage	*TP53* mutations		p53 immunostaining	
	Wild-type	Mutated	Negative	Positive
I	22	8 (26%)	22	8 (27%)
II	7	2 (22%)	5	4 (44%)
III	32	31 (49.2%)	23	40 (63%)
IV	2	3 (60%)	2	3 (66%)
P	0.096		0.01	
Summary	63	44 (41.1%)	53	55 (51.4%)

had a shorter time before relapsing than patients with normal p53 (28 versus 51 months; P = 0.075).

Status of the SNP309 in the* MDM2 *P2 promoter. SNP309 was analyzed in 103 of 107 patients with ovarian carcinoma. We found SNP309 with a relatively high frequency in the heterozygous T/G state (52.4%) and at a lower percentage in the homozygous G/G state (7.8%) compared with healthy volunteers (T/G, 40%; G/G, 12%; ref. 21), although the difference was not significant (P = 0.53). We have previously shown that the *G*-allele of SNP309 predominantly acts in women with an active estrogen signaling pathway (27). Therefore, we analyzed whether the association of SNP309 and the age of onset were affected by the expression of ER in patients with ovarian carcinoma. We found that 43% (46 of 107) of the tumors were ER-negative, 17% (18 of 107) showed low ER expression, and 40% (43 of 107) showed high ER expression (data not shown). In patients with FIGO stage III disease, the occurrence of SNP309 was associated with an onset almost 6 years earlier for patients with detectable expression of the ER (T/T, 70.6 years; T/G + G/G, 64.4 years), although the difference did not reach statistical significance (P = 0.101) and was 8.5 years earlier in patients with strongly elevated ER expression (P = 0.048). In ER-negative patients, there was no difference regarding the age of onset (P = 0.44). These results support the hypothesis that the *G*-allele of SNP309 requires an intact estrogen signaling pathway to accelerate tumorigenesis (27).

P53, SNP309 status, and overall survival. When combining the p53 mutational and protein expression status, patients with

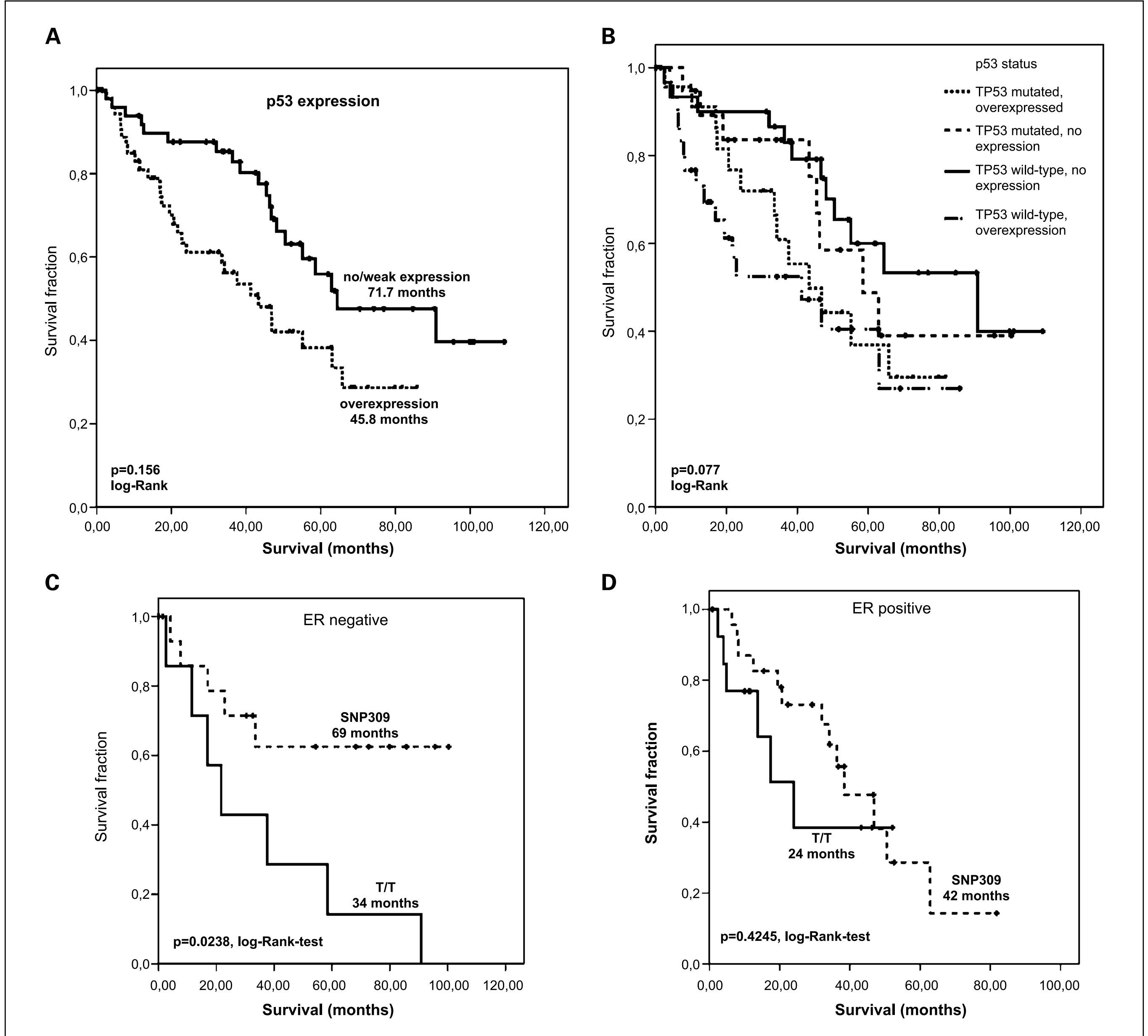

Fig. 2. *A,* overall survival of ovarian cancer patients with different p53 expression status. *B,* overall survival of ovarian cancer patients with combined p53 protein and mutational status (refer to text for further details). *C* and *D,* overall survival of ovarian cancer patients with different SNP309 genotypes and ER expression levels.

Table 4. Prognostic significance of molecular and clinical factors identified by multivariate Cox regression analysis

Variables	Univariate analysis		Multivariate analysis*	
	Unadjusted RR (95% CI)	P	Adjusted RR (95% CI)	P
p53 immunostaining				
Negative	1.00		1.00	
Positive	2.021 (1.13-3.616)	0.018	1.331 (0.703-2.522)	0.38
TP53 mutations				
Wild-type	1.00		1.00	
Mutated	1.051 (0.596-1.851)	0.864	0.648 (0.333-1.26)	0.201
p53 immunohistochemistry + mutation				
Normal	1.00		1.00	
Wild-type, overexpression	2.548 (1.188-5.465)	0.016	1.598 (0.662-3.856)	0.297
Mutated, no expression	1.922 (0.87-4.246)		0.884 (0.336-2.325)	
Mutated, overexpression	1.284 (0.524-3.145)		0.82 (0.299-2.246)	
p53 altered				
Normal	1.00		1.00	
Altered	1.901 (0.985-3.667)	0.045	1.107 (0.498-2.461)	0.804
Arg72Pro SNP				
Arg/Arg	1.00		1.00	
Arg/Pro	0.747 (0.386-1.446)		1.693 (0.769-3.728)	
Pro/Pro	1.371 (0.588-3.200)	0.412	6.452 (1.072-6.452)	0.035
Histology				
Serous	1.00		1.00	
Nonserous	1.938 (1.011-3.717)		1.449 (0.672-3.124)	
Undifferentiated	3.679 (1.284-10.54)	0.032	1.590 (0.419-6.033)	0.496
Ascites				
No	1.00		1.00	
Yes	3.015 (1.27-7.161)	0.01	1.681 (0.695-1.681)	0.249
Relapse				
No	1.00		1.00	
Yes	22.34 (3.072-162.54)	0.002	11.769 (1.374-100.77)	0.024
Residual disease				
None	1.00		1.00	
Any	6.556 (2.976-14.444)	<0.001	5.255 (1.648-29.588)	0.008
FIGO stage				
I	1.00		1.00	
II	1.462 (0.449-4.764)		1.403 (0.378-5.210)	
III	3.175 (1.505-6.697)		1.542 (0.523-4.541)	
IV	9.922 (2.932-33.577)	<0.001	6.983 (1.648-29.588)	0.008

Abbreviations: RR, relative risk; 95% CI, 95% confidence interval.
*Adjusted to FIGO stage and residual disease.

an up-regulation of the wild-type *TP53* gene had the shortest overall survival time (42.8 months), compared with patients with an overexpression of a mutated *TP53* gene (48.2 months) and with patients with no detectable p53 expression (*TP53* wild-type, 74.2 months; *TP53* mutated, 64.4 months); however, the difference in survival time between all groups was only marginally significant ($P = 0.077$, log-rank test; Fig. 2B). The difference in survival time reached statistical significance, however, when comparing p53 expression in patients with a wild-type *TP53* gene ($P = 0.019$, log-rank test). To summarize, patients with normal p53 (wild-type *TP53* gene and no detectable protein expression) had a longer survival time compared with patients whose tumors showed an overexpression of wild-type or mutated *TP53*.

Patients with p53 overexpression, compared with patients with no or weak expression, showed a significantly decreased overall survival time (45.8 versus 71.7 months; $P = 0.016$; log-rank test; Fig. 2A), but there was no difference in the overall survival for patients with wild-type or mutated *TP53* genes ($P = 0.86$; log-rank test). It is noteworthy that patients with FIGO III carcinomas and a wild-type *TP53* gene had a shorter overall survival than patients with a mutated *TP53* gene; this difference nearly reached statistical significance (41 versus 59 months; $P = 0.058$). We made a similar observation when we analyzed the effect of SNP309 on overall survival. The average survival time for women with the T/T genotype was 59 months; for women with the *G*-allele, this was 62 months ($P = 0.994$). In patients with FIGO stage III disease, the *G*-allele was significantly associated with prolonged overall survival (57.5 months) compared with patients with a T/T genotype (35.3 months; $P = 0.045$; log-rank test). When patients with FIGO stage III disease were further divided into groups according to their ER expression status (Fig. 2C and D), we found that ER-negative patients with a T/G or G/G genotype had a significantly increased survival time ($P = 0.024$, log-rank test) compared with patients with a T/T genotype of SNP309 (69 versus 34 months, respectively). In contrast, the *G*-allele did not influence the overall survival for FIGO stage III patients with ER-positive tumors (low expression, $P = 0.424$; strong expression, $P = 0.828$). These observations resulted in a model in which a weakened p53 pathway,

either through a mutant *TP53* or the *G*-allele of SNP309, was associated with a better outcome for patients with ovarian cancer.

In a univariate Cox's regression model (Table 4), we could show that overexpression of wild-type p53 (P = 0.016) and positive p53 immunostaining (P = 0.018) were prognostic factors, whereas the occurrence of *TP53* mutations alone did not increase the risk of tumor-related death (P = 0.864). Other factors with prognostic effects were an undifferentiated histology (P = 0.032), the occurrence of ascites (P = 0.01), residual disease (P < 0.001), FIGO stage (P < 0.001), and the progression of disease (relative risk, 22.3; P = 0.002). In a multivariate Cox regression, however, only FIGO stage (P = 0.008) and residual disease (P = 0.008) were independent prognostic factors for overall survival. Interestingly, the Pro/Pro alleles of the Arg72Pro SNP were correlated with a 6.4-fold increased risk of tumor-related death (P = 0.032) in a multivariate Cox regression that was adjusted for FIGO stage and residual disease.

Discussion

In our study, we analyzed the *TP53* gene, its protein expression, and the status of SNP309 within the p53-sensitive MDM2-P2 promoter in a cohort of 107 ovarian carcinomas. In 39% of the ovarian carcinomas (42 of 107), we found *TP53* mutations that resulted in a change of the amino acid sequence. This percentage is near the range of 40% to 80% which was reported in the literature for ovarian cancer (reviewed in ref. 7). The TP53 immunopositivity of 51% in our study is near the mean value of ~49% within a range of 29% to 62% published for FIGO I to IV paraffin-embedded samples (8 – 12, 32 – 34).

Our results clearly show that immunohistochemistry is not a surrogate marker of the *TP53* gene status. We found that 49.2% of the cases with a wild-type *TP53* gene were also positive for the p53 immunostaining, a percentage that is higher than those previously reported by others (5, 6). Havrilesky et al. (5) and Reles et al. (6) found 28% and 38%, respectively, of tumors with a wild-type sequence and overexpression of the p53 protein. Wild-type p53 is usually very unstable and is maintained at low levels because it is a key regulator of cell growth (35). In this regard, it is surprising that 90% of the wild-type p53-overexpressing tumors were high-grade ovarian carcinomas. The reasons for this abnormal stability are thus far unknown. It is conceivable that there is either a change in the functionality of proteins that interact and control the activity and the levels of p53, such as MDM2 (36) and MDMX (37), or a constitutive phosphorylation of TP53 that prevents an interaction with these negative regulators. It has also been suggested that a yet unknown mediator between ubiquitinylated p53 and the proteasome might be down-regulated in these tumors (38). Wang et al. (39) and Kraus et al. (40) have reported that the stabilization of wild-type p53 correlates with the expression of MDM2 splice variants. To summarize, the reason for the accumulation of wild-type p53 and its biological significance are currently unknown. Further studies are necessary to clarify the cause of the abnormal stability of wild-type p53, because in our study, 82% of the tumors that recur belong to this group whereas only 52% of the tumors with normal p53 (wild-type gene, no expression) had a relapse.

P53 plays a key role in platinum-induced apoptosis. Therefore, one can assume that alterations in p53 might confer a platinum-resistant phenotype. The ability of a tumor cell to respond to a given drug, such as cisplatin, depends on the type of mutations and probably on the status of the Arg72Pro SNP. Vikhanskaya et al. (41) have analyzed several *TP53* hotspot mutants in conjunction with the Arg72Pro SNP and found that cells homozygous for the Arg allele are—albeit slightly—more resistant than cells homozygous for the Pro allele. In our study, there was no significant difference in the time to progression in patients with tumors homozygous for the Arg allele (P = 0.87; log-rank test); in contrast, in patients with Arg72Pro heterozygous tumors, the mean time to progression was 61 months for wild-type *TP53* compared with 19 months when *TP53* was mutated and/or overexpressed (P = 0.02). On the other hand, patients with FIGO stage III disease whose tumors exhibited *TP53* mutations had a longer overall survival than patients with wild-type TP53 (data not shown). This is at least partly consistent with data from Havrilesky et al. (5), who showed that patients with mutant *TP53* had a reduced short-term risk of disease progression. Many chemotherapeutics cause DNA damage and normal p53 may contribute to enhanced DNA repair rather than undergoing apoptosis; this might provide a favorable prognosis for patients with tumors exhibiting specific *TP53* mutations. Other authors also described a better response from tumors with mutated *TP53* to a cisplatin-containing treatment (42, 43). In contrast, Reles et al. (6) reported that *TP53* mutations correlated with early relapse in both early and advanced stage ovarian carcinomas.

There seems to be a trend in which overexpression of p53 (8, 9), rather than mutation of *TP53* (4, 15), is correlated with shortened overall survival. Our results, showing that p53 immunostaining in >10% of the tumor cells is correlated with a decreased overall survival (P = 0.0065) and that the occurrence of *TP53* mutations is not (P = 0.86), are therefore consistent with the findings of other authors. We further showed that patients with altered p53 generally have a worse prognosis (P = 0.047) and a shortened overall survival (P = 0.05) compared with patients with normal p53. Of patients with altered p53, those with an overexpression of wild-type p53 surprisingly have the shortest average overall survival time. Because only a few studies have evaluated both the *TP53* gene and protein expression status (i.e., refs. 5, 6, 44), the predictive value of overexpression of wild-type p53 has thus far been underestimated. Because p53 is stabilized and functionally inactivated by as yet unknown mechanisms, its effect on chemosensitivity and tumor progression may be more important than the loss of p53.

In addition to p53 status, we also analyzed SNP309, a single nucleotide polymorphism that resides within the p53-sensitive P2 promoter of the *MDM2* gene. We have previously shown that the *G*-allele of SNP309 is only associated with an earlier age of tumor onset in females, but not in males, i.e., in diffuse large B-cell lymphoma and soft tissue sarcomas (27). Data from patients with invasive ductal carcinomas of the breast provided further evidence that the *G*-allele requires an intact estrogen-signaling pathway in order to accelerate tumorigenesis (27). Indeed, in our present study, the *G*-allele of SNP309 only associates with an earlier age of onset in high ER-positive (8 years earlier, P = 0.048) but not ER-negative (1 year earlier, P = 0.77) ovarian carcinomas. If one ignores the level of ER expression in ovarian carcinomas, no differences in the age of onset were observed between the different genotypes of SNP309, as has been published recently (45). In another study, Galic and coworkers analyzed the SNP309 status in 150 patients with ovarian cancer (46). In their study, there was also no association of the *G*-allele with the age of onset. The

distribution of the respective genotypes was different from our study. They found that 12% were G/G, 40% were T/G, and 47% were T/T, compared with 7% G/G, 50% T/G, and 43% T/T, respectively, in our study. Furthermore, patients with or without ER expression were not analyzed separately. Our results provide further evidence for the association of the estrogen pathway and the G-allele of SNP309, as has been previously shown for breast cancer (27).

The best outcome was determined for patients with normal TP53 status as opposed to patients with overexpression of p53 regardless of the existence of mutations. Importantly, we identified a large group of patients whose tumors overexpressed p53 despite a wild-type gene status. These patients were characterized by the highest percentage of early relapse and the shortest overall survival. Further studies are mandatory to evaluate the mechanism of wild-type p53 overexpression to circumvent chemoresistance in this subset of ovarian cancer. In addition, our study underscores the importance of assessing the functionality of p53 rather than separately looking at *TP53* mutations and overexpression in order to predict sensitivity to platinum-based chemotherapies and patient outcome.

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Alternative and aberrant splicing of *MDM2* mRNA in human cancer

Frank Bartel,[1,4] Helge Taubert,[2] and Linda C. Harris[1,3]

[1]Department of Molecular Pharmacology, St. Jude Children's Research Hospital, Memphis, Tennessee 38105
[2]Institute of Pathology, Faculty of Medicine, University of Halle-Wittenberg, D-06097 Halle/Saale, Germany
[3]Correspondence: linda.harris@stjude.org
[4]Present address: Institute of Pathology, Faculty of Medicine, University of Halle-Wittenberg, Halle/Saale, Germany

MDM2 has been characterized as a protein that binds to and facilitates degradation of the tumor suppressor p53. Interestingly, more than 40 different splice variants of *MDM2* transcripts have been identified both in tumors and normal tissues, and the majority of these variants do not contain sequence encoding the p53 binding site. This review describes the different splice forms, the tissues in which they have been identified, and their association with tumor progression and prognosis. In addition, we discuss the potential functions of these variants and how they interact with full-length MDM2 protein.

Introduction

The first alternatively spliced *MDM2* transcripts in human tumors were identified in 1996 (Sigalas et al., 1996), but only during the past year have any potential functions been suggested. A problem that has developed is one of consistency. Even though most of the splice variant sequences are in GenBank, some investigators have chosen to give different names to previously published isoforms. This makes it very difficult to determine the frequency of occurrence of specific variants or to compare the variants expressed within different tumor histiotypes. This review briefly summarizes the functions of full-length MDM2 and then describes all the known *MDM2* splice variants and their potential role in human cancer.

Full-length MDM2

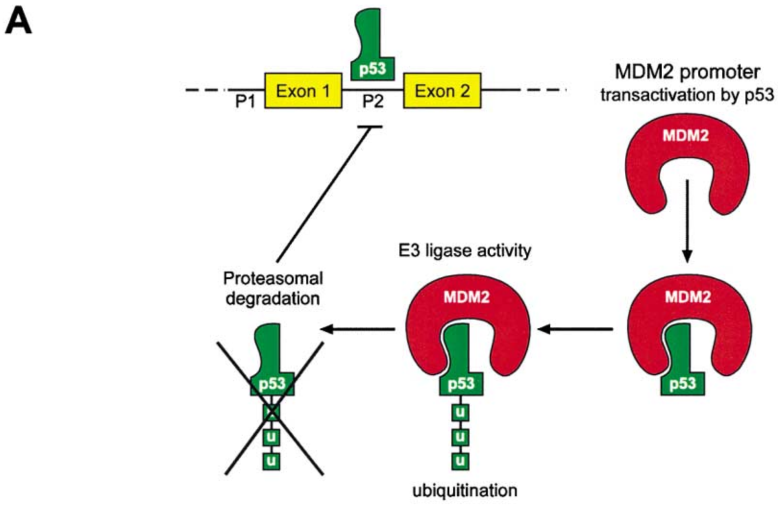
The *MDM2* gene was originally isolated from the spontaneously transformed murine cell line 3T3DM (Cahilly-Snyder et al., 1987; Fakharzadeh et al., 1991). The human homolog of *MDM2*, also referred to as *HDM2*, was cloned by Oliner et al. (1992) from the colon carcinoma cell line CaCo-2. Human *MDM2* is amplified and overexpressed in approximately one-third of human sarcomas, including those of soft tissue and bone. Combined analysis of data obtained from 3889 tumor samples from 28 tumor types revealed an overall *MDM2* amplification frequency of 7% (Momand et al., 1998). To date, *MDM2* amplification has been identified in 19 tumor types with varying frequency. In addition, MDM2 expression can be upregulated independent of gene amplification (Landers et al., 1994; Cordon-Cardo et al., 1994), and oncogenic splice variants have been identified (Sigalas et al., 1996; Matsumoto et al., 1998; Kraus et al., 1999; Pinkas et al., 1999; Bartel et al., 2001; Lukas et al., 2001). Therefore, simple analysis of gene amplification underestimates the involvement of MDM2 in human cancer.

The function of MDM2 was unclear until it was demonstrated to bind to the tumor suppressor p53 and inhibit p53-mediated transactivation (Momand et al., 1992; Figure 1A). Overexpression of MDM2 is a mechanism, independent of gene mutation, by which wild-type p53 function can be inactivated (Chen et al., 1996; Argentini et al., 2001). MDM2 itself can be upregulated by p53, generating a negative feedback loop (Wu et al., 1993; Zauberman et al., 1993). Once MDM2 binds to p53, MDM2 acts as a ubiquitin ligase and facilitates proteasomal degradation of p53 (Haupt et al., 1997; Kubbutat et al., 1997; Honda and Yasuda, 2000; Figure 1A).

As well as its p53-dependent functions, MDM2 is part of a complex network of interactions through which MDM2 affects the cell cycle, apoptosis, and tumorigenesis. Specific regions of MDM2 that interact with p53, CBP/p300, pRB, p73, E2F1, DP1, the L5 ribosomal ribonucleoprotein particle, $p14^{ARF}$, and RNA are indicated in Figure 1B. The specific functions of the MDM2 oncoprotein related to the binding of these different proteins have been discussed in detail in numerous excellent reviews (Freedman et al., 1999; Momand et al., 2000; Juven-Gershon and Oren, 1999).

Human tumors containing both mutant *p53* and *MDM2* amplification are rare (Cordon-Cardo et al., 1994), but the fact that both modifications can occur within the same tumor and that two mechanisms to inactivate wild-type p53 are redundant provides support for a p53-independent function of MDM2. In addition to its p53-associated functions, MDM2 has been reported to transform cells, independent of p53. Dubs-Poterszman et al. (1995) demonstrated transformation of *p53* null cells by expression of MDM2, and Jones et al. (1998) showed that sarcomas develop in the presence or absence of p53 in *MDM2* transgenic mice. The identification of oncogenic splice variants of human *MDM2* transcripts that lack the p53 binding site (Sigalas et al., 1996) provides further evidence of p53-independent functions of MDM2.

Although considerable evidence suggests that MDM2 has transforming potential, conflicting data have generated confusion with respect to the oncogenic function of this protein. Two groups have reported difficulty in transfecting and expressing full-length *MDM2* cDNA in various cell types (Brown et al., 1998; Kubbutat et al., 1999). These results were unexpected and inconsistent with the hypothesis that *MDM2* is exclusively an oncogene. Brown et al. (1998) characterized two growth inhibitory domains within the human MDM2 protein (ID1 and ID2 in Figure 1B). Deletion of either region allowed stable expression of MDM2 in NIH3T3 cells and enhanced the tumorigenic potential of the cells (Brown et al., 1998). Folberg-Blum et al. (2002) demonstrated that MDM2 overexpression targeted to the *Drosophila* wing induced apoptosis of wing imaginal discs, and Dilla et al. (2000) showed that expression of this protein promoted apoptosis in medullary thyroid carcinoma cells. Furthermore, MDM2 is expressed at high levels in terminally differentiated tissues such as skin, brain, and muscle (Piette et al., 1997); such findings are consistent with a growth-inhibitory antioncogenic phenotype. Potentially, some MDM2 proteins



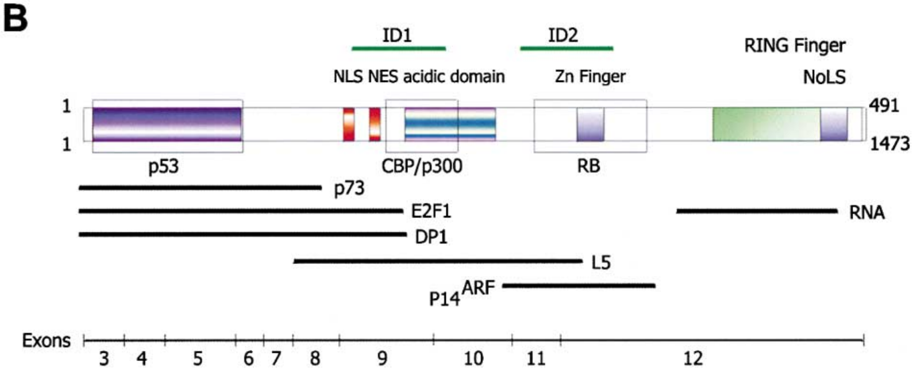


Figure 1. MDM2—structure and function

A: Model depicting the well characterized interactions between MDM2 and p53. p53 activates transcription of MDM2. MDM2 protein in turn binds to p53 and acts as an E3 ubiquitin ligase that targets p53 for degradation by the proteasome, thereby inhibiting its own transcription.
B: Structure of full-length human MDM2 protein and the cDNA that encodes it. The domains of the human MDM2 protein and the binding sites of various proteins are indicated. ID1, ID2: growth inhibitory domains 1 and 2 (Brown et al., 1998). NES: nuclear export sequence. NLS: nuclear localization sequence. NoLS: cryptic nucleolar localization signal.

encoded by splice variants could display an oncogenic function that overrides the growth-inhibiting phenotype and/or the apoptotic phenotype of full-length MDM2 protein. However, data that suggest a completely opposite function have been described for several murine MDM2 splice variants, as discussed below (Dang et al., 2002).

Alternatively and aberrantly spliced variants of *MDM2*

In addition to mutations and chromosomal aberrations, splicing of multiple pre-mRNAs is a mechanism by which gene expression can be regulated or altered. Splicing can occur either "alternatively" at intron-exon borders by using genuine donor and acceptor splice sites, or "aberrantly" at cryptic splice sites within introns or exons. The usage of different promoters can also generate alternative transcripts. For example, *MDM2* gene transcription can occur from two independent promoters. Transcripts that arise from the constitutive P1 promoter lack exon 2, whereas the p53-sensitive P2 promoter generates transcripts lacking exon 1 (Zauberman et al., 1995; Figure 1A). These mRNAs are identical except for the 5′-untranslated region (Barak et al., 1994). However, transcription from P2 is approximately 6-fold greater than that from P1 (Landers et al., 1997). The proteins derived from these two mRNAs are identical, as translation begins in exon 3, but the translation of the shorter P2-derived transcript is more efficient in tumor cells (Brown et al., 1999).

The analysis of expression of the *MDM2* oncogene has revealed the presence of multiple, different sized *MDM2* transcripts and MDM2 protein isoforms in NIH3T3 cells (Haines et al., 1994). After the determination of the exon-intron structure of the murine *MDM2* gene (Oca Luna et al., 1996), it was confirmed that two of these transcripts were alternatively spliced. Different MDM2 isoforms have also been identified in B cell lymphomas isolated from Eμ-Myc transgenic mice (Eischen et al., 1999). Six of these variants have been characterized, and five were shown not to encode portions of the p53 binding domain because of deletions of exons 3, 5, or 4–8 (Dang et al., 2002). The sixth variant did not contain exon 8 and generated no protein when a retrovirus containing its cDNA was used to transduce MEFs. An alternate translation start site at amino acid 198 was utilized by two of the variants generating identical N-terminal truncated MDM2 proteins (Dang et al., 2002). Although none of these murine variants were identical to any found to date in human tumors, the murine variant V3 demonstrated the most similarity to the splice variant MDM2-A (Figure 2). *MDM2* mRNA transcripts of 3.3, 1.6, and 1.5 kb have been detected in tumors derived from a murine mammary tumor model, the shorter transcripts having lost a portion of the C-terminal coding region (Pinkas et al., 1999).

In human breast carcinoma tissue, transcripts of 6.7, 4.7, and 1.9 kb have been detected (Pinkas et al., 1999), with the 1.9 kb mRNA lacking exon 12. Western blot analysis of a panel of human breast carcinomas demonstrated that truncated MDM2 isoforms of 85, 76, and 57 kDa were expressed in addition to the full-length 90 kDa protein (Bueso-Ramos et al., 1996).

A detailed analysis of the *MDM2* mRNA in ovarian and bladder cancers revealed alternative as well as aberrant splicing (Sigalas et al., 1996). Sigalas and coworkers have described five *MDM2* transcripts (MDM2-A, -B, -C, -D, and -E in Figure 2) that lack sequences that encode at least part of the p53 binding domain, the nuclear localization and export sequences, and the acidic domain. In vitro expression studies confirmed that the protein isoforms encoded by four of these splice variants (MDM2-A, -B, -C, and -D in Figure 2) are unable to bind p53. Individual or multiple splice variants have also been detected in glioblastomas (MDM2-A, -B, -C, -D, and -E in Figure 2;

Figure 2. Summary of all known *MDM2* mRNA splice variants and the domains that they encode.

MDM2-FL refers to full-length *MDM2* mRNA. At least some of the splice variants, labeled with "*", can be translated into protein in vitro.
The sequence that is omitted in most splice forms is highlighted in gray. The number in parentheses indicates the reference: (1) Sigalas et al., 1996; (2) Bartel et al., 2001; (3) Bartel et al., 2002; (4) Lukas et al., 2001; (5) Tamborini et al., 2001; (6) Kraus et al., 1999; (7) Schlott et al., 2001; (8) Hori et al., 2000; and (9) F.B., unpublished data.

Matsumoto et al., 1998) and glioblastoma cell lines (MDM2-LN229a, -LN229b, -LN18, -G116, and -G150 in Figure 2; Kraus et al., 1999). In breast carcinomas, previously described splice variants and five additional shorter isoforms (MDM2-281bp, -219bp, -254bp, -DelE, and -DelF in Figure 2) have been detected (Lukas et al., 2001; Hori et al., 2000). Another splice variant, MDM2-Del.G (Hori et al., 2000), which lacks the sequence between nucleotides 182 and 1432 of the coding region of the *MDM2* mRNA, exactly corresponds to the 219 bp form described by Lukas et al. (2001).

Screening 87 adult soft-tissue sarcomas (STS), 85 of which expressed the complete MDM2 coding region, revealed at least 14 additional *MDM2* transcripts in 55% of the cases (Bartel et al., 2001). Of these shorter transcripts, two (MDM2-A and -B in Figure 2) had been described previously (Sigalas et al., 1996), whereas the others, to date, are unique to STS (e.g., MDM2-PM2 and -EU2 in Figure 2). Six additional novel *MDM2* splice variants were identified in primary pediatric rhabdomyosarcoma tumors and cell lines (MDM2-FB25, -26, -28, -29, -30, and -55 in Figure 2; Bartel et al., 2002). These variants include alternatively as well as aberrantly spliced forms.

Some of the aberrantly spliced *MDM2* transcripts (e.g., MDM2-PM2, -EU2, -KB3, and -219bp) have a common splicing pattern that is illustrated in Figure 3. Splicing occurs at cryptic splice donor and acceptor sites in regions with high sequence homology and which occur as many as four times in the coding region of the *MDM2* mRNA. For example, a 10-base repeat sequence (TG<u>G</u>CCAGTAT in exon 5; TG<u>C</u>CCAGTAT of exon 12) is involved in the splicing of the KB3 variant (a splice variant detected in STS; Bartel et al., 2001) and the MDM2-219bp variant first described by Lukas et al. (2001). In addition to the splicing at repetitive sequences, the splice variant *MDM2*-DS3 (Bartel et al., 2001) shows another interesting feature. This splice form contains a novel 87 bp sequence inserted between exons 4 and 5. Although the function is not known, this sequence is similar to an α-exon found in the canine *MDM2* mRNA (Veldhoen et al., 1999). Therefore, MDM2-DS3 represents the first identified example of an expressed human MDM2 splice variant that contains α-exon sequence.

In summary, at least 40 alternatively and aberrantly spliced transcripts of *MDM2* mRNA have been identified in tumors, but it is currently unknown how many of these are actually expressed as protein. Most variant transcripts lack sequence that encodes at least part of the p53 binding domain and the p300 binding domain. The fact that some splice variants have been detected only in a particular tumor type suggests that they might contribute to the transformed phenotype of these tumors, whereas others (e.g., *MDM2*-B) may be associated with tumorigenesis in general or be generated as a consequence of the malignant phenotype.

Relationship of MDM2 expression to tumor stage and prognosis

Results of clinical studies investigating the association between MDM2 expression and tumor prognosis are contradictory. Overexpression of MDM2 in acute lymphoblastic leukemia and STS is associated with an unfavorable prognosis (Cordon-Cardo et al., 1994; Gustafsson et al., 1998; Wurl et al., 1998). In contrast, no relationship has been observed between MDM2 expression in glioblastomas and survival of patients (Newcomb et al., 1998), and *MDM2* gene amplification is a favorable prognostic marker in non-small cell lung carcinoma and STS

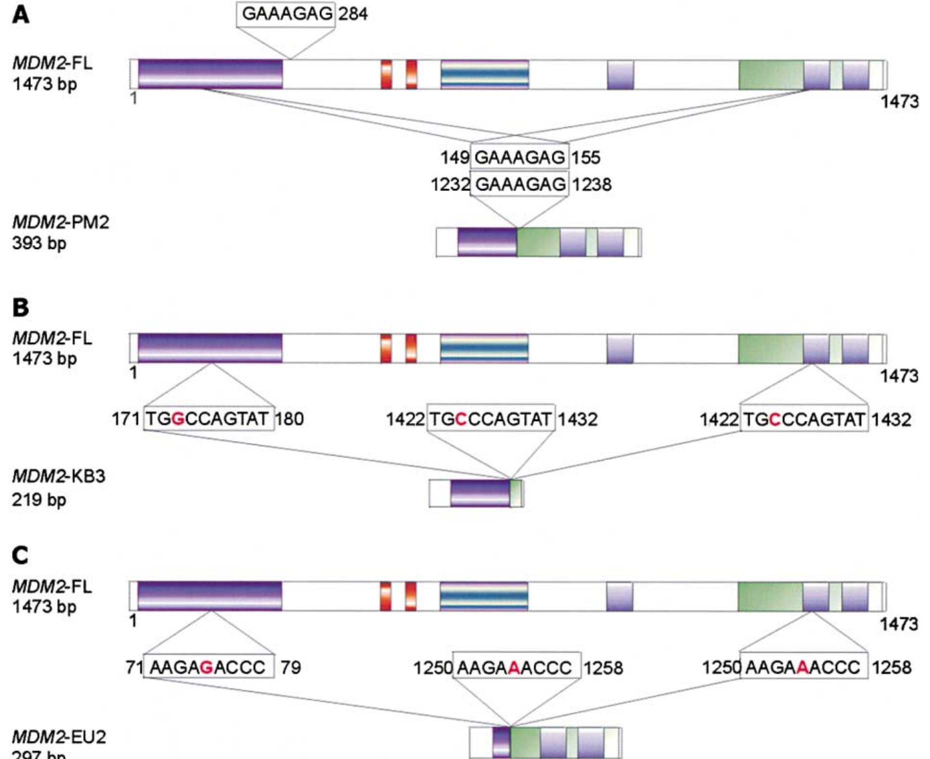


Figure 3. Mechanism of aberrant splicing at repetitive sequences.

MDM2-FL refers to full-length *MDM2* mRNA. The different shaded areas represent the domains of MDM2 as shown in Figure 1 (see text for details).

(Higashiyama et al., 1997; Bartel et al., 2001). In tumors of the head and neck region, the loss of MDM2 expression is associated with a poor prognosis (Millon et al., 2001). A potential explanation of these conflicting findings may be that alternatively or aberrantly spliced MDM2 variants are expressed in certain tumors and, when present, influence prognosis.

Expression of oncogenic splice forms of *MDM2* occurs more frequently in high-grade than low-grade tumors (Matsumoto et al., 1998; Bartel et al., 2001). Expression of aberrantly but not alternatively spliced mRNA in breast carcinoma was associated with a shortened overall patient survival (Lukas et al., 2001), but there was no association with outcome or survival time for patients with STS that expressed either alternatively or aberrantly spliced MDM2 isoforms (Bartel et al., 2001). In giant cell tumors of the bone, splice variants were expressed only in stromal cells, not in the giant cells (Evdokiou et al., 2001). This finding supports the hypothesis that stromal cells comprise the tumor element in these giant cell tumors.

Unfortunately, studies measuring MDM2 protein expression have been carried out using different antibodies, several of which recognize multiple nonspecific proteins in addition to MDM2. Therefore, the information obtained by measuring total MDM2 protein in tumors may be of little value, particularly if the antibodies used for analyses were raised against epitopes not present within the mutant MDM2 proteins. In order to determine whether splice variants are expressed, and to evaluate their relationship to tumorigenesis and prognosis, specific reagents must be developed.

One could speculate that splice variants of MDM2 exhibit an antiapoptotic function, which would help explain why some patients whose tumors contain *p53* mutations and overexpress MDM2 have a worse prognosis than do those whose tumors have only one of these modifications (Wurl et al., 1998; Figure 4). However, as discussed above, MDM2 has been shown to exhibit a p53-independent tumorigenic potential that could generate an additive growth advantage in cells expressing mutant *p53* (Dubs-Poterszman et al., 1995; Jones et al., 1998; Figure 4). It is possible that there are no splice variants expressed in tumors where *MDM2* amplification is associated with a good prognosis (Higashiyama et al., 1997). However, p53 may also facilitate chemotherapy-induced apoptosis in such tumors if MDM2-p53 binding is disrupted (Shieh et al., 1997; Khosravi et al., 1999; Mayo et al., 1997). Additional work will be required to determine which of these hypotheses are correct.

Functions of the MDM2 isoforms

Although more than 40 splice variants of *MDM2* mRNA have been identified, little is known about their functions, and it is important to remember that it is unknown whether all the variant transcripts are translated into protein. Although most variants have lost internal sequences, there does not appear to be a common deleted region that could suggest a common function. Many examples of alternative splicing force the region encoding the C terminus to be out-of-frame. This change potentially leads to the generation of novel amino acid sequences, but because these new sequences differ in every case, they are unlikely to contribute to a common novel function of the proteins. The open reading frames of many variants contain premature stop codons, whose presence suggests the generation of truncated proteins containing only the N-terminal portion of MDM2.

It is important to note that alternatively and aberrantly spliced *MDM2* mRNAs are usually found together with full-length *MDM2* transcripts. This finding is of interest because Evans et al. (2001) have demonstrated that at least one splice variant (MDM2-B, ALT1) can bind to full-length MDM2 protein and sequester it in the cytoplasm. The splice variant MDM2-B is the most frequently expressed form in numerous types of cancer, including ovarian and bladder cancers (Sigalas et al., 1996), breast cancer (Matsumoto et al., 1998; Lukas et al., 2001), STS (Bartel et al., 2001; Tamborini et al., 2001), and giant cell tumors of the bone (Evdokiou et al., 2001). However, Evans et al. (2001) also demonstrated that the binding of MDM2-B to full-length MDM2 increased wild-type p53 activity. A similar observation was made using the murine splice variants identified by Dang et al. (2002). Binding of these variants to full-length MDM2 in MEFs resulted in a release of wild-type p53 such that p53 was not targeted to the proteasome (as shown in Figure 1A), and was free to mediate growth inhibition as depicted in Figure 4. Expression of splice variants is also associated with p53 stabilization in glioblastoma cell lines despite amplifi-

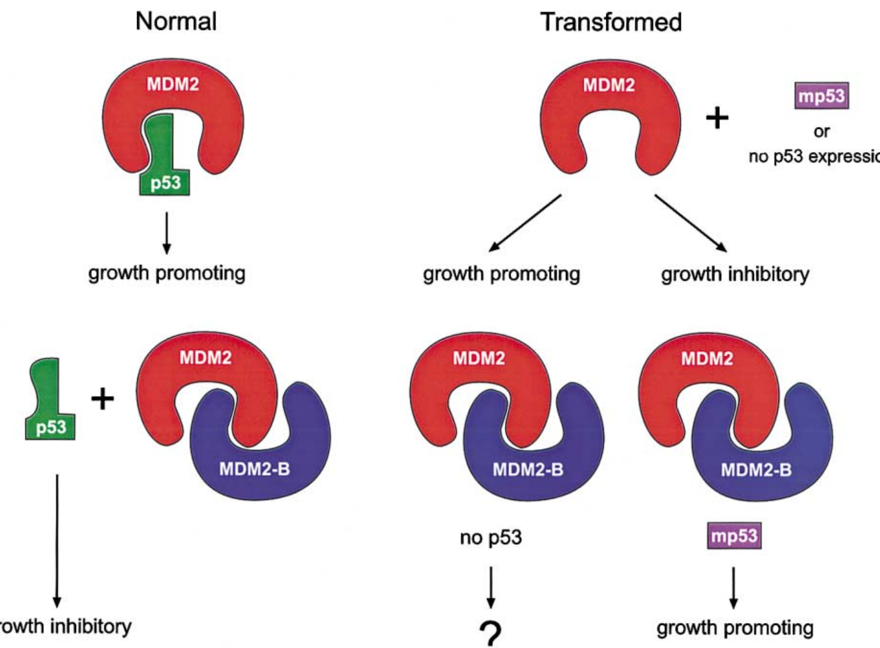


Figure 4. A model suggesting how MDM2 splice variants with an intact C terminus, for example MDM2-B, might exhibit contrasting functions.

In "normal" cells, MDM2 binds to and inhibits the function of wild-type p53, promoting cell growth. When MDM2 splice variants are expressed, they bind to full-length MDM2 protein, releasing p53, resulting in growth inhibition. In transformed cells when p53 may be mutated or not expressed, MDM2 can still promote cell growth. Under these circumstances, there may be no consequence of splice variant expression, because splice variant binding to full-length MDM2 would not release any wild-type p53. Under certain conditions, full-length MDM2 expression in a transformed cell results in apoptosis or growth inhibition. If splice variants are expressed in these cells, they may bind and inhibit the growth-inhibitory function of full-length protein, resulting in an increased rate of proliferation.

cation of the *MDM2* gene (Kraus et al., 1999), and in STS we have found a similar correlation between the expression of *MDM2* splice forms and overexpression of p53 (Bartel et al., 2001). These findings suggest that p53 accumulation arises as a consequence of alternative as well as aberrant MDM2 splicing independent of the mutational status of p53.

Most *MDM2* splice variants described to date lack sequence that encodes at least part of the p53 binding domain. In vitro binding assays have shown that the splice forms MDM2-A, -B, -C, and -D (Figure 2) lack the ability to bind to and inactivate p53 (Sigalas et al., 1996). Despite the fact that the p53 binding domain is retained in a couple of MDM2 isoforms (MDM-E, -FB26) which still have the ability to bind p53, MDM2-mediated degradation of p53 may be inhibited because of the loss of p300 binding (Zhu et al., 2001). Nevertheless, an increased amount of wild-type p53 is inconsistent with a potential transforming phenotype of *MDM2* splice variants.

Sigalas et al. (1996) demonstrated that NIH3T3 cells transfected independently with several splice variants could grow as colonies in soft agar. In support of this transforming function of MDM2 splice variants, unpublished data from S. Jones et al. show that MDM2-B can cause tumors in a transgenic mouse model. In contrast, MDM2-B is expressed in both malignant and normal mammary tissue (Lukas et al., 2001). It appears that MDM2 splice variants display different characteristics in different cellular backgrounds, as shown in Figure 4. Because it is unknown under what conditions alternative functions of full-length MDM2 are active, it is impossible to predict the function of the splice variants in each model system evaluated.

Each of the numerous MDM2 splice forms could be responsible for a distinct phenotype. For example, some isoforms encoded by splice variants may lack the ability to bind to $p14^{ARF}$, whereas other isoforms might bind to this protein and be sequestered in the nucleolus (Weber et al., 2000). Furthermore, the addition of different novel amino acid sequences at the C terminus might generate novel tumorigenic functions. However, many splice variants lack the C terminus, a finding that suggests the importance of the N-terminal portion of the protein. Much work remains before the functions of MDM2 and its isoforms encoded by alternatively spliced variants are understood and before the relationship of this new information to the MDM2-p53-$p14^{ARF}$ pathway is clarified.

Conclusions

The MDM2-p53-$p14^{ARF}$ pathway has been elegantly mapped out by many investigators (including Sherr, 1998), but it now appears that the pathway may not be as straightforward as originally thought. Full-length MDM2 displays both oncogenic and growth inhibitory properties (Figure 4), but how these different functions are controlled is unknown. It appears as though MDM2 function may parallel that of MYC. MYC expression induces apoptosis in cells where the MDM2-p53-$p14^{ARF}$ pathway is functional (Packham and Cleveland, 1995; Zindy et al., 1998). Yet, when this pathway is disrupted, the oncogenic phenotype of MYC is displayed (Sherr, 1998). Similarly, the contrasting functions of full-length MDM2 are probably dependent upon the genetic background of the cells in which they are expressed. At least some MDM2 splice variants encode proteins that possess a transforming function both in vitro and in vivo, but in different model systems, they function to release p53 from full-length MDM2, inducing wild-type p53 activity (Figure 4). Additionally, p53-independent functions of MDM2 and its isoforms encoded by alternatively and aberrantly spliced transcripts might be important in tumorigenesis, but it remains to be seen how these functions are integrated within the well-characterized MDM2-p53-$p14^{ARF}$ tumor surveillance pathway.

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Growth reduction of a xenotransplanted human soft tissue sarcoma by MDM2 antisense therapy via implanted osmotic minipumps

PETER WÜRL[1,5*], FRANK BARTEL[2*], AXEL MEYE[4], MATTHIAS KAPPLER[2], MATTHIAS BACHE[3], HANNELORE SCHMIDT[2], MANFRED SCHÖNFELDER[1] and HELGE TAUBERT[2]

[1]Clinic of Surgery 1 University of Leipzig, Liebigstr. 20a, 04103 Leipzig; [2]Institute of Pathology, [3]Department of Radiotherapy, Martin-Luther-University Halle-Wittenberg, Magdeburger Str. 14, 06097 Halle/Saale; [4]Clinic of Urology, University of Dresden, Fetscherstr. 74, 01307 Dresden, Germany

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Abstract. The *MDM2* oncogene plays an important role in tumorigenesis and especially in soft tissue sarcomas (STS). Overexpression of the MDM2 protein is associated with a poorer prognosis for STS patients. An *MDM2* antisense approach to reduce MDM2 protein levels has been successfully applied on several carcinomas *in vitro* and used on a few *in vivo* cases. However, antisense treatment not only resulted in an MDM2 protein reduction but also in a wild-type *P53* gene (wt-*P53*) mediated tumor growth suppression due to its genetic wt-*P53* status. In this study, we used a clinically relevant xenotransplanted STS model with a mutated *P53* gene (mt-*P53*) in order to exclude the influence of wt-*P53*. The human STSs were surgically implanted and one week later osmotic pumps were implanted intraperitoneally into nude rats releasing MDM2-antisense ODNs (AS ODNs) continuously for one week. As controls *MDM2*-sense ODN (SE ODN) or a 0.9% NaCl solution (saline solution) were administered. After one week animals treated with *MDM2*-AS ODN (100 or 200 µg) showed a reduction in tumor mass in comparison to animals treated with *MDM2*-SE ODN. The reduction in tumor mass was significant in animals treated with *MDM2*-AS ODNs in comparison to the saline solution treated ones (p=0.018 or p=0.007). Furthermore, a significant reduction in macroscopically visible tumor number with *MDM2*-AS ODN treatments (100 or 200 µg) in comparison to *MDM2*-SE ODN or saline solution treatment (both p=0.009) was observed for the first time. As expected a reduction in MDM2 protein expression in the *MDM2*-AS ODN treated tumors when compared to the *MDM2*-SE ODN or saline solution treated tumors was detected in Western blot analyses and immunohistochemically. In addition, an unexpected reduction in mt-P53 protein expression after AS ODN therapy was also observed. In short, we have demonstrated *in vivo* for the first time that *MDM2*-AS ODN treatment of xenotransplanted STS (mt-*P53*) reduces tumor mass, tumor number, MDM2 protein and mt-P53 protein expression. Our findings support the hypothesis that *MDM2*-AS ODN treatment may exert a tumor inhibiting effect on all MDM2 expressing tumors regardless of the P53-status, this in turn may be of general importance in gene therapy of cancer.

Correspondence to: Dr Helge Taubert, Institute of Pathology, Martin-Luther-University Halle-Wittenberg, Magdeburger Strasse 14, D-06097 Halle/Saale, Germany
E-mail: helge.taubert@medizin.uni-halle.de

*Contributed equally

[5]*Present address*: Clinic of Surgery, University of Ulm, Steinhövelstr. 9, 89075 Ulm, Germany

Abbreviations: AS, antisense; SE, sense; HPF, high power-field; STS, soft tissue sarcoma(s); MBO, mixed backbone oligonucleotides; ODN, oligodesoxynucleotides; MDM2, murine double minute gene 2; wt, wild-type; mt, mutant

Key words: MDM-2, antisense MBO ODNs, soft tissue sarcoma, osmotic minipumps, nude rats, RD cell line

Introduction

Soft tissue sarcomas (STS) are a large, histologically diverse group of solid mesenchymal tumors. At the time of diagnosis 10-20% of the patients have distant metastases with an increase up to 50% over a five year period (1,2) connected with a relative resistance to chemotherapy and irradiation. More than 50% of the STS patients die within five years after diagnosis (3,4). Radical surgical resection is the only therapeutic intervention with a chance of cure. But in case of multiple metastases, this therapeutic approach is not able to change the course of the disease (5,6).

In addition to the well known clinical prognostic factors and molecular abnormalities in the tumor suppressor gene *P53* an especially important role of the oncogene *MDM2* (murine double minute gene 2) has been ascertained in STS tumorigenesis (7-9).

There are several lines of evidence for a direct link between an abnormal MDM2 function and sarcomagenesis. STS

possesses an amplification frequency of the *MDM2* gene of about 20-30% that corresponds to the highest amplification rate described for malignant tumors (10). Secondly, overexpression of MDM2 in transgenic mice results in 38% of cases developing sarcomas independent of P53 status (11). Thirdly, MDM2 protein overexpression in STS is correlated with a poorer survival of sarcoma patients (7,8,12).

Based on these findings, an approach targeting the *MDM2* oncogene for STS therapy seems reasonable. Several studies using *MDM2* antisense oligodesoxynucleotides (AS ODNs) have shown that they do have an effect on tumor cell viability, on the rate of apoptosis *in vitro*, on reduction in tumor mass and a prolonged survival in animal models (13-17). However, apart from two previous studies which were conducted by our group on STS cell lines, the efficacy of an *MDM2*-AS ODNs has not yet been investigated in STS (13,18).

In the present study, we have chosen a human xenotransplanted STS as a model for an isolated intraperitoneal tumor spread in nude rats in order to characterize the effect of *MDM2*-AS ODNs *in vivo*. Due to the evident clinical relevance of an intraperitoneal tumor spread, we used a peritoneal sarcoma model to mimic the clinical situation. *MDM2*-AS ODNs were delivered to the peritoneum by a continuous osmotic pump. In order to exclusively analyze the effect of MDM2 inhibition without interference of wild-type P53 (wt-P53) STS transplant tumors originating from the *P53*-mutated RD cell line were studied.

Materials and methods

mdm2 AS ODNs and osmotic minipumps. The AS-, as well as, the SE-control ODNs used in this study have been described elsewhere (19). The sequences of the used ODNs were as follows: *MDM2* AS-ODN 5'-oUogACACCTgTTCT CACoUoCoArC-3', and *MDM2* SE-ODN 5'-oUoGAgACCA gTTgTCAgoUoCoArC-3'. All ODNs were purchased from Tibmolbiol (Berlin, Germany). Shortly before use the lyophilized ODNs were resolved in an appropriate volume of aqua bidest, which allowed us to reach the respective working concentration. The Alzet® osmotic pumps (Alza Pharmaceuticals, Palo Alto, CA, USA) were filled with an equal total volume of 200 µl containing *MDM2*-AS-ODNs, and SE-ODNs or physiological saline solution, respectively. The pumps with a length of 3.0 cm, a diameter of 0.7 cm and a weight of 1.1 g have a nominal pumping rate of 1 µl/h with a nominal pumping duration of 1 week.

Human STS xenotransplant model in nude rat. To establish the experimental animal model, the human embryonal rhabdomyosarcoma cell line RD (20,21) purchased from ATCC (Rockville, MD; code: CCL-136) was used. This cell line contains in one allele a *P53* missense mutation (in codon 248 a CGG→TGG transition), whereas the other *P53* allele is missing (21). It does not carry an amplification of the *MDM2* gene (Bartel, unpublished data) but expresses the MDM2 protein (data not shown). The RD cell line was grown as monolayer (37˚C, 3% CO_2, RPMI-1640 with 15% fetal calf serum) and cells were harvested by trypsinization. Tumor cells ($5x10^6$) were inoculated intramuscularly into the hind legs of male nude rats RH-nu/nu (Harlan-Winkelmann, Borchen, Germany) under sterile conditions. After macroscopic tumor formation (Fig. 1A) the solid tumors were surgically removed. After removing the central part of the tumor the surrounding tissue was minced carefully into 1-2 mm^3 parts and stored at 4˚C. The tissue of several tumors was pooled, and immediately afterwards 500 mg tumor tissue per animal was intraperitoneally implanted into the rats (Fig. 1B). The processing of the tumor material and all operations were done under sterile conditions.

Animal groups and tumor growth inhibition. All investigations concerning this study were performed in accordance with the Declaration of Helsinki and the Interdisciplinary Principles and Guidelines for the Use of Animals in Research, Marketing and Education.

Twenty-four eight-week old nude rats with body weights of 80-100 g were randomly distributed into four groups (seven rats per group, five rats per control group, respectively) and 500 mg minced tumor tissue was distributed into all four quadrants of the peritoneal cavity of the animals. Exactly one week after tumor transplantation, the osmotic pumps filled with ODN solution or saline solution were implanted (Fig. 1C). At the time of laparotomy tumor tissue was visible in all of the animals. The concentrations of the therapeutic ODNs used for this experiment are based on the results of previous *in vitro* experiments (13,18). The animals were randomized into four groups and the pumps for each group contained: a) 100 µg *MDM2*-AS ODN (14.5 µg/d); b) 200 µg *MDM2*-AS ODN (29 µg/d); c) 200 µg *MDM2*-SE ODN (29 µg/d); or d) control group: 0.9% NaCl solution (saline solution).

The incision was ≤2 cm long and placed along the center line for all abdominal operations. All animals were kept in cages (Macrolon III cages with three or four rats per cage) and had access to tap water and standard food *ad libitum* (dark-light cycle of 12 h). The body weight and the tumor size were monitored twice a week.

Two weeks after tumor transplantation all animals were sacrificed and evaluated for the number of macroscopically visible tumors. In addition, tumor mass, body weight and the presence of distant metastases in liver and lung were documented (Fig. 1D). From all animals with macroscopically visible tumors tissue samples from the tumor, the liver and muscle were stored in liquid nitrogen. Along with all of the stored tumors, paraffin-embedded tissue specimens were prepared for histological evaluation and for the determination of the mitotic rate [counts per ten high powerfields (HPF), magnification x40].

Western blot and immunohistochemistry for P53 and MDM2. Thirty micrograms of total protein was subjected to electrophoresis in a 10% polyacrylamide-SDS gel (Minigel system, Biometra, Göttingen, Germany) from all tumor samples. Proteins were transferred to a PVDF Immobilon membrane (Millipore, Eschborn, Germany) at 200 mA for 90 min (Miniblotter, Biometra). After non-specific binding sites were blocked by 0.1% Tween-20 containing 3% bovine serum albumin, the membrane was incubated for 1 h with an anti-P53 antibody (DO-7; dilution 1:500; Dianova, Hamburg, Germany) or with an anti-MDM2 antibody (2A10; dilution

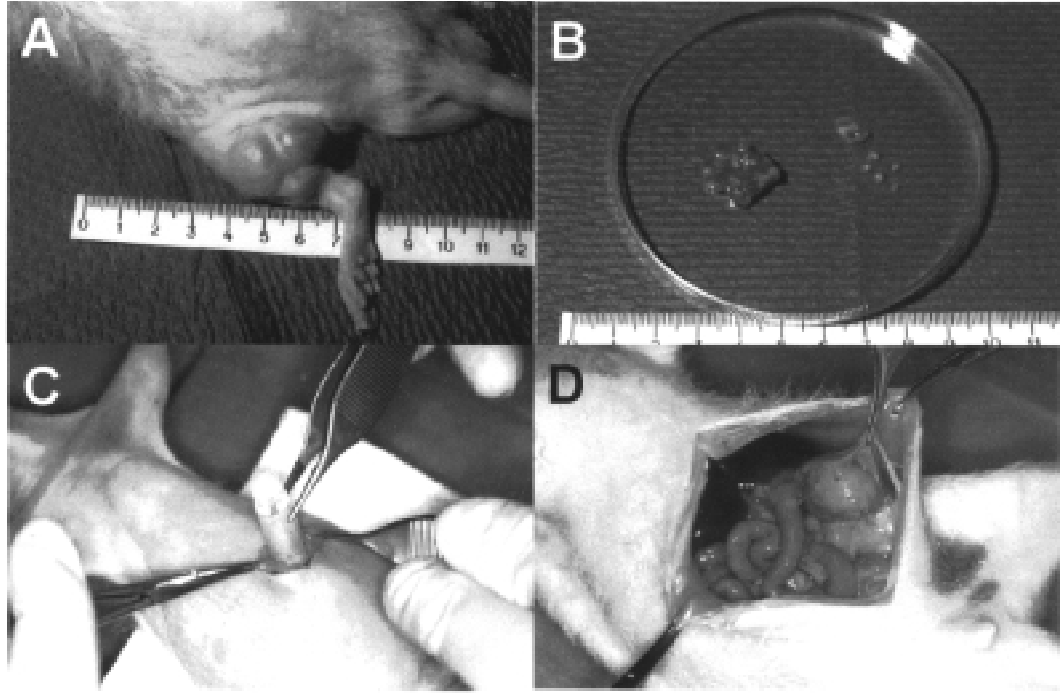


Figure 1. Experimental procedure. (A), Tumor on the left hind leg before harvesting for intraperitoneal transplantation. (B), Minced into 1-2 mm^3 parts for intraperitoneal transplantation (left, tumor mass of 500 mg; right up, ten fragments together; right down, four isolated fragments). (C), Implantation of the osmotic pump in general anesthesia via laparotomy. (D), Tumor in the left middle part of the peritoneal cavity at the end of the experiment (isolated tumor in an animal of the MDM2-AS ODN 100 μg group).

Table I. Total tumor mass and number of macroscopically detectable intraperitoneal tumors for each experimental group.

Therapeutic group	*MDM2*-AS ODN 100 μg	*MDM2*-AS ODN 200 μg	*MDM2*-SE ODN 200 μg	Physiological saline solution
Animals per group	7	7	5	5
Tumor mass Mean (± SD)	1.07 g (±0.55 g)	0.78 g (±0.53 g)	1.74 g (±1.17 g)	2.64 g (±1.33 g)
Tumor number Mean (±SD)	2.4 (±0.97)	2.3 (±1.11)	3.4 (±1.51)	4.4 (±1.14)

Standard deviations are given in parentheses.

1:1000; generous gift from Dr A.J. Levine). The incubation with the primary antibody was followed by a 1-h incubation with horseradish peroxidase-conjugated anti-rabbit IgG or anti-mouse IgG antibody (dilution, 1:1000; Dako, Glostrup, Denmark) at room temperature. For visualization of the bands, the membrane was placed into ECL-substrate (Amersham, Braunschweig, Germany) for 1 min and then exposed to a Biomax film (Kodak, Stuttgart, Germany).

Afterwards, densitometry (Imagemaster VDS 3.0 software, Pharmacia) was performed to determine the relative levels of MDM2 and P53 protein. The relative amounts of MDM2 and P53 were standardized to that of ß-actin from the same sample. The resulting values were classified according to a semi-quantitative scale as no, low, intermediate, or strong expression.

For IHC staining we used the monoclonal MDM2 antibodies (Abs) IF2 (Oncogene Science, Manhasset, NY, USA; epitope N-terminal) and 1B10 (Medac, Hamburg; epitope C-terminal) and the P53 Abs DO-1 and Pab1801 (both Oncogene Science, epitope DO-1 amino acids 20-25, Pab1801 40-65, respectively), and DO-7 (Medac, Hamburg; epitope amino acids 20-25). Four-μm-thick tissue sections were placed on poly-L-lysine coated slides and deparaffinized. For the 1B10 Ab, an antigen retrieval with pressure cooking for 2 min was performed. Endogenous peroxidase activity was inhibited and non-specific binding was inhibited by 5% carrier protein solution for 15 min. Incubation with the primary Abs (working dilution: IF2 20 μg/ml, 1B10 1:200, DO-7 5 μg/ml, DO-1 5 μg/ml) was performed at 4˚C for 12 h. As a negative control, a non-

Table II. p-values of Student's t-test comparing the total tumor masses of therapeutic groups.

Therapeutic group	*MDM2*-AS ODN 100 µg	*MDM2*-AS ODN 200 µg	*MDM2*-SE ODN 200 µg	Physiological saline solution
MDM2-AS ODN 100 µg	-	0.354	0.21	0.018
MDM2-AS ODN 200 µg	0.354	-	0.083	0.007
MDM2-SE ODN 200 µg	0.21	0.083	-	0.294
Physiological saline solution	0.018	0.007	0.294	-

immune mouse serum was applied instead of the primary Abs and normal human liver tissue was stained. Incubation was followed by a washing step and detection of the primary Abs by an LSAB-Kit (Dako). Staining was considered positive if more than 10% of the cells were positive. All slides were examined and scored independently by two investigators.

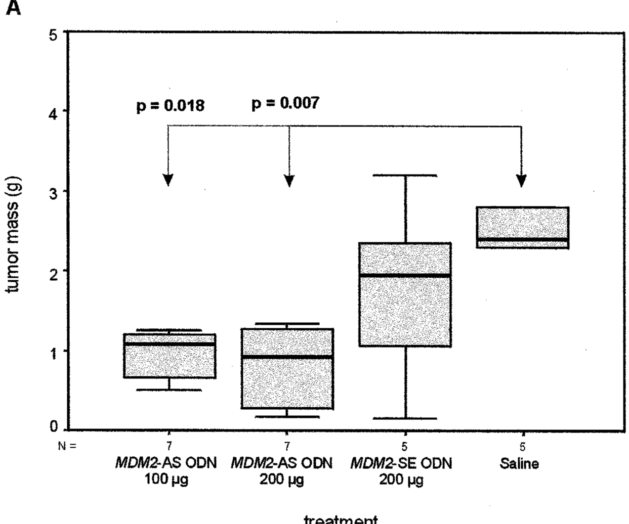
Statistical analysis. After a descriptive statistical approach with calculation of median, mean and standard deviation, analysis was done using the Student's t-test to determine differences between the therapeutic groups. The level of significance was set at $p<0.05$.

Results

AS specific inhibition of tumor growth. MDM2-AS ODN therapy was tested in a nude rate model carrying an intraperitoneal human STS xenograft. For therapeutic treatment an AS ODN, an SE ODN, or physiological saline solution as control were administered by an osmotic pump at a flow rate of 1 µl/h over a period of 7 days. The tumors were surgically removed from the animals after a continuous therapeutic period of one week. Treatment of the tumors with *MDM2*-AS ODN resulted in a significantly reduced tumor mass when both 100 and 200 µg of *MDM2*-AS ODN were administered compared to *MDM2*-SE ODN or saline treated tumors, respectively (p=0.018 and p=0.007, Tables I and II, Fig. 2). On day 7, after treatment with 200 µg of *MDM2*-AS ODN an average of 71% less tumor mass (0.78±0.53 g) was determined when they were compared to the saline treated tumors (2.64±1.33 g), and there was 56% less tumor mass than in SE-ODN treated tumors (1.74±1.17 g) (Fig. 2).

Remarkably, the average number of macroscopically detectable tumors in the *MDM2*-AS ODN treated animals was also reduced (2.3 tumors per rat) compared to the number found in *MDM2*-SE ODN (3.4 tumors, p=0.009) or saline solution treated animals (4.4 tumors, p=0.009) (Tables I and II).

Western blot analysis. The MDM2 protein expression levels were reduced in tumors that were treated with either 100 or



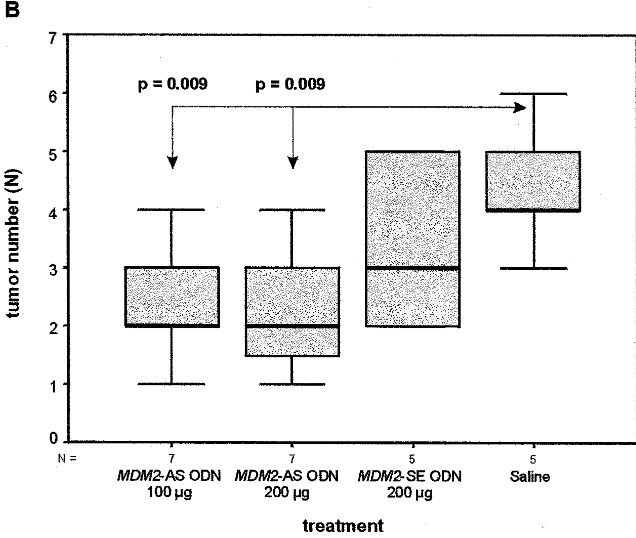


Figure 2. Boxplots showing the reduction of tumor mass (A) as well as tumor number (B) in animals treated with *MDM2*-AS ODNs in comparison to *MDM2*-SE ODN or saline solution treated rats.

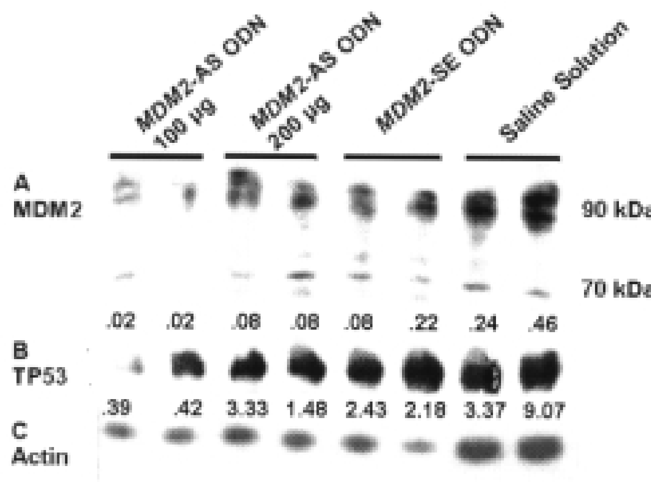


Figure 3. *In vivo* inhibition of MDM2 (A) and mt-P53 (B) protein expression by *MDM2*-AS ODNs. The animals were treated with either 100 or 200 µg of *MDM2*-AS ODN or 200 µg of *MDM2*-SE ODN, respectively. Saline solution treated animals served as a control. After one week, the tumors were removed, protein was isolated, and equal amounts of protein were analyzed by Western blot analysis using a monoclonal anti-MDM2 (2A10), and an anti-P53 antibody (DO-7). The numbers represent ratio of the intensity of the MDM2/actin and the TP53/actin ratio determined by densitometry. The ß-actin protein (C) served as a control, and its expression level was used to standardize the MDM2 and mt-P53 protein expression levels.

200 µg of *MDM2*-AS ODN in comparison to tumors that received an *MDM2*-SE ODN or saline solution treatment (Fig. 3). Only a low MDM2 expression in *MDM2*-AS ODN treated rats was detected when 100 or 200 µg were given per week. In *MDM2*-SE ODN treated animals a marked MDM2 expression, and in the tumors from the control animals treated with saline solution a strong expression of the MDM2 oncoprotein could be detected, which was noticeable for the SE ODN group (p=0.068), and statistically significant for the saline control group (p=0.05). The mt-P53 protein expression detected by Western blot analysis appeared to be low in the groups which were treated with different doses of the *MDM2*-AS ODN, intermediate in the *MDM2*-SE ODN treated group, and strong in the saline control group. The mt-P53 protein expression levels in the group which received treatment with 100 µg *MDM2*-AS ODN were significantly reduced compared to the saline solution control group (p=0.029).

Immunohistochemistry. The counting of mitotic figures per 10 HPF resulted in a minimal number of 52 and a maximum number of 78 mitoses without any differences between the different experimental groups (data not shown). Therefore all tumors are considered as high grade.

Although in all cases single cells were distinctly stained by all three P53 Abs, staining was often below the 10% cut-off level and a specific staining pattern was observed in the different therapy groups (Table III). In both *MDM2*-AS ODN treated groups only the DO-7 antibody detected any P53 protein. In the *MDM2*-SE ODN group a moderate staining with the Pab-1801 and the DO-1 antibodies was also observed in two cases in addition to DO-7 staining. All of the tumors in the saline solution treated control group showed a strong staining with all three P53 Abs.

MDM2 was only detected when immunohistochemical staining was carried out with Ab 1B10 that recognizes a C-terminal epitope. The antibody IF-2 that recognizes an N-terminal epitope did not mark any tumor cells in any of the treatments groups. In the *MDM2*-SE ODN, the *MDM2*-AS ODN (100 µg) and in the *MDM2*-AS ODN (200 µg) groups all except one tumor in the latter group did not show an MDM2 positive staining. On the contrary, in the saline solution treated group all tumors were stained positively by the 1B10 antibody.

Discussion

This report describes the first *in vivo* investigation of *MDM2*-AS ODN in STS. An *MDM2*-AS ODN was tested in a human xenotransplanted STS using a nude rat model of peritoneal tumors. The model mimics the frequent clinical problem of abdominal and intraperitoneal STS that is difficult to treat by conventional surgery, radiation- or chemotherapy. A peritoneal tumor distribution can limit prognosis without the appearance of distant metastases. Therefore, it is necessary to consider alternative therapeutic strategies, for example the use of AS ODNs that target the *MDM2* oncogene.

Table III. Summary of the immunohistochemistry results.

Antibody	*MDM2*-AS-ODNs 100 µg	*MDM2*-AS-ODNs 200 µg	SE-ODNs	Saline solution
Pab-1801	-[a]	-	+	+++
DO-1	-	-	+	+++
DO-7	±	±	++	+++
1B10	-	±	-	+++
IF-2	-	-	-	-

[a]- -, no staining; ± -, low percentage of positively stained cells; + -, moderate; ++ -, increased; +++ -, strong expression.

Several authors have investigated *MDM2*-AS therapy and suggested that it has a potential use in cancer therapy (13-18). *MDM2*-AS ODNs using mixed backbone oligonucleotides (MBOs) has been shown to inhibit MDM2 protein expression and antitumor effects *in vitro* and *in vivo* (15,19).

In this study, the comparison of animals treated with *MDM2*-AS ODNs to those treated with *MDM2*-SE ODNs or saline solution revealed a reduction in tumor mass as well as a decrease in tumor number. This is in agreement with findings published by other authors on size reduction in tumors, a marked delay in tumor growth and a prolongation of animal survival after an *MDM2*-AS treatment (15,19). However, a reduction in tumor number is reported in this study for the first time. Although the difference of the reduction of the tumor mass and tumor number between the AS-treated and the SE-treated animals is noticeably, but not significant (p=0.08 and p=0.17, respectively, Student's t-test), the effect of the treatment with *MDM2*-AS-ODNs is clearly visible. This might be due to a sequence-independent effect of the used ODNs.

A high efficacy of *MDM2*-AS ODN treatment has been described for several cell lines and for tumors with low or with high MDM2 protein expression. Therefore, a reduction of the MDM2 expression level seems beneficial in cancer treatment and does not seem to affect the survival of normal cells (16,22). An explanation for antitumor activity through a decrease of MDM2 protein levels has not been completely resolved. In several tumors with wt-P53 function a reduction in MDM2 expression levels results in an increase of P53 activity and gradually in growth arrest or induction of apoptosis (23). The idea that M*DM2*-AS ODNs might be effective only in cells with a wt-P53 status seems to be true for B-cells of lymphomas (17), but not for others (16). However, *MDM2*-AS ODNs may also affect MDM2 activity directly and induce an antitumor effect.

There are several lines of evidence suggesting that an abnormal MDM2 function in itself can be oncogenic independent of the P53 status. i) There is a transforming phenotype of tumor cells in the absence of P53 and also when MDM2 splice variants are present which have lost their P53 binding site (24,25). ii) In mouse models MDM2 overexpression contributes to tumorigenesis in $P53^{-/-}$ mammary epithelial cells and *MDM2* transgenic mice ($P53^{-/-}$) develop a disproportionally high number of sarcomas (11,26). iii) Transfection of *MDM2* results in MDM2 that can form complexes with the retinoblastoma protein and with the E2F1 and DP1 transcription factors independent of P53. Both complexes can stimulate expression of E2F1-responsive genes and a role in promoting cell cycle progression from G1 to S phase is suggested (27,28). iv) MDM2 is able to inhibit another member of the P53-family, the P73 protein, e.g. at induction of apoptosis (29). Additionally, there are several sarcoma cases described with simultaneous P53 mutations and MDM2 overexpression suggesting that both may interfere with different pathways in tumorigenesis (7,12,30,31). v) This study demonstrates that *MDM2*-AS ODN therapy for STS transplanted tumors, without a functional wt-*P53* gene, can reduce tumor growth and the number of tumor nodules. To our knowledge, this is the first report showing an *in vivo* tumor inhibitory effect of *MDM2*-AS ODNs in a mutated *P53* background. In the other *in vivo* studies mentioned above, the same *MDM2*-AS ODN therapy was evaluated in tumor xenografts with different P53 status, and an induction of P53 expression, a delay in tumor growth/weight and a prolongation of animal survival was shown (15). Interestingly, the entire dose of *MDM2*-AS ODNs per animal in the reports of both Wang *et al* (19) and Tortora *et al* (15) was approximately 10 mg. We applied 100 or 200 µg of *MDM2*-AS ODNs per animal, suggesting that these ODNs are also very effective at lower doses, especially when administered continuously using osmotic minipumps.

As expected the detection rate of MDM2 protein by immunohistochemistry decreased in the *MDM2*-AS ODN treated groups when compared to the saline solution treated group, and this result was even more pronounced by Western blot analyses. Interestingly, concerning immunohistochemistry, the only antibody that provided a staining pattern was the one with C-terminal recognition epitope, whereas the antibody with the N-terminal recognition epitope did not stain any tumor even though positive controls were stained appropriately (results not shown). The biological relevance of this finding is supported by two other observations. In previous studies, we were able to show that positive staining was only detected with an antibody carrying a C-terminal epitope and that staining was correlated to a poor prognosis. Staining with another antibody with an N-terminal epitope demonstrated that there was no association between the staining pattern of this Ab and patient's outcome (32). Furthermore, Schlott *et al* (33) have described that a C-terminal MDM2 protein fragment was only present on the surface of tumor cells but not on the surface of normal hepatic cells, suggesting that C-terminal part of the MDM2 protein may play a specific role in tumorigenesis.

Somewhat surprising is the finding that a mt-P53 protein could not be detected using two P53 Abs (DO-1 and Pab-1801) in the *MDM2*-AS ODN treated groups in comparison to the *MDM2*-SE ODN and the saline solution treated groups, where it was moderate and strong, respectively. Binding of MDM2 protein to wild-type and mutant (mt) P53 has been reported (34), this suggests an increase in wt-P53 and mt-P53 after an *MDM2*-AS ODN treatment. However, for several tumor cell lines a stabilization of MDM2 protein by mt-P53 is reported (35,36). One of these cell lines carries the same *P53*-mutation as the RD cell line (codon 248K) (36). In this report we describe that a decrease in MDM2 protein causes a reduction in P53 protein, suggesting that mutant P53 could be stabilized by MDM2. A reduction in mutant P53 levels would be of interest, since about 50% of tumors carry *P53* mutations, and mutations can lead to the stabilization of P53 protein, which results in higher levels of expression of mutant compared to wild-type P53 (37,38). It is interesting that the biological relevance of antibody staining is dependent on the MDM2 epitope recognized by the applied antibody. In previous studies, we showed that P53 positive tumors detected by the DO-1 and Pab-1801 antibodies were associated with poor prognosis (32).

In conclusion, we have demonstrated that *MDM2*-AS ODNs can induce a reduction in tumor growth and tumor number in an STS tumor xenograft in a nude rat model independent of the P53-status. This finding supports the

suggestion, that *MDM2* AS treatment may exert their effects in all MDM2 expressing tumors regardless of P53-status (16). This observation may have a general importance in gene therapy of cancer.

Acknowledgements

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Subject Review

MDM2 and Its Splice Variant Messenger RNAs: Expression in Tumors and Down-Regulation Using Antisense Oligonucleotides

Frank Bartel,[1] Linda C. Harris,[2] Peter Würl,[3] and Helge Taubert[1]

[1]*Institute of Pathology, Faculty of Medicine, University of Halle-Wittenberg, Halle/Saale, Germany;* [2]*Department of Molecular Pharmacology, St. Jude Children's Research Hospital, Memphis TN; and* [3]*Surgical Clinic I, Faculty of Medicine, University of Ulm, Ulm, Germany*

Abstract

Alternative splicing has an important role in expanding protein diversity. An example of a gene with more than one transcript is the *MDM2* oncogene. To date, more than 40 different splice variants have been isolated from both tumor and normal tissues. Here, we review what is known about the alteration of *MDM2* mRNA expression, focusing on alternative splicing and potential functions of different MDM2 isoforms. We also discuss the progress that has been made in the development of antisense oligonucleotides targeted to MDM2 for use as a potential cancer therapy.

Introduction

MDM2[1] appears to play a role in many normal physiological and pathological pathways. It has been shown that MDM2 not only acts as an oncogene (1–3) but also displays growth-inhibitory functions (4, 5). MDM2 can also function independently of p53; for example, MDM2 interacts with transcription factors of the E2F family and the human TATA binding protein (6, 7) and inhibits retinoblastoma growth-regulatory function (8). A question remains whether the same MDM2 protein is responsible for the diverse functions reported or whether different isoforms or post-translationally modified MDM2 proteins could be involved. We examine here the different MDM2 variants that are likely translated from alternatively spliced *MDM2* pre-mRNAs and how antisense oligonucleotide (AS-ODN) therapies might be useful for down-regulating the expression of both full-length and alternatively spliced variants of *MDM2*. Down-regulation of full-length MDM2 expression in tumors that overexpress MDM2 would be predicted to result in p53 protein stability and sensitization to DNA-damaging agents (chemotherapeutic drugs and radiation) that act via p53-dependent pathways (Fig. 1). In addition, p53-independent functions of MDM2, such as in regulating the cell cycle, would also be abrogated, thereby restoring growth control in the tumor (Fig. 1). Progress has been made in the *in vitro* and *in vivo* uses of MDM2 antisense to down-regulate MDM2 expression.

Splicing of the *MDM2* Messenger RNA

Three human *MDM2* mRNA transcripts of 6.7, 4.7, and 1.9 kb long were reported by Pinkas *et al.* (9) in breast carcinoma cells, with the 1.9-kb mRNA having lost exon 12. In addition, several truncated MDM2 isoforms of 85, 76, and 57 kDa have been described in a panel of human breast carcinomas, together with the full-length 90-kDa protein (10). During the last few years, detailed expression analyses of the *MDM2* mRNA in various cancer types and in normal tissue have revealed alternative as well as aberrant splicing (Fig. 2; reviewed in Ref. 11). The tumor types investigated to date include ovarian and bladder cancer (12), glioblastomas (13), glioblastoma cell lines (14), breast carcinomas (15, 16), soft tissue sarcomas (17–19), giant cell tumors of the bone (20), and Hodgkin's lymphoma (21). The majority of the greater than 40 splice variants that have been detected to date lack sequences that encode at least part of the p53 binding domain, the nuclear localization and export sequences, the p300 binding domain, and the acidic domain. *In vitro* expression studies confirmed that the protein isoforms encoded by at least four of these splice variants (*MDM2*-A, -B, -C, and -D; 12) are unable to bind p53. The splice form *MDM2*-B, which is the most frequently detected transcript variant, has been described in various tumor types as well as in normal tissue, whereas many of the other variants have only been found in one particular cancer type.

A problem that has developed, however, is that some investigators have chosen to give different names to previously published isoforms, although most of the sequences of published splice variants are in Genbank. For example, the recently detected splice form *MDM2*-HD1 (accession no. AJ5505169) corresponds to the PM2 form (accession no. AJ278977), and another splice variant, *MDM2*-Del.G (15), which lacks the sequence between nucleotides 182 and 1432 of the coding region of the *MDM2* mRNA, exactly corresponds to the 219bp form

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Requests for reprints: Frank Bartel, Institute of Pathology, Faculty of Medicine, University of Halle-Wittenberg, D-06097 Halle/Saale, Germany. Phone: 49-345-557-4272; Fax: 49-345-557-1295. E-mail: frank.bartel@medizin.uni-halle.de
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[1]The abbreviations used are *MDM2*, human gene and oncogene; MDM2, human protein and isoform; *mdm2*, mouse gene; Mdm2, mouse protein.

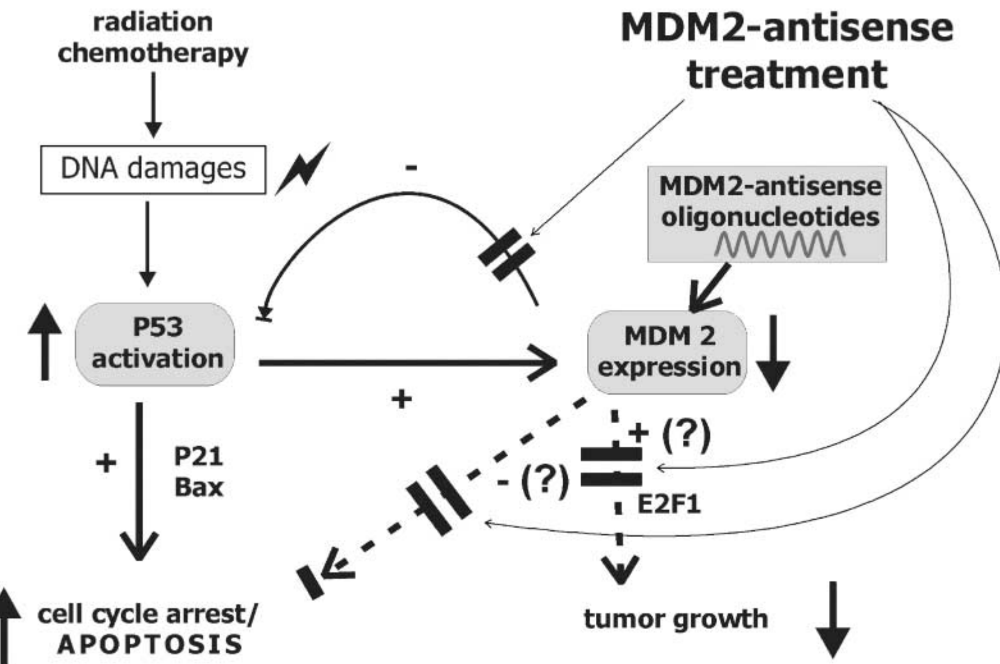

FIGURE 1. Proposed mechanism of action of MDM2-AS-ODNs (modified from Ref. 58). MDM2 and p53 form an autoregulatory feedback loop in which the MDM2 expression is up-regulated by p53 and MDM2 binds to p53, thereby promoting its degradation. In many tumors, however, MDM2 is overexpressed and can limit the effect of p53 activation by DNA-damaging drugs or radiation. By inhibiting the expression of MDM2 by targeting MDM2 mRNA with AS-ODNs, p53 should be activated to induce cell cycle arrest and/or apoptosis. In addition, it is predicted that MDM2-AS-ODN treatment will also inhibit p53-independent functions of MDM2.

described by Lukas *et al.* (16). This makes it difficult to determine the frequency of occurrence of specific isoforms and to compare the variants expressed within different tumor types.

A large proportion of the known *MDM2* transcripts is aberrantly spliced (*e.g.*, MDM2-PM2, -EU2, -KB3, and -219bp). These examples have a common splicing pattern that is illustrated in Fig. 3. Splicing occurs at cryptic splice donor and acceptor sites in regions with high sequence homology and that are present as many as four times within the coding region of the *MDM2* mRNA. For example, a 9-bp repeat sequence (AAGAGACCC in exon 3 and AAGAAACCC of exon 12) is involved in the splicing of the EU2 variant (a splice variant detected in soft tissue sarcomas; 17). Similar findings have been described by Hong and Li (22) for the retinitis pigmentosa GTPase regulator. Here, various portions of a purine-rich region were removed as introns. When analyzing the purine-rich sequences within the *RPGR* gene, several exonic splice enhancers that promote splicing through interaction with splicing factors were found, and Western blot analysis revealed many different sizes of RPGR proteins (22). Similar observations have also been made when MDM2 protein expression is evaluated in primary tumor samples (Bartel, unpublished observations) and in several normal tissues (23).

Given the fact that alternative splicing of the *Drosophila* Dscam pre-mRNA gives rise to over 38,000 transcripts (24), the splicing pattern of *MDM2* mRNA is still somewhat concise. However, the question remains as to why there are so many splice variants. We have described a mechanism by which a diverse collection of mRNAs can be generated from a single gene (Fig. 3), but it is unknown how many of the more than 40 splice variants described for *MDM2* are real and functionally relevant. It has been shown that many of the documented splicing errors can be caused by mutations in the genomic DNA, thereby creating new or destroying normal splice sites (25). Furthermore, mutations within binding sites of splicing regulatory proteins can cause "missplicing" (26, 27). However, whether this is also true for *MDM2* is not known. There are only a few reports describing analysis of mutations within the *MDM2* cDNA, but it is unknown whether these contribute to the diversity of the *MDM2* mRNA transcripts. Point mutations have been described in the zinc finger-encoding region of *MDM2* cDNA isolated from non-Hodgkin's lymphomas, leukemias, and hepatocellular carcinomas (28) and in other domains in liposarcomas (19). However, the mutation frequency of the *MDM2* gene in general is rather low, as other reports detected no mutations in the *MDM2* cDNA isolated from other tumor types (29–31).

The fact that some splice variants have been detected only in particular tumor types suggests that they might contribute to the transformed phenotype of these tumors, whereas others (*e.g.*, *MDM2*-A) may be associated with tumorigenesis in general. However, it is important to note that a considerable number of *MDM2* splice variants have been detected in normal tissues (23), demonstrating that MDM2 isoforms do not always possess oncogenic properties.

Functions of MDM2 Isoforms

There are several variants that share great structural homology (Fig. 2), suggesting that they may perform similar cellular functions. In addition, because multiple isoforms with

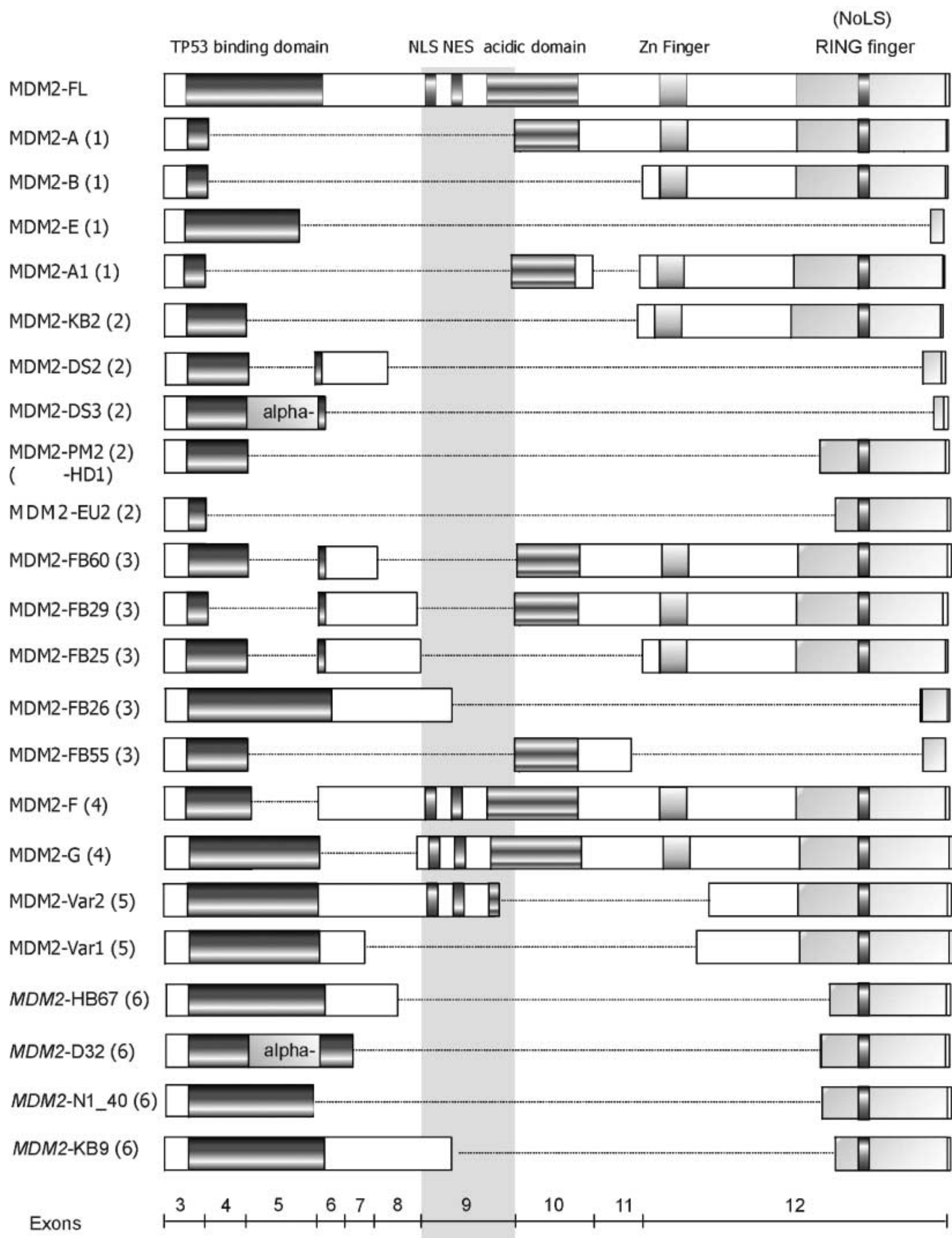

FIGURE 2. Summary of the most frequently expressed MDM2 splice variants and the domains that they encode. The sequence that is spliced out in most of the variants is highlighted with a *light gray box*. *Numbers in parentheses,* (*1*) Sigalas *et al.* (12), (*2*) Bartel *et al.* (17), (*3*) Bartel *et al.* (18), (*4*) Tamborini *et al*. (19), (*5*) Schlott *et al*. (59), and (*6*) Bartel *et al.* (unpublished observations).

similar sequences are often expressed within the same tumor (11), some isoforms are functionally redundant. Many examples of alternative splicing result in the region encoding the COOH terminus to be out of frame. This frame shift leads to the generation of novel amino acid sequences; however, because these new sequences differ in every case and are very short (only eight or nine amino acids), they are unlikely to contribute to a common novel function. However, it is not without precedent that these unique sequences could contribute to a gain of function as has been shown for MDMX-S, an isoform of the MDMX protein (32, 33). The MDMX-S isoform is characterized by a short unique amino acid sequence, which increases 12-fold the affinity of MDMX-S binding to p53 compared with MDMX (33).

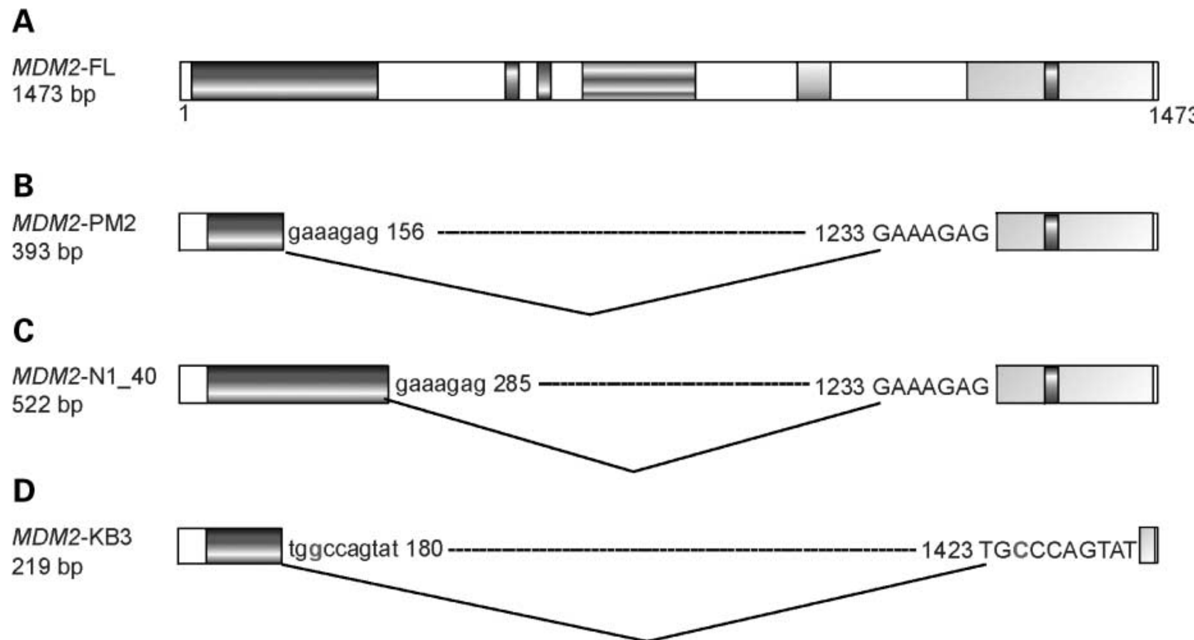

FIGURE 3. Mechanism of aberrant splicing at repetitive sequences. *MDM2-FL,* full-length MDM2; *capital letters,* sequence that is present in the splice variant. A detailed description of the splicing mechanism is given in the text.

Evans *et al.* (34) have shown that at least one splice variant (*MDM2*-B or *ALT1*) encodes a protein that binds full-length *MDM2*, resulting in sequestration of both proteins in the cytoplasm. This finding is important because alternatively and aberrantly spliced *MDM2* mRNAs are usually expressed together with full-length *MDM2* transcripts. *MDM2*-B is the most frequently expressed *MDM2* splice variant. It has been observed in numerous types of cancer, including ovarian and bladder cancers (12), breast cancer (13, 16), soft tissue sarcomas (17, 19), and giant cell tumors of the bone (20), but it also occurs in normal breast tissue (16).

Evans *et al.* (34) demonstrated that binding of *MDM2*-B to full-length MDM2 increased wild-type p53 activity, an observation that was also described by Dang *et al.* (35) with some of the murine variants. On binding of *MDM2* splice variants with an intact COOH-terminal RING finger domain to full-length MDM2 protein, p53 protein becomes stabilized, resulting in a growth-inhibitory phenotype.

A correlation between expression of *MDM2* splice variants and stabilization of wild-type p53 has also been described in glioblastoma cell lines despite an amplified *MDM2* gene (14). In soft tissue sarcomas, the expression of *MDM2* splice forms was also associated with an overexpression of mutant and wild-type p53 (17). These findings suggest that p53 accumulation arises as a consequence of alternative and aberrant *MDM2* splicing independent of the mutational status of p53. Although wild-type p53 overexpression induced by MDM2 isoforms is inconsistent with tumor progression, it is conceivable that the stabilization of mutant p53 might contribute to transformation and tumor growth. It is also possible that *MDM2* splice variant-mediated p53 activation could result in enhanced selective pressure to inactivate the p53 apoptotic pathway, thereby increasing accumulation of other genetic defects and promoting tumorigenesis. However, the association between *MDM2* splice variants and malignancy is controversial. Initial studies by Sigalas *et al.* (12) demonstrated that the expression of *MDM2* splice forms in transfected NIH3T3 cells could grow as colonies in soft agar. These findings have been supported by data from Steinman *et al.* (36) who demonstrated that *MDM2*-B, which is the most prevalent isoform identified in tumors, can cause tumors in a transgenic mouse model. Recent data from Fridman *et al.* (37) show that the murine equivalents of the human *MDM2*-B, -D, and -E splice forms, but not *MDM2*-A, significantly accelerated lymphomagenesis in an Eμ-*myc* transgenic mouse model. The lymphomas produced by the splice variants were aggressive and displayed a similar pathology to lymphomas expressing full-length *MDM2*. These data provide evidence that at least some MDM2 isoforms can contribute to tumor development in an *in vivo* mouse model. However, in contrast to a transforming function, *MDM2*-B is expressed in both normal and malignant mammary tissues (16).

Another aspect to be considered regarding a potential transforming function of the MDM2 isoforms is the amount and/or the translation efficiency of a given mRNA as well as the ratio of two or more splice variants. Because it is unknown under what conditions p53-independent functions of full-length *MDM2* may be active, it is impossible to predict the function of each splice variant in all the model systems evaluated. In many cases, MDM2 isoforms may not be functional and, therefore, would not affect the cell negatively. However, many of the proteins synthesized from alternatively and/or aberrantly spliced mRNAs can play either a transforming role or a "normal" physiological role dependent on the cell type. In addition, recent data from Bartl *et al.* (38) suggest a new RNA-based function of MDM2. The authors describe a 365-bp,

Table 1. Sequence of the Anti-MDM2 Oligonucleotides

Oligonucleotide	Sequence[a]	Target Sequence in Exon	Characteristic	References
Kondo	GACATGTTGGTATTGCACAT	Exon 3	Phosphorothioate oligonucleotide	(39–41)
HDMAS5	GATCACTCCCACCTTCAAGG	Exon 7	Phosphorothioate oligonucleotide	(42, 43)
Anti-MDM2-MBO	UGACACCTGTTCTCACUCAC	Exon 7	Mixed backbone oligonucleotide	(44–46, 48, 51, 55–57)

[a]Underlined sequences, 2′-*O*-methyl-RNA linkages between nucleotides.

alternatively spliced transcript that is composed of the first five *MDM2* exons and is highly stress inducible. It appears to be the major processing product of the *MDM2* mRNA both in normal and in cancer cells (38). Therefore, it seems likely that even transcript variants that are not translated fulfill a function as small non-mRNAs. Future studies are required to clearly define the functions of the many *MDM2* transcript variants and how their expression is controlled.

Down-Regulation of the *MDM2* Messenger RNA by AS-ODNs

The use of AS-ODNs targeted to different *MDM2* mRNAs may be a useful approach to evaluate the functions of alternatively spliced isoforms. In addition, MDM2 antisense designed to down-regulate splice variant and/or full-length *MDM2* mRNAs may be a useful anticancer therapeutic approach (Fig. 1). However, many of the MDM2-AS-ODNs reported to date were designed to target sequences not present within the majority of splice variants and therefore would only down-regulate full-length MDM2 expression. Here, we summarize current progress on the development of MDM2-AS-ODNs as a potential anticancer therapy.

The first study to use MDM2-AS-ODNs (Table 1) to down-regulate *MDM2* expression was published by Kondo *et al.* (39). It was shown that glioblastoma U87-MG cells were more susceptible to cisplatin-induced apoptosis when the cells were cotreated with cisplatin and MDM2-AS-ODNs that targeted the first 20 nucleotides of the open reading frame. A decrease of the MDM2 protein level could be observed, although p53 protein levels remained constant. These findings (39) suggest an important role for MDM2 in the development of resistance to cisplatin. Teoh *et al.* (40) studied the effect of the same oligonucleotide in a series of multiple myeloma cell lines. Treatment with MDM2-AS-ODNs resulted in decreased DNA synthesis and cell viability as well as a G_1 cell cycle arrest associated with pRb-E2F1 binding. Furthermore, apoptosis was induced in the AS-ODN-treated cells (40). In another study using the same MDM2-AS-ODNs described by Kondo *et al.* (39), we observed decreased MDM2 protein expression 24 h after antisense treatment and an 80% decrease in the colony-forming ability of MDM2-AS-ODN–treated undifferentiated sarcoma cells (US8/93) compared with control cells that received a scrambled control oligonucleotide (41). Although *MDM2* splice variants were not evaluated during these studies, the MDM2-AS-ODN used had the potential to down-regulate many of the common splice forms

Table 2. Summary of Published *in Vivo* Applications of Anti-MDM2 Oligonucleotides

Tumor Type	Cell Line	Animal Model	p53 Gene Status	Oligonucleotide, Amount, Schedule	Drugs	Effects of Cotreatment	References
Prostate cancer	DU-145, PC-3	Mice	ND[a]	Anti-MDM2-MBO, 25 mg/kg/day, 4 × 5 days/wk	Irinotecan, paclitaxel, rituxan	Synergistic effect (+ irinotecan) slightly increased activity (+ paclitaxel, rituxan)	(56)
Colon cancer	LS174T	Mice	Wild-type	Anti-MDM2-MBO, 20 mg/kg/day, 5 days/wk	10-Hydroxy-camphothecin, 5-fluorouracil	Synergistically or additive therapeutic effects	(46, 57)
	DLD-1		Mutant				
Glioblastoma multiforme	U87-MG	Mice	Wild-type	Anti-MDM2-MBO, 25 mg/kg/day, 5 days/wk	Irinotecan, paclitaxel	Inhibition of tumor growth, 39- and 63-fold activity of irinotecan and paclitaxel	(48)
Rhabdomyosarcoma	RD	Rat	Mutant	Anti-MDM2-MBO, 100 μg continuously over 1 wk	–	Significantly reduced tumor growth, decreased mutant p53 levels	(51)
Breast cancer	MCF-7	Mice	Wild-type	Anti-MDM2-MBO, 25 mg/kg/day, 3 × 5 days/wk	Irinotecan, paclitaxel, 5-fluorouracil	Synergistically or additive therapeutic effects	(47)
	MDA-MB-468		Mutant				
Colon cancer	GEO	Mice	ND	Anti-MDM2-MBO, 10 mg/kg/day, 2 × 5 days/wk	Cisplatin, topotecan	Potentiation of effects of cisplatin, topotecan	(45)
Osteosarcoma	SJSA	Mice	ND	Anti-MDM2-MBO, 25 mg/kg/day	10-Hydroxy-camphothecin, Adriamycin	Synergistic effect of cotreatmant	(44)
Choriocarcinoma	JAR	Mice	ND	Anti-MDM2-MBO, 25 mg/kg/day	10-Hydroxy-camphothecin, Adriamycin	Synergistic effect of cotreatmant	(44)

[a]ND, not determined

(Fig. 2) in addition to full-length MDM2. However, because splice variant expression was not analyzed during these studies, it is not possible evaluate how potential changes in splice variant expression may have influenced the observed results.

Chen *et al.* (42) investigated the effects of improved phosphorothioate MDM2-AS-ODNs in JAR, SJSA, and MCF-7 cells. The MDM2 protein level was reduced up to 5-fold by using an oligonucleotide termed HDMAS5-ODN (Table 1), resulting in activation of p53 (42). This oligonucleotide was directed against a sequence within exon 7 of the MDM2 mRNA, a region that is omitted in many splice variants. Similar results were observed by Sato *et al.* (43) in osteosarcoma U2-OS cells treated with HDMAS5-ODN in combination with DNA-damaging drugs such as mitomycin C and cisplatin. The drug-mediated cell killing were even more pronounced when the expression of both MDM2 and p21 was blocked by antisense transfection (43). Encouraging *in vitro* results of MDM2-AS-ODN treatment led to the development of xenograft models of different tumors. These xenograft models include osteosarcoma SJSA cells and choriocarcinoma JAR cells (44), colon cancer GEO cells (45), LS174T and DLD-1 cells (46), breast cancer MCF-7 and MDA-MB-468 cells (47), several glioblastoma multiforme cell lines (48), and prostate cancer cell lines (49). In these studies, the human tumor cells were injected into the inguinal area, grown to xenografts, and subsequently treated with either an anti-MDM2 mixed backbone oligonucleotide (anti-MDM2-MBO; Table 1; 44) alone or in combination with chemotherapeutic drugs such as irinotecan, paclitaxel, 5-fluorouracil, or radiation (Table 2; 47–51). MDM2-MBO is another oligonucleotide targeted to exon 7 that is deleted in most splice variants (Fig. 2). In most studies, the MDM2-AS-ODNs were given by i.p. injection at 5 consecutive days. Compared with untreated animals, treatment of the respective xenografts with MDM2-AS-ODNs led to significant antitumor activity in terms of slowed tumor growth and prolonged survival that was attributed to inhibition of MDM2 expression. In cell lines with wild-type p53 such as MCF-7 (47) and U87-MG (48), these antitumor effects were accompanied by elevated levels and increased activity of wild-type p53, whereas in cell lines with mutant p53 such as MDA-MB-468 (47) and T98G (48), the p53 protein levels remained unaffected. In contrast, treatment of an orthotopic xenograft model of rhabdomyosarcoma RD cells (p53mt/−) in the peritoneum of nude rats with MDM2-AS-ODNs resulted in decreased levels of both MDM2 and mutant p53 (51). In general, synergistic effects were observed when cells or xenografts were treated with a combination of MDM2-AS-ODNs and the respective drug or radiation compared with the treatment with either of these agents alone (47–50). However, combination of irinotecan and a MDM2 mismatch control oligonucleotide had the similar effect as the combination of irinotecan and MDM2-AS-ODNs (47–49). The authors speculated that treatment with oligonucleotides, in general, increases the uptake of the active metabolite of irinotecan, SN-38 (46).

Conclusions

It has been shown that MDM2 not only acts as an oncogene but also displays growth-inhibitory functions. However, it is currently unclear how these different functions are controlled or influenced by expression of alternatively spliced isoforms of MDM2. The use of AS-ODNs to inhibit the expression of genes of the p53-MDM2 pathway has the potential to become an exciting new cancer therapy (52, 53). MDM2-AS-ODNs specifically inhibit MDM2 expression, and they employ their antitumor activity via different mechanisms, regardless of p53 status. This is particularly noteworthy given the high mutation frequency of the p53 gene in human cancer (54). The activity of conventional drugs or radiation can be synergistically enhanced if given in combination with MDM2-AS-ODNs, and the effects appear to be independent of p53 gene status or the different MDM2 isoforms that might be expressed. In light of the current findings, effective cancer therapy may involve inhibiting MDM2 expression, in addition to blocking binding of MDM2 to p53, to inhibit all the growth-promoting activities of MDM2. However, whether the AS-ODNs influence expression of any MDM2 splice variants is currently unknown. As specific splice variants have now been shown to play a role in tumorigenesis, knowing whether they are down-regulated together with full-length MDM2 will be important for interpretation of future data.

Acknowledgments

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