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Prognoseparameter bei soliden Tumoren und Systemerkrankungen

Dissertation

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vorgelegt von

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Erklärung

Hiermit versichere ich, Anke Meyer (geb. Schultze; 25.09.1980), dass für das Verfassen der vorliegenden Dissertation „Prognoseparameter bei soliden Tumoren und Systemerkrankungen“ folgende drei Aussagen zutreffen:

1. Ich habe die Arbeit ohne unerlaubte fremde Hilfe angefertigt.
2. Ich habe keine anderen als die von mir angegebenen Quellen und Hilfsmittel benutzt.
3. Ich habe die den benutzten Werken wörtlich oder inhaltlich entnommenen Stellen als solche kenntlich gemacht.

Bremen im Mai 2010

……

Anke Meyer

„Inmitten der Schwierigkeiten liegt die Möglichkeit.“

Albert Einstein

I Inhaltsverzeichnis

II Abkürzungsverzeichnis

°C	Grad Celsius
A	Adenin
AAD	Akute Aortendissektion
AD	Aortendissektion
AG	Aortengewebe
AC	Adenocarcinoma
ADP	Adenosin Diphosphat
BCA	Bicinchoninic acid
BGG	Bovine Gamma Globulin
bp	Basenpaare
CD20	Cluster of Differentiation 20
cDNA	complementary DNA
CREB	cAMP responsive element binding
CRTC1	CREB-regulated transcriptional coactivator 1
CT	Computertomographie
DNA	Desoxyribonucleic acid
E2F1	E-box binding 2 transcription factor 1
ELISA	Enzyme Linked Immunosorbent Assay
EMT	Epitheliale Mesenchymale Transition
ER	Estrogen receptor
ERαS305	Estrogen receptor alpha at serine 305
f	Feminin
FFPE	Formalin fixiert und in Paraffin eingebettet
FISH	Floureszenz-in-situ-Hybridisierung
FKS	Fetales Kälberserum
FNA	Feinnadelaspiration
FV	Follikläre Variante
g	Gravitationsbeschleunigung
h	Hour
H2AX	Histon 2A Variante X
Her-2	Human epidermal growth factor receptor 2
HFPE	HOPE fixiertert und in Paraffin eingebettet
HMG	High mobility group

HMGA	High mobility group protein A
HMGB	High mobility group protein B
HMGN	High mobility group protein N
HOPE	Hepes-Glutamic acid buffer mediated Organic solvent Protection Effect
Hz	Hertz
i	Insullär
IHC	Immunhistochemie
IL-1	Interleukin 1
IL-6	Interleukin 6
IL-8	Interleukin 8
kDa	Kilodalton
kb	Kilobasenpaare
let-7	Letal-7
LPP	LIM domain containing preferred translocation partner
m	maskulin
MAML2	mastermind-like 2
MAP	Mitogen activated protein
MEC	Mucoepidermoid Carcinoma
mg, ml, mm, mM	Milli -gram, -liter, -meter, -mol
µg, µl, µm, µM	Micro -gram, -liter, -meter, -molar
MI	Minimal invasiv
min	Minute
miRNA	Micro RNA
MOPS	3-N-Morpholinopropansulfonsäure
MRT	Magnetresonanztomographie
NADPH	Nicotinsäureamid-Adenin-Dinukleotid-Phosphat
NF-κB	Nuclear factor 'kappa-light-chain-enhancer' of activated B-cells
NHL	Non-Hodgkin Lymphom
NSCLC	Non small cell lung cancer
o	Oncocytär
ORF	Open reading frame
p53	Protein 53
PAI-1	Plasminogen activator inhibitor 1

PBS	Phosphate buffered saline
PCR	Polymerase Chain Reaction
PDGF	Platelet-derived growth factor
PR	Progesterone receptor
RA	Rheumatoide Arthritis
RAGE	Receptor for advanced glycation end products
RNA	Ribonucleic acid (Ribonukleinsäure)
RIPA	Radioimmunoprecipitation Assay
ROC	Reciever operation curve
RT	Raumtemperatur
-RT	Ohne reverse Transkriptase
s	Sekunde
SAP/JNK	Stress-activated phosphor/ c-Jun-N-terminal kinases
SCC	Squamous cell carcinoma
SCN	Struma colloides nodosa
SLE	Systemischer Lupus erythematodes
T	Thymin
t	Trabekulär
TAE	Tris-Acetat-Ethylendiamintetraacetat
TBP	TATA-Box binding Protein
TNF α	Tumor necrosis factor α
TNM	Tumor Nodes Metastases
TLR2	Toll-like receptor 2
TLR4	Toll-like receptor 4
TLR9	Toll-like receptor 9
UNG	Uracil-N-Glykosylase
UG	Unauffälliges Gewebe
UL	Uterusleiomyom
uPA	Urokinase-Typ Plasminogen Aktivator
UTR	Untranslated region

III Abbildungsverzeichnis

1 Einleitung

1.1 Tumordiagnostik

Weltweit wird jedes Jahr bei elf Millionen neuen Patienten die Diagnose Krebs gestellt. In jedem Jahr sterben 6,7 Millionen Menschen infolge ihrer Krebserkrankung, wobei die Tendenz steigt. Diese Zahlen wurden von Parkin et al. im Rahmen einer statistischen Erhebung im Jahr 2002 ermittelt, bei der die Fallzahlen der 25 weltweit am stärksten verbreiteten Tumorarten, zusammengefasst worden sind (Abbildung 1) (Parkin et al., 2005).

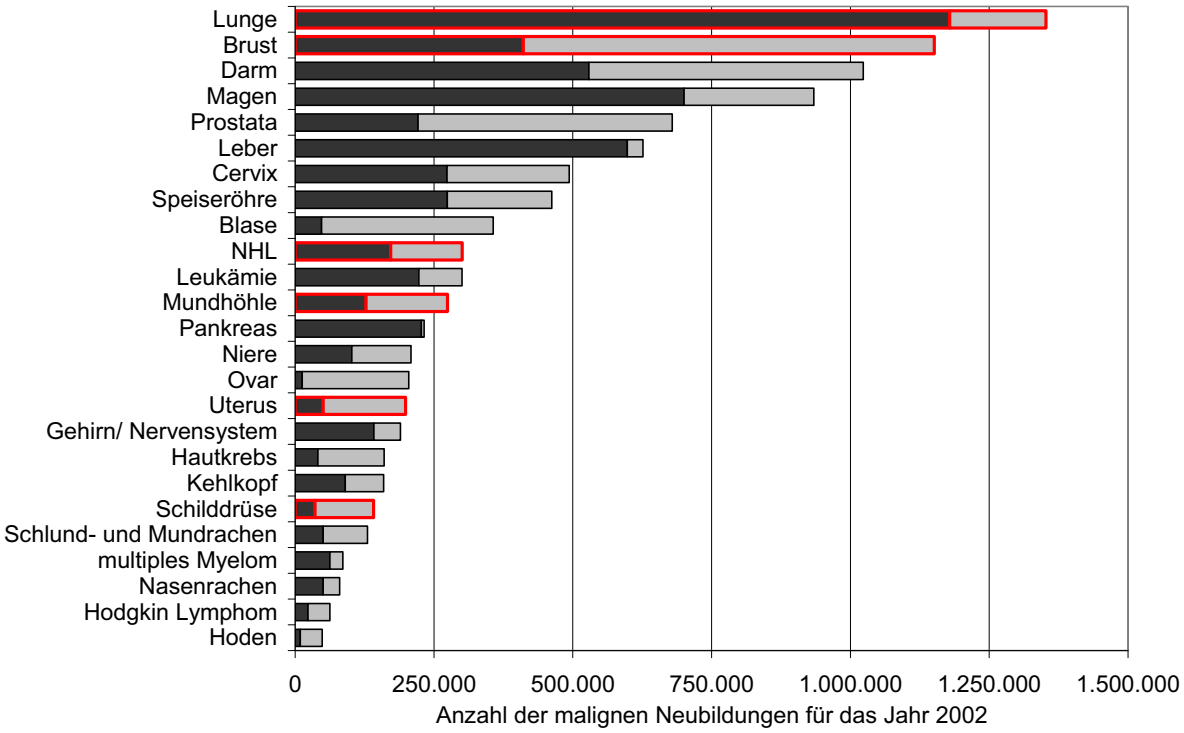

Abbildung 1: Grafische Umsetzung der von Parkin et al. 2005 veröffentlichten Daten zur statistischen Verteilung von malignen Neubildungen. Grau sind die Anzahl der Neubildungen für das Jahr 2002 und in schwarz die Anzahl der verstorbenen Patienten für die jeweilig betroffenen Gewebebereiche im Jahr 2002 abgebildet. Mit rotem Rahmen dargestellt sind in dieser Dissertation näher betrachtete Tumorentitäten.

Aufgrund der bisher entwickelten Diagnose- und Therapiemöglichkeiten ist die Überlebensdauer bei vielen Patientenentitäten deutlich verbessert worden. Dennoch unterliegt die Diagnostik häufig Limitationen. Werden Tumoren nicht rechtzeitig erkannt und richtig klassifiziert, kann einer Vielzahl von Patienten nicht oder nicht optimal geholfen werden (Duffy, 2005; Lang et al., 2005).

Infolge dieser Problemstellung kommt es einerseits dazu, dass nicht alle Tumoren und ihre Rezidive in einem Stadium diagnostiziert werden, in dem sie therapierbar sind, andererseits werden Patienten häufig mit einer stärkeren Medikation behandelt

als es nötig wäre (Baloch & LiVolsi, 2002; LiVolsi, 2003; Suster, 2006). Als anspruchsvoll gestaltet sich beispielsweise die Unterteilung von follikulären Schilddrüsenveränderungen in benigne und maligne Aberrationen.

Aus diesen Gründen ist es notwendig, sich mit weiteren Strategien bezüglich Diagnose, Therapie und Prognose zu beschäftigen. Hierbei rücken, zusätzlich zu den etablierten Diagnosemethoden, verstärkt molekulare Biomarker in den Fokus des wissenschaftlichen, wirtschaftlichen und gesellschaftlichen Interesses (Esserman et al., 2007).

Ansatzpunkte für den Einsatz molekularer Biomarker reichen von Diagnose- über Prognoseparameter (Langelotz et al., 2003) bis hin zur Entwicklung neuer Behandlungsstrategien. Diese werden zunächst im Tiermodell (Scala et al., 2000), dann anhand von humanen Zellen (Pentimalli et al., 2003) und anschließend an realen Patienten (Boo et al., 2005) erprobt. Für eine Vielzahl von Tumorentitäten gibt es bei rechtzeitiger Erkennung und Klassifizierung des Tumors, signifikant bessere Optionen für eine erfolgreiche Behandlung, als bei bisherigem Kenntnisstand.

1.2 High mobility group Proteine

Die Gruppe der High mobility group (HMG) Proteine und deren Gene sind in der Tumordiagnostik mit hohem Potenzial angesehen. Die in den 1970er Jahren von Godwin et al. erstmals beschriebenen HMG Proteine werden aufgrund ihrer unterschiedlichen Sequenzmotive in drei Familien (HMGA, HMGB, HMGN) eingeteilt (Goodwin et al., 1973). Der letzte Buchstabe der Proteinfamilie ergibt sich dabei aus der jeweiligen DNA-Bindungsdomäne, bei der es sich um eine AT-Hook-Domäne (HMGA), eine HMG1-Box (HMGB) oder eine nukleosomale Bindungsdomäne (HMGN) handeln kann (Bustin & Reeves, 1996; Bustin, 2001). Diese Chromatin-assoziierten Nicht-Histon-Proteine üben als sogenannte architektonische Transkriptionsfaktoren u.a. einen Einfluss auf die Genregulation aus. HMG Proteine sind in der Lage, die Bindungsaffinität weiterer Transkriptionsfaktoren an die DNA zu erhöhen oder zu inhibieren. Dies erreichen sie über strukturelle Veränderungen (Bustin & Reeves, 1996) wie z.B. Krümmung, Streckung (Grosschedl et al., 1994) oder Entwindung (Javaherian et al., 1978) der Chromatinorganisation bzw. der DNA. Die HMG Proteine sind somit indirekt an der Regulation einer Vielzahl von Genen und zellulären Prozessen beteiligt, auf die in den folgenden Kapiteln detaillierter eingegangen wird.

1.3 HMGA2

Ein wesentlicher Fokus der vorliegenden Arbeit befasst sich mit der HMGA2 Proteinuntergruppe, da vorherige Studien signifikante Zusammenhänge zwischen der Expressionsstärke dieser Proteine und dem Grad der Malignität verschiedener Tumorentitäten, wie z.B. Mammakarzinomen (Rogalla et al., 1997), Lungenkarzinomen (Sarhadi et al., 2006), Plattenepithelkarzinomen der Mundhöhle (Miyazawa et al., 2004) und Schilddrüsenneoplasien (Chiappetta et al., 1995) nachweisen konnten. Die Expression von HMGA2 Proteinen in transgenen Mäusen führte darüber hinaus zur Ausbildung multipler Tumoren u.a. in der Hirnanhangsdrüse, den Milchdrüsen und der Niere (Fedele et al., 2002). Eine Inhibierung der *HMGA2* Genexpression hingegen resultierte in ausbleibender maligner Transformation von Schilddrüsenzellen *in vitro* (Berlingieri et al., 1995).

Das in der chromosomalen Region 12q13-15 lokalisierte (Schoenmakers et al., 1995), aus fünf Exons bestehende *HMGA2* umspannt genomisch einen Bereich von 142 kb und besitzt mindestens 2 Splicevarianten: *HMGA2a* und *HMGA2b* (Chau et al., 1995). Das hieraus resultierende HMGA2 Protein besteht aus 320 Aminosäuren und kann sowohl mit der DNA (Reeves & Nissen, 1990) als auch mit Proteinen (Vallone et al., 1997) Wechselwirkungen eingehen. Hierbei interagieren die HMGA2 Proteine über ihre AT-Hook Domäne mit geringer Sequenzspezifität mit der kleinen Furche AT-reicher Sequenzen von in B-Form vorliegender genomischer DNA (Reeves & Nissen, 1990). Dies ermöglicht es dem HMGA2 Protein, sehr flexibel mit einer Vielzahl von Strukturen zu interagieren und so Einfluss auf verschiedene zelluläre Funktionen zu nehmen (vgl. Abbildung 2). Hierzu zählen neben der Transkriptionsregulierung (Ashar et al., 1995), die RNA Prozessierung (Sgarra et al., 2005), die DNA Reparatur (Borrmann et al., 2003), die Epitheliale Mesenchymale Transition (EMT) (Thuault et al., 2006), Zellwachstum und -differenzierung sowie die Organisation des Chromatins (Reeves, 2001), die Zellzykluskontrolle (Tessari et al., 2003), die Steuerung der neoplastischen Transformation (Berlingieri et al., 1995) und der Apoptose (Fedele et al., 2001).

Posttranslational finden Modifikationen wie Phosphorylierung, Acetylierung, Methylierung und Poly-ADP-Ribosylierung statt, welche dem HMGA2 Protein eine Vielzahl von Interaktionsmöglichkeiten eröffnen (Reeves, 2001; Reeves & Beckerbauer, 2001). Ein Einfluss einer hohen *HMGA2*-Expression wird in Zusammenhang mit ei-

nem verstärkten Ansprechen auf eine Doxorubicin basierende Therapie diskutiert, deren Wirkung auf der der verstärkten Apoptose sowie der Phosphorylierung von Histon 2A Variante X (H2AX) Ser139 basiert (Boo et al., 2005).

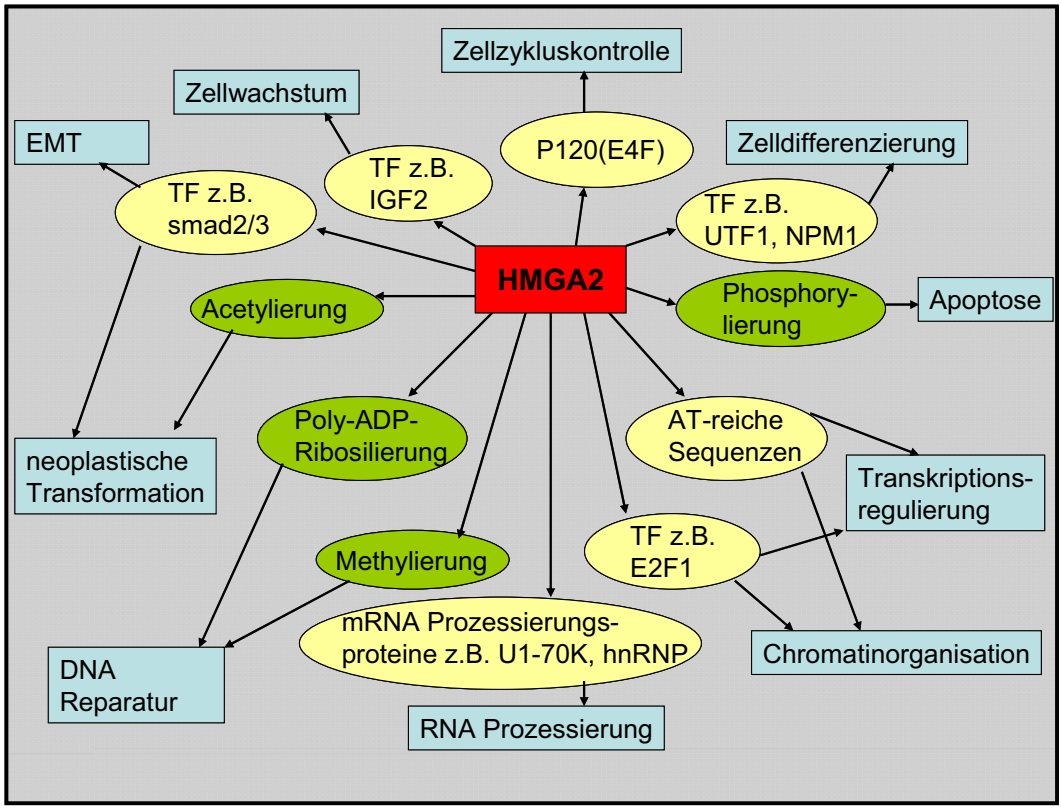

Abbildung 2: Darstellung der im Text näher erläuterten Reaktionswege (grün) und Interaktionspartner (gelb) von HMGA2 sowie deren Funktion (blau).

Weiterhin kann HMGA2 über den Weg der Acetylierung den Transkriptionsfaktor E2F1 aktivieren, welcher in seiner aktiven Form eine neoplastische Transformation der Zelle induzieren kann (Johnson et al., 1994; Fedele et al., 2006). Methylierung (Martens et al., 2005; Agrawal et al., 2007) und Poly-ADP-Ribosilierung (Giancotti et al., 1996; Hassa et al., 2006) von HMGA2 können zu einer geringeren Reparaturrate der DNA, einem niedrigeren Level an Tumor Supressor Genen und gestörten inter- und intrazellulären Reaktionswegen führen, was auf eine starke Involvierung des HMGA2 Proteins in die Tumorentwicklung hinweist.

Die Expression des *HMGA2*-Gens ist während der Embryonalentwicklung und in stark proliferierenden Zellen sehr ausgeprägt, während sie in ausdifferenzierten adulten Zellen nur schwach oder gar nicht vorhanden ist (Zhou et al., 1995; Chiappetta et al., 1996; Meyer et al., 2007).

Auffällig an der Struktur des *HMGA2*-Gens ist das mit 140 kb vergleichsweise große Intron zwischen dem dritten und vierten Exon. Bereits Schoenmakers et al.

beschrieben eine auffällig häufige Bruchfrequenz in dieser Region (Schoenmakers et al., 1995). Chromosomale Rearrangierungen, welche das *HMGA2*-Gen betreffen, haben oftmals in dieser Region einen Ansatzpunkt. Chromosomale Aberrationen resultieren in benignen Veränderungen, wie z.B. für Uterusleiomyome (Heim et al., 1988), Fibroadenome der Brust (Staats et al., 1996), chondroiden Hamartome der Lunge (Kazmierczak et al., 1996) und Lipome (Ashar et al., 1995) beschrieben (Schoenmakers et al., 1995). Die hierbei entstehenden Fusionstranskripte und der einhergehende Verlust der Genexpressionskontrolle führen zu einer unkontrollierten Proliferation (Borrmann et al., 2001; Lee & Dutta, 2007). Bisher genauer analysiert ist das *HMGA2-LIM domain containing preferred translocation partner (LPP)* Transkript, welches zu einer malignen Transformation von Fibroblasten (genauer NIH 3T3 Zellen) führt, die durch den Wildtyp *HMGA2* nicht induziert wird (Fedele et al., 1998).

Einen eindeutigen Hinweis auf die Bedeutung von HMGA2 in Bezug auf Wachstum und Entwicklung liefert die Untersuchung eines 8-jährigen Jungen. Er weist eine perizentrische Inversion auf Chromosom 12 (Bruchpunkte: p11.22 und q14.3) auf, welcher den Bereich des *HMGA2*-Gens mit einschließt und zu einer verstärkten Expression von *HMGA2* führt (Ligon et al., 2005). Der Junge zeigt neben einem verstärkten Wachstum eine Vielzahl benigner Tumoren, was in ähnlicher Weise auch bereits bei Mäusen, welche eine trunkierte Variante des *HMGA2*-Gens exprimieren, gezeigt werden konnte (Battista et al., 1999; Fedele et al., 2002; Zaidi et al., 2006). Eine Deletion des *HMGA2*-Gens führt zu stark vermindertem Wachstum von Mäusen und unterstreicht die Bedeutung des *HMGA2*-Gens (Zhou et al., 1995).

Bei malignen Tumoren hingegen wird von einem Genregulationsmechanismus ausgegangen, welcher die veränderte *HMGA2*-Expression initial induziert, da in diesen Fällen keine durchgehende chromosomale Veränderung ursächlich ist und der Wildtyp keine maligne Transformation auslöst. Über diesen Mechanismus ist bisher jedoch nur wenig bekannt (Fusco & Fedele, 2007). Eine erhöhte *HMGA2*-Expression konnte beispielsweise in Tumoren der Lunge (Sarhadi et al., 2006), des Magens (Yang et al., 2007b), der Bauchspeicheldrüse (Abe et al., 2003), sowie in der Mundhöhle (Miyazawa et al., 2004) nachgewiesen werden.

Im Rahmen der vorliegenden Arbeit sind sowohl benigne als auch maligne Tumorentitäten auf deren *HMGA2* Genexpression hin untersucht worden. Neben dem *HMGA2*-Gen bzw. dem Protein, wurden vergleichend Analysen zu bisher bekannten Parametern tumorspezifisch durchgeführt.

1.4 HMGB1

Der zweite Fokus dieser Arbeit liegt auf dem *HMGB1*-Gen sowie dem HMGB1 Protein. HMGB1 wird im Gegensatz zu den HMGA2 Proteinen ubiquitär exprimiert (Johns, 1982) und tritt sowohl intrazellulär (Johns, 1982) als auch extrazellulär (Hori et al., 1995) auf. Das in der chromosomalen Region 13q12-13 lokalisierte Gen (Ferrari et al., 1996) besteht aus fünf Exons und kodiert für ein ca. 24,9 kDa großes Protein mit zwei DNA bindenden Domänen, der A- und der B-Box (Reeck et al., 1982). Eine unterschiedliche Expression wurde in verschiedenen Differenzierungsstadien der Zelle beschrieben (Falciola et al., 1997).

In das Blickfeld der Tumordiagnostik ist das Gen gerückt, da in verschiedenen Tumoren (unter anderem in Mammakarzinomen (Flohr et al., 2001), Colonkarzinomen (Xiang et al., 1997; Volp et al., 2006) und in Leukämiezellen (Cabart et al., 1995) verstärkte *HMGB1* Expressionen detektiert worden sind. Das HMGB1 Protein hat neben seiner intrazellulären Funktion als Transkriptionsregulator extrazelluläre Funktionen und wird hier als Zytokin-ähnlicher Faktor beschrieben (Czura et al., 2001; Yang et al., 2001; Yang et al., 2005). Extrazelluläres HMGB1 spielt unter anderem eine Rolle bei entzündlichen Prozessen (Andersson et al., 2000; Scaffidi et al., 2002), Angiogenese (Schlueter et al., 2005; van Beijnum et al., 2006), Zelldifferenzierung (Passalacqua et al., 1998), Apoptose (Huttunen et al., 2000; Kuniyasu et al., 2005), Stammzell- und Zellmigration (Palumbo & Bianchi, 2004; Palumbo et al., 2004; Rouhiainen et al., 2004) sowie bei der Metastasierung (Fages et al., 2000; Liotta & Clair, 2000; Taguchi et al., 2000).

Das intrazelluläre HMGB1 beeinflusst durch die beschriebenen HMG Interaktionen mit DNA und Proteinen verschiedene Gene und Reaktionskaskaden (z.B. *p53* (Jayaraman et al., 1998), Steroidhormone (Boonyaratanakornkit et al., 1998), HOX-Proteine (Zappavigna et al., 1996) und TATA-Box binding Protein (TBP) (Sutrias-Grau et al., 1999)). Weiterhin bindet das HMGB1 Protein an irreguläre DNA Strukturen wie z.B. four way junctions (Bianchi et al., 1989; Bianchi et al., 1992), Cisplatin modifizierte DNA (Hughes et al., 1992; Pil & Lippard, 1992; Imamura et al., 2001)

und durch UV-Licht geschädigte DNA (Pasheva et al., 1998). Durch seine Interaktionen mit defekten sowie zu reparierenden DNA Strukturen wird die wegweisende Bedeutung des intrazellulären HMGB1 Proteins deutlich hervorgehoben.

In seiner aktiven Form wird das HMGB1 Protein von Astrocyten (Passalacqua et al., 1998) sowie Makrophagen und Monozyten (Wang et al., 1999) sezerniert. Hierbei werden verschiedene Wege unterschieden. Das HMGB1 Protein kann beispielsweise aktiv phosphoryliert (Youn & Shin, 2006) oder acetyliert (Sterner et al., 1979; Scaffidi et al., 2002; Bonaldi et al., 2003) werden. Durch die Änderung der Ladung kann das HMGB1 Protein die Bindung an das Chromatin verlieren und als zu transportierendes Protein erkannt werden (Gardella et al., 2002; Bonaldi et al., 2003) oder seine Bindungseigenschaften an auffällige DNA-Strukturen verändern (Ugrinova et al., 2001).

HMGB1 wird jedoch auch passiv, unter anderem durch den Verlust der Bindung zum Chromatin, durch nekrotisierte Zellen sowie spontan zerstörte Zellen entlassen (Scaffidi et al., 2002). Ob durch mittels Apoptose zerstörte Zellen gezielt HMGB1 freigesetzen, wird für verschiedene Zelltypen kontrovers diskutiert. Zunächst wurde von Scaffidi et al. belegt, dass apoptotische Zellen kein HMGB1 freisetzen (Scaffidi et al., 2002), im Anschluss konnten Bell et al. für Jurkat Zellen das Gegenteil beweisen (Bell et al., 2006).

Das extrazellulär vorliegende HMGB1 bindet vorwiegend mit der B-Box (Li et al., 2003) an RAGE (Receptor for Advanced Glycation End products) Rezeptoren (Hori et al., 1995) sowie an Toll-like Rezeptoren 2, 4 und 9 (TLR2, TLR4 und TLR9) (Park et al., 2004; Ivanov et al., 2007). Durch die Bindung an den transmembranen RAGE Rezeptor wird dieser aktiviert und setzt unter anderem den Platelet-derived growth factor (PDGF) (He et al., 2007) und proinflammatorische Zytokine, wie Interleukin-8 (IL-8) und Tumor Nekrose Faktor α (TNF α) (Fiuza et al., 2003) frei. Dadurch werden über verschiedene Kinasen wie z.B. p44/42, p38 und SAP/JNK MAP (Liotta & Clair, 2000) bedeutsame Signalwege, welche eine große Rolle bei der Tumor- sowie Metastasenbildung spielen, induziert (Taguchi et al., 2000).

Darüber hinaus bindet HMGB1 extrazellulär an die Rezeptoren TLR2, TLR4 und TLR9, was zu einer verstärkten Expression von *nuclear factor 'kappa-light-chain-enhancer' of activated B-cells (NF-κB)* führt (Park et al., 2004; Park et al., 2006; Ivanov et al., 2007).

Eine HMGB1-Expression über dem Grundniveau induziert eine erhöhte Ausschüttung von proinflammatorischen Zytokinen wie IL-1, IL-6, IL-8 und TNF (Andersson et al., 2000; Wang et al., 2004; Tian et al., 2007). Zusätzlich ist eine Aktivierung der NADPH Oxidase, eingeleitet über eine Bindung von HMGB1 an TLR4, beschrieben, die bei der Immunantwort auf zerstörtes Gewebe einen entscheidenden Einfluss hat (Fan et al., 2007).

Im Hinblick auf das Tumorwachstum ist der parakrine Einfluss von HMGB1 auf Gewebeverbände von großer Bedeutung, da es zur Migration von verschiedenen Zellen z.B. Mesangioblasten (Palumbo et al., 2004), Monozyten (Rouhiainen et al., 2004), Neuriten (Hori et al., 1995) bzw. im speziellen dendritischen Zellen (Yang et al., 2007a) und zur Bildung und Einwanderung von versorgenden Blutgefäßen in Gewebe sowie Tumoren kommt (Schlueter et al., 2005; Mitola et al., 2006) (Abbildung 3).

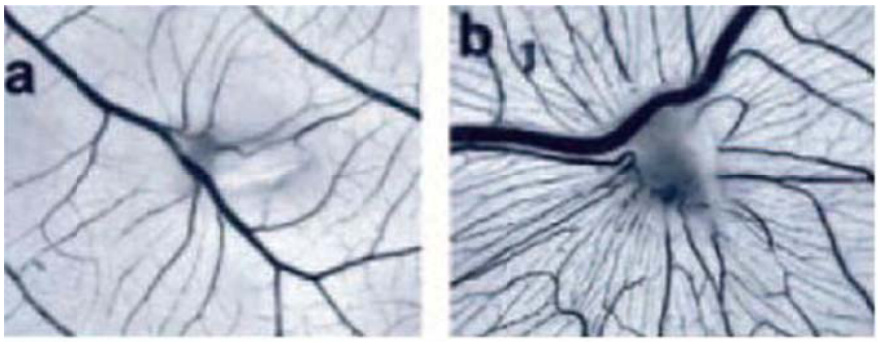

Abbildung 3: In Teilabbildung a) ist ein unbehandelter Hühnerembryo dargestellt, während der in Teilabbildung b) präsentierte Bereich mit HMGB1 Protein behandelt wurde. Die verstärkte Exposition führt zur vermehrten Ausbildung von Blutgefäßen (Mitola et al., 2006).

Als relevant, nicht nur im Hinblick auf die Tumorforschung, stellt sich der Zusammenhang zwischen HMGB1 und Stammzellen dar (Palumbo & Bianchi, 2004), wobei HMGB1 eine Migration von hämatopoetischen Stammzellen herbeiführt und so bei Verletzungen die Geweberegeneration vorantreibt und eine entscheidende Rolle bei der Wundheilung spielt. So konnten beispielsweise bei Mäusen mit HMGB1 stimulierte Myoblasten in geschädigte Muskelregionen einwandern und die Regeneration einleiten, während eine Inhibierung von HMGB1 eine verminderte Anzahl von Blutgefäßen sowie eine verminderte Geweberegeneration zur Folge hatten (De Mori et al., 2007). Diese Verbindung liefert Hinweise auf das hohe Potenzial von HMGB1 in therapeutischen Modellen zusätzlich zu Ansatzpunkten in Tumorprognose und Therapie.

1.5 Zielsetzung

Im Rahmen dieser Arbeit sollen Funktionen und Expressionsmuster von Prognoseparametern, welche als Werkzeuge in der Tumordiagnostik dienen können, spezifisch analysiert und charakterisiert werden.

Der indikationsgerechte Einsatz diagnostischer Verfahren sowie deren angemessene Schnelligkeit und Zuverlässigkeit waren und sind Anspruch einer wissenschaftlich fundierten Medizin. Aus diesem Grund werden im Rahmen dieser Arbeit die Genexpressionen verschiedener potentieller Tumormarker an unterschiedlichen soliden Tumoren sowie malignen Systemerkrankungen untersucht, um die wissenschaftlichen und wirtschaftlichen Aspekte der Verwendung der beschriebenen *HMG*-Gene sowie ihrer Proteine zu analysieren.

Das Ziel dieser Untersuchungen war es, in den beschriebenen Tumorentitäten die Diagnose zu erleichtern, die Aussagen zur Prognose zu verbessern, und bestenfalls auch Möglichkeiten aufzuzeigen, mit deren Hilfe die Therapie verschiedener Tumoren verbessert werden kann.

2 Material und Methoden

2.1 Gewebeproben

Alle untersuchten Gewebeproben (Abbildung 4) wurden nach der Einverständniserklärung des Patienten im Anschluss an die Entnahme umgehend in flüssigem Stickstoff, in ein Fixierungsmedium wie Formalin und HOPE 1 (Hepes-Glutamic acid buffer mediated Organic solvent Protection Effect) oder in Hanks Wachstumsmedium überführt. Nachfolgend wurden, soweit möglich, Gewebeblöcke zur histologischen Untersuchung nach dem in Kapitel 2.3 beschriebenen Verfahren angefertigt. Für die Analysen der Schilddrüsengewebe, der Mammakarzinome, sowie der Herz- und Speicheldrüsengewebe wurden bereits fertige Blöcke durch die in diesem Kapitel aufgelisteten Kliniken zur Verfügung gestellt. Eine Liste über sämtliche in der vorliegenden Arbeit analysierten Gewebeproben befindet sich im Anhang (Kapitel 10.1).

Material	Anzahl (n)	Zielsetzung	Kooperations-partner	Informationen zum Material
Lunge	35	Vergleich der *HMGA2* Expression bezgl. Umgebungsgewebe zu Tumorgewebe und Korrelation mit dem Grading	Kapitel 2.1.1	Kapitel 10.1.1
Schild-drüse	62	Untersuchung der *HMGA2* Expression; Vergleich von Diagnosen und Expressionswerten	Kapitel 2.1.2	Kapitel 10.1.2
Herz	50	Untersuchung der *HMGA2* Expression in Bezug auf das Auftreten von Aortendissektionen	Kapitel 2.1.3	Kapitel 10.1.3
Speichel-drüse	53	Untersuchung der *HMGA2* Expression sowie des Fusionstranskriptes CRTC1-MAML2	Kapitel 2.1.4	Kapitel 10.1.4
Brust	130	Untersuchung der *HMGA2* Expression im Vergleich zu bekannten Tumormarkern	Kapitel 2.1.5	Kapitel 10.1.5
Myom	184	Zytologische und molekulargenetische Charakterisierung	Kapitel 2.1.6	Kapitel 10.1.6
Non-Hodgkin Lymphom	24	Korrelation der HMGB1 Expression mit der Diagnose	Kapitel 2.1.7	Kapitel 10.1.7
Zelllinien	15	Vergleichende Genanalysen und Entwicklung sowie Etablierung von Methoden	Kapitel 2.2	Kapitel 10.1.8
Serum	32	Vergleichende HMGB1 Analyse unter Chemotherapieeinfluss	Kapitel 2.1.8	Kapitel 10.1.9
Exsudat	37	Vergleich der malignen und entzündlichen Ergüsse in Bezug auf die HMGB1 Menge	Kapitel 2.1.9	Kapitel 10.1.10

Abbildung 4: Übersicht über die untersuchten Materialien.

2.1.1 Lungengewebe

Die in dieser Dissertation untersuchten Proben stammen aus der Klinischen und Experimentellen Pathologie des Forschungszentrums Borstel (Borstel, Deutschland).

2.1.2 Schilddrüsengewebe

Das untersuchte Schilddrüsenarchivmaterial wurde von der Pathologieabteilung des Klinikums Bremen-Mitte (Bremen, Deutschland), beziehungsweise von der Pathologie der Asklepios Klinik Hamburg Altona (Hamburg, Deutschland) zur Verfügung gestellt.

2.1.3 Herzgewebe

Die Aortengewebe stammen aus einer Zusammenarbeit mit der Klinik für Herzchirurgie der Universität zu Lübeck (Lübeck, Deutschland).

2.1.4 Speicheldrüsengewebe

Die Speicheldrüsengewebe wurden von der Albertinen-Pathologie, sowie dem Institut für Hämatopathologie (beide Hamburg, Deutschland) bereitgestellt.

2.1.5 Brustgewebe

Das Probenmaterial wurde von verschiedenen nachfolgend aufgelisteten Krankenhäusern bzw. deren Unterabteilungen bereitgestellt:

- Universitätsklinikum Hamburg Eppendorf, Institut für Gynäkopathologie (Hamburg, Deutschland)
- Universität Münster, Institut für Pathologie (Münster, Deutschland)
- Klinikum Bremen Mitte, Zentrum für Pathologie (Bremen, Deutschland)
- Universitätsklinikum Schleswig-Holstein, Institut für Pathologie (Kiel, Deutschland)

2.1.6 Myome

Die analysierten gutartigen Gewebeproben wurden von der Klinik Sankt Joseph Stift und dem DIAKO Bremen (beide Bremen, Deutschland) beigesteuert.

2.1.7 Non-Hodgkin Lymphome und regelgerechte Lymphknoten

Das untersuchte Probenmaterial von Non-Hodgkin Lymphomen überließ die Klinik I für Innere Medizin des Zentrums für integrierte Onkologie der Universitätsklinik Köln (Köln, Deutschland). Die überprüften, unveränderten Lymphknoten wurden vom Klinikum Bremen Nord (Bremen, Deutschland) bereitgestellt.

2.1.8 Blutseren

Die Fälle wurden in Zusammenarbeit mit der Stiftung Tierärztliche Hochschule Hannover (Hannover, Deutschland) analysiert und in der Klinik entnommen.

2.1.9 Exsudate

Die Erguss Proben wurden durch das Klinikum Bremen-Mitte, aus der Medizinischen Klinik I (Bremen, Deutschland), zur Untersuchung bereitgestellt.

2.2 Zelllinien

Die in dieser Arbeit untersuchten Zelllinien wurden in RPMI Medium (Life Technologies, Paisley, England) mit 10%igem fötalem Kälberserum (FKS) (Fisher Scientific, Schwerte, Deutschland) kultiviert. Alle analysierten Zelllinien wurden vom Zentrum für Humangenetik der Universität Bremen zur Verfügung gestellt und anteilig auch dort etabliert.

2.3 Fixierung von Gewebeproben

Referenzproben der bearbeiteten Gewebe wurden in Formalin oder HOPE fixiert und in Wachsblöcke gegossen. An diesen Materialien wurden anschließend zur Verifizierung des Tumor- oder Proteinanteils histologische Untersuchungen durchgeführt.

Die HOPE fixierten und in Paraffin eingebetteten (HFPE) Blöcke wurden mindestens 16 h auf Eis in HOPE-1 Lösung inkubiert, in welche sie direkt nach der OP überführt wurden. In den folgenden Schritten erfolgten einige Veränderungen zu dem von Olert et al. etablierten Protokoll (Olert et al., 2001). Nach Verwerfen der HOPE-1 Lösung folgte eine 2stündige Inkubation in 5 ml Aceton mit 5 µl HOPE-2 Lösung. Im Anschluss daran wurde das Gewebe mit Aceton entwässert. Hierzu wurde das Aceton nach 2 h, sowie anschließend zweimal nach jeweils 1 h, gewechselt. Das Gewebe wurde über Nacht in Paraffin in einer Gewebekassette eingelegt. Der Paraffinblock wurde in eine Metallkassette gegossen und mit Paraffin an der Gewebekassette befestigt.

Die in Formalin fixierten Gewebe sind direkt nach der Entnahme in 5%iges gepuffertes Formalin überführt und danach mindestens 16 h darin inkubiert worden. Das ursprünglich 1893 von Blum entdeckte und von Fox weiterentwickelte Verfahren wurde dabei deutlich angepasst (Blum, 1893; Fox et al., 1985). Die Fixierung erfolgte nach 2-6stündigem Spülen in aufsteigender Isopropanolreihe (jeweils 1 h bei 20%, 40%,

60%, 80%, 90% und 100% Isopropanol). Im Anschluss an eine 8-16stündige Inkubation in 100% Isopropanol, schloss sich dreimal jeweils 1 h Inkubation in Xylol an. Nach jeweils 4, 8 und wieder 4 h in Xylol wurden die Gewebe in Paraffin eingebettet.

Die in HOPE fixierten Gewebeblöcke wurden bei 4°C und die in Formalin fixierten bei RT gelagert.

2.4 RNA-Isolierung

Die Ribonukleinsäuren wurden aus unterschiedlich behandelten Geweben auf verschiedene Art und Weise isoliert. Alle RNAs wurden bis zur weiteren Verwendung bei -80°C gelagert.

2.4.1 RNA-Isolierung aus Zellkultur

Nachdem die Zellen in den Kulturflaschen mit 1xPBS gewaschen wurden, konnten sie mithilfe von 1 ml Trypsin (Cytogen, Sinn, Deutschland) abgelöst und in ein Mikroreaktionsgefäß überführt werden. Nach einem Waschschritt mit 1 ml 1xPBS zum Entfernen des Trypsins wurden die Proben mithilfe der QIAshredder Säulen in 350 µl RLT-Puffer homogenisiert und als nächstes mit dem RNeasy® Mini Kit (beides Qiagen, Hilden, Deutschland) nach Herstellerangaben inklusive DNase I Verdau auf der Säule isoliert. Darauf aufbauend wurde ein zweiter Verdau, welcher in dem angefügten Artikel VIII detaillierter erläutert ist, durchgeführt. Jene im Rahmen dieser Arbeit weiterentwickelte Methode dient zur Erschließung pseudogenreicher Genfamilien für die Untersuchung mittels der sensitiven Real-Time PCR Methode.

2.4.2 RNA-Isolierung aus Frischgewebe

Alle untersuchten Frischgewebe wurden unter Verwendung des RNeasy® Mini Kit (Qiagen, Hilden, Deutschland) nach Herstellerangaben inklusive DNase I Verdau isoliert. Zunächst wurden die Proben mit Hilfe von Kanüle und Skalpell sowie QIAshredder Säulen in 350 µl RLT-Puffer homogenisiert.

Die Technik des Homogenisierens von Gewebe mittels Kanüle und Skalpell erwies sich in der praktischen Umsetzung als problematisch und führte darüber hinaus in einigen Fällen zu geringen RNA-Ausbeuten. Aus diesem Grund wurde die Methode des Gewebeaufschlusses unter zur Hilfenahme des TissueLysers (Qiagen, Hilden, Deutschland) in 600 µl RLT-Puffer bei einem Homogenisierungsschritt von 10 min bei 30 Hz etabliert.

2.4.3 RNA-Isolierung aus HFPE/FFPE Gewebe

Sowohl die Formalin als auch die HOPE fixierten Gewebeproben wurden mit dem High Pure RNA Paraffin Kit (Roche Applied Science, Penzberg, Deutschland) nach Herstellerangaben durchgeführt. Abweichend dazu sind die Zentrifugationszeiten auf 30 s erhöht worden. Eine Entparaffinierung erfolgte mittels Xylol- und Ethanolwaschschritten. Hierbei wurden jeweils 6 Schnitte à 5 µm Dicke zweimal mit 800 µl Xylol 10 min entwachst und nachfolgend durch Zugabe von 400 µl Ethanol absolut gefällt, bei RT und 16.100 xg 3 min zentrifugiert und der Überstand verworfen. Im Anschluss daran wurde das Xylol aus den Proben anhand von 2 Ethanol absolut Waschschritten mit je 1 ml für 10 min entfernt, wobei der Überstand erneut verworfen worden ist.

2.4.4 miRNA-Isolierung aus Frischgewebe

Zur Isolierung der miRNA kam die Phenol-Chloroform-Extraktion zum Einsatz (Chomczynski & Sacchi, 1987). Zunächst wurden 50-60 mg Aortengewebe in 1 ml TRIzol® (Invitrogen, Karlsruhe, Deutschland) 10 min bei 30 Hz im TissueLyser homogenisiert und 5 min bei RT inkubiert. Danach wurden 200 µl Chloroform zugegeben und die Lösung durch kräftiges Schütteln vermischt. Es folgte eine 3minütige Inkubation bei RT, woran sich eine 15minütige Zentrifugation bei 12.000 xg und 4°C zur Phasentrennung anschloss. Die wässrige, RNA enthaltende obere Phase wurde in einem neuen 2 ml Reaktionsgefäß mithilfe von 500 µl Isopropanol absolut gefällt. Im Anschluss an ein fünfmaliges Invertieren wurden die Proben 10 min bei RT inkubiert. Nach einer 10minütigen Zentrifugation bei 4°C und 12.000 xg und Entfernen des Überstandes, konnte das Pellet mit 1 ml 75% Ethanol versetzt werden. Dieses wurde nach einer Zentrifugation für 5 min bei 4°C und 7.500 xg wieder entfernt. Danach wurde erneut 1 ml 75% Ethanol zum Pellet gegeben und gut vermischt. Es schloss sich eine Zentrifugation (5 min, 7.500 xg und 4°C) an, nach welcher der Überstand verworfen wurde. Das getrocknete Pellet wurde in 80 µl RNase freiem Wasser gelöst.

2.5 Proteinisolierung

Die Isolierung der Proteine erfolgte mittels RIPA-Puffer (modified radioimmunoprecipitation) inklusive einer Proteinaseinhibitormischung (die Mischung setzte sich zusammen aus 10 verschiedenen Proteinaseinhibitoren der „complete“ Reihe der Firma Roche Applied Science, Penzberg, Deutschland), welche zur Stabilisierung der Proteine diente. Die Zusammensetzung des RIPA-Puffers wurde wie folgt gewählt:

50 mM Tris-HCl, 150 mM NaCl, 1% (v/v) NP-40, 0,5% (w/v) Natriumdeoxycholat, 1 mM EDTA. Die Proben wurden im Verhältnis 1:2 mit RIPA-Puffer versetzt und für 1 min homogenisiert. Nach einer 45minütigen Inkubation im Schütteleisbad wurden die Proben 15 min bei 14.000 xg und 4°C zentrifugiert. Der die Proteine enthaltende Überstand wurde bis zur weiteren Verwendung bei -80°C gelagert.

2.6 Konzentrations- und Reinheitsabschätzung

2.6.1 Konzentrations- und Reinheitsabschätzung von Nukleinsäuren

Die Konzentrations- und Reinheitsbestimmungen der RNA erfolgten mittels Spektralphotometer (Biophotometer, Eppendorf, Hamburg, Deutschland). Zur Verifizierung der Fragmentlängen und der RNA Integrität wurden RNA Gelelektrophoresen unter denaturierenden Bedingungen durchgeführt. Hierzu wurden mit Agarose 1,2%ige Gele mit 10% MOPS (3-N-Morpholinopropansulfonsäure) und, nach Abkühlen auf 65°C, mit 2% 37%igem Formaldehyd sowie 3,5 x 10^{-3} ng/ml Ethidiumbromid versetzt. Nach einer 30minütigen Äquilibrierung in 1 x MOPS fand die Auftrennung der mit RNA-Beladepuffer versetzen Proben (Einsatzmenge 1 µg) bei 7-9 V/cm statt. Eine Auswertung der Ergebnisse erfolgte auf dem UV-Tisch (Biostep, Jahnsdorf, Deutschland). Die Dokumentation erfolgte mit der Digitalkamera (Olympus, Hamburg, Deutschland) und der Computersoftware Argus (Biostep, Jahnsdorf, Deutschland).

2.6.2 Konzentrationsabschätzung von Proteinen

Die Proteingesamtmenge wurde unter zur Hilfenahme des Pierce® BCA Protein Assay Kit (Pierce Biotechnology, Rockford, USA) detektiert. Bei einer Wellenlänge von 562 nm wurde die Konzentration durch eine mit Standards (BSA und BGG: 20-2000 µg/ml) ermittelte Gleichung ermittelt. Zur Erhöhung der Genauigkeit wurden die Proben in dreifacher Bestimmung gemessen.

Zur Bestimmung der HMGB1 Proteinmenge in Proteinlösungen und Seren wurden Enzyme Linked Immunosorbent Assay (ELISA) Experimente durchgeführt.

Die HMGB1 Konzentration wurde mithilfe eines kommerziell verfügbaren ELISA-Tests (Shino-Test Corporation, Kanagawa, Japan) ermittelt. Hierbei wurden jeweils 10 µl der Probe in 3facher Bestimmung in die mit anti-HMGB1-Antikörpern beschichtete Mikrotiterplatte vorgelegt und mit 100 µl Probenverdünnungslösung versetzt. Die enzymatische Farbreaktion wurde bei einer Wellenlänge von 450 nm unter Verwen-

dung eines Synergie HT (BioTek, Winooski, USA) gemessen und auf die durch Standards berechnete Geradengleichung bezogen.

2.7 Synthese von komplementärer DNA (cDNA)

Zur Untersuchung der Genexpression aus den isolierten RNA Proben musste die RNA zunächst mithilfe des von Gerard und Kollegen beschriebenen Prinzips durch eine RNA abhängigen DNA Polymerase M-MLV in cDNA umgeschrieben werden (Gerard et al., 1997).

Die Synthese erfolgte nach dreierlei Schemata, je nachdem, ob die Messung der Transkriptmengen für miRNA bzw. RNA mittels absoluter oder relativer Quantifizierung stattfinden sollte. Alle cDNAs wurden bei -20°C gelagert.

2.7.1 Synthese von cDNA für die relative Quantifizierung

Für die cDNA, die während der relativen Quantifizierung verwendet wurde, war es notwendig ein Hexamergemisch zu verwenden, welches es ermöglicht die gesamte RNA in cDNA umzuschreiben, da an der gleichen cDNA die Expression sowohl von 18S rRNA als auch von verschiedenen Genen nachgewiesen werden sollte.

Bei Ansätzen, an die sich eine relative Quantifizierung (siehe Kapitel 2.8.2) anschloss, wurden ca. 250 ng gesamt RNA mit je 1 µl random hexamers (150 ng/µl) und 1 µl dNTP-Mix (10 mM) versetzt. Durch Erhitzen auf 65°C für 5 min wurden die Sekundärstrukturen zerstört. Im Anschluss wurden die Proben mindestens 1 min auf Eis inkubiert. Es folgte die Zugabe von 4 µl 5x Erststrangpuffer, 2 µl DTT (0,1 M), 1 µl RNaseOUT (40 U) sowie 1 µl M-MLV (200 U) Reverse Transkriptase (alle Reagenzien Invitrogen, Karlsruhe, Deutschland). Es wurde ebenfalls eine Negativkontrolle mit Aqua bidest. anstelle von RNA, sowie eine Negativkontrolle für jede Probe ohne das Enzym M-MLV Reverse Transkriptase (-RT) nach dem gleichen Schema pipettiert. Es folgte eine Inkubation für 10 min bei 25°C und für 50 min bei 37°C mit einer anschließenden Inaktivierung des Enzyms für 15 min bei 70°C.

2.7.2 Synthese von cDNA für die absolute Quantifizierung

Für Proben, die während der absoluten Quantifizierung gemessen wurden, galten einige Änderungen. Anstelle des random hexamer Gemisches kamen genspezifische Primer (2 µM) zum Einsatz. Im Einzelnen handelte es sich um die Sequenzen: *HMGA2* (reverse: 5’ GCCATTTCCTAGGTCTGCCTC 3’ (Gross et al., 2003)) und

HMGB1 (reverse: 5' TCCTTTGCCCATGTTTAATTATTTTC 3'). Weiterhin wurde der Inkubationsschritt von 10 min bei 25°C, welcher der Anlagerung des Hexamergemisches dient, ausgelassen. Zur Kontrolle der Gleichmäßigkeit der cDNA-Synthese Reaktion wurde in jeder cDNA-Synthesereaktion der 10^6-Standard als interne Kontrolle mitgeführt und in der anschließenden PCR überprüft.

2.7.3 Synthese von cDNA für miRNA Quantifizierungen

Bei der cDNA Synthese für die miRNA Proben gibt es abweichend kleinere Änderungen im Protokoll. Zunächst wurden 15 µl Ansätze nach Vorgabe des TaqMan® MicroRNA Reverse Transcription Kits erstellt, die aus 0,15 µl dNTPs (10 mM), 1 µl MultipleScribe RT (50 U/µl), 1,5 µl 10x RT-Puffer, 0,19 µl RNase Inhibitor (20 U/µl), 3 µl genspezifischen Primern (*RNU6B* und *let-7d*, beide Applied Biosystems, Darmstadt, Deutschland), 4,16 µl Aqua bidest. sowie 5 µl der zu untersuchenden RNA Probe (10 ng gesamt RNA) bestanden. Die Proben wurden gründlich vermischt und nach einer 5minütigen Inkubation auf Eis 30 min bei 16°C, 30 min bei 42°C und 5 min bei 85°C im Thermocycler (Eppendorf, Hamburg, Deutschland) inkubiert.

2.8 Amplifikation von DNA Fragmenten (Polymerase Chain Reaction-PCR)

Die in dieser Untersuchung durchgeführten Arbeiten wurden alle mittels der quantitativen Real-Time PCR, basierend auf dem TaqMan® Prinzip, durchgeführt. Dieses wurde erstmals von Livak und Kollegen 1995 beschrieben (Livak et al., 1995). Die Untersuchungen gliedern sich in zwei nachfolgend erläuterte Gruppen; die der absoluten und der relativen Quantifizierung. Für die Real-Time PCR Reaktionen wurden das ABI Prism 7000 Sequence Detection System sowie das 7300 Real-Time PCR System (beide Applied Biosystems, Darmstadt, Deutschland) verwendet.

2.8.1 Absolute Quantifizierung mittels Real-Time PCR

Bei der absoluten Quantifizierung wurden mithilfe von Standardisierungsreihen zwischen 1 x10^2-1 x10^9 Kopien die Anzahl der gesuchten Transkriptmengen ermittelt. Sowohl die Bestimmung der Messpunkte der Standards als auch die der Proben erfolgte in Triplikaten. Es wurden ausschließlich Ergebnisse mit einer PCR-Effizienz von mindestens 90% genutzt. Weiterhin wurde darauf geachtet, dass die Messungenauigkeiten innerhalb der Mehrfachbestimmungen einen Ct-Wert Unterschied von 0,15 nicht überschritten. Für jede Probe wurde explizit für jedes untersuchte Gen ebenfalls die in 2.7 definierte -RT Probe überprüft. Ferner wurden für jedes Gen

Negativkontrollen mit H_2O anstelle der Probe sowohl auf Ebene der cDNA-Synthese als auch auf Ebene der Real-Time PCR durchgeführt. Der Ansatz einer jeden Probe betrug 25 µl und enthielt neben 25 ng des zu untersuchenden RNA-Äquivalents 600 nM der genspezifischen Primer und 200 nM der jeweiligen Sonden sowie 1x Universal Mastermix (incl. Uracil-N-Glykosylase (UNG); Applied Biosystems, Darmstadt, Deutschland) und nukleasefreies Wasser. Die in 96-well Format angesetzten Proben wurden durch eine optischen Klebefolie verschlossen und 1 min bei 1.000 xg zentrifugiert. Hieran knüpfte der PCR-Lauf mit folgenden Bedingungen an: 2 min bei 50°C zur Aktivierung der UNG mit anschließender Denaturierung der Probe, Inaktivierung der UNG, sowie Aktivierung der Hot Star *Taq* Polymerase für 10 min bei 95°C. Es folgten 45-50 Zyklen, in denen sich 15 s bei 95°C zur Denaturierung und 1 min bei 60°C für Annealing und Elongation abwechselten. Eine Erfassung der gemessenen Fluoreszenzveränderung stand am Ende eines jeden Zyklus.

Die Auswertung der Daten bezieht sich auf eine bestimmte Transkriptmenge in insgesamt 250 ng gesamt RNA. Die Auswertung erfolgt unter Berücksichtigung der in der cDNA-Synthese analysierten Faktoren sowie der mittels Standardisierungsreihe errechneten Geradengleichung.

2.8.2 Relative Quantifizierung mittels Real-Time PCR

Die bei der relativen Quantifizierung gemessenen Werte beziehen sich im Gegensatz zu den bei der absoluten Quantifizierung ermittelten Werten auf eine interne endogene Kontrolle (Heid et al., 1996). In den hier präsentierten Untersuchungen wurde mithilfe der 18S rRNA mit einer hohen Konstanz zwischen Geweben, sowie 90-100% PCR-Effizienz richtlinienkonforme Ergebnisse erzielt (AppliedBiosystems, 2001).

Die Messungen erfolgten immer in einer Dreifachbestimmung. Die in Punkt 2.8.1 erläuterten Kontrollen dienten zur Überprüfung der analysierten Proben.

Es konnten aufgrund der relativen Quantifizierung auch Proben untersucht werden, welche aufgrund starker Degradierung oder geringer Konzentration für eine absolute Quantifizierung nicht in Frage gekommen wären. Neben den 2 µl cDNA variabler Konzentration wurden 600 nM genspezifischer Primer sowie 200 nM der jeweiligen Sonde oder ersatzweise 1x genspezifischer Assay (Applied Biosystems, Darmstadt, Deutschland) in die Reaktion mit 1x Universal Mastermix incl. UNG eingesetzt. Das Reaktionsvolumen wurde mit 7 µl nukleasefreiem Wasser auf 20 µl aufgefüllt. Die ebenfalls in 96-well Format angesetzten Reaktionen wurden, nach Verschluss mit

einer optischen Klebefolie, für 2 min bei 50°C, 10 min bei 95°C und nachfolgenden 50 Zyklen 15 s bei 95°C und 1 min bei 60°C inkubiert. Die Messung der Fluoreszenzveränderung fand am Ende eines jeden Zyklus statt und die Auswertung der relativen Quantifizierung erfolgte per ΔΔ Ct-Methode (Livak & Schmittgen, 2001). Die Ergebnisse wurden als x-fache Schwankung im Vergleich zu einer beliebigen Referenz dargestellt. Bei der Auswertung wurden weder Werte berücksichtigt, bei denen eine Schwankung im Bereich der endogenen Kontrolle detektiert wurde, noch Proben bei denen Schwankungen innerhalb der Mehrfachbestimmungen von über 0,15 Ct-Werten vorlagen. Eine Assayeffizienz von mindestens 90% ist ebenfalls vorausgesetzt worden.

2.8.3 Relative miRNA Quantifizierung mittels Real-Time PCR

Zur miRNA Quantifizierung unter Verwendung eines TaqMan® MicroRNA Assay wurde der *let-7d* Assay mithilfe der endogenen Kontrolle RNU6B (beide Applied Biosystems, Darmstadt, Deutschland) nach der ΔΔ Ct Methode quantifiziert. Hierzu wurden 1 µl des jeweiligen TaqMan miRNA Assays mit 10 µl Universal Mastermix, 7,67 µl Aqua bidest. und 1,33 µl cDNA für jeden Ansatz vermischt. Die Messungen erfolgten wie bereits für die absolute und relative Quantifizierung beschrieben in Triplikaten unter Berücksichtigung der bereits beschriebenen Kontrollen und Richtlinien.

2.9 Immunhistochemie (IHC)

Für die immunhistochemischen Färbungen wurden jeweils Proben in 4-5 µm Dicke mit einem Mikrotom (pfm, Köln, Deutschland) erstellt. Es sind jeweils Negativkontrollen ohne primären Antikörper durchgeführt worden. Nachfolgend wurde je nach Fixierungsmethode in zwei verschiedene Protokolle unterschieden.

Die in HOPE fixierten Lungengewebe wurden zunächst 1 h bei 37°C getrocknet und anschließend mithilfe von Diethylether zweimal 30 s entwachst. Nach einer 30minütigen Rehydrierung bei 4°C in 50% Aceton wurden die Schnitte zunächst 10 min in einer Küvette mit dH_2O inkl. 1% TritonX-100 und danach in reinem dH_2O inkubiert. Im Anschluss an eine 20minütige Inkubation in 1xPBS wurde die endogene Peroxidase (0,3% in H_2O_2 in 1xPBS; bzw. 1:20 H_2O_2 in Methanol) 10 min blockiert. Nachdem die Lösung unter Nutzung von H_2O entfernt und die Schnitte 10 min in 1xPBS äquilibriert worden waren, wurden die Proben mit dem primären Antikörper (HMGA2: sc-23684; HMGB1: sc-12523; beide Santa Cruz Biotechnology, Santa

Cruz, USA) beschichtet und 1 h in einer feuchten Kammer bei RT inkubiert. Nach Entfernen des primären Antikörpers mittels zweimal 2 min Inkubationen in 1xPBS, erfolgte die Zugabe des sekundären Antikörpers, welcher nach 20 min ebenfalls mit 1xPBS entfernt wurde. Daraufhin wurde der Objektträger mit Peroxidase Label für 20 min inkubiert. Nachdem die Proben erneut zweimal mit 1xPBS gewaschen worden waren, kam die Zugabe des Substrates. Das überschüssige Substrat wurde nach 10 min mithilfe von dH_2O und 1xPBS entfernt wurde.

Eine Gegenfärbung erfolgte mittels Mayer´s Hämalaun, und die Schnitte wurden in dem hydrophilen Medium AquaMount (FisherScientific, Schwerte, Deutschland) eingedeckt.

Das Wachs der Formalin fixierten Schnitte wurde im Gegensatz zu den HOPE fixierten mithilfe eines zweimaligen Waschschrittes in Xylol entfernt, nachdem sie 2 h bei 56°C getrocknet worden waren. Eine Rehydrierung erfolgte unter Nutzung einer absteigenden Ethanolreihe (96%, 80%, 70%; jeweils 2 min). Nachdem das Ethanol mittels dH_2O entfernt und 10 min in einer weiteren Küvette inkubiert worden war, fand eine 20minütige Inkubation in 1xPBS statt. Die nachfolgenden Schritte der IHC an Formalin fixierten Geweben entsprechen den bereits für HOPE fixiertes Gewebe beschriebenen beginnend mit der 10minütigen Blockierung der endogenen Peroxidase. Abweichend hierzu wurden dem Protokoll bei unspezifischen Signalen eine 20minütige Inkubation in 10 mM Citratpuffer zur Antigendemaskierung hinzugefügt. Die Auswertung der immunohistochemischen Untersuchungen erfolgte nach dem immunohistochemischen Score System (IRS). Die IRS setzt sich aus dem prozentualen Anteil der positiven Tumorzellen und der Intensität ihrer Anfärbung zusammen (Remmele & Stegner, 1987).

2.10 Statistische Auswertungen

Die statistischen Analysen students T-Test, Mann-Whitney U Test, Spearman- und Pearson Korrelation erfolgten mit dem WinStat Programm (R. Fitch Software, Krozingen, Deutschland) wobei Analysen mit $p<0,05$ als signifikant erachtet wurden. Die überdies aufgeführten statistischen Analysen wie ROC-Analysen, Kaplan Meier Überlebens Analysen und Cox-Regressionsanalysen wurden in Zusammenarbeit mit Herrn Wosniok sowie Herrn Prof. Wischnewsky (beide Universität Bremen, Bremen, Deutschland) erstellt. Die Analysen erfolgten uni- sowie multivariat.

3 Ergebnisse

Die im Rahmen dieser Arbeit erstellten Resultate gliedern sich in zwei Hauptteile. In den Kapiteln 3.1 und 3.2 werden die Ergebnisse der im Rahmen dieser Arbeit optimierten *HMGA2* Genexpressionsanalysen vorgestellt. Besonders hervorzuheben ist hierbei die Etablierung einer Methode zur Quantifizierung der Genexpression mittels Real-Time PCR an Archivmaterial.

Im zweiten Teil werden in den Kapiteln 3.3 und 3.4 die Ergebnisse der Quantifizierung von HMGB1 mittels Real-Time PCR und ELISA präsentiert. Hier konnte ein Zusammenhang zwischen HMGB1 und systemischen Erkrankungen und deren Therapieansprechen hergestellt werden.

3.1 Quantitative *HMGA2* Genexpressionsanalysen

Zur Bestimmung der *HMGA2* Genexpression konnten verschiedene solide Tumoren wie nicht kleinzellige Bronchialkarzinome (Kapitel 3.1.1), Schilddrüsenneoplasien (Kapitel 3.1.2), Speicheldrüsentumore (Kapitel 3.1.4), Mammatumore (Kapitel 3.1.5) und Uterusleiomyome (Kapitel 3.1.6) sowie veränderte Herzgewebe als Untersuchungsmaterial verwendet werden (Kapitel 3.1.3). Allen *HMGA2* Untersuchungsergebnissen gemein ist die deutliche Differenz der Gesamtexpression in Neoplasien und unveränderten Geweben.

3.1.1 *HMGA2* Genexpression an nicht kleinzelligen Bronchialkarzinomen

I) Meyer et al., 2007, Mol Carcinog.

Das Bronchialkarzinom ist weltweit der Tumor, der von allen Krebsarten am häufigsten zum Tode führt. Die Karzinome wachsen sehr schnell und bilden in vielen Fällen Metastasen aus, weshalb sie häufig erst in einem fortgeschrittenen Stadium diagnostiziert werden. Um die Diagnose bei Bronchialkarzinomen zu verbessern, ist eine Aufklärung der molekularen Mechanismen, welche in Tumorentwicklung und Progression involviert sind, essentiell. In vorangegangen Untersuchungen der *HMGA2*-Expression konnte *HMGA2* mittels semiquantitativer Methoden lediglich in Bronchialkarzinomen und nicht im unveränderten Umgebungsgewebe detektiert werden.

Im Rahmen dieser Untersuchungsreihe wurden Tumorproben sowie zugehörige nicht maligne Gewebe von 17 Adenokarzinompatienten (AC) sowie 17 Plattenepithelkarzinompatienten (SCC) sowohl durch quantitative Real-Time PCR als auch mittels IHC untersucht. Die in allen untersuchten Proben detektierbare *HMGA2*-Expression

schwankte zwischen 289 und 525.947 Kopien pro 250 ng gesamt RNA in nicht verändertem Umgebungsgewebe und zwischen 5.422 und 16.991.545 Kopien im Tumorgewebe. In 97% der Fälle (33/34) konnte eine statistisch hoch signifikante (AC $p<0,0001$; SCC $p<0,0001$) Überexpression des *HMGA2*-Gens im Tumorgewebe detektiert werden. Die per Real-Time PCR ermittelten Expressionsunterschiede konnten durch die IHC bestätigt werden. Dies deutet darauf hin, dass die Veränderung der *HMGA2*-Expression in nicht kleinzelligen Bronchialkarzinomen eine große Rolle bei der malignen Transformation der Zelle spielt. Da keine Korrelation zu bisher gebräuchlichen Markern wie z.B. Grading oder Staging nachgewiesen werden konnte, ist davon auszugehen, dass mit der *HMGA2*-Expression ein Informationsgewinn zu erzielen ist.

3.1.2 *HMGA2* Genexpressionanalysen an Schilddrüsenneoplasien

II) Belge[1], Meyer[1], Klemke[1] et al., 2008, Genes Chromosomes Cancer.

In der vorliegenden Untersuchung wurden Schilddrüsenveränderungen von 64 Patienten analysiert. In den FFPE-Proben konnte eine stark unterschiedliche Genexpression bzw. Proteinexpression von *HMGA2*/HMGA2 sowohl mittels Real-Time PCR als auch durch IHC belegt werden. In allen untersuchten Schilddrüsenneoplasien konnte eine *HMGA2*-Expression nachgewiesen werden. Die stärkste Expressionsänderung im Vergleich zum gemittelten nicht veränderten Gewebe konnte mit einer 432fachen Überexpression bei einem follikulärem Karzinom detektiert werden. Es wurde mittels ROC Analysen ein Schwellenwert zur Unterscheidung zwischen benignen und malignen Schilddrüsenveränderungen bei einem Wert von 3,99fach errechnet, wobei eine Sensitivität von 95,9% und eine Spezifität von 93,9% vorlag. Mithilfe des studentischen T-Tests konnte zwischen den Kontrollgeweben und den Adenomen keine signifikante Expressionsänderung festgestellt werden, wobei zwischen den Kontrollgeweben und den Karzinomen ein mit $p<0,001$ hoch signifikanter Unterschied detektiert wurde.

Besonderes Augenmerk bei dieser Studie fällt auf die Tatsache, dass diese Unterschiede an vollständigen Schnitten ermittelt wurden und auch bei einem kleinen Tumoranteil ein Signal messbar ist. In diesen Fällen wurde die Expression von *HMGA2* in den Tumorzellen auch auf Proteinebene per IHC bestätigt.

Die Unterscheidung zwischen follikulären Adenomen und Karzinomen gestaltet sich bisher schwierig. Mithilfe der durchgeführten *HMGA2* Untersuchung konnte ein

möglicher Diagnoseparameter für eine spezifischere Differenzierung zwischen den Gruppen aufgezeigt werden. Weiterhin spiegelt die andauernde Expressionssteigerung den kontinuierlichen Weg der malignen Transformation wider. Dies deutet darauf hin, dass dem *HMGA2*-Gen sowie dem korrespondierenden Protein während der malignen Transformation der Zelle bei Schilddrüsenneoplasien eine entscheidende Rolle zuteil wird. Zusammenfassend ist HMGA2 in Schilddrüsenneoplasien sowohl ein signifikanter Marker, um zwischen benignen und malignen Fällen zu unterscheiden, als auch ein Indikator für den kontinuierlichen Prozess der malignen Zelltransformation.

3.1.3 *HMGA2* Genexpressionsanalysen an Herzgeweben

III) Belge et al., zur Veröffentlichung vorbereitet.

Das *HMGA2*-Gen, welches vorwiegend in der embryonalen Entwicklung, jedoch auch in Stamm- und Tumorzellen exprimiert wird, spielt ebenfalls eine Rolle bei der EMT. Um festzustellen, ob ein Zusammenhang zwischen der *HMGA2* Genexpression und Aortenveränderungen besteht, wurde diese bei 56 Patienten untersucht. In Aortendissektionen vom Typ A (n=19) konnte eine 24fache Erhöhung ($p<0,05$) im Vergleich zum Aortengewebe ohne Dissektion hergestellt werden. Ein Zusammenhang zwischen der Genexpression von *let-7d* und *HMGA2* in Aortendissektionen konnte nicht detektiert werden. Durch immunhistochemische Methoden konnten HMGA2 positive Zellen innerhalb der Endothelzellen des *vasa vasorum* ermittelt werden. Dies bringt die Vermutung mit sich, dass es bei Aortendissektionen mit einer hohen *HMGA2*-Expression zu einer Redifferenzierung des Gewebes kommt und jenes somit seine Funktionen nicht mehr wahrnehmen kann und einreißt, was dann innerhalb kürzester Zeit zum Tode führt. Der initiale Anstieg der *HMGA2*-Expression könnte ein Hinweis auf eine bevorstehende Ruptur und Geweberückbildung sein, so dass *HMGA2* als Marker für gefährdete Patientengruppen hilfreiche Dienste leisten könnte. Bisher gibt es im Bereich der Detektion von bevorstehenden Aortendissektionen keine sichere Analysemethode, was die Dringlichkeit der Erforschung neuer Optionen unterstreicht.

3.1.4 *HMGA2* Expressionsanalysen an Speicheldrüsentumoren

IV) Fehr et al., 2009, Genes Chromosomes Cancer.

Die Diagnose, Prognose und genaue Klassifizierung von Speicheldrüsentumoren gestaltet sich häufig schwierig, weshalb im Rahmen der *HMGA2* Expressions-

analysen ebenfalls Untersuchungen an Mucoepidermoid Karzinomen (MEC) der Speicheldrüse von insgesamt 53 Patienten sowie 4 unveränderten Referenzgeweben durchgeführt wurden.

Zusätzlich ist überprüft worden, ob in den Proben ein *CRTC1-MAML2* Fusionstranskript vorliegt. Bei Speicheldrüsentumoren weist das Vorhandensein des Fusionstrankriptes auf eine deutlich bessere Prognose hin, wobei das Fehlen dieses Transkriptes eine deutlich schlechtere Aussicht beinhaltet. Die hier untersuchten Fälle wiesen zu 74% ein Fusionstranskript auf, wobei die *HMGA2* Genexpression in dieser Gruppe bei einer 67±13fachen Erhöhung im Vergleich zum Referenzgewebe liegt. Bei den 26%, in denen kein Fusionstranskript detektiert werden konnte, lag hingegen die Expression von *HMGA2* bei 302±124fach über dem Normalwert.

Eine signifikante Trennung p=0,04 konnte auch mittels der *HMGA2*-Expression in den verschiedenen Gradingstufen ermittelt werden. Diese Untersuchungen stützen den vermuteten Zusammenhang zwischen der *HMGA2* Genexpression und der Malignität des Tumors. Die Theorie einer erhöhten Expression durch einen regulatorischen Mechanismus, wurde durch einzelne FISH-Untersuchungen untermauert. Es konnte sowohl bei Fusionstranskript positiven als auch negativen Fällen keine Amplifikation oder Rearrangierung detektiert werden.

Insgesamt konnten zwei Gruppen von MEC beschrieben werden, wobei die erste Gruppe Karzinome mit einem Fusionstranskript beinhaltet, welche möglicherweise durch ihr neu entstandenes Fusionstranskript mit dem NOTCH- oder CREB Signalweg interagieren, so dass es zur Ausbildung von Tumoren kommt. Die zweite Gruppe beinhaltet die für das Fusionstranskript negativen Fälle mit hoher Expression von *HMGA2*, welche eine deutlich schlechtere Prognose aufweisen. Die Quantifizierung der Gruppen bietet eine große Chance, da Patienten so in Zukunft besser und zielgerichteter, beispielsweise über eine Anti-*HMGA2* Therapie, behandelt werden können.

3.1.5 *HMGA2* Genexpressionsanalysen an Mammakarzinomen

V) Meyer[1], Wischnewsky[1], zur Veröffentlichung vorbereitet).

Bei Mammakarzinomen gibt es eine „niedrig Risiko Gruppe“ von Patientinnen, welche sich durch nicht befallene Lymphknoten und positive Hormonrezeptoren auszeichnet. Etwa 30% dieser Patientinnen erleiden jedoch postoperativ ein Rezidiv, das mit bisherigen Möglichkeiten nicht prognostiziert werden kann. Aus diesem Grund erhalten alle Patientinnen vorsorglich eine zusätzliche medikamentöse Therapie.

Es wurde bei einem Patientenkollektiv von 130 Patientinnen die *HMGA2* Genexpression vergleichend zu den etablierten Prognoseparametern Größe, Alter, Grading, ER, PR, *Her2*, *uPA* und *PAI-1* bestimmt. Ab einem *HMGA2* Expressionswert von 4,6fach über dem Kalibrator wurden die Patientinnen der Gruppe mit erhöhter *HMGA2*-Expression zugeordnet, welche eine signifikant schlechtere Chance auf Rezidiv freies (p=0,003) bzw. Gesamtüberleben (p=0,001) aufwiesen. Die Adjustierung der Parameter mittels Cox Regressionsanalyse konnte den Zusammenhang zwischen der *HMGA2* Genexpression und der Prognose der Patientinnen weiter unterstützen.

Mit diesen Untersuchungen wurde belegt, dass *HMGA2* ein bedeutender und von bisherigen Einflussgrößen unabhängiger Diagnose- und Prognoseparameter ist. Mit Hilfe der *HMGA2* Genexpressionsmessungen können die betroffenen Patientinnen deutlich besser klassifiziert werden. Die biologischen Zusammenhänge des HMGA2 mit der Phosphorylierung lassen vermuten, dass eine Doxorubicin basierende Therapie schlechter bei Patienten mit höherem HMGA2 Gehalt in den Zellen wirken wird.

3.1.6 *HMGA2* Genexpressionsanalysen an Uterusleiomyomen

VI) Klemke[1], Meyer[1], et al., 2008, Genes Chromosomes Cancer.

Uterusleiomyome treten bei 30% der Frauen im Alter ab 30 Jahren auf. Die Entstehungsursache wurde bisher nicht hinreichend geklärt. Auch eine Vorhersage über Wachstumsgeschwindigkeiten und Dignität des Myoms konnte bisher nicht vollständig aufgeklärt werden.

Eine verstärkte *HMGA2*-Expression könnte bei der Entwicklung von Uterusleiomyomen eine Schlüsselrolle spielen. Eine Veränderung der chromosomalen Region des *HMGA2*-Gens konnte in vorherigen Untersuchungen beschrieben werden. In dieser Studie wurden 180 verschiedene Uterusleiomyome sowohl zytologisch als auch molekularbiologisch charakterisiert. Zusätzlich wurden 51 dazugehörige Myometrien auf ihre *HMGA2* Genexpression hin untersucht. In 7% der Fälle konnte eine chromosomale Veränderung der Region 12q13~15 beschrieben werden, welche mit einer erhöhten *HMGA2*-Expression korreliert. Außerdem wurde festgestellt, dass auch unabhängig von Aberrationen die Genexpression in Uterusleiomyomen im Vergleich zu Myometrien deutlich erhöht ist. Diese Untersuchung unterstreicht die Bedeutung von HMGA2 in der Tumorgenese von Uterusleiomyomen.

3.2 Quantitative Analyse des *HMGA2* 3´UTR

Im Bereich des 3´UTR befinden sich häufig regulatorische Elemente für die entsprechenden Gensequenzen. In Kapitel 3.2.1 werden die Ergebnisse an Leiomyomen des Uterus dargestellt.

3.2.1 Untersuchung des *HMGA2* 3´UTR an Uterusleiomyomen

VII) Klemke[1] et al., 2010, Cancer Genetics and Cytogenetics.

Im Abschnitt 3.1.11 wurde eine Erhöhung der *HMGA2*-Expression in Uterusleiomyomen sowohl im Vergleich zum umgebenden Myometrium als auch innerhalb der Myomgruppe zwischen 12q13~15 aberranten sowie allen anderen aberranten Fällen beschrieben. Im Rahmen dieser Untersuchung wurde überprüft, ob eine verstärkte Expression von *HMGA2* auf eine Veränderung im Bereich des 3´UTR zurückzuführen ist. Hierzu wurden die miRNAs der *let-7* Familie analysiert, welche nachweislich die *HMGA2*-Expression beeinflussen. Insgesamt wurden zwölf Myome mit bekannter Translokation im Bereich 12q13~15 vergleichend untersucht. In drei Fällen weist eine deutlich abweichende Expression von *let-7* auf trunkierte Varianten des Gens hin. Durch eine fehlende Regulation der *HMGA2* Genexpression könnte die Erhöhung erklärt werden. Die weiteren neun Fälle weisen sowohl im 3´UTR als auch im ORF eine gleichmäßige Expression auf, so dass in diesen Fällen die Veränderung der Genexpression durch einen anderen zellulären Mechanismus erklärt werden müsste.

3.3 Quantitative *HMGB1* Genexpressionsanalysen

Das *HMGB1*-Gen beinhaltet eine Vielzahl von Pseudogenen, wobei aktuell bis zu 65 beschrieben sind. Hieraus ergibt sich die Problematik von falsch positiven Signalen. Die im Folgenden dokumentierten Ergebnisse basieren auf einer, im Rahmen dieser Arbeit, verbesserten Methodik (Kapitel 3.3.1).

3.3.1 *HMGB1*-Expression in Non-Hodgkin Lymphomen

VIII) Meyer et al., 2008, Leukemia & Lymphoma.

Bei Non-Hodgkin Lymphomen handelt es sich um Zellen des lymphatischen Systems, welche maligne Veränderungen aufweisen. Sie leiten sich aus ihrer Entstehungsgeschichte ab und sind unterteilt in B- oder T-Zell Lymphome. In dieser Untersuchung wurden 18 Proben verschiedener Untergruppen sowie 6 unveränderte Lymphknoten bezüglich ihrer *HMGB1*-Expression analysiert. Intrazellulär ist das DNA bindende Protein HMGB1 in der Lage die Expression von verschiedenen Genen zu

beeinflussen, wohingegen es extrazellulär als Zytokin ähnliches Alarmsignal agiert. Weiterhin unterdrückt das Protein die Apoptose und fördert das Wachsen von Blutgefäßen, wodurch eine maligne Entartung des Gewebeverbandes zusätzlich unterstützt wird. Diese erstmalig zur *HMGB1* Untersuchung erschlossen Proben, zeigten Veränderungen in Bezug auf das gemittelte Normalgewebe zwischen einer 3fachen Abnahme der Expression und einer 8fachen Zunahme der Expression. Die *HMGB1* Expressionsunterschiede wurden ferner mithilfe immunhistochemischer Untersuchungen belegt. Durch diese Arbeit sind Ansatzpunkte für Therapien aufgedeckt worden, mit deren Hilfe, der Patientengruppe mit hoher *HMGB1*-Expression geholfen werden könnte. Aus diesem Grund handelt es sich bei dieser Studie eventuell um einen Vorläufertest der nach klinischen Studien direkt zum Einsatz kommen kann.

3.4 HMGB1 Proteinanalysen

Neben dem *HMGB1*-Gen wurde auch das daraus resultierende HMGB1 Protein untersucht. Hierzu wurden ELISA Messungen durchgeführt, mit deren Hilfe Unterschiede in der Expression des ubiquitär vorliegenden HMGB1 in caninen Lymphomen unter Therapieeinfluss (Kapitel 3.4.1) und in humanen Ergüssen (Kapitel 3.4.2) ermittelt werden konnten.

3.4.1 Verlaufsanalyse von HMGB1 unter Chemotherapieeinfluss

IX) Meyer et al., 2010, Veterinary and Comparative Oncology.

Nachdem im Rahmen der *HMGB1* Expressionsanalysen in humanen Lymphomen deutliche Unterschiede detektiert werden konnten, sollte innerhalb dieser Studie der Einfluss von Chemotherapeutika auf den Verlauf der Expression von HMGB1 analysiert werden. Hierzu wurden die HMGB1 Blutserumkonzentrationen in 16 Hunden mit gesichertem Lymphombefund zu verschiedenen Zeitpunkten (Woche 1, Woche 2, Woche 6, Woche 12) unter kombinierter Chemotherapie untersucht und mit einer Gruppe von 16 Hunden ohne Befund verglichen.

Es konnte ein signifikanter ($p=0,005$) Unterschied zwischen der erkrankten und gesunden Gruppe detektiert werden. Ein Zusammenhang zwischen der HMGB1 Konzentration und den klassischen klinischen Parametern wurde nicht ermittelt. In 10 Fällen konnte eine deutliche Abnahme der HMGB1 Konzentration über die Zeit festgestellt werden. 4 Fälle mit einem initial niedrigen HMGB1 Level wiesen in Woche 2 einen Anstieg von HMGB1 auf, während die Konzentration in den verbleibenden 2

Fällen über die 12 Wochen konstant blieb. Weiterhin scheint ein Zusammenhang zwischen dem Ansprechen auf die Chemotherapie und dem initialen HMGB1 Level zu bestehen, wobei die Fälle mit einer hohen Initialkonzentration weniger gut abschneiden. Zusätzlich liefern die Ergebnisse Hinweise auf einen Bezug ($p=0,027$) zwischen dem Verhältnis zwischen W12/W6 und der Dauer der Remission, wobei das remmissionsfreie Überleben bei einem Wert über 122% höher ist.

Durch die regelmäßige Kontrolle der Blutseren von Lymphompatienten könnten sich während der laufenden Therapie erste Hinweise auf den Erfolg dieser ergeben. In der klinischen Praxis könnte die Beobachtung bezüglich eines Rückfalls zukünftig über den HMGB1 Status verfolgt werden.

3.4.2 Untersuchung der HMGB1 Konzentration in humanen Ergüssen

X) Winter N[1], Meyer A[1] et al., 2009, Anticancer Research.

Im Rahmen dieser Untersuchungen wurde der Einfluss des HMGB1 Proteins, welches extrazellulär unter anderem den NF-κB Signalweg aktiviert und somit die Angiogenese bzw. inflammatorische Prozesse vorantreiben kann, in Ergüssen überprüft.

Neben 6 Patientenproben mit Aszitese rguss wurden 30 Proben von Pleuraergüssen (insgesamt 11 Transudate, 12 maligne Exsudate und 13 inflammatorische Exsudate) sowohl auf die Gesamtproteinmenge als auch auf die HMGB1 Proteinmenge hin untersucht. Insgesamt konnte eine Schwankung des HMGB1 Proteingesamtanteils zwischen 0,0004% und 0,0025% detektiert werden. Die HMGB1 Konzentration ist sowohl in entzündlichen als auch in malignen Exsudaten signifikant ($p<0,001$) höher als in Transudaten. Da die Gesamtproteinmenge in Transudaten generell niedriger ist, wurde auch der Gesamtproteinanteil von HMGB1 statistisch ausgewertet, welcher bei Transudaten durchschnittlich bei 0,0005% und bei Exsudaten bei einem Anteil von durchschnittlich 0,0015% liegt. Da hier ebenfalls mit $p<0,001$ ein hoch signifikanter Unterschied detektiert werden konnte, ist die Herkunft der Differenzen aufgrund verschiedener Zellmengen ausgeschlossen.

Mithilfe des ELISA Testverfahrens könnten sich Möglichkeiten sowohl für die bessere Charakterisierung der Proben als auch für die Therapie ergeben. Mit den bisher verwendeten, von Light und Kollegen ermittelten, „Light Kriterien“ ist es nicht möglich diese Gruppen zu differenzieren. Dementsprechend ermöglicht die vorliegende Untersuchung Chancen, für Patientenkollektive, welche z.B. von HMGB1 Antikörperstrategien, die die Zytokinwirkung des HMGB1 einschränken, profitieren.

4 Diskussion

Die Problemstellung dieser Arbeit war die Klassifizierung von Prognoseparametern bei soliden Tumoren und malignen Systemerkrankungen. Mit den bisher bekannten Verfahren konnte einem Großteil der Betroffenen nicht oder nur unzureichend geholfen werden. Da weltweit etwa 11 Millionen Menschen jährlich neu zu der Gruppe der Betroffenen zählen besteht ein akuter Forschungsbedarf in diesem Bereich.

Bisherige Expressionsanalysen wurden vorwiegend an direkt präpariertem und in Stickstoff gelagertem Gewebe durchgeführt, so dass eine Aussage über den Anteil der Tumorzellen in den Proben bei Mischgewebe hypothetisch geblieben ist. Aufgrund der begrenzten Sensitivität bisher eingesetzter Verfahren (Immunhistochemie und Blotting Techniken) wurden deren Ergebnisse durch sensitivere Methoden wie z.B. der Real-Time PCR verifiziert, um detailliertere Aussagen treffen zu können. Im Zuge dieser Promotionsarbeit konnten so neue Erkenntnisse im Bereich der Erforschung der HMGA2 und HMGB1 Proteine gewonnen werden. Mithilfe eines standardisierten Verfahrens soll die Prozedur transparenter gestaltet und vereinfacht werden, so dass sie im Rahmen von Routinelabormethoden auch im Krankenhaus vor Ort zur schnellen Diagnostik eingesetzt werden kann. Hierzu wären folgende Schritte notwendig:

- ⇒ Etablierung einer quanitativen Real-Time PCR an Archivmaterial
- ⇒ Bestimmung der Expressionsstärke der Prognoseparameter
- ⇒ Statistische Erfassung von Zusammenhängen in der Expressionsstärke mit bekannten Größen

Hierbei wird das Ausmaß der Expression der *HMG*-Gene im Zusammenhang mit der Tumorentstehung sowie ihrer Progression diskutiert. Zusätzliche Vorteile dieser Vorgehensweise liegen in einem sauberen Cutpoint und der fehlenden Inter Observer Varianz, welche bei anderen Methoden wie der IHC ein Problem darstellen kann. Mit der hier etablierten sensitiven Methodik wurden signifikante Unterschiede in der Expression des *HMGA2*- und des *HMGB1*-Gens zwischen den analysierten Proben detektiert. Auch unter Anwendung quantitativer Bestimmungen der Proteinkonzentration anhand von ELISA-Untersuchungen konnten eindeutige Schwankungen in der HMGB1 Konzentration festgestellt werden.

4.1 HMGA2

Die Expression des *HMGA2*-Gens wurde mittels quantitativer Real-Time PCR bei Herzgewebe-Veränderungen wie Aortendissektionen (Belge et al., zur Veröffentlichung eingereicht) sowie in benignen (Uterusleiomyomen (UL) (Klemke et al., 2009; Klemke et al., 2010) und malignen Läsionen (Bronchialkarzinomen (Meyer et al., 2007), Schilddrüsenveränderungen (Belge et al., 2008), Speicheldrüsentumoren (Fehr et al., 2009) und Mammakarzinomen (Meyer et al., in Vorbereitung)) untersucht.

Mithilfe dieser Untersuchungen soll auf eine individualisierte Therapie hingewirkt werden. Diese würde viele Vorteile beinhalten:

- ⇒ Minimale Exposition durch Therapeutika
- ⇒ Erhaltung/Verbesserung der Lebensqualität
- ⇒ Erhöhung der Heilungswahrscheinlichkeit
- ⇒ Zeit- und Kostenersparnis bei Patient und Klinik

4.1.2 HMGA2 in Uterusleiomyomen

Bei benignen Veränderungen konnte die stärkste Erhöhung der *HMGA2*-Expression in UL mit Translokationen im Bereich 12q13-15, in dem das *HMGA2*-Gen lokalisiert ist, detektiert werden (Klemke et al., 2009). Diese Ergebnisse decken sich mit bekannten Beobachtungen (Hennig et al., 1997; Gross et al., 2003; Peng et al., 2008). Die in dieser Arbeit realisierten Messungen zeigen nicht nur in Myomen mit einer 12q13-15 Translokation eine erhöhte Expression von *HMGA2,* sondern auch generell im Myom im Vergleich zum Myometrium (Klemke et al., 2009). Deshalb ist der bisher vermutete Zusammenhang zwischen der Translokation als initialem Ereignis für die erhöhte Expression zu überdenken, da diese ohne Translokation ebenfalls auftritt. Unterstützend dazu wurde nicht in allen Fällen mit einer 12q14-15 Translokation eine über das Grundniveau hinausgehende Expression von *HMGA2* detektiert. Zur Kontrolle weiterer regulatorischer Zusammenhänge wurde der 3´UTR Bereich der UL im Hinblick auf die Expression der *let-7* miRNAs genauer untersucht (Klemke et al., 2010). Hierbei konnte festgestellt werden, dass es sich bei 3 von 12 untersuchten UL mit einer hohen *HMGA2*-Expression um eine trunkierte Variante des *HMGA2* mit fehlenden Bindungsstellen für das regulatorische *let-7* handelt. Die erhöhte *HMGA2*-Expression in 3 Fällen könnte also am Fehlen des negativ regulierenden *let-7*

festgemacht werden. Gleichzeitig konnte diese Änderung jedoch bei 9 Myomen nicht detektiert werden, was auf einen anderen oder zusätzlichen Regulationsmechanismus hinweist. Hierbei könnte es sich z.B. um eine Promotormutation handeln. Weiterhin wären auch veränderte Funktionen von Enhancern, Silencern oder weiterer Interaktionspartner von HMGA2 möglich, welche die Expression beeinflussen. Vorstellbar ist ebenfalls, dass über das verstärkte Vorhandensein des HMGA1 Proteins die Synthese des HMGA2 Proteins angeregt wird. Hierzu könnten ergänzende Experimente zur Quantifizierung von HMGA1 herangezogen werden.

4.1.2 HMGA2 in Bronchialkarzinomen

Bronchialkarzinome stellen mit 1,3 Millionen Neuerkrankungen pro Jahr und einer Mortalität von 87% eine der größten medizinischen Herausforderungen dar (Parkin et al., 2005). Die verstärkte Expression von *HMGA2* in Bronchialkarzinomen (Meyer et al., 2007) bestätigt die Untersuchungen von Sarhadi et al. und wird durch neuere Studien gestützt (Sarhadi et al., 2006; Di Cello et al., 2008; Kumar et al., 2008). In allen Arbeiten konnte ein direkter Zusammenhang zwischen der Aggressivität des Tumors und der Expressionsstärke nachgewiesen werden.

In den untersuchten unauffälligen Gewebebereichen konnte wie schon bei den UL eine basale Expression von *HMGA2* detektiert werden. Aufgrund der Ergebnisse der weniger sensitiven semiquantitativen Methoden war bisher davon ausgegangen worden, dass es nur in embryonalen bzw. tumorösen Zellen zu einer Expression von *HMGA2* kommt (Rogalla et al., 1998). Nach heutigen, u.a. durch diese Arbeit erworbenen, Erkenntnissen ist diese basale Expression jedoch von entscheidender Bedeutung für die Zellsynthese und Differenzierung (Monzen et al., 2008; Richter et al., 2009; Belge et al., zur Veröffentlichung eingereicht). Folgerichtig muss eine quantitative Aussage über die Expression getroffen werden, was die Bedeutung der etablierten quantitativen Messmethoden verdeutlicht.

Aufbauend auf die hier gewonnen Informationen bezüglich der erhöhten Expression von *HMGA2* in tumorösen Bereichen und den neuen Erkenntnissen der Regulation von *HMGA2* über die *let-7* Genfamilie kann als zukünftiger Schritt eine individuell gesteuerte, lokale Anpassung der Genexpression für die Patienten in Betracht gezogen werden. Erste Versuche von Kumar und Kollegen, in die Tumorgenese von Mäusen einzugreifen, zeigten bereits Erfolg versprechende Resultate (Kumar et al., 2008).

Im Rahmen der einfachen Tumorfrüherkennung ist es denkbar, in Zukunft Sputum-Proben von Hochrisikopatienten in regelmäßigen Abständen auf die *HMGA2*-Expression zu testen und bei Überschreiten eines Grenzwertes weiteren Maßnahmen, wie z.B. Computertomographie (CT), Magnetresonanztomographie (MRT) bzw. bei Bedarf Lungenbiopsie einzuleiten. Für bisherige Methoden war das Verhältnis zwischen dem Anteil von Tumorzellen zu normalen Zellen zu klein, um die Tumorzellen verlässlich nachzuweisen. Da die Real-Time PCR auch kleinste *HMGA2* Mengen detektiert, kann hier ein einfacher den Patienten nicht unnötig belastender, Test etabliert werden.

4.1.3 HMGA2 in Schilddrüsenneoplasien

Insgesamt erkranken 4-7% der Weltbevölkerung im Laufe des Lebens an Schilddrüsenläsionen. Sie unterteilen sich in benigne Hyperplasien und Adenome, sowie maligne papilläre, follikuläre und anaplastische Tumoren der Schilddrüse. Benigne Veränderungen sind mit 70-80% deutlich häufiger als die malignen (Baloch et al., 2002).

Zur Zeit wird bei auffälligem Ultraschall eine Feinnadelpunktion (FNA) durchgeführt (Pacini & DeGroot, 2001). Mithilfe dieser werden die Zellen analysiert und klassifiziert. Das Problem, vor dem Ärzte und Patienten bei Schilddrüsenveränderungen stehen, ist, dass sich follikuläre Adenome von Karzinomen zytologisch oft nicht unterscheiden lassen. Der Unterschied, der sich bisher lediglich durch Kapsel- und Gefäßeinbrüche definiert (Baloch et al., 2002; Smith et al., 2005), ist in durch FNA gewonnenen losen Zellverbänden nicht detektierbar. Bei unklarer Diagnose wird zur Sicherheit stets der ungünstigste Fall angenommen, was zur Folge hat, dass in 60-70% zusätzliche Therapien in die Wege geleitet werden (Tamez-Perez et al., 2007).

An dieser Stelle könnte ein neuer Marker wie *HMGA2* Abhilfe schaffen, da mittels des Unterschiedes der *HMGA2*-Expression zwischen benignen und malignen Schilddrüsenveränderungen differenziert werden kann. Dies konnte in eigenen Untersuchungen erstmalig gezeigt (Belge et al., 2008) und in neueren Studien eindeutig belegt werden (Chiappetta et al., 2008; Arora et al., 2009; Zeiger, 2009).

Der nächste Schritt zur Etablierung der *HMGA2*-Expression als molekularen Marker bei Schilddrüsenneoplasien würde darin liegen, parallele Untersuchungen zur routinemäßigen FNA durchzuführen. Hierbei wären immunohistochemische und auch auf Real-Time PCR basierende Techniken denkbar. Die Ergebnisse dieser Arbeit demonstrieren, dass beide Verfahren an histologischen Schnittpräparaten bereits

erfolgreich etabliert wurden. Aufbauend darauf könnte eine immunohistochemische Untersuchung an einem FNA Ausstrichpräparat verwirklicht werden. Es ist ebenfalls möglich, unter Verwendung der sensitiven Methode der Real-Time PCR, Ergebnisse aus sehr geringen Zellmengen zu gewinnen, so dass beispielsweise zur Extraktion der RNA lediglich die zur FNA benutzte Kanüle ausgespült werden müsste. Hierbei käme es zu keiner zusätzlichen Belastung des Patienten.

4.1.4 HMGA2 in Aortendissektionen

Ein besonders lebensbedrohliches Ereigniss für den Menschen ist die AAD, die mit 0,00004% Erkrankungswahrscheinlickeit pro Individuum jedoch relativ selten auftrit (Grundmann et al., 2006; Isselbacher, 2007). Diese Erkrankung zeichnet sich zu 90% durch einen Einriss der Intima mit nachfolgendem Bluteinstrom in die Aortenwand aus (Nienaber & Eagle, 2003). Eine diagnostische Herausforderung ist die Vorhersage einer solchen Dissektion. Sobald sie eintritt, ist ein Handeln nur noch schwer möglich und die Mortalitätsrate dadurch mit bis zu 90% sehr hoch (Grundmann et al., 2006). Die im Rahmen dieser Dissertationsschrift veröffentlichten Daten weisen erstmals einen direkten Zusammenhang zwischen dem Auftreten der AAD und der *HMGA2* Genexpression auf.

Der Zusammenhang könnte in einer durch EMT verursachten Reembryonalisierung des Gewebes mit anschließendem Funktionsverlust begründet sein. In vorherigen Untersuchungen konnte bereits belegt werden, dass HMGA2 auch eine Rolle bei der EMT spielt (Thuault et al., 2006). Der Zusammenhang zwischen HMGA2, der EMT sowie der Tumorprogression konnte an Tumoren des Pankreas belegt werden (Watanabe et al., 2009).

Anhand des Nachweises der Korrelation zwischen der Expression von *HMGA2* und dem Auftreten der AAD, könnte bei Risikopatienten die *HMGA2*-Expression kontinuierlich überwacht werden und bei einem Anstieg und demnach erhöhtem Risiko entsprechend frühzeitig gehandelt werden. Da eine ständige Überprüfung des Aortengewebes des Patienten jedoch nicht praktikabel erscheint, sollte diese drastische Maßnahme lediglich für die Hochrisikopatienten (z.B. familiäre Prädispositionen wie Marfan Syndrom und Ehlers-Danlos Syndrom (Nienaber & Eagle, 2003)) in Betracht gezogen werden. Ein Ansatz für folgende Projekte wäre die Überprüfung der *HMGA2*-Expression an Blut aus der Aorta, da sich eventuell in den abgetragenen Zellen bereits erhöhte Mengen an *HMGA2* detektieren lassen.

Alternativ könnten auch durch normale Blutabnahme ausreichende Mengen an *HMGA2* detektiert werden. Hierbei bleibt jedoch die Herausforderung, dass diese auch aus anderen Bereichen des Körpers stammen könnten und nicht zwingend in Zusammenhang mit einer möglichen AAD stehen müssen. Sollte sich diese Hypothese als anwendbar erweisen, könnte dieser Nachweis einer Vielzahl von Patienten das Leben retten.

4.1.5 HMGA2 in Speicheldrüsentumoren

Zu den häufigsten Speicheldrüsentumoren gehören mit ca. 5-16% die MEC (Spiro, 1986; Seifert et al., 1990). Diese unterschiedlich differenzierten, plattenepithelialen und schleimbildenden Tumorzellen werden in niedrig- und hochdifferenzierte Tumoren unterschieden (Healey et al., 1970). Die 5 Jahres-Überlebenswahrscheinlichkeit liegt aktuell bei durchschnittlich 62,3% (Ozawa et al., 2008).

Die bei Warthins Tumoren auftretende Translokation t(11;19)(q21;p13) (Enlund et al., 2004), welche die Gene *CRTC1* auf Chromosom 19 und *MAML2* auf Chromosom 11 umfasst, konnte hier auch in einer Subgruppe der MEC beschrieben werden (Fehr et al., 2009). Diese gehören vorwiegend in die Gruppe mit niedrigem oder mittlerem Malignitätsgrad (Behboudi et al., 2006). Tirado und Kollegen beschrieben 2007 eine verstärkte Ausbildung von Metastasen bei Tumoren ohne Fusionstranskript (Tirado et al., 2007). Darüber hinaus konnte gezeigt werden, dass die Anwesenheit des Fusionstranskriptes mit einer signifikant geringeren *HMGA2* Genexpressionsstärke korreliert (Fehr et al., 2009).

Die in dieser Arbeit präsentierten Ergebnisse geben Aufschluss über die Diversität innerhalb der MEC. Aufgrund der unterschiedlichen Tumorbiologie erscheint es sinnvoll, die Gruppe der MEC neu zu strukturieren. Die MEC lassen sich aufgrund der hier gewonnen neuen Erkenntnisse zwei Untergruppen zuordnen:

⇒ Gruppe 1 mit niedriger *HMGA2*-Expression sowie einem *CRCT1-MAML2* Fusionstranskript → günstige Prognose

⇒ Gruppe 2 mit hoher *HMGA2*-Expression sowie ohne einem *CRCT1-MAML2* Fusionstranskript → ungünstige Prognose

Die ermittelten Daten liefern Ansatzpunkte für eine klinische Studie, in der die Daten verifiziert werden müssen. Die ersten Erkenntnisse lassen jedoch hoffen, dass zu-

künftig auch grenzwertig zu klassifizierende Fälle besser diagnostizierbar sind und Therapien individueller für den Patienten umgesetzt werden können.

4.1.6 HMGA2 in Mammakarzinomen

Mammakarzinome sind nach Bronchialkarzinomen die zweithäufigste Tumorerkrankung insgesamt und machen bei Frauen 23% aller Krebserkrankungen aus (Parkin et al., 2005). Bisherige Untersuchungen äußern sich widersprüchlich über die Verwendung von *HMGA2* als Tumormarker in Proben von Mammakarzinompatientinnen. So konnten beispielsweise Langelotz und Kollegen im Jahr 2003 einen signifikanten Zusammenhang zwischen der Stärke der *HMGA2*-Expression in Blut von Tumorpatientinnen im Vergleich zu Gesunden nachweisen. Fabjani und Kollegen hingegen konnten im Jahre 2005 diese Idee durch Verwendung einer sensitiveren Methode widerlegten, da sie im Blut von allen untersuchten Patientinnen *HMGA2* detektieren konnten und somit *HMGA2* als reinen Marker, der nur bei Vorhandensein von Tumorzellen auftritt, ausschlossen (Langelotz et al., 2003; Fabjani et al., 2005).

Wie bereits in Abschnitt 3.1 und 3.2 beschrieben, ist es mit den durch diese Arbeit etablierten Methoden ebenfalls möglich, in allen Geweben eine basale *HMGA2*-Expression zu detektieren. Die Besonderheit liegt jedoch in der Höhe des *HMGA2* Expressionsanstiegs, der den weiteren Krankheitsverlauf in hohem Maße zu beeinflussen scheint. So wurden Proben von 130 Probandinnen auf die *HMGA2*-Expression hin untersucht. Es konnte innerhalb dieses Niedrig-Risiko Kollektivs (in Bezug auf Rezidiv freiem bzw. Gesamtüberleben) eine Untergruppe von bisher verdeckten und nicht diagnostizierbaren Hoch-Risiko Patientinnen identifiziert werden. Die Vergleiche wurden mittels Cox Regressionsanalyse, welche die Patientenverteilung innerhalb der weiteren gemessenen Parameter (Alter, Tumorgröße, Grading, ER, PR, *uPA*, *PAI-1*) normalisierend berücksichtigt, durchgeführt. Hierbei konnten mithilfe der *HMGA2* Expressionsmessung sowohl bezüglich des Gesamtüberlebens ($p=0,001$), als auch bezüglich des rezidiv-freien Überlebens ($p=0,003$), hoch signifikante Unterschiede ermittelt werden.

Weiterhin konnte mittels multifaktorieller Cox-Regressionsanalyse bewiesen werden, dass es sich bei der *HMGA2*-Expression um einen von bisherigen Parametern unabhängigen potentiellen Prognoseparameter handelt, der zusätzliche Informationen zum Patienten offenbart. Daraus ergibt sich, dass bei zukünftigen *HMGA2* Messungen an neuen Patienten eine individualisierte Aussage zur Überlebens-

wahrscheinlichkeit getroffen werden kann. Die ermittelte Hazard Ratio, innerhalb von 5 Jahren zu versterben, liegt bei 16.9 (p=0.0003).

Auch innerhalb dieser Tumorentität bietet sich eine neue Strukturierung des Patientenkollektivs, in eine Gruppe mit hoher *HMGA2* Expression mit schlechterer Prognose, und eine zweite Gruppe mit niedriger *HMGA2* Expression und guter Prognose, an.

4.1.7 HMGA2 in Bezug zur praktischen Anwendbarkeit

Sollten sich die Ergebnisse der beschriebenen Verfahren auch in Kliniken und durch andere Institute bestätigen lassen, wären eine Umgruppierung von Patientenkollektiven sowie eine Anpassung der Therapie für die in den vorhergehenden Abschnitten beschriebenen Tumorentitäten in Betracht zu ziehen.

Eine Therapieanpassung wäre beispielsweise bei Mammakarzinomen denkbar, da bereits Interaktionen von HMGA2 mit Therapeutika nachgewiesen werden konnten. So stellten beispielsweise Boo und Kollegen einen Zusammenhang zwischen der Stärke der *HMGA2*-Expression und der Sensitivität der Zellen für das Taxan Doxorubicin her (Boo et al., 2005). In Zellkulturversuchen wurde gezeigt, dass bei Zugabe von 7 µmol/l Doxorubicin nahezu alle Tumorzellen mit einer erhöhten *HMGA2*-Expression eine 5fach erhöhte Apoptoserate, sowie eine deutliche Anhäufung der Zellen in der G_2-M Phase aufwiesen (Boo et al., 2005).

Bei dem im Fall von Rezeptorpositivität eingesetzten Hormonrezeptormodulator Tamoxifen gelang es Holm und Kollegen, bei phosphoryliertem ERαS305 Rezeptor eine Resistenz gegen das Medikament zu detektieren (Holm et al., 2009). Es wäre vorstellbar, dass diese Phosphorylierung durch ein vermehrtes Auftreten von HMGA2 induziert ist, wie es bei Histon 2A Variante X (H2AX) bereits beschrieben wurde (Boo et al., 2005). Diese Hypothese sollte in einer vergleichenden Analyse verifiziert werden. Weiterhin bleibt noch zu untersuchen, inwieweit die Erkenntnisse bezüglich der *HMGA2*-Expression sich auch auf andere, z.B. Hoch-Risiko Brustkrebspatientinnen (Nodal positiv; Rezeptor negativ), anwenden lassen.

Neben der Interaktion durch Phosphorylierung spielt das HMGA2 Protein auch eine Rolle bei der Acetylierung. Hier konnten Fedele und Kollegen nachweisen, dass das E2F1 Protein, welches für die Zellzykluskontrolle von Bedeutung ist, durch HMGA2 acetyliert wird (Fedele et al., 2006). Es ist bereits seit längerem bekannt, dass ein

erhöhtes Aufkommen des E2F1 Proteins eine neoplastische Zelltransformation auslösen kann (Johnson et al., 1994). Dieser Zusammenhang kann, wie auch die Veränderung der Methylierungsstruktur, bei allen untersuchten Geweben wichtig sein. So wird die Differenzierung von Nervenzellen, welche über viel HMGA2 verfügen, u.a. über die Methylierung gesteuert (Sanosaka et al., 2008). Eine Hypomethylierung führt beispielsweise zu einer verstärkten Expression von Proto-Onkogenen und erhöhten Mutationsraten, während eine Hypermethylierung in einer verringerten Expression von Tumorsupressor Genen und verminderter DNA Reparatur resultiert (Agrawal et al., 2007). Diese Untersuchungen korrelieren mit den Erkenntnissen von Premkumar et al. 2008, welche für Patientinnen mit einer geringeren DNA-Methylierung eine bessere Prognose ermitteln konnten (Premkumar et al., 2008).

Im Rahmen dieser Dissertation wurden Epithel-, Drüsen und Muskelzellen und somit gänzlich verschiedene Zelltypen untersucht, wobei die *HMGA2*-Expression sich durchgängig als auffällig erwies. Auch in anderen Tumoren, z.B. des Pankreas (Hristov et al., 2009), des Colons (Huang et al., 2009), der Retina (Venkatesan et al., 2009) und des Kehlkopfs (Ma et al., 2009) konnten Auffälligkeiten in der *HMGA2*-Expression ermittelt werden, die mit den unter Kapitel 3 präsentierten Erkenntnissen übereinstimmen. Hieraus kann geschlussfolgert werden, dass es sich bei der Veränderung der *HMGA2*-Expression um einen generellen Mechanismus handelt und dass die weitere Erforschung prediktiven Nutzen für eine Reihe von benignen und malignen Neubildungen haben könnte. Es konnte bewiesen werden, dass es mithilfe der Quantifizierung des *HMGA2* Gens möglich ist, Diagnosen zu stellen, die vorher nicht oder nur unzureichend erstellt werden konnten. Des Weiteren konnten im Rahmen der Untersuchung an Mammakarzinom Archivmaterial hoch signifikante Aussagen in Bezug auf die Prognose getroffen werden.

Zu klären bleibt, wie es in den Fällen ohne chromosomale Veränderungen zu einer gezielten Aktivierung des *HMGA2*-Gens bzw. zu der Induzierung der EMT kommt. Mit diesem Wissen könnte diese Aktivierung kontrolliert unterbunden und eine Tumorentstehung bzw. Progression verhindert werden. Vermutlich könnte die Zellrückentwicklung während der EMT, wie auch bei Tumoren entdeckt, mit einer unkontrollierten Stammzelle starten. Die Theorie der entarteten Stammzelle findet für unterschiedliche Tumorentitäten aktuell steigenden Anklang, wobei Bonnet und Dick bereits 1997 den erstmaligen Nachweis von carcinogenen Stammzellen erbrachten indem sie die Entstehung der Leukämie näher betrachteten (Bonnet & Dick, 1997).

Diese vermeintlich unreifen Zelltypen gewinnen erneut die Möglichkeit zu Migration (Thuault et al., 2008), Proliferation und Differenzierung (Kakarala & Wicha, 2008). Das in embryonalen und proliferativen Geweben verstärkt auftretende HMGA2 scheint hierbei eine generelle, nicht Zelltyp spezifische, Rolle zu spielen. Außer Kontrolle geratene Stammzellen bergen für den Organismus dramatische Risiken:

⇒ Kontrollverlust von Zellen

⇒ Kontrollverlust von Gewebebereichen

⇒ Kontrollverlust des Informationsaustausches

⇒ Kontrollverlust des Zellwachstums

⇒ Kontrollverlust der Apoptose

⇒ Kontrollverlust der Migration

⇒ Verlust der eigentlichen Funktion

Diese führen nicht selten zur Ausbildung von soliden Tumoren bzw. systemischen Erkrankungen, welche weltweit zu den häufigsten Todesursachen gehören.

Anhand der in dieser Dissertation durchgeführten Arbeiten bestätigt sich, dass es sich bei dem *HMGA2*-Gen um einen unabhängigen und konstanten Marker handelt, welcher Entscheidungen ermöglicht, die ohne das Wissen um die *HMGA2*-Expression nicht gefällt werden könnten. Dieser wertvolle Schritt der Erforschung des *HMGA2*-Gens sowie des daraus resultierenden Proteins erwirkt eine weitergehende Analyse, um die mit dem *HMGA2*-Gen verbundenen molekularen Mechanismen genauer zu verstehen. Mit diesem Wissen könnte gezielt und mit wenigen Nebenwirkungen in den spezifischen Organismus eingegriffen werden um die bisher bestehenden Diagnose-, Prognose- und Therapieansätze zu verbessern bzw. zu ersetzen.

4.2 HMGB1

Zur Untersuchung der Proliferation von HMGB1 wurden neue Messmethoden für die Quantifizierung von Proben etabliert (Meyer et al., 2008). Ferner wurde die Konzentration von HMGB1 in humanen Exsudaten (Winter et al., 2009) sowie in Seren von caninen Lymphompatienten (Meyer et al., 2010) untersucht.

Große Ähnlichkeiten zwischen humanem und caninem HMGB1 (95,4%) (Murua Escobar et al., 2003) ermöglichen vergleichende Analysen zur Tumorgenese an dem Modelltier Hund. Im Vergleich zum Nagetiermodell bietet das Modelltier Hund zur

Erforschung der genetischen Grundlagen deutliche Vorteile. Zum einen hat der Hund in der Regel die gleichen Lebensbedingungen und ist ähnlichen Umwelteinflüssen ausgesetzt (Vail & MacEwen, 2000). Zum anderen entstehen die Tumoren des Hundes, im Vergleich zur Maus, auch spontan und mit einer höheren Inzidenz als beim Menschen. Weiterhin hat die Tumorgenese, aufgrund der geringeren Lebensspanne des Hundes, einen schnelleren Verlauf, so dass bei ähnlicher Biologie und Histopathologie die Tumorentstehung und Progression in einem weitaus kürzeren Zeitraum beobachtet werden kann (MacEwen, 1990). Bereits 1990 beschrieb MacEwen die Ähnlichkeit der Tumorbiologie bei Non-Hodgkin Lymphomen (NHL) zwischen Mensch und Hund (MacEwen, 1990), so dass dieser sich für die nachfolgend beschriebene Untersuchung der NHL besonders gut als Vergleichsorganismus eignet.

4.2.1 *HMGB1* in NHL

Zur Etablierung eines robusten Messsystems für das pseudogenreiche *HMGB1* wurden die Methode der quantitativen *HMGB1* Messung mittels Real-Time PCR durch einen zusätzlichen DNase1 Verdau modifiziert. Dieser erlaubt die nahezu restlose Entfernung der, für falsch positive Signale sorgenden, Pseudogene. Mithilfe der so entwickelten Methodenvarianz wurden Proben aus einem Kollektiv von humanen NHL (Meyer et al., 2008) untersucht.

NHL entstehen unter anderem durch ein Ausbleiben der Apoptose und stehen aus diesem Grund in einem möglichen Zusammenhang mit HMGB1, welches in Krebszellen die Apoptose verhindert (Huttunen et al., 2000), während das Wachstum von Makrophagen unterdrückt und deren Apoptoserate erhöht werden (Kuniyasu et al., 2005). Deshalb hat das HMGB1 Protein in der Tumorprogression von Lymphomen signifikanten Einfluss und stellt ein lohnendes Ziel für eine Therapie mittels Viren oder Biologicals dar. Biologicals sind (z.B. Wirkstoff Rituximab (Maloney et al., 1997a; Maloney et al., 1997b)) bei Untergruppen von NHL Patienten bereits eine wirkungsvolle Ergänzung neben der konventionellen Strahlen- und Chemotherapie bei CD20 positiven Zellen (Cvetkovic & Perry, 2006).

Zu berücksichtigen bleibt jedoch, dass eine Behandlung, die sich gegen das HMGB1 Protein oder seine Expression richtet, nur gezielt in dem betroffenen Areal erfolgen sollte, da Mäuse ohne HMGB1 aufgrund seiner vielfältigen Funktionen nicht lebensfähig sind (Calogero et al., 1999). Der extrem sensible Bereich, in dem das HMGB1 Expressionsniveau existenziell ist, wird an den Untersuchungen von Wang und

Kollegen deutlich. Sie konnten an Mäusen nachweisen, dass eine erhöhte Menge HMGB1 ebenfalls letal wirkt (Wang et al., 1999).

In allen im Rahmen dieser Dissertation durchgeführten Untersuchungen konnte das ubiquitär exprimierte HMGB1 Protein nachgewiesen und mithilfe immunohistochemischer Analysen bei erhöhter Expression speziell den Tumorzellen zugeordnet werden. In den per qRT-PCR auf *HMGB1* untersuchten humanen malignen NHL Proben konnten deutliche Schwankungen im Bereich der *HMGB1*-Expression ermittelt werden. Signifikante Unterschiede zwischen niedrig und hoch malignen Fällen konnten jedoch, möglicherweise durch die geringe Fallzahl bedingt, nicht festgestellt werden. Da HMGB1 in direktem Zusammenhang mit der Apoptose (Huttunen et al., 2000) sowie für die Tumorprogression relevanten Prozessen steht, wurde der Expressionsverlauf von HMGB1 unter Chemotherapieeinfluss näher analysiert. Hierzu wurden vom Zeitpunkt vor Therapie ausgehend über den gesamten Behandlungszeitraum regelmäßig (Woche 1, Woche 2, Woche 6 und Woche 12) Blutseren der caninen Probanden mittels ELISA auf ihre HMGB1-Expression hin untersucht.

Im Rahmen dieser Arbeit wurden erstmalig Untersuchungen, die einen Überblick über den gesamten Behandlungsverlauf geben, realisiert. Eine signifikant höhere Menge an HMGB1 konnte sowohl zu Beginn der Therapie als auch in der Gruppe der Lymphompatienten ermittelt werden. In vier Fällen mit einer initial niedrigeren HMGB1-Expression kommt es in Woche 2 zu einer erhöhten Expression von HMGB1, welche in Woche 6 bereits unter Anfangsniveau sinkt. Aufgrund der gemessenen Zeitintervalle lässt sich nicht final klären, ob dieses Phänomen sich auch in anderen Proben in einem früheren Zeitraum wiederfindet. Es wäre jedoch ebenfalls denkbar, dass unterschiedliche Polymorphismen des *HMGB1*-Gens eine Rolle bei der Gesamtexpression spielen (Kornblit et al., 2007). Zu berücksichtigen bleibt allerdings, dass Zellen auf therapeutischen Stress unterschiedlich reagieren und somit auch hier der Grund für die Variationen liegen kann (Kepp et al., 2009).

Ein für die Zukunft erstrebenswerter Ansatz wäre die Untersuchung der HMGB1-Expression in kürzeren Zeitintervallen, um eventuelle Veränderungen genauer zuordnen zu können. Besonders zu Beginn der Therapie scheint es deutliche Schwankungen der HMGB1-Expression zu geben, welche möglicherweise mit dem Therapieerfolg korrelieren. Bei einem Verhältnis von Woche 12 zu Woche 6 von unter 122% konnte eine signifikant längere Zeit bis zur kompletten Remission beobachtet

werden. Der Zeitverlauf liefert deutliche Hinweise auf einen Zusammenhang zwischen Expression und Remission. Könnte diese Vermutung mit weiteren Fällen auch ohne Remission bzw. partieller Remission bestätigt werden, wäre eine Überwachung der HMGB1 Expressionswerte in Blutseren eine einfache Bestätigung des Erfolgs der gewählten Therapie. Ein stressinduzierter Anstieg von HMGB1 wäre hierbei auch für Patienten mit erfolgreicher Chemotherapie zu erwarten.

Therapierelevant wird dieses Wissen bei den Therapeutika Cisplatin und Carboplatin, welche neben zahlreichen Nebenwirkungen deutliche Erfolge aufweisen (Von Hoff et al., 1979), weshalb eine Verwendung dieser Medikamente auch aktuell dennoch häufig in Betracht gezogen wird (Azzoli et al., 2009; Tsang et al., 2009). Bei hoher HMGB1 Expressionsrate ist für den gleichen Therapieerfolg weniger Cisplatin im Körper des Patienten notwendig (He et al., 2000), wodurch sowohl der Umfang der Nebenwirkungen und das Risiko einer Überdosierung als auch die Kosten für Medikamente kontrolliert gesenkt werden könnten. Mithilfe von *Hmgb1*$^{-/-}$ sowie *Hmgb1*$^{+/+}$ Mausmodellen konnte eine erhöhte Sensitivität für die Medikamente 5-fluorouracil, cytosine arabinoside und mercaptopurine bei den für HMGB1 positiven Modellen ermittelt werden, welche u.a. für die Behandlung von NHL relevant sind (Krynetskaia et al., 2008). HMGB1 negative Zellen weisen im Vergleich dazu deutlich weniger phosphorylierte Tumorsupressorgene (z.B. *p53, H2AX*) auf. Sie sprechen schlechter auf die Chemotherapie an, wohingegen eine Zugabe von HMGB1 zur Phosphorylierung und damit zur gesteigerten Aktivität von z.B. p53 führt (Krynetskaia et al., 2008).

Neben der Interaktion von HMGB1 mit Chemotherapeutikern lösen auch Steroide über einen bisher nicht genauer untersuchten Mechanismus eine erhöhte HMGB1 Expression aus (Chau et al., 1998; He et al., 2000). Dadurch ist zumindest für die Tumoren, in denen die entsprechenden Hormonrezeptoren vorhanden sind, ein Mittel zum Eingreifen in den HMGB1 Zyklus gegeben. Die erhöhte Expression von HMGB1 ist ebenfalls als Reaktion auf Zytokine beschrieben worden. Die Menge an HMGB1 nimmt sowohl im Zytoplasma als auch im Zellkern unter Einfluss des Zytokins IFN-γ zu (Grundtman et al., 2009). Als Zytokin nimmt HMGB1 Einfluss auf die endokrine und parakrine Steuerung inflammatorischer Signalkaskaden (Wang et al., 2001; Yang et al., 2001) und ist demnach neben Tumoren auch bei Erkrankungen wie rheumatoider Arthritis (RA) und systemischer Lupus erythematodes (SLE) relevant. Sobald seine Interaktionen erforscht und ein gezieltes Eingreifen in die Synthese des Proteins möglich ist, könnte das HMGB1 Protein in einem deutlich breiteren

medizinischen Feld als der Onkologie wertvolle Dienste leisten. Einen Hinweis liefert das Phänomen der Verbesserung des Gesundheitszustandes von RA und SLE Patientinnen während der Schwangerschaft (Zrour et al., 2009). Eine die HMGB1-Expression günstig beeinflussende Hormonumstellung, könnte für ein Abklingen entzündlicher Signalkaskaden im Körper der Frauen ursächlich sein. Sollte sich diese Theorie als haltbar erweisen, könnte der durch die Schwangerschaft ausgelöste Wechsel des Hormonhaushalts eventuell für diese Gruppe kopiert werden. Aufgrund der bekannten Probleme bezüglich des Überlebens mit zu viel (Wang et al., 1999) oder ohne (Calogero et al., 1999) HMGB1 im Organismus, muss diese Steuerung jedoch genau überwacht werden.

4.2.2 *HMGB1* in Ergüssen

Neben Lymphomen wurden Exsudate und Transudate unterschiedlichen Ursprungs auf ihren Gesamtproteinanteil bzw. auf die Menge an HMGB1 hin untersucht. Die Hypothese, das HMGB1 Protein vermehrt in Ergüssen von Patienten mit malignen Erkrankungen zu detektieren, welche den Erguss auch ausgelöst haben können, hat sich bestätigt. Es wurde in diesem Ansatz sowohl das extrazelluläre als auch das in abgetragenen Zellen aus den Ergüssen vorhandene HMGB1 quantifiziert. Da Transudate im Vergleich zu Exsudaten zellärmer sind, wurde der Anteil des HMGB1 Proteins in Relation zur Gesamtproteinmenge untersucht. Mithilfe der neuen Information über die HMGB1-Expression in humanen Exsudaten könnte ein Beitrag zur Diagnose geleistet werden.

Interessant ist in diesem Fall neben dem intrazellulären HMGB1 im Besonderen das in Exsudaten auftretende extrazelluläre HMGB1. Extrazelluläres HMGB1, z.B. abgegeben durch Makrophagen (Wang et al., 1999), Monozyten (Wang et al., 1999), dendritische Zellen (Passalacqua et al., 1998) oder Thrombozyten (Rouhiainen et al., 2000), führt unter anderem zur Aktivierung von Makrophagen (Han et al., 2008), welche normalerweise verstärkt als Reaktion auf Verletzungen, Infektionen oder Fremdkörper ausgesandt werden. Ursache für Ergüsse können neben Infektionen auch Tumoren sein. Das in einzelnen Ergüssen verstärkt messbare HMGB1 könnte aus Tumorzellen stammen und somit wäre es möglich Patienten genauer auf unentdeckte Tumoren zu untersuchen. Weiterhin steuert das HMGB1 Zellwachstum und -migration sowie Synthese von versorgenden Blutgefäßen, so dass bei einem vermehrten Auftreten im Erguss ein Handlungsbedarf bestehen könnte. Daher kann

eine Überwachung des Patienten bezüglich des HMGB1-Status angeraten sein. Die so gewonnenen Informationen können für den Patienten positiv genutzt werden indem Therapiemöglichkeiten eröffnet bzw. bei niedrigen Werten schlimmere Diagnosen ausgeschlossen werden können. Die HMGB1 Aktivität wird zellulär u.a. über den Oxidationsstatus (Hoppe et al., 2006; Sahu et al., 2008) oder die Proteinhalbwertszeit geregelt, so dass diese Ansatzpunkte für eine gezielte Steuerung der HMGB1 Aktivität liefern könnten.

4.3 Potenzial der analysierten Prognoseparameter

Die Proteine HMGA2 und HMGB1 weisen ein hohes prediktives Potenzial auf und das Wissen um ihre Expression kann helfen, gezielt bekannte Therapien auszuwählen, neue Ansatzpunkte für Therapeutika aufzudecken und bisher unbekannte Aussagen zu Diagnose und Prognose zu treffen. Die Kernaussagen, welche beschrieben werden konnten, sind:

- In allen untersuchten Geweben konnte *HMGA2* gegensätzlich zu bisherigen Annahmen detektiert werden. In Tumorzellen ist die *HMGA2* Expression zumeist erhöht.
- Die erhöhte *HMGA2*-Expression geht ursächlich nicht immer auf eine Translokation 12q14-15 zurück. Weiterhin konnte *let-7* als alleiniger Regulator von *HMGA2* ausgeschlossen werden.
- Benigne und maligne follikuläre Schilddrüsenneoplasien können mithilfe der Analyse der *HMGA2*-Expression unterschieden werden.
- Bei MEC können, vor dem Hintergrund der *CRCT1-MAML2* Fusion und der *HMGA2*-Expression, zwei Untergruppen mit unterschiedlicher Prognose differenziert werden.
- In der Gruppe der nodal negativen Hormonrezeptor positven Brustkrebspatientinnen gibt es ein Hoch-Risiko Kollektiv mit hoher *HMGA2* Expression. Eine Vorhersage der Wahrscheinlichkeit für Rezidiv freies bzw. Gesamtüberleben ist hoch signifikant.
- Interaktionen von HMGA2 mit verschiedenen Therapeutika sind bekannt. Durch das Wissen um die *HMGA2* Expression könnte eine Belastung der Patienten, durch unnötige Exposition mit Therapeutika, welche bekanntermaßen

nicht wirken, verhindert werden. Weiterhin könnten durch gezielte Therapien Kosten reduziert werden.

- Die Messung mittels Real-Time PCR lässt Aussagen in einer deutlich größeren Gruppe zu, so dass ein zusätzlicher Aufbau sich auch in Laboren, welche molekularbiologische Methoden wie IHC und ELISA bereits anwenden, positiv bemerkbar machen würde.
- Das Ausmaß der HMGB1-Expression schwankt sowohl in Lymphomen als auch in Ergüssen deutlich.
- Die Veränderung der Expression von HMGB1 unter Chemotherapieeinfluss scheint ein Indikator für den Therapieerfolg zu sein.
- Eine gezielte Dosierung von Medikamenten wie z.B. Cisplatin könnte aufgrund der etablierten Methodik zur exakten Quantifizierung von HMGB1 erfolgen.

Die im Rahmen dieser Untersuchung gewonnen Erkenntnisse ermöglichen es, zugunsten des Patientenwohls eine verbesserte Diagnose zu stellen. Insgesamt können durch die Angabe der Erfolgs- und Überlebenswahrscheinlichkeiten Klassifizierungen optimiert, unnötige Operationen vermieden, sowie medikamentöse Behandlungen eingespart werden.

5 Zusammenfassung

Solide Tumoren und maligne Systemerkrankungen sind mit weit über 11 Millionen Neuerkrankungen pro Jahr zwei der größten Herausforderungen der Medizin weltweit. Durch die in dieser Dissertationsschrift entwickelten Methoden und aufgedeckten Zusammenhänge zwischen HMG Proteinen und den Erkrankungen konnte ein wesentlicher Schritt zur Eindämmung dieser Problematik geleistet werden.

Mithilfe der veränderten Methodik wurden erstmals pseudogenreiche Genfamilien für die Genexpressionsmessung erschlossen. Weiterhin wurden vielfältige Expressionsanalysen auf Gen- und Proteinebene durchgeführt, wodurch neue Eindrücke der Expressionsmuster von den untersuchten Prognoseparametern (*HMGA2*, *HMGB1*, *uPA*, *PAI1*, *Her2neu*) generiert und analysiert werden konnten.

Auffällig bei der *HMGA2*-Expression ist, dass im Gegensatz zur bisherigen Annahme, in allen Fällen eine basale Expression von *HMGA2* existiert, welche aufgrund der sensitiveren Methodik erstmalig bewiesen werden konnte. Dies deutet darauf hin, dass nicht wie bisher angenommen die Re-Expression von *HMGA2* generell der Auslöser für die maligne Zellentartung ist. Es konnte ein signifikanter Zusammenhang zwischen der Expressionsstärke und der Malignität von Bronchial-, Schilddrüsen-, Speicheldrüsen- und Mammakarzinomen detektiert werden. Eine Veränderung im chromosomalen Bereich 12q14 führte bei UL nachweislich zu einer verstärkten Expression von *HMGA2*, wobei die Steuerung nicht ausschließlich über die *let-7* miRNAs erfolgt. Die Unterscheidung von benignen und malignen follikulären Schilddrüsenneoplasien sowie die Risikoabschätzung zum Überleben bei Mammakarzinompatienten wurden mithilfe der *HMGA2* Expressionsanalyse erreicht.

Die *HMGB1* Genexpressionsmessungen an NHL lieferten erstmalig differenzierte Unterschiede in der Proteinmenge. Die anschließende Untersuchung caniner Lymphomseren unter Therapieeinfluss ergab eine Veränderung der HMGB1 Menge, welche mit dem Therapieerfolg in direktem Zusammenhang steht. In humanen malignen Ergüssen konnte eine erhöhte HMGB1 Menge detektiert werden.

Hieraus ergibt sich die Schlussfolgerung, dass die HMG Proteine einen signifikanten Einfluss innerhalb der Tumorgenese einnehmen. Die durchgeführten Untersuchungen bieten Grundlagen für die Anwendung neuer Messverfahren und zahlreiche Ansatzpunkte für klinische Studien, so dass diese Arbeit ein Grundstein für die Verbesserung der Diagnose, Prognose und Therapie in malignen Tumoren darstellt.

6 Summary

Solid tumors and malignant systemic diseases are two of the most severe burdens of the world. The incidence rate of more than 11 million cases per year is considerably high. For this reason new methods and knowledge are required. By the methods developed and relations given in this dissertation a big step forward in cancer research is realized.

New possibilities to measure gene expression of pseudogene rich gene families were established. Furthermore multifaceted gene expression profiles for prognostic parameters such as *HMGA2*, *HMGB1*, *uPA*, *PAI1*, *Her2neu* were established, analysed, and discussed.

Contrary to the existing theory of expression limited to embryonic or malignant tissues *HMGA2* was detected in all samples analysed. Hence, the theory of occurring HMGA2 playing a general role in malignant cell transformation must be reconsiderd. Nevertheless, a correlation between the amount of HMGA2 protein and the grade of malignancy could be described in lung, thyroid, salivary gland, and breast cancers. Chromosomal modifications in the region 12q14 of the *HMGA2* gene lead to a significant higher expression of *HMGA2*. Expression level changes were not detectable in all cases by *let-7* miRNAs. Consequently, further controlling pathways must exist. The first time ever possibility to distinguish between benign and malign thyroid neoplasias was established by the *HMGA2* gene expression measurement described. In addition, this work is an enabler for the prediction of the chance of RFI and OS for node negative and hormone receptor positive breast cancer patients.

For the first time it is described, that there are varying *HMGB1* amounts in NHL. In the analysed canine sample set, an influence of chemotherapy on therapy success was observed. The high peak of HMGB1 expression in the beginning of therapy correlates with the success of the treatment. Also in human pleural and peritoneal effusions, different amounts of HMGB1 were detected. The expression of HMGB1 increased with higher grades of malignancy. Also in inflammatory cases a higher expression of HMGB1 was revealed.

The presented work underlines the significantly high influence of HMG proteins in cancer development. New approaches and starting points for further analyses are identified and are the basis to upgrade diagnosis, prognosis, and therapy of cancer patients.

7 Literatur

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9 Publikationsübersicht

In der folgenden Übersicht sind die der vorliegenden Arbeit zugrunde liegenden Publikationen in der Reihenfolge, in der sie im Ergebnisteil erscheinen, aufgeführt.

* Diese Autoren sind gleichberechtigte Erstautoren der Publikation

I.) Meyer B, Loeschke S, Schultze A, Weigel T, Sandkamp M, Goldmann T, Vollmer E, Bullerdiek J. *HMGA2* overexpression in non-small cell lung cancer. *Molecular carcinogenesis* 2007. 46(7):503-511.

II.) Belge G*, Meyer A*, Klemke M*, Burchardt K, Stern C, Wosniok W, Loeschke S, Bullerdiek J. Upregulation of *HMGA2* in thyroid carcinomas: a novel molecular marker to distinguish between benign and malignant neoplasias. *Genes Chromosomes Cancer* 2008. 47(1):56-63.

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IV.) Fehr A, Meyer A, Heidorn K, Röser K, Löning T, Bullerdiek J. A Link between the Expression of the Stem Cell Marker *HMGA2*, Grading and the Fusion *CRTC1-MAML2* in Mucoepidermoid Carcinoma. *Genes Chromosomes and Cancer* 2009. 48(9):777-785.

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VI.) Klemke M*, Meyer A*, Hashemi Nezhad M, Bartnitzke S, Drieschner N, Frantzen C, Schmidt EH, Belge G, Bullerdiek J. Overexpression of *HMGA2* in Uterine Leiomyomas Points to its General Role for the Pathogenesis of the Disease. *Genes Chromosomes Cancer* 2009. 48(2):171-178.

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VIII.) Meyer A, Staratschek-Jox A, Springwald A, Wenk H, Wolf J, Wickenhauser C, Bullerdiek J. Non-Hodgkin´s lymphoma expressing high levels of the danger-signalling protein HMGB1. *Leukemia & lymphoma* 2008. 49(6):1184-1189.

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3) Winter N, Meyer A, Richter A, Krisponeit D, Bullerdiek J. Erhöhter HMGB1-Level in entzündlichen und malignen Ergüssen. Posterbeitrag 25. 11. Bremer Krebskongress, 2009.
4) Hashemi Nezhad M, Drieschner N, Helms S, Meyer A, Tadayyon M, Klemke M, Belge G, Bartnitzke S, Burchardt K, Frantzen C, Schmidt EH, Bullerdiek J. 6p21 rearrangements in uterine leiomyomas targeting *HMGA1*. *Cancer Genetics and Cytogenetics* 2010. 203(2):247-252.
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9.1 Genexpressionsanalysen von *HMGA2* an Bronchialkarzinomen

I.)

HMGA2 overexpression in non-small cell lung cancer.

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- Zuarbeiten zur Ausarbeitung der Publikation

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HMGA2 Overexpression in Non-Small Cell Lung Cancer

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Lung cancer is still the leading cause of death from cancer worldwide primarily because of the fact that most lung cancers are diagnosed at advanced stages. Overexpression of the high mobility group protein HMGA2 has been observed in a variety of malignant tumors and often correlates with poor prognosis. Herein, *HMGA2* expression levels were analyzed in matching cancerous and non-cancerous lung samples of 17 patients with adenocarcinoma (AC) and 17 patients with squamous cell carcinoma (SCC) with real-time quantitative RT-PCR (qRT-PCR). Transcript levels were compared to results obtained by immunohistochemistry (IHC). *HMGA2* expression was detectable by qRT-PCR in all samples tested and varied from 5422 to 16 991 545 copies per 250 ng total RNA in the carcinoma samples and from 289 to 525 947 copies in the non-cancerous tissue samples. In 33/34 non-small cell lung cancer (NSCLC) samples tested, an overexpression of *HMGA2* was revealed with statistically highly significant differences between non-neoplastic and tumor samples for both AC ($P < 0.0001$) as well as for SCC ($P < 0.0001$). Expression varies strongly and is increased up to 911-fold for AC and up to 2504-fold for SCC, respectively, with statistically significant higher increase in SCC ($P < 0.05$). The results presented herein indicate that *HMGA2* overexpression is a common event in NSCLC and could serve as molecular marker for lung cancer.

Key words: non-small cell lung cancer; HMGA2; quantitative real-time RT-PCR; immunohistochemistry

INTRODUCTION

The high mobility group A (HMGA) proteins are small non-histone chromosomal proteins which are characterized by three highly conserved DNA binding motifs called ''AT-hooks'' and an acidic tail. They preferentially bind to the minor groove of AT-rich B-form DNA by recognizing a particular DNA structure rather than a specific nucleotide sequence [1]. Members of the HMGA protein family are often referred to as ''architectural transcription factors'' [2] because of their ability to regulate expression of a large number of target genes through alteration of chromatin structure [3].

The human HMGA protein family comprises three major members: HMGA1a, HMGA1b, and HMGA2. The proteins HMGA1a and HMGA1b are splicing variants derived from the same gene located at chromosomal locus 6p21 [4,5]. The related HMGA2 protein is encoded by a separate gene assigned to chromosomal locus 12q14-15 [6,7].

The *HMGA* expression level is maximal during embryonic development but is low, or even undetectable, in fully differentiated or non-dividing adult cells and tissues [8–11]. However, in a variety of neoplasms, re-expression of the *HMGA* genes has been detected. Recent findings suggest that overexpression of HMGA proteins is associated with enhanced sensitivity to UV-light and several cytostatic drugs because of diminished cellular DNA repair activity. The inhibition of DNA repair conferred by HMGA might lead to accumulation of mutations and genomic instabilities frequently observed in many types of human cancers [12–15].

Transcriptional reactivation of the *HMGA* genes because of chromosomal translocations was found in many human benign tumors mostly of mesenchymal origin, as for example lipomas, uterine leiomyomas, pleomorphic adenomas of the salivary glands, and pulmonary chondroid hamartomas [6,11,16–20]. As for *HMGA2*, translocations often affect the third intron leading to expression of fusion transcripts containing the three AT-hooks and ectopic sequences from different origin [21]. Transgenic mice expressing a truncated form of the HMGA2 protein comprising the first three binding domains

Abbreviations: NSCLC, non-small cell lung cancer; AC, adenocarcinoma; SCC, squamous cell carcinoma; HMGA, high mobility group protein A; qRT-PCR, real-time quantitative reverse transcriptase polymerase chain reaction; IHC, immunohistochemistry.

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exhibit a giant phenotype with high incidence of lipomas. These results indicate that primarily truncation and/or aberrant expression of *HMGA2* rather than its fusion to other genes is responsible for cell transformation [22,23]. In contrast, it was shown that inactivation of the *HMGA2* gene causes reduced growth (''pygmy phenotype'') in mice [24].

In malign tumors, gene regulatory mechanisms seem to be involved in reactivation of *HMGA* expression. Overexpression of *HMGA1* was observed in a variety of cancers, as for example, in colorectal cancer [25], prostate cancer [26], thyroid cancer [27], breast cancer [28], and cervical cancer [29]. Examples of malignant tumors showing *HMGA2* overexpression are breast cancer [30], pancreatic cancer [31], non-small cell lung cancer (NSCLC) [32], and leukemia [33]. Nevertheless, so far to the best of our knowledge, only one study has used real-time quantitative reverse-transcriptase polymerase chain reaction (qRT-PCR) to quantitate *HMGA2* expression in human malignancies. Miyazawa et al. (2004) [34] detected a 84–315-fold higher *HMGA2* expression level in five squamous cell carcinoma (SCC) tissues of the oral cavity than in normal gingival. As to benign tumors, there is also one study by qRT-PCR. Gross et al. (2003) [35] detected strongly elevated *HMGA2* levels in 10 uterine leiomyomata (UL) with 12q15 rearrangement compared to karyotypically normal UL and normal myometrium. Re-expression of *HMGA* in malign neoplasias is frequently correlated with an increased degree of malignancy, for example, a higher metastatic potential, and is therefore considered as valuable diagnostic and prognostic marker [27–29,34]. Moreover, HMGA proteins seem to be causally involved in neoplastic transformation [36,37].

As mentioned above, in previous work from our group with conventional RT-PCR, *HMGA2* expression was detected in 14/19 NSCLC cases examined whereas in the surrounding non-malignant tissue, *HMGA2* was undetectable [32]. In NSCLC, some recurrent molecular genetic changes have been identified, such as deregulation of *myc* [38], activation of the *MET/HGF* pathway [39], and inactivation of the *RB1/TP16/CCND1* pathway [40,41]. Furthermore, the different types of NSCLC show characteristic genetic modulations as for instance *EGFR* and *TP53* co-expression in SCC [42] and *HER2/neu* overexpression [43] and recurrent *KRAS* mutations frequently observed in AC [44]. Because most of these genetic changes are detected only in a limited number of lung cancer cases, a more common molecular marker would be most valuable for early diagnosis of lung cancer necessary to achieve higher survival rates and for predictions concerning postoperative outcome in resectable tumors.

Aim of this study was to quantitate *HMGA2* expression levels in NSCLC samples with the sensitive quantitative real-time RT-PCR (qRT-PCR). A total of 34 lung cancer samples (17 AC and 17 SCC) and the matching non-neoplastic lung tissue samples from the same patients were analyzed for *HMGA2* expression and transcript levels were compared to results obtained by immunohistochemistry (IHC).

MATERIALS AND METHODS

Tissue Samples

All cancerous and matching non-cancerous samples used for this study (Tables 1 and 2) were provided by the Clinical and Experimental Pathology of the Research Centre Borstel, Borstel, Germany where initial H&E staining and histologic diagnoses were performed. Samples were frozen in liquid nitrogen and stored at −80°C until use.

RNA-Isolation

Total RNA was purified according to the ''RNeasy mini protocol for isolation of total RNA from heart, muscle, and skin tissue'' (Qiagen, Hilden, Germany) including Proteinase K and on-column DNase I digestion. RNA was quantitated spectrophotometrically in triplicate.

Reverse Transcription

cDNA-syntheses were performed with 2 pmol gene-specific reverse primer (5′ GCCATTTCC-TAGGTCTGCCTC 3′) [35] with 250 ng of total RNA and 200 units M-MLV Reverse Transcriptase (Invitrogen, Karlsruhe, Germany) according to the manufacturer's instructions.

Real-Time Quantitative RT-PCR

RT-PCR amplifications were carried out with the ABI Prism 7000 Sequence Detection System (Applied Biosystems, Darmstadt, Germany) or the Applied Biosystems 7300 Real-Time PCR System (Applied Biosystems), respectively. *HMGA2* expression analyses were performed in triplicate in a total volume of 25 μL using 2 μL of each cDNA corresponding to 25 ng of total RNA. cDNA was amplified using 600 nM of forward primer (5′-AGTCCCTCTAAAG-CAGCTCAAAAG-3′) and reverse primer (see above), 200 nM fluorogenic probe (5′ 6-FAM-AGAAG-CCACTGGAGAAAAACGGCCA-TAMRA-3′) [35] in conjunction with universal PCR Mastermix (Applied Biosystems). For each run, non-template controls and no reverse transcriptase reactions were included. PCR conditions were as follows: 2 min at 50°C and 10 min at 95°C, followed by 40 cycles with 15 s at 95°C and 1 min at 60°C. RNA levels were quantitated relative to amplicon-specific standard curves (absolute quantification) and copy numbers were normalized to total RNA concentration and expressed as copy number per 250 ng total RNA [45].

Table 1. *HMGA2* Expression in Matching Adenocarcinoma and Non-Neoplastic Samples of Lung Cancer Patients

Case No.	Histologic type	Sex	Age	Grade	TNM	Stage	Copy no. qRT-PCR†	IHC‡
1	AC*	M	61	1	$T_2N_2M_0$	3A	3.85E + 05	−
	Non-neoplastic						7.52E + 03	−
2	AC	M	73	3	T_2N_2MX	3A	7.15E + 03	−
	Non-neoplastic						2.89E + 02	−
3	AC	M	70	3	$T_2N_2M_0$	3A	8.32E + 06	+
	Non-neoplastic						5.26E + 05	−
4	AC	M	62	2	$T_2N_1M_0$	2B	6.16E + 05	−
	Non-neoplastic						6.95E + 04	−
5	AC	W	63	3	$T_2N_2M_0$	3A	5.42E + 03	−
	Non-neoplastic						1.66E + 03	−
6	AC	W	70	2	$T_2N_0M_0$	1B	1.24E + 05	−
	Non-neoplastic						8.20E + 03	−
7	AC	M	57	3	T_1N_1MX	2A	1.27E + 05	−
	Non-neoplastic						2.33E + 03	−
8	AC	M	61	2	$T_2N_1M_1$	4	1.65E + 04	−
	Non-neoplastic						2.54E + 03	−
9	AC	W	61	3	$T_3N_1M_0$	3A	5.58E + 06	+
	Non-neoplastic						1.92E + 05	−
10	AC	M	65	2	T_2N_0MX	1B	3.13E + 04	n.d.
	Non-neoplastic						4.09E + 04	n.d.
11	AC	M	71	2	T_2N_1MX	2B	1.89E + 06	n.d.
	Non-neoplastic						4.17E + 04	n.d.
12	AC	M	60	3	T_4N_1MX	3B	1.15E + 07	n.d.
	Non-neoplastic						9.07E + 03	n.d.
13	AC	W	52	3	T_2N_0MX	1B	5.92E + 05	n.d.
	Non-neoplastic						3.87E + 04	n.d.
14	AC	M	56	3	T_3N_1MX	3A	3.76E + 06	n.d.
	Non-neoplastic						4.13E + 03	n.d.
15	AC	M	62	2	T_2N_0MX	1B	1.11E + 07	n.d.
	Non-neoplastic						1.30E + 05	n.d.
16	AC	M	66	3	T_2N_3MX	3B	4.67E + 06	n.d.
	Non-neoplastic						2.92E + 04	n.d.
17	AC	W	64	1	T_2N_0MX	1B	4.03E + 05	n.d.
	Non-neoplastic						3.95E + 05	n.d.

*AC, adenocarcinoma.
†Expression results of quantitative real-time RT-PCR (qRT-PCR) are presented as copies per 250 ng total RNA.
‡Immunohistochemistry (IHC) staining was determined using the modified ''Remmele-Score'': −, no intranuclear staining; +, intranuclear staining in 10–50% of the tumor cells; ++, intranuclear staining in more than 80% of the tumor cells; n.d., not determined.

HOPE-Fixation

A part of each frozen tissue sample was incubated in HOPE I solution (DCS, Hamburg, Germany) overnight on ice. Subsequent incubation in acetone with 1:1000 HOPE II solution (DCS) was carried out on ice for 2 h, followed by three successive incubations in pure acetone on ice for 2 h. Afterwards, the tissue samples were incubated overnight in pure paraffin wax (Medite, Burgdorf, Germany) at 57°C, subsequently embedded in paraffin, and stored at 4°C.

H&E Staining of the Embedded Tissue

From the HOPE-fixed paraffin-embedded tissue, 5 μm sections were cut and deparaffinized twice in diethylether (Riedel de Haen, Seelze, Germany) for 30 s. Rehydration was carried out in 95%, 90%, 80% acetone, 10 min each, and 70% acetone for 20 min. Sections were then incubated in bidest. water/Triton X-100 (Merck, Darmstadt, Germany), 1:5000, for 10 min followed by another 10 min incubation in bidest. water. After air drying, samples were stained for 30 s in Mayer's Hemalaun (Merck) and washed in bidest. water for 30 s. ''Blueing'' took place for 1–2 min in running tap water. Subsequently samples were incubated in alcoholic eosin-solution for 1 min and washed in bidest. water for 1 min. After dehydration in 70%, 80%, and 96% ethanol for 2 min each, agitated washing in xylol for 1 min, and another washing in xylol for 5 min, sections were mounted with Histokitt II (Roth, Karlsruhe, Germany).

Immunohistochemistry

Sections were cut and rehydration was performed as described above (H&E staining). After the bidest.

Table 2. *HMGA2* Expression in Matching Squamous Cell Carcinoma and Non-Neoplastic Samples of Lung Cancer Patients

Case No.	Histologic type	Sex	Age	Grade	TNM	Stage	Copy no. qRT-PCR†	IHC‡
1	SCC*	M	70	2	$T_2N_1M_0$	2B	3.53E + 06	–
	Non-neoplastic						3.37E + 05	–
2	SCC	M	68	2	$T_4N_1M_0$	3B	7.78E + 06	++
	Non-neoplastic						8.95E + 03	–
3	SCC	M	64	3	$T_1N_0M_0$	1A	3.62E + 05	–
	Non-neoplastic						8.61E + 03	–
4	SCC	W	62	1	$T_4N_1M_0$	3B	1.37E + 07	++
	Non-neoplastic						5.53E + 04	–
5	SCC	M	62	2	$T_3N_0M_0$	2B	1.64E + 05	–
	Non-neoplastic						1.59E + 04	–
6	SCC	M	74	1	$T_4N_1M_0$	3B	1.32E + 06	++
	Non-neoplastic						1.18E + 04	–
7	SCC	M	69	1	$T_2N_0M_0$	1B	1.15E + 07	++
	Non-neoplastic						1.49E + 04	–
8	SCC	W	62	3	$T_4N_0M_0$	3B	3.14E + 04	–
	Non-neoplastic						5.80E + 03	–
9	SCC	M	59	1	T_2N_1MX	2B	1.24E + 06	–
	Non-neoplastic						4.42E + 03	–
10	SCC	M	68	1	T_2N_0MX	1B	2.09E + 05	n.d.
	Non-neoplastic						1.01E + 03	n.d.
11	SCC	M	60	1	T_2N_0MX	1B	7.43E + 06	n.d.
	Non-neoplastic						6.99E + 04	n.d.
12	SCC	M	63	3	T_2N_2MX	3A	8.31E + 06	n.d.
	Non-neoplastic						2.23E + 04	n.d.
13	SCC	M	75	3	T_2N_0MX	1B	1.70E + 07	n.d.
	Non-neoplastic						6.79E + 03	n.d.
14	SCC	M	68	2	T_2N_0MX	1B	3.32E + 06	n.d.
	Non-neoplastic						3.35E + 04	n.d.
15	SCC	M	65	2	T_2N_0MX	1B	1.17E + 06	n.d.
	Non-neoplastic						2.70E + 05	n.d.
16	SCC	M	73	2	T_2N_2MX	3A	1.87E + 05	n.d.
	Non-neoplastic						4.49E + 03	n.d.
17	SCC	M	50	2	T_2N_1MX	2B	5.01E + 06	n.d.
	Non-neoplastic						1.70E + 05	n.d.

*SCC, squamous cell carcinoma.
†Expression results of quantitative real-time RT-PCR (qRT-PCR) are presented as copies per 250 ng total RNA.
‡Immunohistochemistry (IHC) staining was determined using the modified ''Remmele-Score'': –, no intranuclear staining; +, intranuclear staining in 10–50% of the tumor cells; ++, intranuclear staining in more than 80% of the tumor cells; n.d., not determined.

water bath, sections were incubated in 0.3% H_2O_2 for 10 min and rinsed for at least 20 min in 1× PBS. In a humid chamber, they were incubated for 1 h with 1:50 in Ab-dilution buffer (DCS) diluted primary anti-HMGA2 antibody (goat polyclonal antibody raised against a peptide mapping within an internal region of human HMGA2, S-15, sc-23684, Santa Cruz Biotechnology, Santa Cruz, CA). After washing in 1× PBS for 2 min, incubation with the secondary biotinylated anti-goat antibody (Biogenex, San Ramon, CA) was carried out for 20 min in a humid chamber. Sections were then washed for 2 min in PBS and incubated for another 20 min with Streptavidin-HRP label (DCS). After three further washing steps for 2 min each in PBS, sections were transferred to ready-to-use AEC substrate solution (DCS) for 10 min and then washed for 2 min in PBS. Counterstaining was performed with Mayer's Hemalaun and slides were mounted with Aquamount (Merck). Positive and negative controls were run as usual.

Statistical Analysis

The paired *t*-test was performed to analyze the expression differences between cancerous and non-cancerous tumor samples. Because the expression data did not show normal distribution, data were transformed by logarithmic transformation prior to analysis. Correlation analyses were performed with Pearson correlation coefficients. *P*-values below 0.05 were considered significant.

RESULTS

Histological Examination of Tissue Samples

Despite initial freezing of the material, morphology was excellent after HOPE-fixation and H&E

staining of the tumor and non-tumor specimens. The tumor specimens consisted of varying rates of tumor- and non-tumor-cells. The non-tumor specimens were all completely tumor-free. Sometimes, a mild activation of type II pneumocytes was observed.

HMGA2 Expression Analyses by Real-Time Quantitative RT-PCR

Cancerous and matching non-cancerous samples were obtained from 17 patients with AC and 17 patients with SCC of the lung. By quantitative real-time RT-PCR, *HMGA2* expression could be detected in all samples tested (Tables 1 and 2). The expression varied from 5 422 to 16 991 545 copies per 250 ng total RNA in the carcinoma samples and from 289 to 525 947 copies for the non-cancerous tissue samples. Comparing matching cancerous and non-cancerous lung specimens, a statistically significant overexpression of *HMGA2* was apparent in the AC (Figure 1A) ($P < 0.0001$) as well as in the SCC tested (Figure 1B) ($P < 0.0001$). Apart from one sample were the expression in the normal tissue exceeded the level observed in the matching AC 1.3-fold, the increase detected between non-neoplastic and malign tissue in the AC and SCC ranged from 1.02- to 911.02-fold (mean 158.41-fold) and from 4.34- to 2503.68-fold (mean 336.26-fold), respectively. Statistical analysis revealed a significantly higher increase in the SCC samples than in the AC samples ($P < 0.05$). Comparison of the copy-number of *HMGA2* mRNA in the tumor samples alone or the ratio of copies in the tumor/copies in the corresponding non-neoplastic tissue to clinico-pathologic findings (Tables 1 and 2) revealed no clear relationship of *HMGA2* expression level with tumor classification, stage, or grading. However, concerning the AC, highest expression (11 487 392 copies per 250 ng total RNA) was found in the sole malignancy classified as T4.

HMGA2 Expression Analysis by Immunohistochemistry

The immunohistochemical staining for HMGA2 was analyzed after HOPE-fixation in the specimens AC 1–9 and SCC 1–9. The results confirmed the expression pattern obtained by real-time quantitative RT-PCR as immunostaining of HMGA2 was identified in two AC and four SCC samples representing six of the seven tumor samples with highest copy-number levels (Tables 1 and 2). All non-neoplastic tissues were negative (Figure 2A and C). The positive AC samples showed moderate intranuclear staining in 10–50% of the tumor cells (Figure 2B) while strong intranuclear staining was detected in more than 80% of the tumor cells seen in positive SCC samples (Figure 2D). The intensity of staining was determined with the modified ''Remmele-Score'' [46].

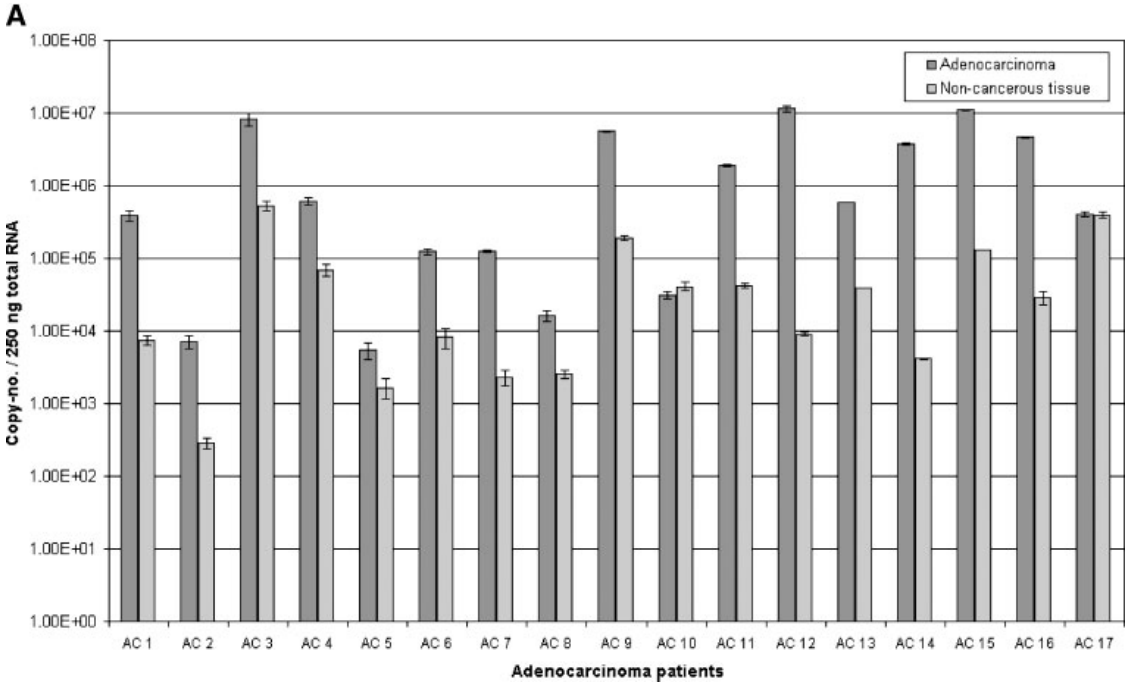

Figure 1. Results of the *HMGA2* expression analyses obtained by real-time quantitative RT-PCR in (A) adenocarcinomas (AC) and (B) squamous cell carcinomas (SCC) of the lung. Dark gray bars represent *HMGA2* copy-no. per 250 ng total RNA for cancerous and light gray bars for the matching non-cancerous samples.

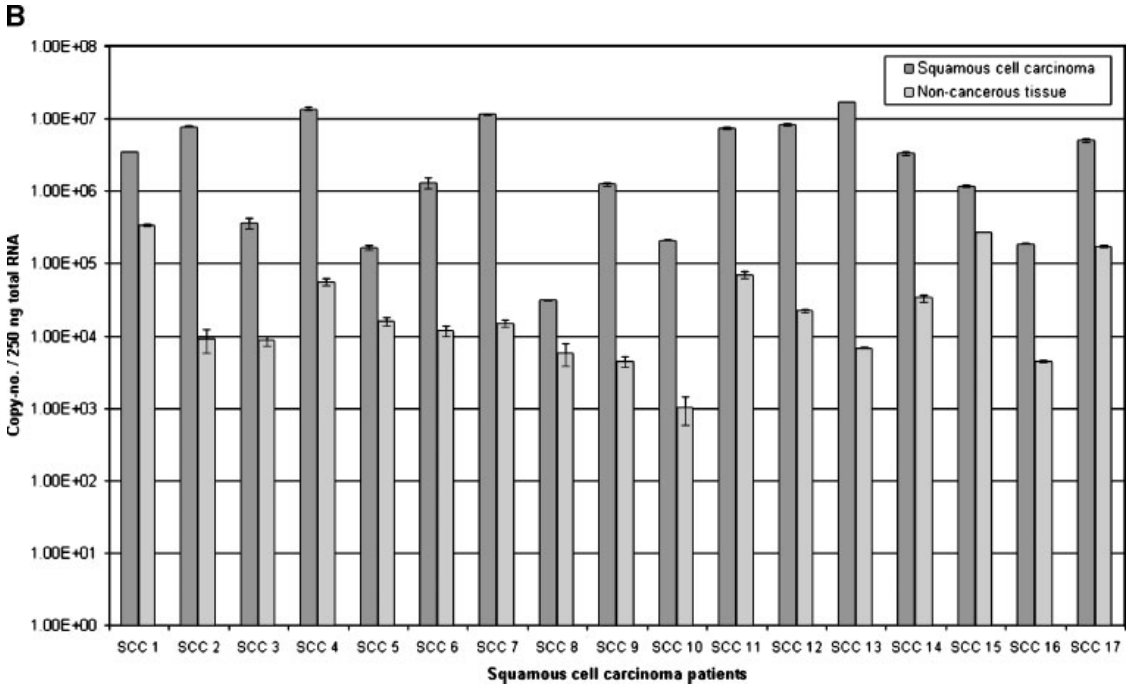

Figure 1. (*Continued*)

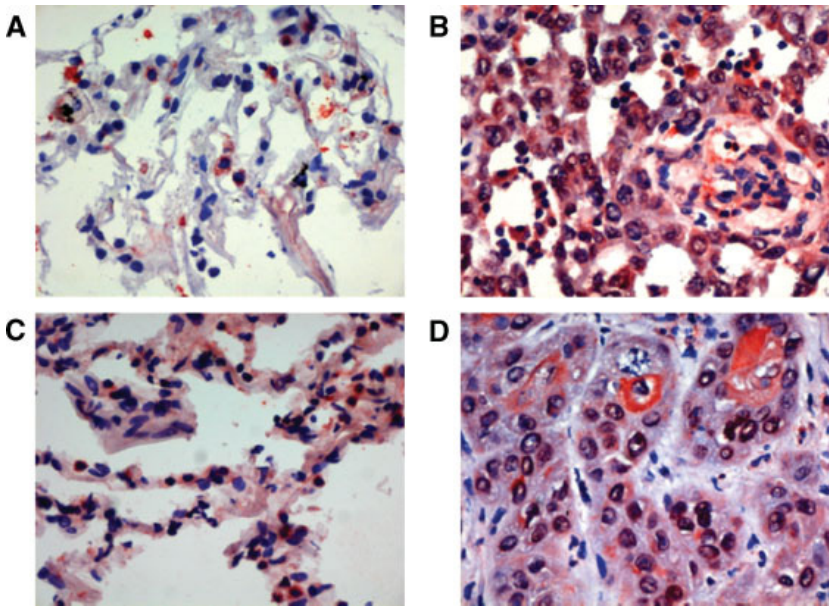

Figure 2. Immunohistochemical staining results using a goat polyclonal antibody raised against a peptide mapping within an internal region of human HMGA2, S-15, sc-23684, Santa Cruz Biotechnology hybridized to the matching non-cancerous and cancerous tissue of adenocarcinoma patient AC 3 and squamous cell carcinoma patient SSC 6. No intranuclear staining was seen in non-neoplastic lung tissue of adenocarcinoma (A) and squamous cell carcinoma patients (C), while intranuclear staining was observed in 10–50% of tumor cells of the positive adenocarcinoma sample (B) and more than 80% of tumor cells of the positive squamous cell carcinoma sample (D) of the lung. Magnification: 200×.

DISCUSSION

To the best of our knowledge this is the first study describing the results of quantitation of *HMGA2* expression in lung cancer and in a larger series of malignant tumors at all.

Lung cancer is still the leading cause of death from cancer primarily because of the fact that the majority of lung cancers are diagnosed at advanced stages [47]. Elucidation of the molecular mechanisms underlying its development and progression could lead to an earlier diagnosis and more effective therapeutic approaches [48]. As previously reported by our group, *HMGA2* expression was detected by conventional RT-PCR in 14/19 NSCLC samples while no expression was found in the non-malignant adjacent lung or pleural tissues examined, suggesting *HMGA2* expression as potential indicator for lung cancer [32]. Aim of this study was to quantitate the *HMGA2* expression in NSCLC. Real-time quantitative RT-PCR was conducted on a total of 68 tissue samples representing matching cancerous and non-cancerous samples from 17 AC and 17 SCC patients. The qRT-PCR results were confirmed by results from IHC. The comparison between qRT-PCR and IHC shows that the PCR approach is far more sensitive than the IHC. In this series, 289 copies of *HMGA2* could be detected in 250 ng total RNA by PCR, whereas positive IHC results were obtained only in samples showing more than 1.3×10^6 *HMGA2* mRNA copies per 250 ng total RNA. In contrast to the previous study [32], herein *HMGA2* expression was detected not only in all cancerous samples but also in the non-neoplastic lung tissues. This is probably because of the higher sensitivity of the real-time quantitative RT-PCR compared to the hemi-nested RT-PCR approach used in the former study. Consistent with the present results, *HMGA2* could be detected by qRT-PCR in normal myometrium [35] and in normal keratinocytes of the oral cavity [34] indicating a basal *HMGA2* expression in these non-neoplastic tissues as well. Because all non-cancerous specimens were completely tumor-free, the strong variation of *HMGA2* expression levels from 289 to 525 947 copies per 250 ng total RNA measured in the normal tissue is remarkable. As described by several groups, multiple genetic alterations similar to those found in lung cancer have frequently been detected in histologically normal lung tissue of smokers and/or lung cancer patients [50,51]. Hence, the elevated *HMGA2* expression levels measured in several non-cancerous samples could result from these genetic changes which might represent early stages in the tumorigenesis of lung cancer.

To date, TNM stage is still the most important prognostic factor for prediction of survival rates in NSCLC [52]. However, to explain the large variability in postoperative outcome of patients with clinically resectable tumors, the identification of new prognostic parameters is required. Comparing matching tumor and non-tumorous samples from the same NSCLC patient the quantitative analysis revealed a statistically highly significant overexpression in 33/34 lung cancer cases with a significantly higher increase in SCC than in AC samples. A strong intertumoral variance of *HMGA2* expression levels was detected and for the AC, highest expression was found in the sole malignancy classified as T4. Because the *HMGA2* gene has shown to be of prognostic relevance in several other cancer entities [30,34,53], these findings indicate that *HMGA2* overexpression is a common event in NSCLC and *HMGA2* expression can be considered as molecular biological factor for NSCLC.

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9.2 Genexpressionsanalysen von *HMGA2* an Schilddrüsenneoplasien

II.)

Upregulation of *HMGA2* in thyroid carcinomas: a novel molecular marker to distinguish between benign and malignant neoplasias.

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Genes Chromosomes Cancer 47(1):56-63 (2008)

Eigenanteil an dieser Publikation:

- Etablierung einer Methode zur relativen Quantifizierung von *HMGA2* an FFPE Material
- Durchführung und Planung der praktischen Arbeiten in Zusammenarbeit mit Herrn Klemke
- Verfassen der Publikation in Zusammenarbeit mit Herrn Belge

GENES, CHROMOSOMES & CANCER 47:56–63 (2008)

Upregulation of *HMGA2* in Thyroid Carcinomas: A Novel Molecular Marker to Distinguish Between Benign and Malignant Follicular Neoplasias

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The identification of molecular markers allowing to differentiate between benign and malignant thyroid tumors remains a diagnostic challenge. Herein, we have used the expression of the high mobility group protein gene *HMGA2* and its protein, respectively, as a possible marker detecting malignant growth of thyroid tumors. HMGA2 belongs to the high mobility group proteins, i.e. small, highly charged DNA-binding proteins. While *HMGA2* is highly expressed in most embryonic tissues, its expression in adult tissues is very low. However, a reactivation of *HMGA2* expression has been described for various malignant tumors and often correlates with the aggressiveness of the tumors. The aim of this study was to investigate whether the *HMGA2* expression can be used to detect malignant thyroid tumors. RNA from 64 formalin-fixed paraffin-embedded thyroid tissues including normal tissue ($n = 3$), thyroiditis ($n = 2$), and follicular adenomas ($n = 19$) as well as follicular ($n = 9$), papillary ($n = 28$), and anaplastic ($n = 3$) carcinomas was reverse transcribed. Finally, real-time quantitative RT-PCR was performed. Expression differences of up to 400-fold were detected between benign and malignant thyroid tumors. Based on *HMGA2* expression alone, it was possible to distinguish between benign and malignant thyroid tissues with a sensitivity of 95.9% and a specificity of 93.9%. There was a highly significant ($P < 0.001$) difference with histology of the tumors being the gold standard between the benign lesions and malignant tumors. Our results show that even as a stand-alone marker *HMGA2* expression has a high potential to improve diagnoses of follicular neoplasms of the thyroid.

INTRODUCTION

Thyroid nodules are frequently found in both sexes. While by palpation it is detected in 4–8% of adults, ultrasound examination reveals a much higher frequency of about 40% corresponding to roughly 50% of thyroid nodules found at autopsy. As to those nodules selected for fine needle aspiration (FNA), the average cancer rate is ~10–13% and does not increase with the number of nodules per patient (Frates et al., 2005). In patients with multiple nodules the cancer is located in the dominant or larger nodule in only two thirds of the cases (Frates et al., 2005). FNA is now the widely accepted method for screening of suspect lesions for cancer. However, the histological diagnosis of follicular-patterned thyroid lesions is often difficult and cytological samples obtained by FNA are even more difficult to diagnose (Greaves et al., 2000; Baloch et al., 2002; Smith et al., 2005). These difficulties are reflected by papers entitled "The demise of follicular carcinoma of the thyroid (LiVolsi and Asa, 1994)", "Can we agree to disagree" (LiVolsi, 2003), or "Follicular-patterned lesions of the thyroid: the bane of the pathologist" (Baloch and LiVolsi, 2002). A particular problem in clinical practice is the cytological differentiation between follicular thyroid carcinoma (FTC) and benign follicular thyroid adenoma (FTA), since the cell morphology is often nearly identical. Thus, FNA followed by cytological examination often will not lead to a precise diagnosis of these lesions. To find diagnostic markers that would enable a better classification of thyroid tumors, we quantified *HMGA2* gene expression in benign as well as malignant thyroid lesions and normal thyroid tissue samples by quantitative real-time reverse-transcription-PCR (qRT-PCR). The high mobility group A (HMGA)

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proteins are small chromatin associated nonhistone proteins that act as architectural transcription factors (Wolffe, 1994; Bustin and Reeves, 1996). HMGA proteins have three DNA-binding domains designated as AT-hooks, enabling their binding to the minor groove of AT-rich DNA, and an acidic C-tail responsible for protein–protein interactions (Reeves and Nissen, 1990; Chau et al., 1995; Harrer et al., 2004; Reeves and Beckerbauer, 2005). The interaction of HMGA with DNA leads to changes in chromatin structure involving HMGA proteins in the regulation of the expression of a high number of target genes. The oncogenic potential of *HMGA2* was first described by Ashar et al. (1995) and Schoenmakers et al. (1995). The *HMGA2* expression level is very high during embryonic development whereas it is almost undetectable in differentiated cells and tissues (Chiappetta et al., 1996; Rogalla et al., 1996; Hirning-Folz et al., 1998). A strong association between the overexpression of *HMGA2* and an adverse prognosis has been demonstrated for carcinomas of the breast (Rogalla et al., 1997), lung (Sarhadi et al., 2006), and squamous cell carcinoma of the oral cavity (Miyazawa et al., 2004).

Nevertheless, so far only few of these studies have used qRT-PCR to quantify *HMGA2* expression in human neoplasias. In the present study, we analyzed the expression level of *HMGA2* in paraffin-embedded thyroid tissues, using real-time quantitative RT-PCR. The *HMGA2* gene expression of 9 FTCs, 28 PTCs, 3 ATCs, 19 adenomas, and 3 normal thyroid tissue samples was tested by quantitative real-time RT-PCR. To confirm the qRT-PCR results on the protein level, immunohistochemistry was performed on two samples from apparently normal thyroid tissue, five adenomas, seven FTCs, and five PTCs with an antibody raised against HMGA2.

MATERIALS AND METHODS

Patients

All patients underwent surgery between 1995 and 2005 in the general hospitals Bremen-Mitte or Hamburg Altona. In this study 64 thyroid samples from 44 female and 17 male patients were analyzed: 19 were adenomas, 28 papillary thyroid carcinomas (PTC), 9 follicular carcinomas (FTC), and 3 anaplastic carcinomas (ATC) (Table 1). In addition, two samples from patients with lymphofollicular thyroiditis were included. The control group consisted of three nonmalignant surrounding tissues. The age of patients ranged from 21 to 85 years. The average thyroid nodule size was 3.1 cm (range 0.3–14.0 cm).

Sample Preparation

Six 5-µm sections of each FFPE tissue were deparaffinized in xylene prior to RNA isolation. Haematoxylin–eosin stains were prepared from consecutive sections following those used for RNA isolation. To validate the constant expression level of the endogenous control, RNA was isolated from a primary cell culture derived from apparently normal thyroid tissue, cell cultures of six adenomas (S325, S40.2, S211, S121, S731, and S734.1), and two carcinomas (ARO and WRO) with the RNeasy Mini Kit (Qiagen GmbH, Hilden, Germany) according to the manufacturer's instructions.

RNA-Isolation

Total RNA was extracted using the high pure RNA paraffin kit for isolation of total RNA from FFPE tissues (Roche, Penzberg, Germany) including a Proteinase K and DNase I digestion according to the manufacturer's instructions. The RNA concentration and the ratio of absorbance at 260–280 nm was quantitated by spectrophotometry.

Reverse Transcription

About 250 ng of total RNA was reverse transcribed with 200 U of M-MLV reverse transcriptase (Invitrogen, Karlsruhe, Germany) and 150 ng random hexamers according to the manufacturer's instructions. RNA was denatured before transcription at 65°C for 10 min and subsequent cooling on ice for 1 min. After adding the enzyme to the RNA primer mixes, samples were incubated for 10 min at 25°C to allow annealing of the random hexamers. Reverse transription was performed at 37°C for 50 min followed by inactivation of the reverse transcriptase at 70°C for 10 min.

Real-Time Quantitative RT-PCR

RT-PCR amplification was performed using the ABI Prism 7300 sequence detection system (Applied Biosystems, Darmstadt, Germany). Because of different degradation of RNA isolated from formalin-fixed samples, a relative quantification method with 18S rRNA as endogenous control was used.

HMGA2 and 18S rRNA expression analyses were performed in triplicate in a total volume of 20 µl using 2 µl of each cDNA corresponding to 25 ng of total RNA. TaqMan primers and probe for *HMGA2* were ordered as assay on demand (No. Hs00171569_m1; Applied Biosystems, Darmstadt, Germany). The

TABLE 1. Histology of Thyroid Lesions Used for *HMGA2* Expression Analyses

Case no.	Age (years)	Sex	Histology	Tumor diameter (cm)	TNM classification and/or grading
1	58	f	Thyroiditis	–	–
2	40	m	Adenoma	7.5	–
3	47	m	Adenoma	6.8	–
4	25	f	Adenoma	2.5	–
5	85	m	PTC	4.0	pT3a
6	53	f	Adenoma	6.5	–
7	44	m	Adenoma	4.0	–
8	48	f	Adenoma	5.0	–
ST			Normal thyroid tissue	–	–
9	63	f	Thyroiditis		–
10	29	f	Adenoma	7.0	–
11	54	f	Adenoma	1.0	–
12	71	f	Adenoma	1.7	–
13	35	m	Adenoma	6.5	–
14	51	f	Adenoma	6.0	–
15	42	m	Adenoma	3.5	–
16	42	f	Adenoma	1.0	–
17	34	f	FTC	2.2	G1
18	61	f	Adenoma	4.0	–
19	44	m	Adenoma	1.0	–
20	39	f	Adenoma	1.5	–
21	30	f	Adenoma	0.3	–
22	40	f	Adenoma	3.8	–
23	42	f	Adenoma	4.0	–
24	35	f	MI-FTC (t)	2.1	pT2 N0 MX
25	61	m	FTC (o)	8.0	pT4
26	72	f	FV-PTC	1.0	pT1
27	84	f	FV-PTC	6.0	pT3 pNX
28	38	f	PTC	0.6	pT1
29	50	f	PTC	2.2	pT2
30	21	f	FV-PTC	1.0	pT1 pNX pMX
31	31	m	PTC	2.5	pT2 pN0
32	46	f	PTC	1.5	pT1; G2
33	65	f	ATC	2.0	pT4 N0; GIV
34	54	f	PTC	0.6	pT1 pNX pMX
35	85	f	PTC	1.5	G2
36	85	f	PTC	14.0	pT3; G3
37	42	m	FV-PTC	0.7	pT1 N0 MX
38	66	f	MI-FTC	2.0	pT1
39	72	f	FV-PTC	1.0	pT3
40	38	m	PTC	0.8	pT1; G1
41	76	f	ATC	3.8	pT4b
42	33	f	PTC	2.5	pT2; G2
43	69	m	FV-PTC	0.5	pT1 NX MX
44	42	f	FV-PTC	1.4	pT2a; G2
45	30	f	PTC	2.5	pT2 NX
46	49	f	PTC	1.2	pT2
47	25	f	FV-PTC	2.3	pT2 pN0
48	48	f	PTC	0.8	pT1; G1-2
49	76	f	ATC	1.7	pT4b
50	67	m	FTC (t)	5.5	pT3 pNX pM1
51	59	f	FTC	1.2	pT1; G2
52	34	f	PTC	2.3	pT2 pN1 pMX
53	57	f	PTC	0.9	pT1
54	31	m	PTC	2.0	pT3 pN1
55	27	m	FV-PTC	2.5	pT2
56	66	f	PTC	2.0	pT3; G2
57	41	m	PTC	2.0	pT1; G2
58	37	f	FTC	2.8	pT2 N0
59	69	m	FTC	3.0	pT4
60	79	f	PTC	2.0	pT3 pNX; G1
61	71	f	FTC (o), with partially insular pattern	9.0	pT4 NX MX

ST, surrounding tissues; PTC, papillary thyroid carcinoma; FTC, follicular thyroid carcinoma; MI-FTC, minimally invasive FTC; ATC, anaplastic thyroid carcinoma; FV-PTC, follicular variant PTC; (o), oncocytic; (t), trabecular.

HMGA2-specific primers in this assay are spanning the boundary between Exons 1 and 2. Primers and probe for 18S rRNA were 5′-GGATCCATTGGA GGGCAAGT-3′ (forward), 5′-AATATACGCTATTG GAGCTGGAATTAC-3′ (reverse), and FAM-TGC CAGCAGCCGC-MGB TAMRA (probe) (Antonov et al., 2005). For each run nontemplate controls (NTC) and reactions without reverse transcriptase (-RT) were included. PCR conditions were as follows: 2 min at 50°C and 10 min at 95°C, followed by 50 cycles with 15 sec at 95°C and 1 min at 60°C.

Analysis of Gene Expression

The relative expression was calculated by the ΔC_t method. The results were compared with those obtained by conventional histology, which is the current "gold standard" in diagnosis of thyroid lesions. In addition, correlation analyses as well as normal distribution parameter estimations were performed to determine the optimal decision limit to distinguish benign from malignant tumors. Sensitivity and specificity calculations were done on logarithms of expression to obtain normally distributed values. Moreover, Student's *t* test was performed to determine the significance of observed differences in *HMGA2* expression in benign and malignant tissues.

Immunohistochemical Analysis of HMGA2 Expression

For the immunohistochemical analysis of HMGA2 expression, formalin-fixed and paraffin-embedded tissue sections (6 μm) were deparaffinized, rehydrated, and washed with PBS before immunoperoxidase staining. Slides were incubated

overnight at 4°C in a humidified chamber with goat anti-HMGA2 (Santa Cruz Biotechnology, Santa Cruz, California, USA). Biotinylated anti-goat link was used as secondary antibody (30 min). Slides were then incubated with avidin biotin enzyme label (Vector Laboratories, California, USA) for 30 min and developed with AEC peroxidase substrate (Camon Laborservice GmbH, Wiesbaden, Germany) for 10 min. Finally, slides were counter-stained with haematoxylin.

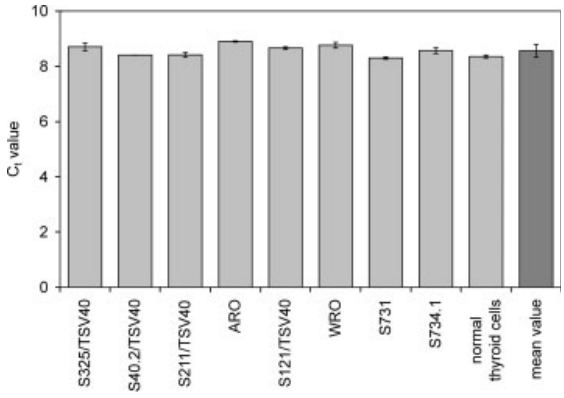

Figure 1. C_t values of 18S rRNA in different types of thyroid cells. Adenoma-derived cell lines: S325/TSV40, S40.2/TSV40, S211/TSV40, S121/TSV40, S731, S734.1; ARO: cell line of an anaplastic carcinoma; WRO: cell line of a follicular carcinoma. Dark gray bar: mean value.

RESULTS

The expression of 18S rRNA in thyroid cell cultures (one normal thyroid, six adenomas and two carcinomas) showed only a low variation, the mean C_t value was 8.56 ± 0.21 (Fig. 1). Therefore, 18S rRNA was chosen as endogenous control for relative quantification.

In all samples an expression of *HMGA2* was detected (Fig. 2). The highest expression was found in a FTC with partially insular pattern with a 432-fold *HMGA2* expression in relation to normal tissue. In contrast, inflammatory diseases or adenomas showed a significantly lower level of *HMGA2* expression. One FTC was gross dissected to quantify the *HMGA2* expression in the tumor and the surrounding tissue separately. The *HMGA2* expression in the surrounding tissue was not higher than in normal thyroid tissues, whereas *HMGA2* was overexpressed 47.2-fold in the tumor.

The possible correlation between the expression of *HMGA2* mRNA and the malignant transformation of the follicular epithelium is illustrated in Figure 2 showing all cases analyzed in increasing order of *HMGA2* expression.

A normal distribution was fitted to the expression data to estimate the decision limit, sensitivity, and specificity. The decision limit for the discrimination of benign and malignant tissues is 3.99 with a sensitivity of 95.9% and a specificity of 93.9%.

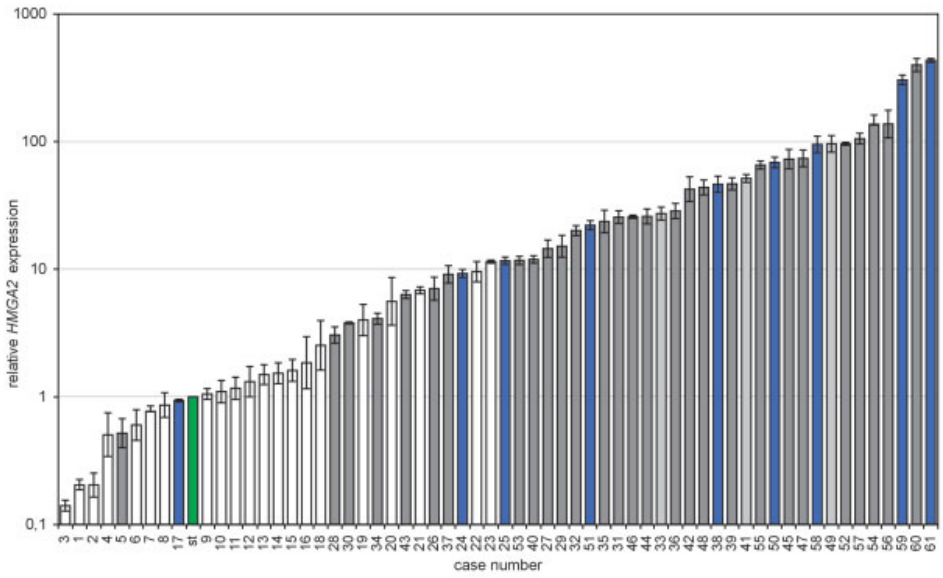

Figure 2. *HMGA2* expression in thyroid lesions. Results of the relative *HMGA2* quantification. The mean value of three surrounding thyroid tissues (ST, green bar) serves as calibrator. Different thyroid lesions are represented by bar colors: blue: FTC, dark gray: PTC, light gray: ATC, white: follicular adenoma or thyroiditis.

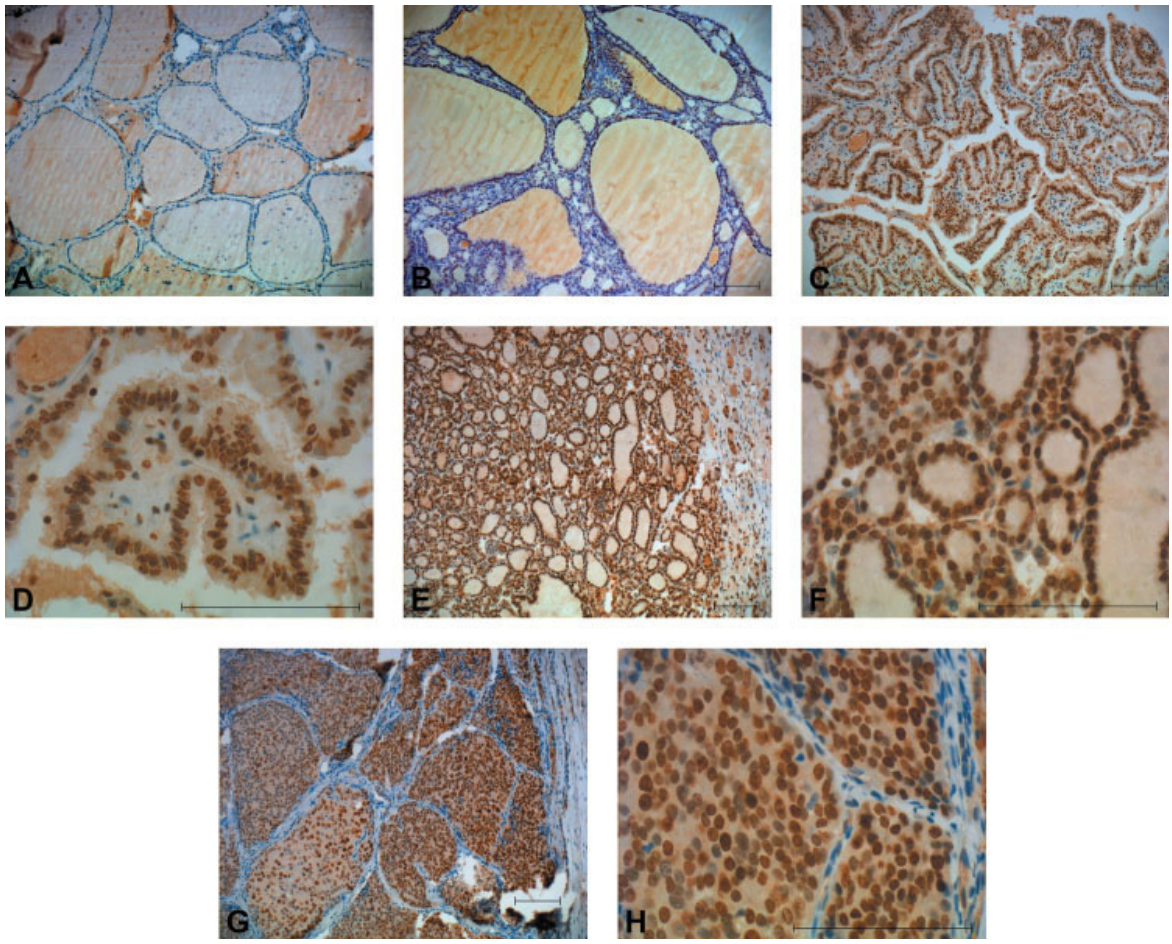

Figure 3. Immunostaining of thyroid tissues with an antibody raised against the HMGA2 protein. (A) Normal thyroid tissue, ×100; (B) Follicular adenoma (Case no. 2), ×100; (C) and (D) Papillary thyroid carcinoma (Case no. 54), ×100 and ×400, respectively; (E) and (F) Follicular thyroid carcinoma (Case no. 38), ×100 and ×400, respectively; (G) and (H) Follicular thyroid carcinoma with partially insular pattern (Case no. 61), ×100 and ×400, respectively. Bars indicate 100 μm. Brown staining of nuclei was noted in cases revealing high *HMGA2* expression by real-time PCR.

As to the adenomas, the *HMGA2* expression level of benign tumors was not significantly elevated as ascertained by Student's *t* test ($P = 0.42$). A maximum value of 11-fold overexpression was found in an atypical adenoma. The majority of the malignant samples had expression levels of more than the 20-fold of normal tissue values. Values up to a 400-fold overexpression were detected. Finally, mean values in the groups (normal, benign, and malignant tissues) were compared by *t* tests. There was no significant difference between the normal and benign tissue groups ($P = 0.42$), while the differences between the normal and malignant tissue group as well as between the benign and malignant tissue group were highly significant ($P < 0.001$).

In the next step, the group of follicular carcinomas and follicular variants of papillary carcinomas was compared with follicular adenomas. The decision limit to separate these two groups is 6.3 with a sensitivity of 81.2% and a specificity of 88.3%. A significant, but weak correlation between patients' age and relative *HMGA2* expression (Pearson correlation coefficient = 0.25340, $P = 0.048$) was observed, whereas no significant correlations were found between the tumor size or patients' age and the expression level.

It should be noted that the qRT-PCR results are based on whole sections. Thus, the area covered by the tumor itself is different among the cases. To check if the results even can be expected to improve when detecting HMGA2 expression only in the tumor cells, IHC has been performed in a number of cases.

A strong nuclear positivity was observed in all carcinomas, whereas no immunoreactivity was observed in normal thyrocytes, tumor cells of adenomas, cells of the stromal component of carcinomas, and lymphocytes, respectively (Fig. 3).

DISCUSSION

Currently, FNA biopsy with subsequent cytologic diagnosis is the most important procedure in preoperative thyroid tumor diagnosis besides scintigraphy. However, it is virtually impossible to differentiate between follicular carcinomas and adenomas based on samples obtained by FNA alone. Only in 20–30% of the cases of surgically removed thyroid glands due to suspicious cytological results is a follicular malignancy diagnosed at postoperative histology (Goellner et al., 1987; Chiappetta et al., 1998; Greaves et al., 2000; Baloch et al., 2002). With regard to the limitations of FNA cytology, reliable markers that allow to differentiate between different thyroid nodules are still an unmet challenge especially for follicular thyroid neoplasias. Also, there is a high demand to stratify the group of papillary cancers with an adverse prognosis. Herein, we have described our results obtained with the expression of *HMGA2* as a standalone molecular marker.

The aim of this study was to evaluate the potential of *HMGA2* expression for the diagnosis of thyroid tumors. As a rule, the expression level of benign tumors was comparable with those of normal tissues. In contrast, we were able to demonstrate that *HMGA2* is significantly overexpressed in most thyroid carcinomas as compared with benign tumors. Values up to 400-fold overexpression were detected.

Even with archival material it was possible to identify malignancies with a sensitivity of 95.9% based on *HMGA2* expression alone taking histology as the gold standard. Furthermore, it should be noted that the quantitative real-time PCR results are based on whole sections. Thus, as shown by the IHC data, if only the area covered by the tumor is studied, even greater differences between benign and malignant samples should be seen.

Generally, *HMGA2* expression either as a standalone parameter or combined with others is a promising marker with the potential to improve preoperative diagnoses and treatment of thyroid nodules. Besides its utility for clinical diagnostics of follicular thyroid tumors, the increased expression of *HMGA2* may also improve the understanding of the molecular mechanisms involved in the genesis of thyroid malignancies.

As an alternative to quantitative real-time PCR, immunohistochemistry is a suitable method to detect the HMGA2 protein in nuclei of malignant thyroid tumors. As shown by immunostaining with an HMGA2-specific antibody (Fig. 3), malignant epithelial tumor cells exhibit a strong nuclear reaction, whereas normal thyrocytes and adenomas as well as stromal components of carcinomas do not show immunoreactivity, thus confirming the results obtained by quantitative real-time PCR. Therefore, HMGA2-specific immunohistochemistry can improve the diagnosis of thyroid tumors.

Furthermore, in papillary thyroid carcinomas (PTCs), the *HMGA2* overexpression varied over a broad range. Although PTCs have a favorable prognosis in general, there are subgroups in which the prognosis is worse. Current methods fail to identify these subgroups prior to surgical removal. Because *HMGA2* expression seems to be a more general marker of increased malignancy in a variety of malignant neoplasms (e.g. lung cancer (Sarhadi et al., 2006) and squamous cell carcinomas of the oral cavity (Miyazawa et al., 2004)), it is tempting to speculate that the range of *HMGA2* overexpression in PTCs reliably identifies the subgroup of PTCs with a poor prognosis. Interestingly, in a recent study by Rodrigues et al. (2007) *HMGA2* was found to be one of the most overexpressed genes in a group of aneuploid papillary thyroid cancers.

Previous studies have identified a variety of genes as potential markers of malignant thyroid neoplasms and in the most recent studies often a combination of several genes is recommended. Kebebew et al. (2006) investigated 19 cases each of hyperplastic nodules, follicular adenomas, follicular thyroid cancers, follicular variants of papillary cancers, and papillary thyroid cancers. Their study is based on the expression of three cell-cycle regulatory genes, *MCM5*, *MCM7*, and *RAD9*, as molecular markers of malignancy. While the combined use of these genes provided a sensitivity of 98.2%, the specificity did not exceed 65.7%. As to single genes, overlaps in expression levels in benign and malignant tumors ranged between 27.3 and 38.9% (Kebebew et al., 2006).

Another multi-gene assay focussing on follicular neoplasias has been suggested by Weber et al. (2005). In this study, both immunostaining and quantitative RT-PCR for three genes were performed. However, minimally invasive FTCs representing the most important diagnostic challenge were excluded from this study. In contrast, this subtype as well as atypical adenomas were included in a study by Fryknäs et al. (2006). Several genes were identified, which are differentially expressed in adenomas and FTCs. Interestingly, *HMGA2* was included in the cDNA microarray, but not included in the panel of finally selected genes. The *FHL1* expression, which is described more in detail, was significantly lower in (minimally inva-

sive) FTCs than in (atypical) adenomas. However, *FHL1* expression levels of benign and malignant follicular neoplasias overlap to a great extent, so that the *FHL1* expression alone does not seem to allow a sufficient differentiation between these groups (Fryknäs et al., 2006).

Moreover, several proteins have been suggested as targets for immunostaining, e.g. galectin-3 (GAL3) and human bone marrow endothelial cell (HBME)-1. Although immunohistochemistry can be an adjunct in the diagnostic process, the staining pattern can vary in subvariants of differentiated thyroid carcinomas representing a particular problem in borderline tumors where a reliable marker is highly needed (Castro and Gharib, 2005; Sheils, 2005). It has been reported, that under certain circumstances, e.g. Hashimoto thyroiditis with coexistent solitary or multiple nodules, the usefulness of *GAL3* expression analysis is limited (Niedziela et al., 2002). While the *GAL3* mRNA and protein level is increased in the majority of PTCs, follicular thyroid tumors were negative with regard to *GAL3* mRNA. This observation raises doubts on the usefulness of this marker, since it seems to be impossible to distinguish adenomas from follicular cancers (Feilchenfeldt et al., 2003).

Other studies suggested the expression of *HMGA1* as a suitable genetic marker to distinguish benign from malignant thyroid tumors (Chiappetta et al., 1995, 1998; Berlingieri et al., 2002; Czyz et al., 2004). As for the RT-PCR analyses, one main disadvantage of *HMGA1* is the existence of several retropseudogenes, which can lead to false-positive results in quantification experiments when RNA extractions are contaminated with genomic DNA. In contrast to *HMGA1*, no retropseudogenes of *HMGA2* are known (Blank et al., 2000; Rogalla et al., 2001; Strichman-Almashanu et al., 2003).

Thus, to the best of our knowledge, *HMGA2* expression is currently the best known single molecular marker to distinguish between benign and malignant thyroid neoplasms.

An additional point of interest are papillary thyroid microcarcinomas (PTMC), defined as being less than or equal to 1.0 cm in diameter. However, the clinical relevance of these tumors still remains a matter of debate and markers to judge the aggressiveness are missing (Frates et al., 2005; Burman, 2006; Roti et al., 2006). Herein, we quantified the expression of *HMGA2* in 10 cases of papillary thyroid microcarcinoma (Case nos. 26, 28, 30, 34, 37, 39, 40, 43, 48, and 53), and found an overexpression in comparison with normal thyroid tissue. Nevertheless, the expression levels found in the individual PTMCs varied over a broad range. Thus, we assume that *HMGA2* expression might be a potent genetic marker to discriminate PTMCs displaying aggressive behavior from those with an indolent clinical course, but clinical trials are necessary to verify the utility of *HMGA2* expression analysis in the diagnosis of PTMC. Further analyses on fresh frozen tissues or FNA biopsies are necessary to elucidate if the accuracy of this new marker is sufficient even to identify the challenging minimally invasive subtype of FTCs.

In summary, *HMGA2* expression is a molecular marker that does not only distinguish between benign and malignant growth but also reflects the continuous process underlying the malignant transformation of thyroid nodules. Besides real-time quantitative PCR, immunohistochemistry is an adequate method to investigate the HMGA2 status of thyroid tissue samples.

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9.3 Genexpressionsanalysen von *HMGA2* an Herzgeweben

III.)

Upregulation of the *high mobility group AT-hook 2* gene in acute aortic dissection with epithelial-mesenchymal-transition.

Belge G, Meyer A, Stegen AI, Radtke A, Richardt D, Nimzyk R, Nigam V, Sievers HH, Tiemann M, Bullerdiek J, Mohamed S

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Eigenanteil an dieser Publikation:

- Planung, Durchführung und Auswertung der Real-Time PCR Versuche
- Erstellen der Übersichtsfärbungen
- Zuarbeiten zum Verfassen der Publikation

Upregulation of the high mobility group AT-hook 2 gene in acute aortic dissection is potentially associated with epithelial-mesenchymal-transition

Short running title: HMGA2 upregulation in the aorta

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Abstract

Background—The *high mobility group AT-hook 2* (*HMGA2*) gene is known to be expressed abundantly during embryogenesis associated with transforming growth factor (TGF-β) signaling. It is proposed that *HMGA2* regulates genes involved in epithelial-mesenchymal-transition (EMT), including the transcriptional repressor snail homolog 1 (SNAI1). We analyzed the expression profile of the *HMGA2* gene in different human aortic diseases.

Methods and Results—Aortic specimens were collected during surgery from 51 patients (mean age 57.9±15.3) including 19 with acute aortic dissection (AAD, type A, 9m/10f), 26 with aortic aneurysm (14 patients with bicuspid aortic valve, 12 patients tricuspid aortic valve) and two with Marfan syndrome. Aortic valve specimens obtained from four patients were also included. Quantitative Real-Time-polymerase chain reaction (qRT-PCR) was done for *HMGA2* and immunohistochemical analyses were performed for HMGA2 and SNAI1. The expression of *let-7d* microRNA, assumed to play a role in regulation of *HMGA2*, was quantified.

The *HMGA2* gene was significantly higher expressed in aortic dissection compared with all other samples (193.1 vs 8.1 fold, $P = 0.00000014$). No significant correlation between *let-7d* microRNA and *HMGA2* was detected among all investigated samples. Immunohistochemical investigation showed that HMGA2 and SNAI1 proteins were mainly detected in the endothelial cells of the *vasa vasorum*.

Conclusions—*HMGA2* is upregulated and could be used as a biomarker of acute aortic dissection. HMGA2 and SNAI1 proteins were detected mainly in endothelium of the *vasa vasorum*. It seems that *HMGA2*-overexpression in AAD occurs in a *let-7d* independent manner associated with EMT of the *vasa vasorum*

Keywords: HMGA2 ■ Acute aortic dissection ■ EMT ■ *vasa vasorum*

Introduction

The *high mobility group AT-hook 2* (*HMGA2*) gene has the ability to bind to AT-rich DNA sequences, induce global changes in chromatin structure and thereby regulate transcription[1;2]. It is regulated by transforming growth factor beta (TGF-β) induced smad-pathway and initiates epithelial-mesenchymal-transition (EMT)[2; 3]. EMT describes a process that reorganizes epithelial cells to become migratory mesenchymal cells[3]. This process is critical to normal embryogenesis and a defining structural feature of organ development. Among the regulators of EMT is the transcriptional repressor snail homolog 1 (SNAI1), a major effector downstream of HMGA2[4, 5]. SNAI1 acts as a strong repressor of epithelial-specific genes like *E-cadherin* and therefore promotes EMT. It is considered as a marker for EMT[6, 7].

Normally, the *HMGA2* expression level is very high during embryonic development whereas it is low or, even undetectable in differentiated adult cells[8, 9]. The expression of *HMGA2* is controlled by the microRNAs of the *let-7* family by targeting its 3'UTR[10]. Reactivation of *HMGA2* due to cytogenetically detectable aberrations of chromosomal region 12q13-15 is a fragment finding in a variety of benign tumors of mesenchymal origin. Pathogenetically, re-expression of *HMGA2* has been implicated in the formation of arteriosclerotic plaques and aortic restenosis as well[11-16]. However, the involvement of *HMGA2* in acute aortic dissection (AAD) is unknown. AAD is a life-threatening situation with high morbidity and mortality rates, and it is most often an unpredictable event[17-19]. People commonly at risk of the disease include those with connective tissue disorders such as Marfan syndrome, Ehlers–Danlos syndrome, Erdheim Gsell medial necrosis, but also bicuspid aortic valve.

In this study we compared the *HMGA2* level in different human aortic diseases. AAD patients, who do not suffer from Marfan syndrome, were opposed to patients suffering from thoracic aortic aneurysm (TAA) or Marfan syndrome. Although many studies have clarified

the involvement of TGF-β in patients with Marfan syndrome, little research was done about the downstream effectors of TGF-β in AAD.

The interaction between TGF-β1 and HMGA2 is essential during the EMT, meanwhile, the *HMGA2* gene is expected to have a role in adult tissues as well. Therefore, we examined *HMGA2* expression in different aortic diseases. We also studied the *let-7* miRNA expression in patients with AAD and compared it to the expression of *HMGA2* to reveal possible dysregulations of *HMGA2* and its inhibitory miRNA. Furthermore the *HMGA2* expression in different aortic tissues of the same AAD patients was determined and protein levels of HMGA2 and SNAI1 were investigated to indicate a possible appearance of EMT.

Materials and methods

Tissue samples:

The study protocol was approved by the institutional ethics committee and written informed consent from all patients was obtained. In cases of aortic valve or ascending aorta replacement, diseased aortic tissue was collected during surgery and carefully divided into two parts. One part was immediately snap-frozen in liquid nitrogen and preserved at -80ºC and then used later for gene expression and protein analysis. The second part was fixed with 4.5% paraformaldehyde and then embedded in paraffin; patients' demographics are shown in Table 1.

Table 1. Patient demographics and clinical characteristics.

Disease	Type A Dissection, N = 19	Ascending Aneurysms, N = 26	Marfan Syndrome, N = 2	Valve Replacement, N = 4
Demographic				
Age (years)	59.6 ± 16.4	59.3 ± 13.8	31.5 ± 6.4	54.0 ± 9.2
Gender (male/female)	9m/10f	22m/4f	2m	4m
Body mass index	26.2 ± 4.0	26.1 ± 3.4	25.5 ± 7.8	27.7 ± 5.5
Aortic diameter (mm)	n.a.	53.3 ± 7.1	70.0 ± 14.1	38.0 ± 1.6
Bicuspid aortic valve	N = 2	N = 14	0	N = 3

n.a. = not available

RNA-Isolation, reverse transcription, and qRT-PCR for HMGA2 measurement:

Total RNA was purified according to the RNeasy® Mini Kit protocol for isolation of total RNA from heart, muscle and skin tissue (Qiagen, Hilden, Germany) including on-column DNaseI digest and homogenisation with TissueLyser. The input amount of material was about 5-10 mg. RNA was quantified and with 5 µg RNA a second DNaseI (6.75 U) digestion for 15 min at room temperature and a cleanup according to the RNeasy Mini Kit protocol was performed to remove contaminating DNA as complete as possible.

Up to 250 ng of total RNA was reverse transcribed with 200 U of M-MLV Reverse Transcriptase and 150 ng random hexamers according to the manufacturer's instructions (Invitrogen, Karlsruhe, Germany).

RT-PCR amplification was performed using the ABI Prism 7300 Sequence Detection System (Applied Biosystems, Darmstadt, Germany). The relative quantification method was practiced and 18S rRNA was used as endogenous control.

The expression of 18S rRNA in all the samples showed only a slight variation, the mean Ct value was 9.94 ± 0.66, moreover the slope of log input amount vs. ΔC_T between the assays was about 0.008 and therefore also acceptable. *HMGA2* and 18S rRNA expression analyses were performed in triplicates in a total volume of 20 µl using 2 µl of each cDNA corresponding to 25 ng of total RNA. For the measurement of gene expression the following sequences (TaqMan probe; Applied Biosystems: *HMGA2* assay number Hs00171569 with an amplicon size of 65bp) was used. The primer pair and probe for 18S rRNA were as follows: forward primer: AATTTCACATAGCCCACTTACATTTACA; reverse primer: TTGATTCTAATAATCCCATGCTTTGA; TaqMan probe with an amplicon size of 65bp: 6-FAM-ACTGAAGAGTAATCAATCTA-MGB. Provision for the higher expression of 18S rRNA the cDNA for these samples was diluted 1:10. For each run non-template controls (NTC) and for each sample reactions without reverse transcriptase (- RT) were included. PCR conditions were as follows: 2 min at 50°C and 10 min at 95°C, followed by 50 cycles with 15 sec at 95°C and 1 min at 60°C.

RNA-Isolation, reverse transcription, and qRT-PCR for let-7d measurement:

Tissue (80 mg) was homogenised with a Tissue Lyser (Qiagen, Hilden, Germany) in 1000 µl of TRIzol®Reagent and following the manufacturer′s instructions (Invitrogen, Karlsruhe, Germany). RNA was resuspended in 80 µl of nuclease-free water. Purity and concentration of the samples were determined by photometry.

Real-time RT PCR was performed in two steps using TaqMan® MicroRNA Assays (Applied Biosystems, Darmstadt, Germany). 20 ng of each miRNA *let-7d* and endogenous control *RNU6B* were reversed, transcribed with gene specific stem-loop primer according to the manufacturer's protocol using the TaqMan® MicroRNA Reverse Transcription Kit (Applied Biosystems, Darmstadt, Germany). Additionally, 0.9 µg/µl whole pooled fetal RNA (Stratagene, Karlsruhe, Germany) and was included. Real-time PCR was conducted with 2x Universal Master Mix and 20x TaqMan®. MicroRNA Assays were purchased as pre-optimized Assays (using the Applied Biosystems 7300 Real-time PCR System (all Applied Biosystems, Darmstadt, Germany). Each reaction was run in triplicates, containing 1.33 µl of cDNA in a final reaction volume of 20 µl. Additionally each run included NTCs and again for each sample - RT. Data were analysed using 7300 system software (Applied Biosystems, Darmstadt, Germany) and the linear regression plot for ΔC_T analyzes between *let-7d* and *RNU6B* was about 0.1 whereas the *RNU6B* expression was constant in Cycle 25.0 ± 0.7.
To prevent false results based on a rapid degradation of miRNAs and resulting cDNAs, isolation of RNA, reverse transcription and Real-time PCR were conducted in one day.

Immunohistochemical Analysis: Immunohistochemical analyses were done for HMGA2, SNAI1, the endothelial cell marker CD34 and MKI-67, a nuclear protein associated with cell proliferation. Formalin-fixed and paraffin embedded tissue sections (4 µm) were deparaffinized, rehydrated, and washed with PBS before immunoperoxidase staining. Slides were incubated overnight at 4°C in a humidified chamber with 1:20 rabbit anti-HMGA2 (sc-30223, Santa Cruz Biotechnology, Santa Cruz, California, USA), 1:30 rabbit anti-SNAI1 (sc-28199, Santa Cruz Biotechnology, Santa Cruz, California, USA), 1:50 mouse anti-CD34 (NCL-END, Leica Biosystems, Newcastle, United Kingdom) and 1:400 mouse anti-MKI-67 (M7240, Dako, Glostrup, Denmark). Biotinylated anti-rabbit link or anti-mouse link was used as secondary antibody (for 30 min). Slides were then incubated with avidin biotin enzyme label (Vector Laboratories, California, USA) for 30 min and developed with AEC peroxidase substrate (Camon Laborservice GmbH, Wiesbaden, Germany) for 10 min. Finally, slides were counter-stained with haematoxylin.

Statistics:
Differences among groups were assessed with the Mann-Whitney-U test. Significant differences were assumed at $P \leq 0.05$.

Results

Measurement of HMGA2 gene expression in aortic tissue using qRT-qPCR

The levels of *HMGA2* mRNA were quantified using Real-time quantitative PCR. Gene expression was measured in aortic tissue of 19 patients with AAD, 26 samples from TAA and two patients suffering from Marfan syndrome (MS), additionally aortic valve tissue of 4 patients, who did not suffer from dissection, was included. Figure 1 demonstrates the log of quantified *HMGA2* expression using the ΔC_t method.

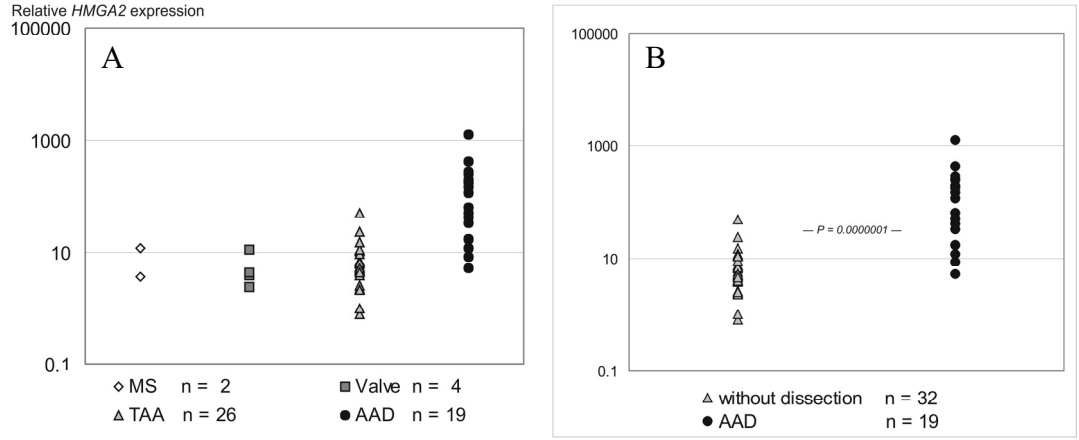

Figure 1. Logarithmic data of *HMGA2* expression in tissue samples of different aortic diseases.
A, Comparison of HMGA2 expression between patients with Marfan syndrome, tissue of aortic valves, patients with thoracic aortic aneurysm and AAD patients. B, HMGA2 expression in different aortic tissues compared to AAD. Samples from Marfan syndrome patients, TAA patients, and aortic valve tissue (n = 32) were grouped together and compared with samples obtained from acute aortic dissection (n = 19), P = 0.0000001.

Expression of *HMGA2* in non-dissection aortic disease samples was moderate (Figure 1 a). Samples obtained from thoracic aortic aneurysm featured a mean level of 8.6±9.7 fold expression when compared to calibrator sample. Samples of Marfan sydrome patients' aortic tissue showed a mean expression level of 7.9±5.8 fold and aortic valve tissue was 5.5±3.9 fold. Differences between these groups were assessed by Mann-Whitney-U-test and no significant variation was detected (MS vs aortic valve, $P = 0.64$; MS vs TAA, $P = 0.86$; valve vs TAA, $P = 0.39$). These groups were considered to feature a similar *HMGA2* expression and were unified in a group termed "without dissection".

HMGA2 expression ranged from 5.2 to 1212.4 fold (mean level = 193.1±272.8) in tissues of AAD patients (Figure 1 b). The difference between AAD samples and the "without dissection" group proofed to be highly significant in Mann-Whitney-U test with $P = 1.44*10^{-7}$

but differences between AAD and the non-dissection sub groups were also significant (AAD vs TAA, $P = 6.14*10^{-7}$; AAD vs MS (two patients), $P = 0.04$; AAD vs valve, $P = 0.003$).
The existence of valve malformations like bicuspid aortic valves (BAV) had no significant impact on *HMGA2* expression in TAA patients (BAV vs TAV: 0.47). Involvement of factors like age, BMI or aortic diameter was also considered, but none of them showed a significant correlation to *HMGA2* expression in the different groups (data not shown).
Tissue sets of 7 AAD patients were used to study possible differences in *HMGA2* expression between different areas of the aorta. Each set contained tissue slices of *tunica adventitia* aortic endothelium, *tunica media*, and whole aorta. Expression in these areas was measured using Real-time quantitative PCR (Figure 2).

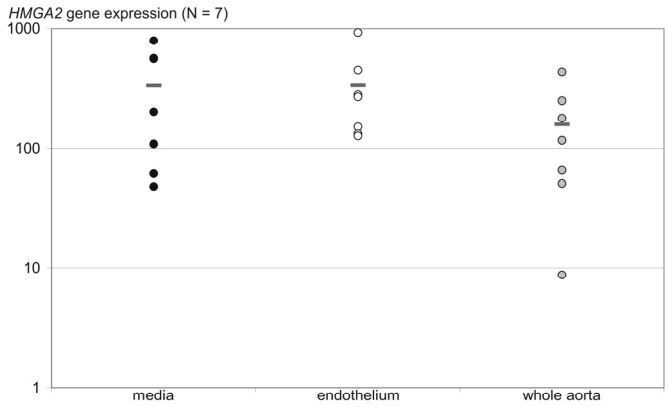

Figure 2. Logarithmic data of *HMGA2* expression in tissue sets of 7 AAD patients. *Expression was assessed in slides of aortic tunica intima endothelium, tunica media tissue and whole aorta. Differences were calculated with Mann-Whitney U-test, P = not significant.*

Real-time quantification showed similar *HMGA2* expression levels in *tunica media* and *tunica adventitia* endothelium of AAD patients (mean value 332.9±298.9 vs. 333.6±286.2). Mean value of expression in whole aorta was 158.0±147.5. However Mann-Whitney-U test proved these differences to be not significant (media vs whole aorta: $P = 0.34$, endothelium vs whole aorta: $P = 0.09$, media vs endothelium: $P = 0.66$).

Immunohistochemical Analysis

Immunohistochemical analysis was performed to determine localization of HMGA2, SNAI1, CD34 and MKI-67 proteins within the aorta. Tissue slides of AAD patients (n = 11) and slides of tissue without AAD (n = 11) were incubated with the antibodies. Most of the cases derived from tissue without AAD were negative for HMGA2 (Figure 3A), whereas several of the AAD cases were clearly positive for HMGA2 (Figure 3B).

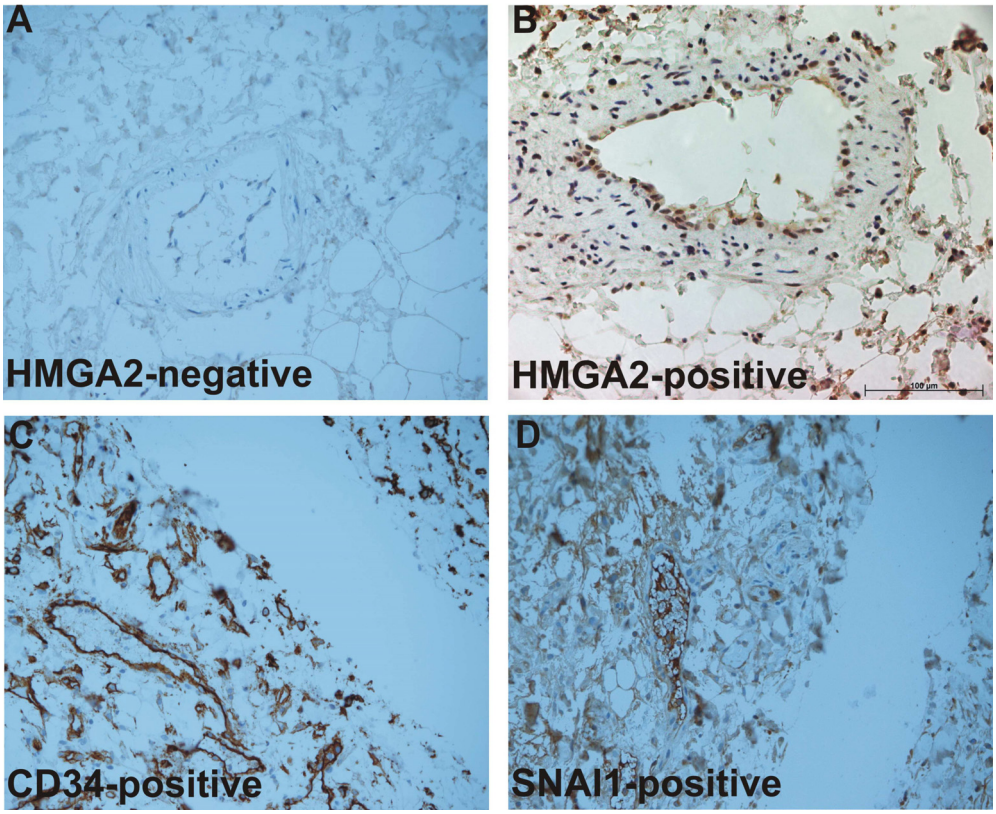

Figure 3. Immunohistochemical staining of aortic tissue slides.
Blue staining indicates HMGA2-negative nuclei while brownish staining suggests HMGA2-positive cells. A, HMGA2-negative aortic tissue obtained from patient without AAD. B, Enlargement of HMGA2-positive endothelium of vasa vasorum of AAD patient. C, immunohistochemistry of AAD aorta were stained using CD34 antibodies. D, Staining of AAD aorta with SNAI1 antibodies. Brownish staining indicates cells positive for the protein in question. Tissue slides were counter-stained with haematoxylin.

HMGA2-positive cells were mainly found in the cells surrounding of *vasa vasorum* (Figure 3B). These cells were proven to be endothelium by immunohistochemistry with endothelial marker CD34 (Figure 3C) but also SNAI1 was found here (Figure 3D). No increased cell proliferation was detected using human MKI-67 antibody (data not shown).

HMGA2 and SNAI1 were found in 7 of 11 cases originated from AAD patients, whereas only two out of 11 cases from other aortic diseases including aortic aneurysm were positive.

Detection of let-7d in aortic tissue of AAD

The levels of *let-7* variant *d* were quantified in 17 AAD tissue samples to study whether *HMGA2* expression in AAD is regulated by this miRNA. Figure 4 compares the *let-7d* expression calculated by ΔC_t method with the quantified *HMGA2* expression in the same samples. Figure 4A shows that the expression of *let-7d* was low compared to expression of *HMGA2* in the same samples. The correlation between the two sample sets was very poor at $R^2 = 0.0447$, $P = 0.86$ (Figure 4B) indicating that there is no relationship between *HMGA2* gene expression in AAD tissue and *let-7d.*

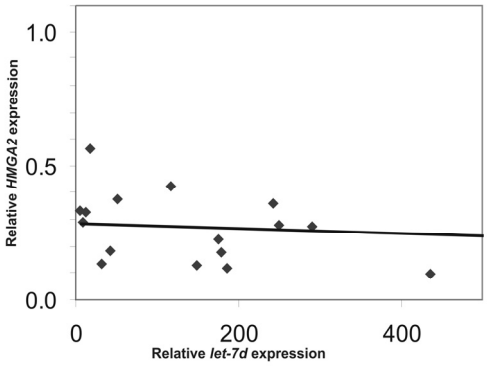

Figure 4: Relative *HMGA2* and *let-7d* expression.
Comparison of quantified relative HMGA2 and let-7d expression of 17 patients with AAD, two patients with TAA, and control fetal RNA revealed no significance. The correlation between the two sample sets (let-7d and HMGA2 expression of 17 AAD patients) was very poor at $R^2 = 0.0447$, $P = 0.86$.

Discussion

Symptoms of AAD are sudden chest pain that may radiate to the back, nausea, sweating and difficulty breathing. These symptoms can be easily confused with those of cardiac infarction, often complicating the diagnosis and delaying the treatment[20]. The gold standard techniques for the diagnosis of AAD are represented by imaging analysis such as computed tomography, magnetic resonance imaging, transesophageal echocardiography, and angiography. These methods are not always at hand in the emergency situation and some of them are time-consuming or not applicable for hemodynamically unstable patients[21, 22]. Since the dissection is associated with degradation of the aortic wall, inflammation, and cell proliferation, the

identification of biochemical and genetic markers able to readily and rapidly diagnose is highly required[23].

An increased *HMGA2* expression can be observed in many cancers[24] but the gene was not investigated in aortic dissections until now. In this study we present evidence that the *HMGA2* gene is significantly upregulated in patients with type A aortic dissection ($P = 0.00000014$), when compared to other aortic diseases like thoracic aortic aneurysm and Marfan syndrome or to aortic valve tissue. These findings hint a possible use for HMGA2 as a biomarker for Type A dissections. In clinical investigation of dissection of the aorta, some biochemical candidate biomarkers are suggested, e.g. circulating smooth muscle myosin heavy chain, d-dimer, and soluble aortic elastin, however, each of them has some limitation. Thus, a specific blood laboratory marker to detect AAD on a daily basis is still absent[23, 25]. Further studies might reveal HMGA2 to fill this gap.

During aortic dissection an initial tear appears in the inner layer of the aorta. The blood passes through the tear separating the *tunica intima* from the *tunica media* or *tunica adventitia*, creating a false lumen[26]. We compared *HMGA2* expression in tissue slices originated from endothelium of the *tunica adventitia* with slices of *tunica media* of AAD patients to verify any differences in gene regulation. No significant up- or downregulation of *HMGA2* could be observed. When slices were compared to each other and to slices of the whole aorta, expression was equally high.

In immunohistochemical analysis we determined the expression site of HMGA2 in AAD tissue. The protein is mainly found in cells surrounding *vasa vasorum*. These *vasa vasorum* penetrate the *tunica media* and *tunica adventitia* for nutrition of the aortic layers. Immunohistochemistry of the endothelial marker CD34 revealed the HMGA2 positive cells to be endothelium, while the EMT marker protein SNAI1 was also found mainly in these cells. These findings indicate a possible EMT of *vasa vasorum* endothelial cells. Absence of MKI-67 in the endothelium of *vasa vasorum* suggests that no increased cell proliferation was detectable (data not shown). This means HMGA2 and SNAI1 are expressed independently of cell proliferation.

Together these results suggest a possible weakening of the *vasa vasorum* endothelium by HMGA2 induced transition of endothelial cells to mesenchymal cells. There is evidence in the literature that impaired *vasa vasorum* are involved in development of aortic dissection. Aortic intramural hematoma are considered to be an predecessor of dissection and originate from ruptured *vasa vasorum*[27, 28].

Let-7d microRNA was quantified in AAD patients and compared to *HMGA2* mRNA expression. A quantification on mRNA level seems reasonable since human microRNAs, unlike their *C. elegans* or *Drosophila* counterparts, tend to degrade their target mRNAs completely[29]. Moreover a negative correlation between *let-7a* miRNA and *HMGA2* was already found in gastric cancer cell lines[5]. It seems that *HMGA2* is expressed in a *let-7d* independent manner in these patients. A possible explanation is the *HMGA2* regulation by another *let-7* variant. It might also be reasonable to assume that *let-7d* independent *HMGA2* expression occurs only in patients with AAD and that a dysfunction of the *let-7* regulatory effect might be the reason for the over expression of *HMGA2* in AAD. Alternatively, in these cases upregulation occurs at the transcriptional level.

In Marfan syndrome *Fibrillin-1* (*FBN1*), a gene coding for a microfibrillar protein of the extracellular-matrix, was found to be mutated[30, 31].

Microfibrills, composed of Fibrillin-1, associate with the latent TGF-β binding protein (LTBP) which controls the availability of TGF-β in the extracellular-matrix. Mutation of *FBN1* leads to dysregulation of the TGF-β pathway and is therefore considered to be a critical event in the formation of AAD in Marfan syndrome[32, 33]. The patients in this study showed no mutation related alterations in *FBN1* mRNA levels when investigated in a recent survey[34], therefore a dysregulation of TGF-β is unlikely. Since HMGA2 acts downstream of TGF-β in EMT, the weakening of *vasa vasorum* by transition of endothelial cells might be a reasonable explanation for AAD in these patients.

Study Limitations

Our study is limited as we can not confirm whether HMGA2s' upregulation is the reason for AAD in these patients or a consequence of injury and repair activity. It is possible that EMT occurs as a repair mechanism in reaction to AAD. Further evidence regarding the involvement of the HMGA2 pathway in AAD could be obtained in animal model by future studies. Nonetheless we could prove the overexpression of HMGA2 merely in *vasa vasorum*. The study is also limited as we have not verified if an elevation of HMGA2 is detectable in human body fluids to clarify its use as a AAD biomarker.

Conclusions

Our results revealed for the first time an overexpression of the *HMGA2* gene in patients with AAD. This expression seems to be independent from regulatory miRNA *let-7d* and to occur mainly in *vasa vasorum*, although no differences in expression could be detected between endothelium of the *tunica adventitia* and tissue of *tunica media*. Since the EMT causing transcription factor SNAI1 was also expressed in the endothelium of *vasa vasorum*, EMT of these cells, induced by elevated HMGA2 levels seems possible.

Acknowledgements

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Disclosures

S.A.M. and H.H.S. are co inventors on a pending German patent application (DE102009030808.3) that discloses the development of tests to diagnose aortic syndroms.

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Table and Figure Legends

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9.4 Genexpressionsanalysen von *HMGA2* an Speicheldrüsen

IV.)

A Link between the Expression of the Stem Cell Marker *HMGA2*, Grading and the Fusion *CRTC1-MAML2* in Mucoepidermoid Carcinoma.

Fehr A, Meyer A, Heidorn K, Röser K, Löning T, Bullerdiek J

Genes Chromosomes and Cancer 48(9):777-785 (2009)

Eigenanteil an dieser Publikation:

- Anteilig Planung, Durchführung und Auswertung der Ergebnisse
- Zuarbeiten zum Verfassen der Publikation

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A Link Between the Expression of the Stem Cell Marker *HMGA2*, Grading, and the Fusion *CRTC1-MAML2* in Mucoepidermoid Carcinoma

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Recently, the concept of cancer stem cells and their expression of embryonic stem cell markers has gained considerable experimental support. In this study, we examined the expression of one such marker, the *high-mobility group AT-hook 2 gene (HMGA2)* mRNA, in 53 formalin-fixed, paraffin-embedded mucoepidermoid carcinomas (MEC) and four normal parotid tissues using quantitative real-time RT-PCR (qPCR). MECs are often characterized by the fusion gene *CRTC1-MAML2,* the detection of which is an important tool for the diagnosis and prognosis of MEC. For detection of the *CRTC1-MAML2* fusion transcript, we performed RT-PCR. The mean expression level of *HMGA2* was higher in fusion negative (302.8 ± 124.4; $n = 14$) than in positive tumors (67.3 ± 13.1; $n = 39$). Furthermore, the fusion-negative tumors were often high-grade tumors and the *HMGA2* expression level rose with the tumor grade (low: 43.7 ± 11.0, intermediate: 126.2 ± 28.3, and high: 271.2 ± 126.5). A significant difference was found in the *HMGA2* expression levels between the different grading groups (one-way ANOVA, $P = 0.04$) and among the fusion-negative and -positive tumors (*t*-test, $P = 0.05$), indicating that the expression level of *HMGA2* was closely linked to grading, the presence/absence of the *CRTC1-MAML2* fusion, and the tumor behavior of MECs. These findings offer further evidence for the theory that the MEC group comprises two subgroups: one group with the *CRTC1-MAML2* fusion, which is a group with a moderate aggressiveness and prognosis, and the other group lacking that fusion corresponding to an increased stemness, and thus, higher aggressiveness and worse prognosis.

INTRODUCTION

In malignant neoplasms, a strong association between the overexpression of *high mobility group AT-hook 2 gene (HMGA2)*, the malignant phenotype, and an adverse prognosis has been demonstrated for a variety of malignancies (Rommel et al., 1997; Rogalla et al., 1997, 1998; Sezer et al., 2000; Gross et al., 2003; Langelotz et al., 2003; Miyazawa et al., 2004; Sarhadi et al., 2006; Meyer et al., 2007a,b; Winkler et al., 2007; Belge et al., 2008).

An overexpression has also been described for a variety of benign tumors, such as tumors of the salivary gland (Schoenmakers et al., 1995; Kazmierczak et al., 1996). HMGA2 is a chromatin-associated nonhistone protein that binds through its AT binding motifs to the minor groove of AT-rich DNA strands and acts as an architectural transcription factor (Reeves and Nissen, 1990; Bustin and Reeves, 1996; Zhou and Chada, 1998). The *HMGA2* gene is expressed predominantly during embryogenesis but is normally repressed in differentiated cells and tissues (Chiappetta et al., 1996; Rogalla et al., 1996; Hirning-Folz et al., 1998).

In this study, the expression of *HMGA2* in mucoepidermoid carcinomas (MECs) of either of two genetic types was compared with the expression in normal salivary gland tissues. MECs are the most frequent malignant tumors of the salivary glands (Goode and El Naggar, 2005). The histopathologic grade of MECs is an established

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predictor of prognosis and influences the treatment of the disease (Nance et al., 2008). A frequent subgroup of MECs is characterized by a recurring t(11;19)(q21;p13) translocation that has also been found in rare cases of Warthińs tumors and clear cell hidradenoma, which is often the sole cytogenetic alteration in this diseases (Enlund et al., 2004; Behboudi et al., 2006; El-Naggar, 2006; Winnes et al., 2007; Fehr et al., 2008a). Recent cloning of the translocation breakpoint in MEC cell lines identified a fusion gene comprised of exon 1 of the *CREB-regulated transcriptional coactivator CRTC1* (also known as *MECT1*, *TORC1*, or *WAMTP1*) gene at 19p13 and exon 2–5 of the *mastermind-like gene MAML2* at 11q21 (Tonon et al., 2003; Enlund et al., 2004). Previous functional studies have shown that the expression of *CRTC1-MAML2* is essential for the growth of translocation t(11;19)-positive MEC cell lines and that the N-terminal CREB-binding domain of CRTC1 is fundamental for the transforming activity of the fusion protein (Coxon et al., 2005; Wu et al., 2005; Komiya et al., 2006). Also, the fusion transcript *CRTC1-MAML2* has been linked with low- or intermediate-grade tumors and a good prognosis (Behboudi et al., 2006; Okabe et al., 2006). Notably, the t(11;19) and the underlying *CRTC1-MAML2* fusion are not restricted to MECs of the salivary glands but have been found in MECs of other organs (Stenman et al., 1998; Liu and Adams, 2007; Tirado et al., 2007), as well as in Warthin's tumors and clear cell hidradenoma (Bullerdiek et al., 1988; Enlund et al., 2004; Behboudi et al., 2005; Winnes et al., 2007; Fehr et al., 2008a). Furthermore, a variant of the *CRTC1-MAML2* fusion, *CRTC3-MAML2*, has been described (Fehr et al., 2008b).

HMGA2 is a gene abundantly expressed in embryonic stem cells. In malignant tumors, its reappearance has been linked to a more aggressive behavior, epithelial to mesenchymal transitions, and increased stemness of the cancer cell population. Accordingly, high expression of *HMGA2* usually correlates with a worse prognosis. Translocations affecting the HMGA2 locus also have been described in pleomorphic adenomas (PAs) of the salivary glands (Schoenmakers et al., 1995; Geurts et al., 1997, 1998), and amplifications of the gene can be found in subgroups of those tumors as well (Roijer et al., 1999, 2002). Persson et al. (2009) have shown that *HMGA2* may be important for the malignant transformation of PA to carcinoma ex PA (Ca-ex-PA).

The aim of this study was to analyze the *HMGA2* mRNA expression level in positive and negative MECs to find out if there was a correlation between expression level, tumor grade, and the presence/absence of the *CRTC1-MAML2* fusion transcript.

MATERIALS AND METHODS

Tumor Material

For this study, 53 formalin-fixed, paraffin-embedded (FFPE) MEC tissues were obtained from the Salivary Gland Registry of Oral Pathology at the University Medical Center Hamburg-Eppendorf and the Albertinen-Pathology, Hamburg, Germany. All tumors were carefully re-examined and the diagnosis of MEC confirmed according to the criteria of the World Health Organization (Goode and El Naggar, 2005). Four snap-frozen normal parotid tissues were used as a calibrator for the qPCR, and a MEC cell line (NCI-H292) positive for *CRTC1-MAML2* was used as a positive control for RT-PCR. For a summary of clinical and pathological data, see Table 1.

RNA Isolation

To validate the constant expression level of the endogenous control, total RNA was extracted from snap-frozen normal tissues with the TissueLyser and the RNeasy® Mini Kit according to the manufacturer's protocol (Qiagen, Hilden, Germany). As the positive control for RT-PCR, RNA was isolated from NCI-H292 using TRIzol® LS reagent (Invitrogen, Karlsruhe, Germany). RNA was extracted from six 5 μm sections of FFPE tumors. The tissue was deparaffinated with xylene and the total RNA isolated with the High Pure RNA Paraffin Kit according to the manufacturer's instructions (Roche, Penzberg, Germany).

QPCR

cDNA synthesis of tumor and normal parotid RNA were performed with 150 ng of random hexamer primer, 250 ng of total RNA, 40 U of RNaseOUT™, and 200 U of M-MLV Reverse Transcriptase™ (Invitrogen, Karlsruhe, Germany) according to the manufacturer's instructions.

Quantitative real-time PCR amplifications were performed with the ABI Prism 7300 Real-Time PCR System (Applied Biosystems, Darmstadt,

TABLE 1. Summary of Clinical and Histopathologic Data, *CRTC1-MAML2* Status, and *HMGA2* Expression in 53 Mucoepidermoid Carcinomas

Case no.	Age (years)	Sex	Histologic grade	*CRTC1-MAML2*[a]	RQ[b]
1	60	F	Low	+	199.0
2	80	F	Low	+	139.7
3	52	M	Low	+	85.4
4	71	M	High	+	22.6
5	72	F	Low	+	7.0
6	77	F	Low	+	7.6
7	79	F	Intermediate	+	83.3
8	74	F	Low	+	20.8
9	49	F	Low	−	57.6
10	63	M	High	−	1273.4
11	17	M	Low	+	7.3
12	79	F	Intermediate	+	149.0
13	38	M	Low	+	10.9
14	72	M	Intermediate	+	337.7
15	42	M	Low	+	41.8
16	42	M	High	−	66.2
17	75	F	Low	+	2.9
18	49	F	Intermediate	+	55.9
19	15	M	Low	−	24.9
20	65	M	High	−	1343.0
21	58	F	Low	+	36.3
22	40	M	Low	+	3.2
23	25	F	Intermediate	−	44.4
24	61	F	Low	+	25.1
25	62	M	Intermediate	+	81.2
26	47	M	Low	+	3.9
27	48	F	Low	−	88.5
28	37	M	Intermediate	−	101.8
29	61	F	Low	+	67.4
30	54	M	Intermediate	−	36.1
31	55	M	High	−	16.4
32	65	M	Intermediate	−	402.4
33	46	M	High	+	60.1
34	51	M	High	+	47.0
35	45	M	Low	+	7.8
36	34	F	Intermediate	+	140.0
37	45	M	Intermediate	+	6.3
38	75	F	Intermediate	+	36.4
39	76	F	High	+	24.9
40	65	M	High	+	139.4
41	71	F	Intermediate	+	17.8
42	–	M	High	−	693.0
43	49	M	High	−	37.1
44	44	M	High	+	15.2
45	49	F	Intermediate	+	281.8
46	58	F	Intermediate	+	256.1
47	62	M	Low	+	44.4
48	22	M	Intermediate	+	41.4
49	29	F	Intermediate	+	74.0
50	69	M	Intermediate	+	5.6
51	73	F	High	+	4.1
52	66	M	Low	+	35.1
53	80	F	High	−	53.8

[a]+, *CRTC1-MAML2*-positive; −. *CRTC1-MAML2*-negative.
[b]RQ, relative *HMGA2* expression level.

Germany). Because of different degradation of RNA isolated from formalin-fixed samples, the relative quantification method was used. In this study, 18S rRNA acted as an endogenous control (Antonov et al., 2005) and *HMGA2* as the target gene. The primer pair of the *HMGA2* assay (TaqMan® Assay no. Hs00171569_m1/Applied Biosystems) spans from exon 1 to 2 and amplifies a 65-bp fragment. *HMGA2* and 18S rRNA expression analyses were performed in triplicate in a total volume of 20 µl using 2 µl of each cDNA corresponding to 25 ng of total RNA for *HMGA2* and 2.5 ng for 18S rRNA quantification. For each PCR run, nontemplate controls and reactions without reverse transcriptase were included. We used tissue from four normal salivary glands to calculate the calibrator value (mean value of the probes). PCR conditions were as follows: 2 min at 50°C and 10 min at 95°C, followed by 50 cycles with 15 sec at 95°C and 1 min at 60°C. The relative expression levels of all probes were calculated using the ΔΔCt method and SDS Software v1.2.3 (Applied Biosystems).

RT-PCR

For cDNA synthesis of MEC RNA, 1 µg of total RNA was used for reverse transcription using 200 U of M-MLV Reverse Transcriptase™ with random primers according to the manual (Invitrogen, Karlsruhe, Germany). For cDNA synthesis of PCR-positive control (NCI-H292), 2 µg of total RNA was reverse transcribed using 200 U of M-MLV Reverse Transcriptase™ (Invitrogen) according to the manufacturer's instructions, using a poly(A)-oligo$(dt)_{17}$ primer.

Primers for amplification of the *CRTC1-MAML2* fragment with an expected size of 105 bp were *CRTC1* 5′-GCCTTCGAGGAGGTCATGA-3′ and *MAML2* 5′-CTTGCTGTTGGCAGGAGA-3′. The PCR amplification of the cDNA was performed in a final volume of 50 µl containing 5 µl template cDNA, 5 µl 10× PCR buffer without Mg^{2+}, 0.5 µM of each primer, 200 µM dNTP mix, 1.5 mM $MgCl_2$, and 0.5 µl Taq DNA Polymerase (5 U/µl) (Invitrogen). RT-PCR was performed using a denaturation step at 94°C for 3 min, followed by 35 cycles of 94°C for 45 sec, 55°C for 30 sec and 72°C for 30 sec. A final extension step was carried out at 72°C for 10 min.

To check the quality of the cDNA, a *glyceraldehyde-3-phosphate dehydrogenase (GAPDH)* fragment with an expected size of 184 bp was also amplified with the forward primer 5′-

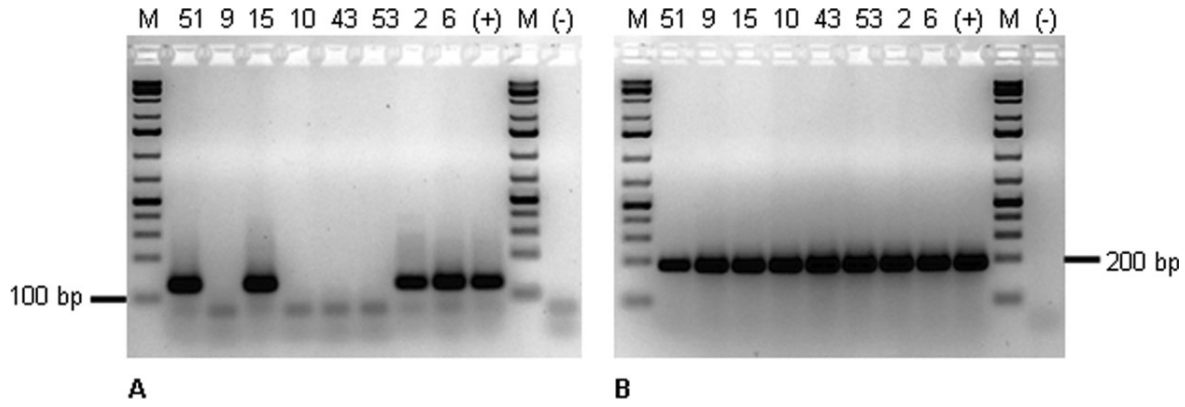

Figure 1. Results of RT-PCR on mucoepidermoid carcinomas (A) from *CRTC1-MAML2* PCR and (B) from *GAPDH* PCR; not all data shown; (+), PCR - positive control; (−), PCR - negative control.

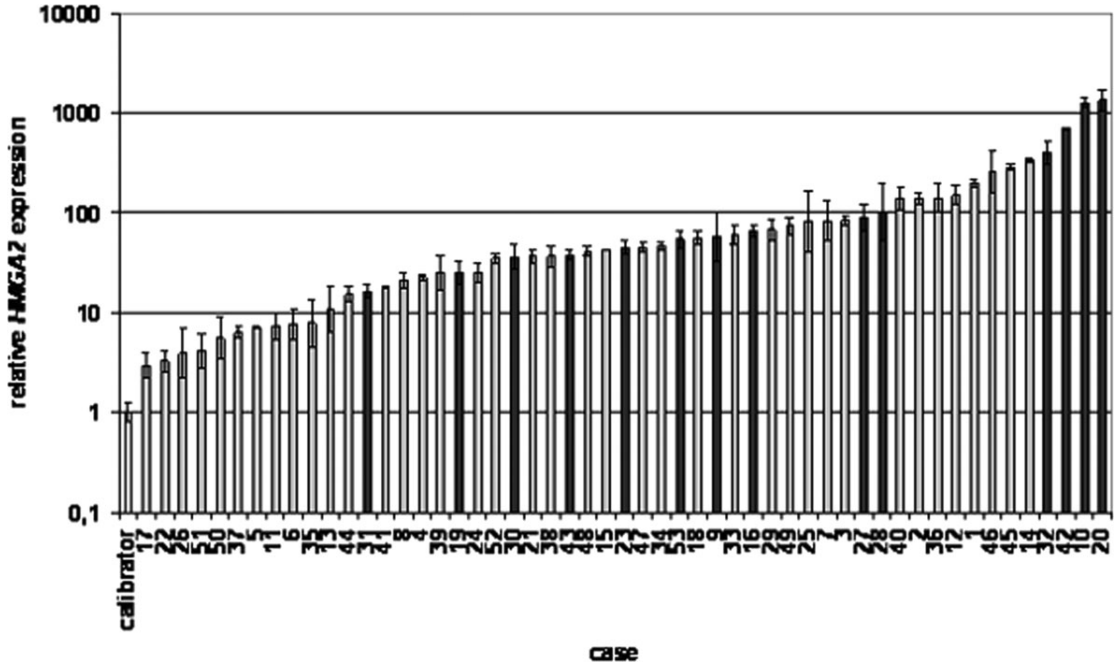

Figure 2. Results of relative *HMGA2* quantification in mucoepidermoid carcinomas. The mean value of three normal tissues serves as calibrator (white bar). Different *CRTC1-MAML2* results are represented by bar colors: dark gray: *CRTC1-MAML2* - negative, pale-gray: *CRTC1-MAML2* - positive.

TCCATGACAACTTTGGTATC-3′ and the reverse primer 5′-TTCAGCTCAGGGATGACCT T-3′ After an initial denaturation at 94°C for 3 min, PCR runs were performed over 35 cycles of amplification, using the following conditions: 94°C for 45 sec, 62°C for 45 sec, and 72°C for 30 sec; the run was completed with a final extension for 10 min at 72°C.

Statistical Analyses

Data were statistically analyzed with a one-tailed Student's *t*-test, two-sided Fisher's test, and one-way ANOVA test. A *P*-value of <0.05 was considered a statistically significant difference.

RESULTS

Detection of the *CRTC1-MAML2* Fusion Transcript in Archival Specimens

Of a total of 53 MECs, 74% ($n = 39$) showed evidence of a *CRTC1-MAML2* fusion. The fusion gene was mainly found in low-grade (46%) and intermediate-grade (36%) tumors; only 18% of the *CRTC1-MAML2*-positive samples were high-

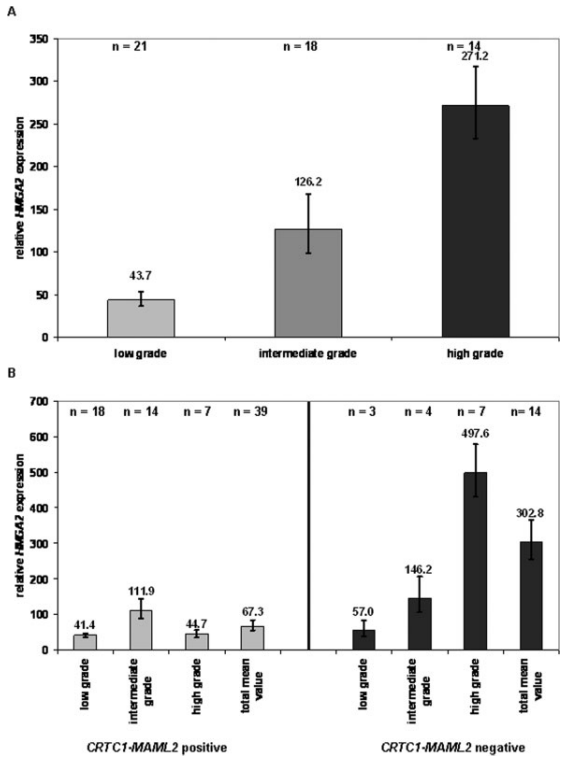

Figure 3. Mean values of *HMGA2* expression in all mucoepidermoid carcinomas (A) in relation to the grading and (B) in relation to the grade and the presence/absence of *CRTC1-MAML2*.

grade tumors. Three cases of 14 *CRTC1-MAML2*-negative samples were from low-grade (21%), four cases from intermediate-grade (29%), and seven cases from high-grade tumors (50%). The results of the *CRTC1-MAML2* RT-PCR are summarized in Table 1. Figure 1 shows the results of (A) the *CRTC1-MAML2* RT-PCR and (B) the *GAPDH* RT-PCR.

HMGA2 Expression in *CRTC1-MAML2*-Positive and -Negative Tumors

The expression of 18S rRNA from snap-frozen parotid tissue showed only low variation, with a mean Ct value of 8.12 ± 0.12. Therefore, 18S rRNA was chosen as the endogenous control for relative quantification. In all tumor samples, an increased expression of *HMGA2* was detected. The highest expression was found in a few fusion transcript-negative high-grade tumors (Fig. 2). In the total cohort of MECs, the mean *HMGA2* expression value rose with the tumor grade (low: 43.7 ± 11.0; intermediate: 126.2 ± 28.3; high: 271.2 ± 126.5) (Fig. 3A). Expression was higher in *CRTC1-MAML2*-negative (low: 57 ± 18.4; intermediate: 146.2 ± 86.7; high: 497.6 ± 227.9) than in fusion-positive tumors (low: 41.4 ± 12.5; intermediate: 111.9 ± 28.8; high: 44.7 ± 17.3) (Fig. 3B).

HMGA2 expression differences between the groups were analyzed using one-tailed Student's *t*-test (Table 2) and one-way ANOVA. One-way ANOVA revealed a significant difference between the *HMGA2* medians of the three grading stages ($P = 0.04$).

TABLE 2. Results of Student's *t*-Test Analyses of *HMGA2* Expression Levels

Parameter	*P*-value
All probes	
Normal tissue vs. total cohort of MEC	$P = 0.0004$
Fusion genes	
Normal tissue vs. *CRTC1-MAML2*-positive	$P \leq 0.0001$
Normal tissue *vs.* *CRTC1-MAML2*-negative	$P = 0.02$
CRTC1-MAML2-positive vs. *CRTC1-MAML2*-negative	$P = 0.05$
Histologic grade	
High-grade (*CRTC1-MAML2*-positive) vs. High-grade (*CRTC1-MAML2*-negative)	$P = 0.04$

TABLE 3. Clinicopathologic Correlations with *CRTC1-MAML2* Fusion Transcript Status in MECs

Parameter	Positive ($n = 39$)	Negative ($n = 13/14$)	*P*-value
Age			
>40	5	3	$P = 0.40$
<40	34	10	
Gender			
Male	19	10	$P = 0.20$
Female	20	4	
Grading			
Low/Intermediate	32	7	$P = 0.03$
High	7	7	

Clinicopathologic Correlation of *CRTC1-MAML2* Fusion Transcript in MECs

Group results were analyzed using a two-sided Fisher's test (Table 3). Although no correlation among gender, age, and fusion transcript status was evident, a correlation between the grading stages low/intermediate and high was found ($P = 0.03$).

DISCUSSION

The Mitelman Database lists a large group of a variety of tumors with alterations of the chromosomal region 12q13-15 (Mitelman Database of Chromosome Aberrations in Cancer, 2009), including a group of salivary gland tumors

(Stenman and Mark, 1983; Bullerdiek et al., 1987, 1993; Wanschura et al., 1995; Kasamatsu et al., 2005; Rutherford et al., 2005). These alterations commonly result in deregulation of *HMGA2*-containing oncogenes (Schoenmakers et al., 1995; Geurts et al., 1997, 1998; Persson et al., 2009). Several authors have described a strong association between the overexpression of *HMGA2*, the malignant phenotype, and an adverse prognosis of carcinomas (Rommel et al., 1997; Rogalla et al., 1997, 1998; Sezer et al., 2000; Gross et al., 2003; Langelotz et al., 2003; Miyazawa et al., 2004; Sarhadi et al., 2006; Meyer et al., 2007a,b; Winkler et al., 2007; Belge et al., 2008).

In this study, the *HMGA2* mRNA expression in two different subtypes of MECs was analyzed to examine possible correlations between expression level, tumor grade, and the presence/absence of the *CRTC1-MAML2* fusion. We quantified the *HMGA2* expression level in 53 MECs of the two types by qPCR. The results of qPCR showed highly significant differences in the expression level of *HMGA2* between the calibrator (median of four normal tissues) and the tumor tissues ($P = 0.0004$). Furthermore, there was a significantly higher level of *HMGA2* expression in high-grade tumors when compared with low-grade tumors.

The *CRTC1-MAML2* fusion gene was significantly more often detected in low-/intermediate-grade tumors than in tumor tissues of high grade. We also noted a lack of correlation among gender ($P = 0.2$), age ($P = 0.4$), and fusion transcript status. Previous studies reported similar findings (Okabe et al., 2006; Tirado et al., 2007). Behboudi et al. (2006) showed that the clinical prognosis for low-grade MECs of the salivary glands was much better than for high-grade tumors, and Tirado et al. (2007) described *CRTC1-MAML2*-negative tumors to be more likely to develop distant metastases. These observations were confirmed by our results: *CRTC1-MAML2*-negative tumors were frequently high-grade tumors (7 of 14 cases, 50%), and the *HMGA2* level was significantly higher in *CRTC1-MAML2*-negative tumors than in *CRTC1-MAML2*-positive tumors.

The results of this study confirmed that the *CRTC1-MAML2* transcript was present in the majority of MECs. These results correlate well with previously reported studies (Martins et al., 2004; Behboudi et al., 2006; Tirado et al., 2007; Fehr et al., 2008b). The *CRTC1-MAML2*-positive tumors in this study frequently had a low or intermediate grade (32 of 39 cases, 82%), whereas positive high-grade tumors were relatively rare (7 of 39 cases, 18%); these results correlate with previously reported results (Tirado et al., 2007; Fehr et al., 2008b).

The one-way ANOVA test revealed a significant variation between the *HMGA2* medians of the three grading stages. The histopathologic grade of a MEC is an established predictor of prognosis and treatment. Auclair et al. (1992) found that the 5-year mortality rates for MECs were 3, 10, and 46% for low-, intermediate-, and high-grade tumors, respectively (Goode et al., 1998). Because of the more aggressive behavior of high-grade compared with low-grade MECs, a more intensive treatment has been recommended for the former group (Nance et al., 2008). In comparison to the calibrator, all examined tumors showed an increased *HMGA2* expression level of 3- to 1343-fold. Furthermore there was a correlation between the *HMGA2* expression level, the presence/absence of the *CRTC1-MAML2* fusion gene, and the grade of the tumor. These results indicated that the expression level of *HMGA2* was closely linked to grading and the *CRTC1-MAML2* fusion, as well as with the tumor behavior of MECs.

The general association between high *HMGA2* level and the aggressiveness of solid tumors could be an interesting starting point for a possible *HMGA2* silencing therapy; previous reports have discussed this form of therapy for thyroid neoplasms, liposarcomas, and ovarian cancer. The authors and others have silenced the *HMGA2* gene, both in vitro and in vivo, which results in growth inhibition of the tumor and increased apoptosis (Berlingieri et al., 1995; Pentimalli et al., 2003; Malek et al., 2008).

This leads to considerations on the correct classification of MECs. The present classification of MEC includes two subgroups: a large group with the translocation t(11;19) and the *CRTC1-MAML2* fusion (with a moderate aggressiveness and prognosis), and a smaller group lacking this fusion (more aggressive and with a worse prognosis). Previous functional studies have shown that the expression of *CRTC1-MAML2* is essential for the growth of t(11;19)-positive MEC cell lines and that the N-terminal CREB-binding domain is fundamental for the transforming activity of the chimeric protein (Coxon et al., 2005; Wu et al., 2005; Komiya et al., 2006). Like *Drosophila Mastermind* and *MAML1*, full-length *MAML2* acts as a CSL-dependent transcriptional coactivator of Notch (Wu et al., 2000, 2002; Lin et al., 2002) In contrast, CRTC1-MAML2 activates the

transcription of Notch target genes independent of Notch ligands and CSL binding sites. In addition, the recent identification of the CRTC1 protein as a potent coactivator for genes that are regulated by cAMP-responsive elements suggests that *CRTC1-MAML2* may disrupt both Notch and CREB signaling pathways and induce tumorigenesis (Conkright et al., 2003; Iourgenko et al., 2003; Wu et al., 2005). The second group, which lacks the *CRTC1-MAML2* fusion, may represent a distinct category of poorly differentiated carcinomas of non-MEC etiology (Behboudi et al., 2006; Okabe et al., 2006; Tirado et al., 2007) with aberrantly high *HMGA2* levels. These two groups might have different origins, with the subgroup characterized by a *CRTC1-MAML2* fusion representing a histogenetic link within Warthin's tumor (Bell et al., 2008). One possible origin for the second subgroup could be the translocation t(3;8)(p21;q12) (Bullerdiek et al., 1990) in a MEC. Interestingly, this translocation is typically found in PAs of the salivary glands (Mark and Dahlenfors, 1986; Mark et al., 1988; Fonseca et al., 2008), and Persson et al. (2009) have hypothesized that amplification of *HMGA2* may cause the malignant transformation of a benign PA into a Ca-ex-PA. However, for this study on MECs, we think that the elevated mRNA levels are as a rule due to a global dedifferentiation observed in the tumors rather than to mutations affecting HMGA2. This was suggested by the result of FISH analyses on a few of the tumors, with and without the *CRTC1-MAML2* fusion, using a HMGA2 break-apart probe. The FISH analysis showed neither amplification nor rearrangements of the HMGA2 locus (data not shown). However, we cannot rule out minor changes of the HMGA2 locus which the FISH break-apart probe cannot detect.

In summary, this study indicated that the MEC group comprises two subgroups. The first had the *CRTC1-MAML2* fusion that may affect the Notch and CREB pathways, and is a group with a moderate aggressiveness and prognosis. The second subgroup had aberrant *HMGA2* expression levels and has a higher aggressiveness and worse prognosis. This aberrant *HMGA2* expression level makes *HMGA2* an interesting therapeutic target against high-grade MECs.

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9.5 Genexpressionsanalysen von *HMGA2* an Mammakarzinomen

V.)

HMGA2 gene expression is an independent prognostic parameter for recurrence free survival and overall survival in primary breast cancer.

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***HMGA2* gene expression is an independent prognostic parameter for recurrence free survival and overall survival in primary breast cancer**

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Abstract

Background: Overexpression of high mobility group protein A2 (HMGA2) is known to be associated with neoplastic transformation and poor prognosis in tumors, and with sensitization of cancer cells to DNA-damaging drugs. In this retrospective study including 130 invasive primary breast cancer samples (BC) we analyzed the prognostic value of *HMGA2* expression for recurrence free survival (RFS) and overall survival (OAS) and compared it with the prognostic value of *uPA/PAI-1*.

Methods: The *HMGA2* expression was determined by relative quantification against 18S rRNA. Standard survival analysis for RFS and OAS was performed using Kaplan-Meier/Cox-regression.

Results: Median age was 58 years, (range 31-84). 68.5% of patients had stage I, 27.7% stage II and 3.8% stage III cancer. The mean observation time was 45.1 (SD=33.6) months. There was a strong correlation between *HMGA2* overexpression and reduced RFS and OAS. Hazard Ratio (HR) for RFS stratified for *HMGA2* overexpression and adjusted for age, stage, and grading was 3.08 (95% CI: 1.48-6.41; p<0.01). HR for OAS stratified for *HMGA2* overexpression and adjusted for age, stage, and grading was 9.57 (95% CI: 2.72-33.60; p<0.001). In a Cox-model with *uPA, PAI-1*, *HMGA2*, grading, stage, and age *HMGA2* was still the most important prognostic parameter for RFS (HR= 3.22; 95% CI: 1.49-6.93; p=0.01) and OAS (HR= 9.35; 95% CI: 2.66-32.83; p<0.001).

Conclusion: Patients with primary node-negative (N0) BC highly overexpressing *HMGA2* belong to a high-risk group. *HMGA2* alone or in combination with *uPA/PAI-1* is an independent prognostic factor and an excellent prognostic predictor for primary BC.

Key words: breast cancer, *HMGA2*, prognosis, quantitative real-time PCR, recurrence, survival

Introduction

The expression of HMGA2 has been linked to invasive behavior and epithelial-mesenchymal transition (EMT) in many malignancies as e.g. squamous cell carcinomas of oral cavity[1] as well as lung[2], pancreatic[3], breast[4, 5], colorectal[6], prostate[7], and thyroid cancer[8, 9]. Clinically, the expression correlates with stage of malignancy[4], invasiveness[10], poor prognosis[1, 4, 11], and reduced survival[1, 11], but increased expression levels of *HMGA2* can be detected in several benign tumors of mesenchymal and epithelial origin[12] as well. While chromosomal translocations are the major cause of the *HMGA2* upregulation in benign tumors[13], in malignant tumors the mechanisms of the increased *HMGA2* expression are yet unclear[14].

The study of Rogalla et al.[4] shows overexpression of *HMGA2* in breast cancer samples correlating with histologic grade, but no reports of the *HMGA2* expression based on quantitative real-time PCR have been published so far. Here, we have performed a qRT-PCR based approach with 130 samples of breast cancer. The primary endpoint of this phase II diagnostic study [15] is RFS respectively OAS and its correlation to the expression of *HMGA2*.

In general hormone receptor positive patients will benefit from an endocrine therapy. Nevertheless, about 30% of patients relapse[16, 17] and there is a high demand for more precise prognostic and predictive molecular markers.

In this study the *HMGA2* expression of 130 invasive ductal breast cancer tumors was analyzed. Beside the established prognostic factors like tumor size (level of evidence (LOE)1), nodal state (LOE 1), grading (LOE 2), ER/PgR (LOE 2) and age (LOE 2), up to now only two molecular markers for node-negative breast cancer patients have reached level of evidence 1[18] and thus uPA (plasminogen activator) and PAI-1 (Urokinase-type plasminogen inhibitor) attain clinical relevance.

To assess the potential of *HMGA2* expression as an additional molecular marker, these genes have been analysed.

Material and Methods

Formalin-fixed, paraffin-embedded tissue sections from 130 patients with invasive primary BC were used for real-time PCR analyses.

RNA isolation: RNA was prepared and extracted as described before[8].

cDNA synthesis: Reverse transcription reactions were performed according to the manufacturer's instructions (Invitrogen, Karlsruhe, Germany) in a volume of 20 µl containing 250 ng of total RNA.

Real-time PCR: Taqman® real-time RT-PCR was performed using the 2x Taqman Universal PCR Mastermix with an ABI 7300 Cycler (Applied Biosystems, Darmstadt, Germany). Reactions were measured in triplicates in a volume of 20 µl with specific primers and probes (HMGA2: Hs00171569_m1; uPA: Hs01547054_m1; PAI-1: Hs00167155_m1, Applied Biosystems, Darmstadt, Germany). 18S rRNA was used as the endogenous control as described before[8]. The assays show efficiencies between 95-100% and amplicons with a lenght of 61 bp to 82 bp were used to avoid amplification problems with degraded RNA from the formalin-fixed tissue sections. For all amplicons a cDNA component control and a control without reverse transcriptase were included.

Statistical analyzes: Receiver operating characteristic analysis (ROC) was performed to determine a cut point for *HMGA2* overexpression in the breast cancer samples. The primary endpoints are RFI and OAS. For each group we calculated the number of cases, mean, standard deviation, standard error of the mean, minimum, maximum, 95% confidence interval (CI) for the mean, medians (interquartile range, IQR). Levene's test was used to assess the homogeneity of variance. Furthermore,

analysis-of-variance table and robust tests of the equality of means (medians) were used. We applied the Kolmogorov-Smirnov goodness-of-fit test to check normal distribution. In addition to the standard Kolmogorov-Smirnov (K-S) test, we used the Anderson-Darling test and the Cramer-Smirnov-Von-Mises test, a modification of the K-S test, giving more weight to the tails of the distribution than the K-S test. Standard survival analysis using a Kaplan-Meier approach was performed for assessing RFI and OS. The log-rank test was used to provide a formal statistical assessment of the differences between treatment arms with respect to RFS, OAS etc. The Cox proportional hazards model was used to estimate the HR and confidence intervals. Comparisons of categorical variables between groups were made by using χ^2 tests. Multivariate Cox proportional hazards regression models were used to adjust for differing risk factor distributions between groups. The proportional hazards assumption was assessed by including the product of the individual terms with time in the models. *P* values ≤0.05 were considered statistically significant. In addition Kohonen nets, Chi-squared automatic interaction detection to identify optimal splits and classification and regression trees for binary splits were used for multivariate analysis.

Results

Expressions *of HMGA2, uPA* and *PAI-1* could be detected in 130 (128; 126) of the 130 cases. The median observation time was 48 (mean 45.1; standard deviation 33.6) months. Median age was 58 years (range 31-84). The patients´ characteristics are summarized in table 1.

An optimal cut point of 4.6 for *HMGA2* with respect to RFS was obtained by ROC (area under curve: 0.65; standard error: 0.06; asymptotic significance: p<0.02; 95% CI: (0.53, 0.77)). According to the results of the ROC analysis a relative expression of *HMGA2* ≥ 4.6 will be defined as overexpressed (*HMGA2* +). There was a strong correlation between overexpression of *HMGA2* and reduced RFS and OAS. HR for RFS stratified for *HMGA2* expression and adjusted for age, stage and grading was 3.08 (95% CI: 1.48-6.41; p<0.01) (Figure 1). HR for OAS stratified for *HMGA2* expression and adjusted for age, stage and grading was 9.57 (95% CI: 2.72-33.60; p<0.001) (Figure 2).

5-year RFS is 82% for *HMGA2*- and 56% for *HMGA2*+, respectively. The 5-year OAS is 96% for *HMGA2*- and 68% for *HMGA2*+. In the subgroup of stage 1 patients HR for RFS stratified for *HMGA2* expression and adjusted for age and grading was 4.80 (95% CI: 1.49-15.46; p<0.01).

In a multivariate Cox-model with *uPA*, *PAI-1*, age, stage, and grading *uPA* is the most important parameter for RFS (p=0.001; HR = 4.30, 95% CI: 1.84-10.03) and stage 1 patients (p<0.03; HR = 4.23, 95% CI: 1.16-15.41). Adding *HMGA2* mRNA as a parameter to this model (*uPA*, *PAI-1*, age, stage, and grading) *HMGA2* remains the only variable in the equation for RFS with HR = 3.08 (95% CI: 1.52-7.00; p<0.01). The same applies to stage 1 patients with HR = 5.20 (95% CI: 1.60-16.94; p<0.01). Considering the hormone receptor positive (HR+) patients we obtained a hazard ratio of 3.73 for RFS (95% CI: 1.67-8.33; p=0.001) and hazard ratio of 13.53 for OAS

(95% CI: 3.03-60.47; p=0.001) both stratified for *HMGA2* expression and adjusted for age, stage, grading, uPA and PAI.

Discussion

For patients with lymph node-negative hormone receptor positive breast cancer disease, the clinical benefits of current treatment are relatively low as they show cure rates of 60-70%[14]. Consequently a large number of patients are exposed to unnecessary and toxic side effects. To avoid this, strong prognostic markers and new therapeutically targets are needed to distinguish between patients with low and high risk of disease recurrence or death.

Next to the various diagnostic markers established, genes and proteins of the high mobility group family have reached an increasing level of interest since the 1990's as they are discussed to carry the potential to act as diagnostic markers with a higher sensitivity compared to the commonly used ones[8, 19, 20]. It could be possible to subdivide patient subgroups by molecular biology and to adjust specific diagnosis and treatments. Identification of those prognostic and predictive factors that indicate the tumor state of primary BC patients is very important for the selection of node negative high risk patients who may benefit most from a modified therapy.

Controversy in a recent investigation by Chiapetta et al. in which familiar breast cancer is analysed BRCA2 mutations and a high HMGA1 expression measured by IHC correlates with a better overall survival[21]. This observation was limited only to the group of BRCA2 positive patients. In the recent investigation detection of BC patients with high *HMGA2*-expression levels in their primary tumor tissue have a significantly lower chance for cure than patients with low expression levels of *HMGA2* patients, this is a diagnostic challenge.

By the results obtained in this multicenter, retrospective phase I-II diagnostic trial[15] it could be demonstrated that the prognostic value of *HMGA2* is statistically significant with respect to RFI (p=0.003) and OS (p=0.001). It is an independent prognostic parameter for grade, stage, hormone receptor status, size of the tumor, *uPA*, and

PAI-1-expression. Furthermore it was revealed, that *HMGA2* is clinically relevant with a hazard ratio of 9.6. The hazard ratios showed, that BC patients with no *HMGA2*-overexpression profit in all obtained time slots starting from the very beginning. The gene expression information of *HMGA2* offers the possibility of new patient information which is up to now not available. In comparison to Recurrence Score Algorithm described in the oncotype B-14-trail[22] (HR 3.2) the gene expression analyzes of *HMGA2* (HR 3.7) in N_0 and hormone receptor positive cases for RFI is shown to be of the same value. In a further prospective investigation the results of comparable studies would also be considered.

The multicenter investigations could proof the independence of *HMGA2* from up to date valuable parameter tested in parallel. The information output regarding the RFI and OS of the patients was proved to be higher using *HMGA2* alone or in combination with these parameters. Furthermore the initially described functions of HMGA2 lead to the hypothesis of connections between therapeutics and the success of the therapy outcome. These results connected with the linked function of HMGA2 in cell cycle development could be expected. HMGA2 itself interacts with the transcriptional repressor $p120^{E4F}$, resulting in the induction of the cyclin A gene[23]. Cyclin A is a key factor controlling cell cycle progression both at the S phase entry and at the G_2/M transition by binding to cdk2 and cdc2 kinases[23]. By transient *HMGA2* gene silencing Malek and colleagues were able to increase the percentage of cells in early and late phase of apoptosis cells and the number of dead cells due to necrosis in ovarian cancer cells[24].

Taken together, these informations indicate an influence of HMGA2 to the control of growing speed and invasiveness of the tumor cells (e.g., inactivation of p53- induced apoptosis). The HMGA2 protein itself is controlled by different mechanisms. For example the ectopic expression of *let-7* reduce *HMGA2*-expression via degradation

of its mRNA[25]. Beside this for example the induction of let-7 RNA or knockdown of *HMGA2*-expression via gene silencing is a control mechanism[24, 25]. By elimination or knockdown of the HMGA2 protein several problems might occur. It is possible that the described influence on patients´ therapy outcome is reduced, while HMGA2 is blocked. Finally the control of cell death via necrosis and apoptosis could increase the elimination of tumor affected cells.

As the expression of *HMGA2* is only high in tumor affected cells[1, 8], these techniques will mostly influence tumor cells which will ideally reduce adverse effects. Basically several new side-effects might occur, but they are not proven up to know. It is now known that a low basic expression level of HMGA2 protein can be detected in nearly all tissues. Considering known interactions of HMGA2 with several genes and pathways side-effects can not be excluded. There is a dependency of *HMGA2* and *let-7* expression, which could be indirectly affected by blocking *HMGA2*-expression.

Beside this, HMGA2 influences the TGF-ß signaling pathway via the protein Snail and thus induces epithelial-mesenchymal transition (EMT)[10]. Furthermore, Li and colleagues identified HMGA2 as a regulator of genes linked to mesenchymal cell differentiation, cell proliferation and adipogenesis in human embryonic stem cells[26]. Interestingly this reembryonalisation could be speculated to be the reason for the increasing expression of *HMGA2* which is even normal in embryonic tissues. However, avoiding EMT should be possible in a limited area such as the human breast.

HMGA2-mediated displacement of HDAC1 from pRB protein leads to the activation of the E2F1[28]. Usually the transcription factor E2F1 has an important role in the control of cell proliferation and is associated with cellular proliferation in BC[27] and induces neoplastic transformation[28]. Consequently, an interruption of this pathway will lead to a decreased cell number which is a welcome side effect and might be

realized by blocking the expression of *HMGA2*. It is further thinkable to reduce the HMGA2 effect by competitive inhibition, which would allow for avoidance of strong and up to now unknown side effects.

These results show the power of the analyzed patient collective. However, there were also weak-points in this study.This investigation showed a relatively high failure rate of 7%. A reduction of this rate might be achieved by upgrading the analyzing methods. To ensure that the investigations are referred to tumor cells it could be reasonable to perform a micro dissection. Adding this method to the protocol, RNA dilution side effects might be deleted. With respect to the high expression of *HMGA2*, the signal can be easily detected and this method is abdicable.

Nevertheless 130 cases were well characterized. The importance of new biomarkers for node negative BC patients were identified and the high additional impact of information gained from *HMGA2* gene expression analyzes was demonstrated.

This investigation proves the high information output of *HMGA2* gene expression measurements. This is a very good possibility to predict patients outcome and this may help to identify the best possible treatment.

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Table 1: Characteristics of 130 invasive primary BC patients.

	total	HMGA2 -	HMGA2 +	p-value
number of patients	130	84 (64.6%)	46 (35.4%)	
age				
median	58	60	56	
range	31 - 84	31 - 84	32 - 84	
age classes				
≤ 40	10 (7.7%)	6 (7.1%)	4 (8.7%)	0.703
40 – 55	43 (33.1%)	26 (31.0%)	17 (37.0%)	
> 55	77 (59.2%)	52 (61.9%)	25 (54.3%)	
stage				
stage 1	89 (68.5%)	59 (70.2%)	30 (65.2%)	
stage 2	36 (27.7%)	23 (27.4%)	13 (28.3%)	0.486
stage 3	5 (3.8%)	2 (2.4%)	3 (6.5%)	
grading				
G1	10 (7.7%)	8 (9.5%)	2 (4.3%)	
G2	87 (66.9%)	55 (65.5%)	32 (69.6%)	0.633
G3	32 (24.6%)	20 (23.8%)	12 (26.1%)	
unknown	1 (0.8%)	1 (1.2%)	0 (0%)	
HR-status				
negative	5 (3.8%)	2 (2.4%)	3 (6.5%)	
ER or PgR positive	16 (12.3%)	10 (11.9%)	6 (13.0%)	0.483
ER and PgR positive	109 (83.9%)	72 (85.7%)	37 (80.5%)	

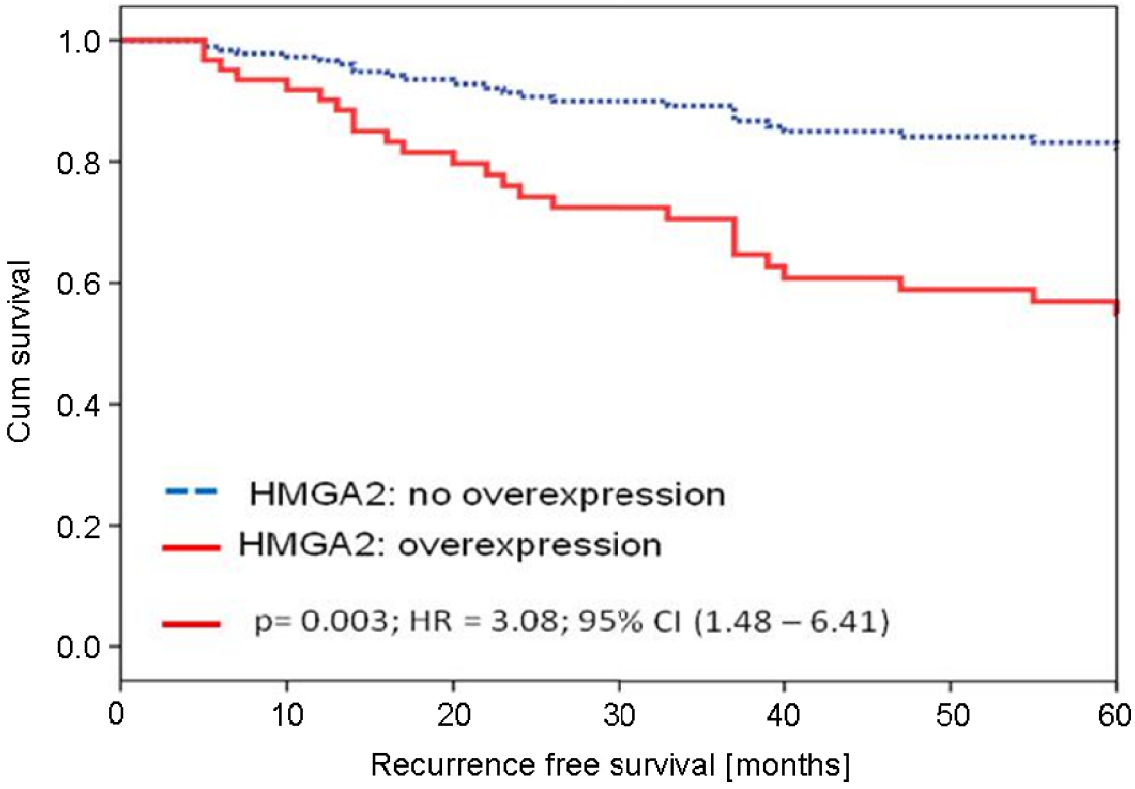

Figure 1: Recurrence free survival (RFS) stratified for *HMGA2* expression and adjusted for age, stage, and grading.

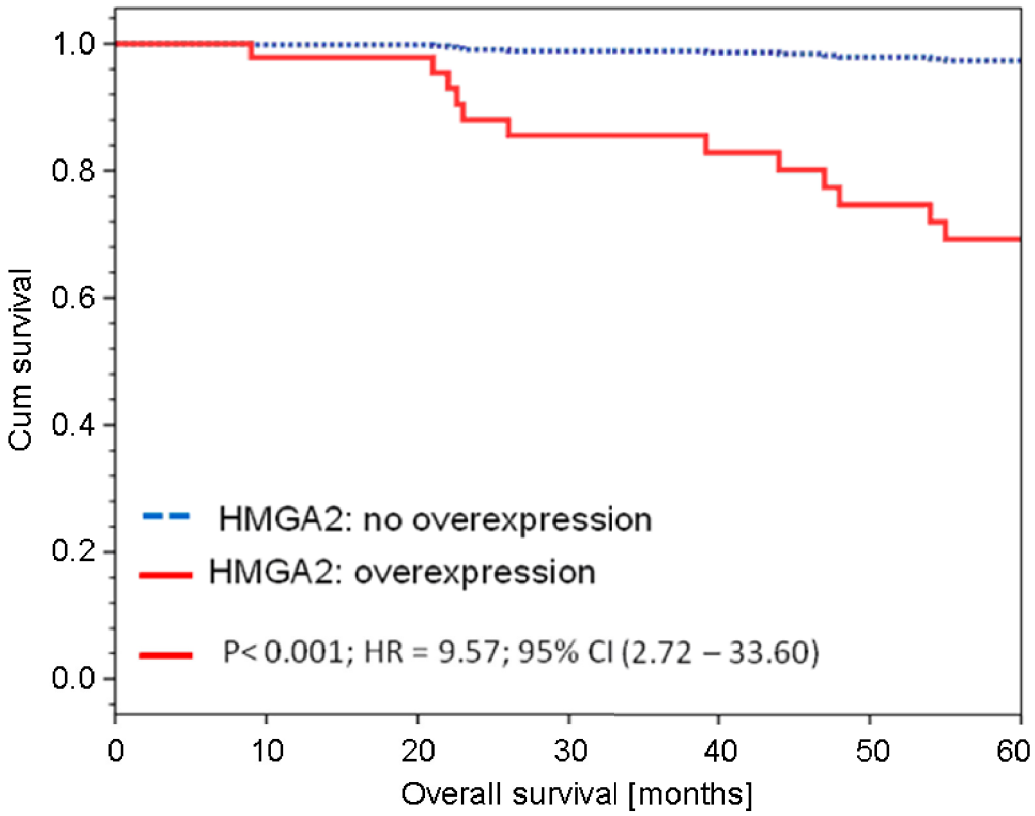

Figure 2: Overall survival (OAS) stratified for *HMGA2* expression and adjusted for age, stage, and grading.

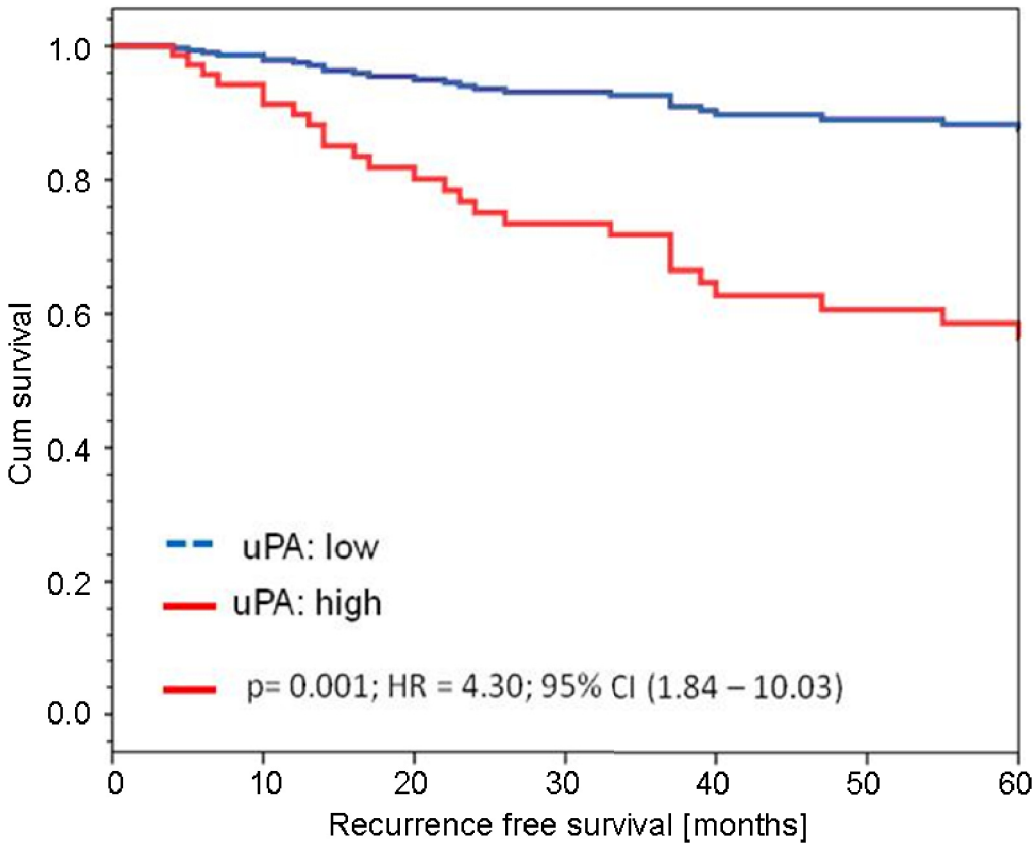

Figure 3: Recurrence free survival (RFS) stratified for *uPA* expression and adjusted for age, stage, and grading.

9.6 Genexpressionsanalysen von *HMGA2* an Uterusleiomyomen

VI.)

Overexpression of *HMGA2* in Uterine Leiomyomas Points to its General Role for the Pathogenesis of the Disease.

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*diese Autoren sind gleichberechtigte Erstautoren des Artikels

Genes Chromosomes Cancer 48(2):171-178 (2009)

Eigenanteil an dieser Publikation:

- Etablierung der Methode
- Durchführung, Planung und Auswertung der Genexpressionsanalysen
- Anlegen einer Myomdaten- und Gewebebank
- Erstellen der Publikation in Zusammenarbeit mit Herrn Klemke

GENES, CHROMOSOMES & CANCER 48:171–178 (2009)

Overexpression of *HMGA2* in Uterine Leiomyomas Points to its General Role for the Pathogenesis of the Disease

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An overexpression of *HMGA2* is supposed to be a key event in the genesis of leiomyoma with chromosomal rearrangements affecting the region 12q14-15 targeting the *HMGA2* gene, but gene expression data regarding differences between uterine leiomyomas with and those without 12q14-15 aberrations are insufficient. To address the question whether *HMGA2* is only upregulated in the 12q14-15 subgroup, the expression of *HMGA2* was analyzed in a comprehensive set of leiomyomas ($n = 180$) including tumors with 12q14-15 chromosomal aberrations ($n = 13$) and matching myometrial tissues ($n = 51$) by quantitative RT-PCR. The highest expression levels for *HMGA2* were observed in tumors with rearrangements affecting the region 12q14-15, but although *HMGA2* is expressed at lower levels in leiomyomas without such aberrations, the comparison between the expression in myomas and matching myometrial tissues indicates a general upregulation of *HMGA2* regardless of the presence or absence of such chromosomal abnormalities. The significant ($P < 0.05$) overexpression of *HMGA2* also in the group of fibroids without chromosomal aberrations of the 12q14-15 region suggests a general role of *HMGA2* in the development of the disease.

INTRODUCTION

Uterine leiomyomas (UL, fibroids) are the most frequent gynecological tumors and, despite being benign, constitute an enormous public health burden. Among the symptoms caused by uterine leiomyomas are menorrhagia, abdominal pain, and infertility (Stewart, 2001). Their actual prevalence is still a matter of debate and seems to vary among populations, but at least one third of women aged 30 years or older have one or more UL (Cramer and Patel, 1990; Baird et al., 2003; Heinemann et al., 2003). Their incidence seems to be higher in African American than in European American or European women (Marshall et al., 1998).

Currently, mutations of the two human genes encoding high mobility group proteins of the HMGA type, i.e., *HMGA1* and *HMGA2* have been assumed to be causally linked with the development of subsets of uterine leiomyomas. Both genes encode members of the so-called high mobility group proteins. HMGA proteins are capable of binding to the minor groove of AT-rich DNA with three DNA-binding domains (so-called AT-hooks), thus inducing conformational changes in chromatin structure and enabling the regulation of the expression of various target genes. In addition, they can interact with other proteins by means of their acidic domain (Fusco and Fedele, 2007). *HMGA1* and *HMGA2* map to chromosomal bands that are targeted by nonrandom structural chromosomal abnormalities found in uterine leiomyomas, i.e., 6p21 for *HMGA1* (Kazmierczak et al., 1996) and 12q14-15 for *HMGA2* (Ashar et al., 1995; Schoenmakers et al., 1995). Usually, these regions are affected by chromosomal translocations but inversions can occur as well with structural chromosomal aberrations affecting 12q14-15 being much more frequent than those affecting 6p21 (Nilbert and Heim, 1990). The molecular alterations resulting from the cytogenetic deviations generally seem to

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include an upregulation of the genes (Tallini et al., 2000; Gross et al., 2003). With regard to *HMGA2*, a considerable fraction of the chromosomal breakpoints has been assigned to regions outside the open reading frame of the gene, thus primarily affecting its expression rather than its protein sequence (Quade et al., 2003). Thus, overexpression of *HMGA2* seems to be sufficient to trigger tumorigenesis. It is obvious that an enhanced level of *HMGA2* is pathogenetically relevant in a subset of some 10–20% of uterine leiomyomas (Hennig et al., 1999), but it remains an open question whether or not an increased level of *HMGA2*, compared to normal myometrium, may characterize also leiomyomas that do not have *HMGA2* rearrangements. A recent study by Peng et al., (2008) suggests that upregulation of *HMGA2* may be a more general phenomenon in UL but the analyzed tumors were not genetically classified and no matched samples were analyzed individually by quantitative RT-PCR. Herein, we have quantitated the level of *HMGA2* mRNA in a large series of uterine leiomyomas.

MATERIALS AND METHODS

Tissue Samples

Samples of uterine leiomyomas and myometrium were snap frozen in liquid nitrogen immediately after surgery and stored at −80°C. In case of UL another part of the tumor was used for cell culturing and karyotyping. Informed consent was obtained from all patients.

For fluorescence in situ hybridization (FISH) analyses HOPE (HEPES-glutamic acid buffer mediated organic solvent protection effect)-fixed, paraffin-embedded tissue sections were used. The tissues were cut into 5 μm sections which were subsequently used for FISH analyses.

RNA Isolation, Reverse Transcription, and Quantitative RT-PCR

RNA was isolated from fresh-frozen tissue samples with the RNeasy Mini Kit (Qiagen, Hilden, Germany) including DNase treatment according to the manufacturer's instructions and quantitated by spectrophotometry. After reverse transcription of 250 ng of total RNA using M-MLV RT (Invitrogen, Karlsruhe, Germany) and random hexamers, the *HMGA2* mRNA levels were determined by relative quantification referring to the expression of 18S rRNA. Real-time PCR was performed on a 7300 Real-Time PCR System with Assay No. Hs00171569_m1 (Applied Biosystems, Darmstadt, Germany) for the detection of *HMGA2* and primers and probe for 18S rRNA as described previously (Belge et al., 2008).

Analysis of Gene Expression

The relative expression was calculated by the ΔC_t method, using 18S rRNA as endogenous control and by choosing the *HMGA2* expression of a myometrial sample as calibrator. The significance of differential *HMGA2* expression between the different groups (myometrium, myoma with and without 12q14-15 aberrations) was determined by Student's *t*-test.

Cell Culture

After surgery, samples of primary tumors were stored in Hank's solution with antibiotics (200 IU/ml penicillin, 200 μg/ml streptomycin). For cell culture the tumor samples were minced and treated with 0.26% (200U/ml) collagenase (Serva, Heidelberg, Germany) for 5–8 hr. After centrifugation, the pellet was resuspended in culture medium (TC 199 with Earle's salts supplemented with 20% fetal bovine serum, 200 IU/ml penicillin, 200 μg/ml streptomycin) and incubated at 37°C and 5% CO_2.

Chromosome Analyses

For chromosome analyses exponentially growing cultures of leiomyoma cells were used. Metaphase chromosome spreads were prepared by using colcemid (0.06 μg/ml for 1 hr) to arrest cultured cells during mitosis. A hypotonic solution (culture medium and aqua bidest in a 1:6 ratio) and the fixative (methanol and acetic acid in a 3:1 ratio) were then applied sequentially. Finally, the chromosome suspension was dropped onto glass slides. The chromosomes were GTG-banded according to routine techniques. Karyotype description followed ISCN (2005).

HMGA2-Specific Break-Apart Probes and FISH

For FISH three BAC clones were used as break-apart probes. RP11-745O10 (AC078927) and RP11-293H23 (AC012264) are located distal (3′) to *HMGA2*. RP11-269K4 (AQ478964 and AZ516203) is located proximal (5′) to *HMGA2*. Labeling was performed by nick translation (Roche Diagnostics, Mannheim, Germany) either with digoxigenin (RP-269K4) or biotin (RP11-

745O10 and RP11-293H23). For each FISH experiment 2 ng/μl of the distally located probes (RP11-745O10 and RP11-293H23) and 3 ng/μl of RP11-296K4 were used in 15 μl hybridization solution containing 50% formamide, 2×SSC, 10% dextrane sulfate and 105 ng/μl COT human DNA.

FISH analysis on metaphase preparations was performed after GTG banding of the metaphase spreads. Treatment of metaphases and subsequent FISH experiments were performed as described previously (Kievits et al., 1990) with a few modifications. For one slide 25 μl of hybridization mixture were used. Codenaturation was performed on a Mastercycler gradient (Eppendorf, Hamburg, Germany) for 3 min at 80°C followed by O/N hybridization in a humidified chamber at 37°C. Posthybridization was performed at 61°C for 5 min in 0.1×SSC. Subsequent treatment of slides was performed as described previously (Kievits et al., 1990). For detection of the hybridized probes antidigoxigenin fluorescein fab fragments (Roche Diagnostics) and Cy3-conjugated streptavidin (Dianova, Hamburg, Germany) were used. Slides were counterstained with DAPI (0.75 μg/ml) (Vector Laboratories, Burlingame, CA).

For FISH, formalin-fixed, paraffin-embedded (FFPE) tissue sections were deparaffinized with diethylether. Protease digestion was done with a pepsin ready-to-use solution (DCS, Hamburg, Germany) for 12–17 min. After dehydration in a 70%, 80%, and 95% ethanol series the sections were postfixed with 1% formaldehyde in 1×PBS for 15 min. Prior to codenaturation the sections were dehydrated again. Codenaturation was performed on a Mastercycler gradient (Eppendorf) for 5 min at 85°C followed by O/N hybridization in a humidified chamber at 37°C. Posthybridization was performed at 42°C or 61°C for 2 min in 0.4×SSC/0.3%NP-40. Subsequent treatment of slides and detection of hybridized probes were performed as described for FISH on metaphase preparations.

Slides were examined in an Axioskop 2 plus fluorescence microscope (Zeiss, Göttingen, Germany). Images were captured with an AxioCam MRm digital camera and were edited with AxioVision (Zeiss). For metaphase preparations, 10 metaphases were examined. For analysis of FFPE tissue sections at least 100 nonoverlapping nuclei from different (at least three) areas of the tumors were scored. Nuclei with two colocalized red/green signals (RG) were scored as normal. Nuclei with one colocalized red/green signal, one single red, and one single green signal (1RG1R1G) were scored as positive for *HMGA2* rearrangement.

RESULTS

For this study 180 uterine leiomyomas from 100 patients have been investigated by qRT-PCR for the expression level of *HMGA2*. A total of 57 myometrium samples from uteri removed because of the occurrence of UL were investigated as well. For 51 of these samples, matching tissue from one or more leiomyomas was available. All UL have been karyotyped successfully based on at least 10 G-banded metaphases showing a resolution of 400 bands per haploid set or higher.

Based on cytogenetics the group of UL was further subdivided into those showing aberrations of chromosomal region 12q14-15 ($n = 13$; Table 1) and those with an apparently normal karyotype or other clonal aberrations ($n = 167$), respectively.

As to these three groups, i.e., myometrium, UL with 12q14-15 changes, and other UL *HMGA2* expression was determined by qRT-PCR using fresh-frozen samples. The average relative *HMGA2* mRNA expression was 1.99 for the myometria and 261.41 for all UL. When distinguishing between both subgroups of UL outlined above average expression levels were 3213.78 for UL with aberrations in the chromosomal region 12q14-15 and 31.59 for those without changes in this region, respectively.

Thus, even within the group of UL without cytogenetically detectable rearrangements of the *HMGA2* locus at 12q14-15 *HMGA2* mRNA was expressed at a higher level than in myometrium (Fig. 1). Differences between all leiomyomas and myometrium as well as between leiomyomas without 12q14-15 aberrations and myometrium were statistically significant ($P < 0.005$ and $P < 0.05$, respectively). Furthermore, an individual analysis of the matched samples (51 myometrial tissues and 107 corresponding UL) was performed. The mean *HMGA2* expression was 11.37 in karyotypically normal UL ($n = 101$) and 1.77 in the corresponding myometrial tissues ($n = 51$).

The results clearly show that in nearly all cases within each of the paired samples the leiomyomas showed higher *HMGA2* expression than the corresponding myometrium (Fig. 2).

One case with a normal karyotype and an unexpectedly high *HMGA2* expression as well as

TABLE 1. Karyotypes of the 13 Leiomyomas with Chromosome 12 Aberrations and Results of Interphase FISH with *HMGA2* Specific Break-Apart Probes

Karyotype	Relative *HMGA2* expression	FISH results (2RG/1RG1R1G)[a]
46,XX,inv(5)(q15q31∼ 33),t(12;14)(q15;q24)[13]	8.6	98/1
46,XX,t(12;15;14)(q15;q26;q24)[20]/46,XX[1]	302.3	–
46,XX[36]	894.5	72/23
–	993.3	41/51
46,XX,der(1)r(1;2),t(12;14)(q15;q24)[4]/46,XX,t(12;14)(q15;q24)[13]	1047.8	m
46,XX,t(12;14)(q15;q24)[9]/46,XX[3]	1327.3	–
46,XX,r(1),t(1;12;14)(p36.3;q14;q24)[19]	1722.5	–
46,XX,t(2;12)(q33;q13)[17]	2381.6	–
46,XX,der(12),der(14)?ins(14;12)[8]/46,idem,r(1)[4]	3444.3	–
46,XX,t(3;5;12)(q23∼ 25;p13∼ 15;q13∼ 15)[11]/45,XX,idem,-22 [10]	4450.7	–
46,XX,t(12;14)(q15;q24)[5]/46,XX[9]	5906.1	–
45,XX,t(12;14)(q15;q24),der(14)t(12;14)(q15;q24),-22[15]	7760.3	–
45,XX,t(12;14)(q15;q24),der(14)t(12;14)(q15;q24),-22[15]	11539.7	–

[a]Percentage of nuclei either with two colocalized signals (2RG) or with one colocalized, one single red and one single green signal (1RG1R1G) indicating a *HMGA2* rearrangement.
m, FISH was performed on metaphase preparations (Fig. 4). All 10 metaphases showed a breakpoint upstream of *HMGA2*.

a second myoma with a cytogenetically visible t(12;14) and a rather low *HMGA2* expression were checked by interphase FISH. In the cytogenetically normal myoma, FISH showed *HMGA2* disruption in 23% of the cells (Fig. 3, Table 1). The tumor with visible t(12;14) but low expression of *HMGA2* showed two colocalized signals in 98% of the nuclei, indicating an intact *HMGA2* locus.

Metaphase FISH was also done on one case with a t(12;14)(q15;q24) (Fig. 4). Interestingly, the probe located proximal to *HMGA2* (RP11-269K4) showed three signals: on the normal chromosome 12, the derivative chromosome 12, and the derivative chromosome 14 (Fig. 4C), indicating a breakpoint located 5′ of *HMGA2*. The approximately 16kb distance between the probe RP11-269K4 and the 5′ end of *HMGA2* suggests that the breakpoint was located approximately 20kb upstream of *HMGA2*.

DISCUSSION

Despite their high prevalence the etiology and pathogenesis of UL remain poorly understood. Mutations of the gene encoding the high mobility group protein HMGA2 have been suggested to cause a subset of uterine leiomyomas (Schoenmakers et al., 1995; Hennig et al., 1999). As a

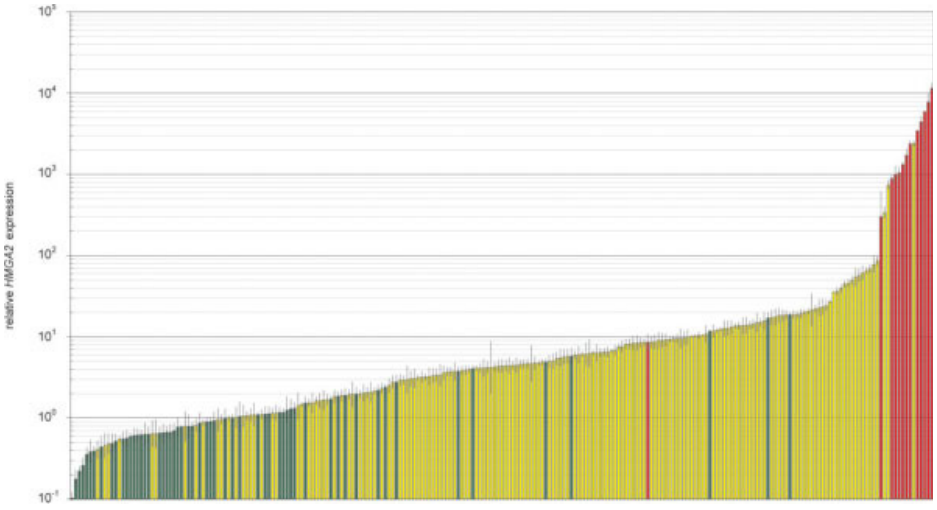

Figure 1. Relative quantification of the *HMGA2* expression in uterine leiomyomas and myometrial tissues. Green bars: Myometrium; yellow bars: UL without cytogenetically detectable aberrations of chromosomal region 12q14-15; red bars: UL with 12q14-15 aberrations.

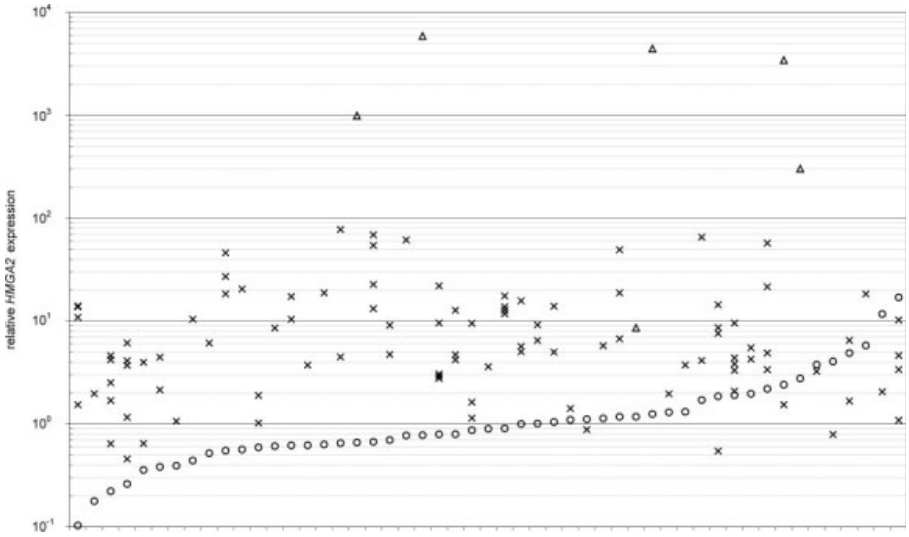

Figure 2. *HMGA2* expression in UL (crosses) and matching myometrial tissues (open circles) in increasing order of expression in myometrial tissues. Triangles indicate UL with 12q14-15 aberrations.

rule, this subset is characterized by cytogenetically visible structural chromosome alterations affecting chromosomal region 12q14-15. *HMGA2* has been identified as the target of these alterations (Schoenmakers et al., 1995) and despite a wide distribution of breakpoints, intragenic as well as extragenic (Kazmierczak et al., 1995; Hennig et al., 1996; Schoenmakers et al., 1999; Kurose et al., 2000; Mine et al., 2001; Takahashi et al., 2001; Quade et al., 2003), the key mechanism by which the chromosomal alterations contribute to tumorigenesis seems to be an upregulation of the *HMGA2* gene leading to overexpression of the full-length transcript or a truncated or chimeric protein. Nevertheless, the majority of UL lack cytogenetically visible chromosome alterations and also by molecular-cytogenetic methods there is no evidence that submicroscopic alterations of the *HMGA2* locus occur in a considerable number of these cases (Weremowicz and Morton, 1999). On the other hand, *HMGA2* overexpression could play a more general role in the development of UL, and not only in the subgroup characterized by 12q14-15 alterations. Roughly 10 years ago the hypothesis was advanced that *HMGA2* overexpression induces an embryonic chromatin configuration in cells, thus re-endowing them with a stem-cell like behavior (Bullerdiek, 1997). This assumption was further supported by recent studies on the *HMGA2* expression in embryonic stem cells (Li et al., 2006, 2007).

Here, we have shown that also UL without cytogenetically detectable 12q14-15 rearrangements overexpress *HMGA2*. This supports the assumption that an elevated level of a stem-cell chromatin associated protein is one of the key events in the genesis of UL. Of particular note, the expression of *HMGA2* in myomas almost always exceeded that of the corresponding myometrium (Fig. 2). Apparently, the basic level of *HMGA2* varies among the samples. Possibly, this could reflect changes throughout the menstrual

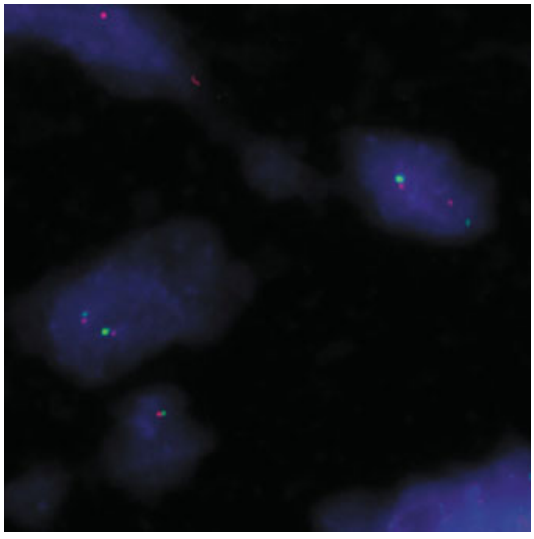

Figure 3. Dual-color FISH performed on interphase nuclei of a leiomyoma with cytogenetically normal karyotype. One nucleus showing two colocalized red/green signals (2RG, left) and a second nucleus with one colocalized red/green and one single red and green signal (1RG1R1G, right), respectively, indicating a rearrangement of *HMGA2*.

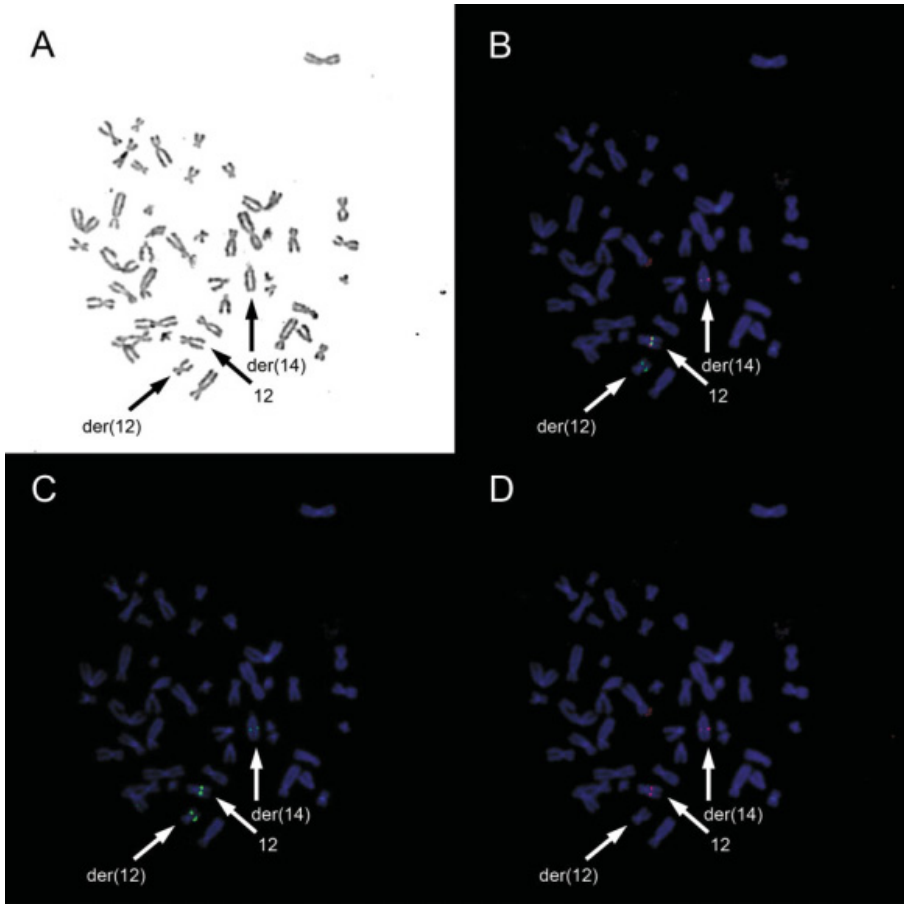

Figure 4. FISH with *HMGA2* break-apart probes in a UL with t(12;14)(q15;q24). A: G-banded metaphase prior to FISH. B: The same metaphase after dual-color FISH indicating a rearrangement of the *HMGA2* locus. C: Corresponding figure only showing the green fluorescent probe (RP11-269K4) located proximal (5′) to *HMGA2*. D: Corresponding figure only showing the red fluorescent probes (RP11-745O10 and RP11-293H23) located distal (3′) to *HMGA2*.

cycle. Such alterations in gene expression patterns have been described, e.g., by Kayisli et al. (2007). Herein, the term overexpression refers to an expression exceeding that of the matching myometrium. Despite the monoclonal origin of UL (Townsend et al., 1970; Mashal et al., 1994; Hashimoto et al., 1995; Zhang et al., 2006) overexpression of *HMGA2* must not necessarily be due to mutations affecting the gene itself. It has recently been described that *HMGA2* is regulated by microRNAs of the let-7 family (Lee and Dutta, 2007; Mayr et al., 2007; Park et al., 2007; Shell et al., 2007; Kumar et al., 2008; Motoyama et al., 2008; Peng et al., 2008). Thus, having now identified the overexpression of *HMGA2* also in UL without 12q14-15 alterations being visible at the microscopic level, future studies should also address mutations of let-7 genes and their binding sites within the 3′ UTR of *HMGA2*.

To further validate the cytogenetic results in cases with high *HMGA2* expression and no visible translocation or vice versa, FISH was performed with *HMGA2* break-apart probes. In the first case, separate signals from BAC clones located 5′ and 3′ of *HMGA2* occurred in 23% of the cells, indicating a translocation with a breakpoint within or in close proximity of *HMGA2*. Besides a cryptic *HMGA2* rearrangement undetectable by classical cytogenetics, a selection of cells without translocation during cell culture may explain the observation that the karyotype was apparently normal. However, the fact that chromosome 12 was found to be aberrant in almost one fourth of the cells by FISH is concordant with the high *HMGA2* expression despite an apparently normal karyotype.

In the second case showing a low *HMGA2* expression despite a visible t(12;14) the signals

were colocalized in 98% of the cells suggesting that the breakpoint was localized outside the region covered by the FISH probes. This unusually large distance between the breakpoint and *HMGA2* may explain why the observed *HMGA2* expression was unexpectedly low in one UL with a t(12;14). On the other hand, as indicated by FISH on metaphases of one UL with t(12;14)(q15;q24), an extragenic breakpoint upstream but in closer proximity of *HMGA2* can be sufficient to trigger the observed overexpression. Breakpoints located 5′ of *HMGA2* in UL with t(12;14) have also been reported by Quade et al. (2003).

The *HMGA2* expression in uterine leiomyomas has also been quantified in a study by Gross et al. (2003). Eleven karyotypically normal UL plus four matching myometrial samples as well as 10 UL with 12q14-15 rearrangements plus three myometrial tissues were analyzed by qRT-PCR, and a significantly higher *HMGA2* expression in UL with 12q14-15 rearrangements was noted. However, in contrast to the present study, no significant differences between the expression levels in karyotypically normal UL and matching myometrial samples were observed.

In summary, our results confirm the strongly increased *HMGA2* expression in UL with 12q14-15 rearrangements. Moreover, the expression levels detected in 101 UL from 51 patients and 51 matching myometrial samples indicate a general increase of *HMGA2* mRNA also in karyotypically normal tumors.

ACKNOWLEDGMENTS

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9.7 Analyse des 3´UTR an Uterusleiomyomen

VII.)

Loss of *let-7* Binding Sites Resulting from Truncations of the 3´UTR of *HMGA2* mRNA in Uterine Leiomyomas.

Klemke M, Meyer A, Hashemi Nezhad M, Belge G, Bartnitzke S, Bullerdiek J.

Cancer Genetics and Cytogenetics 196(2):119-123 (2010)

Eigenanteil an dieser Publikation:

- Anlegen einer Myomdaten- und Gewebebank
- Ermittlung der *HMGA2* Genexpression
- Zuarbeiten zur Publikation

ELSEVIER

Cancer Genetics and Cytogenetics 196 (2010) 119–123

Cancer Genetics and Cytogenetics

Loss of *let-7* binding sites resulting from truncations of the 3' untranslated region of *HMGA2* mRNA in uterine leiomyomas

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Abstract A subset of uterine leiomyomas (UL) shows chromosomal rearrangements of the region 12q14~q15, leading to an overexpression of the high-mobility group protein A2 gene (*HMGA2*). Recent studies identified microRNAs of the *let-7* family as post-transcriptional regulators of *HMGA2*. Intragenic chromosomal breakpoints might cause truncated *HMGA2* transcripts lacking part of the 3' UTR. The corresponding loss of *let-7* complementary sites (LCS) located in the 3' UTR would therefore stabilize *HMGA2* mRNA. The aim of this study was to check UL with rearrangements of the chromosomal region 12q14~15 for truncated *HMGA2* transcripts by real-time reverse-transcription polymerase chain reaction. In 8/13 leiomyomas with aberrations of chromosomal region 12q15, the results showed the presence of the complete 3' UTR with all LCS. A differential expression with highly reduced 3' untranslated region levels was found in 5/13 myomas. In two of these, full-length transcripts were almost undetectable. Truncated transcripts were apparently predominant in roughly one-third of UL with chromosomal rearrangements affecting the *HMGA2* locus, where they lead to a higher stability of its transcripts and subsequently contribute to the overexpression of the protein. The assay used is also generally suited to detect submicroscopic alterations leading to truncated transcripts of *HMGA2*.

1. Introduction

A subgroup of uterine leiomyomas (UL) is characterized by rearrangements of chromosomal segment 12q14~q15. Roughly 15 years ago, a causal link among these aberrations, the deregulation of the gene encoding the high-mobility group AT-hook 2 (*HMGA2*), and the pathogenesis of UL has been shown [1–3]. *HMGA2* was found to be targeted by breakpoints that are located either intragenically or extragenically 3' or 5' of the gene. This type of abnormality is shared by a variety of other mainly benign tumors of mesenchymal origin (e.g., lipomas or pulmonary chondroid hamartomas) [1,2,4,5]. Initially, the transcriptional deregulation of *HMGA2* by the rearrangements of controlling elements and/or fusion genes was thought to be the relevant molecular alteration resulting from the chromosomal rearrangements. Because *HMGA2* is abundantly expressed during prenatal development and because its rearrangements often leave the open reading frame (ORF) intact, it is tempting to assume that the increased protein level alone is sufficient to cause or contribute to UL development [6]. Accordingly, the 12q14~q15 rearrangements are always associated with a drastically increased level of *HMGA2* mRNA [7–9]. However, there have been recent descriptions of another mechanism by which the chromosomal deviations can lead to a higher stability of the *HMGA2* transcript and, subsequently, a higher protein level as well. The 3' untranslated region (UTR) of *HMGA2* was shown to harbor multiple binding sites for microRNAs of the *let-7* family [10–14]. Truncations of the *HMGA2* transcript resulting from intragenic breakpoints can thus reduce the sensitivity of the transcript against microRNAs of the *let-7* family, finally leading to a higher protein level in the corresponding cells. This also explains an earlier observation that constructs containing a truncated *HMGA2* 3' UTR are more stable than those with a wild-type UTR [15]. While this mechanism is experimentally well documented, it is unclear to which extent truncation of *HMGA2*

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mRNA coincides with the chromosomal rearrangements of the gene locus and could amplify the effect of simple transcriptional up-regulation. To address this question, we have quantified and compared the level of wild-type and truncated *HMGA2* mRNA from 13 primary UL and 2 cell lines derived from UL with 12q14~q15 rearrangements by real-time reverse-transcription polymerase chain reaction (RT-PCR).

2. Materials and methods

2.1. Tumor samples and cell culture

Tissue samples of uterine leiomyomas were collected immediately after surgery, snap-frozen in liquid nitrogen, and stored at −80 °C for RNA isolation. Samples used for cell culturing were treated as described previously [9].

Immortalized cells from a lipoma with a t(3;12)(q27~q28;q14~q15) accompanied by a partial genomic deletion of the *HMGA2* locus were used as a control because the presence of fusion transcripts consisting of exons 1−3 of *HMGA2* and exons 9−11 of *LPP* was shown for this case [16]. Cells were cultured in medium 199 with Earle's salts containing 1% fetal calf serum (FCS) for 24 hours and in FCS-free medium for an additional 24 hours to avoid the stimulating effects of FCS on the expression of *HMGA2* from the nonrearranged allele. Before the isolation of total RNA, cells were lysed directly in the cell culture flask.

2.2. Methods

Total RNA was isolated from tissue samples and cell cultures using the RNeasy Mini Kit (Qiagen, Hilden, Germany). In addition to the on-column DNase I digestion, a second digestion was performed in solution before reverse transcription, since the PCR reaction with the primer set binding in the 3' UTR of *HMGA2* is highly sensitive to contaminations with genomic DNA.

Reverse transcription of 250 ng RNA was carried out with Moloney Murine Leukemia Virus (M-MLV) reverse transcriptase and random hexamers (Invitrogen, Karlsruhe, Germany) according to the manufacturer's recommendations. Controls without enzyme (NoRT) were included for each sample to ensure the absence of DNA contaminations, which would introduce a bias to the results of the 3' UTR−specific primer set.

Quantitative real-time RT-PCR was performed on a 7300 Real-Time PCR System (Applied Biosystems, Darmstadt, Germany) with TaqMan Universal PCR Mastermix. Of each cDNA, 2 µL served as template in a final reaction volume of 20 µL. Reaction conditions were as follows: 2 minutes at 50 °C, 10 minutes at 95 °C, and 50 cycles of 15 seconds at 95 °C and 1 minute at 60 °C. A commercial *HMGA2*-specific assay (assay ID Hs00171569_m1; Applied Biosystems) with primers binding in exons 1 and 2 was used to detect transcripts irrespectively of a possible downstream truncation. In addition, for full-length transcripts with an intact 3' UTR, a set of primers and probe complementary to the distal 3' end of the mRNA downstream of all *let-7* complementary sites (Fig. 1) was designed (forward primer: 5'-TGTATTAT CACTGTCTGTTCTGCACAA-3', reverse primer: 5'-TGG AACTGTAACAAAGAGCAGGAA-3', probe: 6FAM-CAG CCTCTGTGATCCCCATGTGTTTTG-TAMRA). A differential expression of full-length and truncated *HMGA2* transcripts has been reported recently for lipomas with t(3;12) [17]. Herein, for evaluating the 3' UTR−specific primer/probe set and graphic display of the results, the same method with slight modifications has been used. To evaluate the primer and probe set designed for the distal 3' UTR of *HMGA2*, a cell line of a lipoma with t(3;12) was tested for differential expression. In a previous study [16], we had reported the presence of *HMGA2-LPP* fusion transcripts consisting of exons 1−3 of *HMGA2* and exons 9−11 of *LPP* in this cell line.

HPRT1 was chosen as an endogenous control and was detected with the following primer/probe set: forward primer: 5'-GGCAGTATAATCCAAAGATGGTCAA-3', reverse primer: 5'-GTCTGGCTTATATCCAACACTTCGT-3', probe: 6FAM-CAAGCTTGCTGGTGAAAAGGACCCC-TAMRA. All reactions were run in triplicate. Due to the low *HMGA2* expression in normal tissues, the Ct values of the 3' UTR−specific PCR were much higher in myometrial tissues than in myomas with aberrations affecting the chromosomal region 12q15, making the use of normal tissue as a calibrator unsuitable. Therefore, the myoma with the smallest difference in dCt values between both PCRs (case 4) was chosen as a calibrator. Although the chromosomal region 12q13~q15 is rearranged in this case, the closely related dCt values indicate the predominance of full-length transcripts. Thus, an overestimation of the relative 3' UTR expression resulting from the alternative use of myometrial tissues was avoided. The $\log_{10}$ of the relative expression was used for graphic display (Fig. 2).

3. Results

In a total of 234 uterine leiomyomas from 124 patients, chromosome analysis of GTG-banded metaphases revealed rearrangements of the chromosomal region 12q14~15 in 12 cases (Table 1), which were subjected to real-time RT-PCR analysis. One additional myoma (case 13) had an apparently normal karyotype, but fluorescence *in situ*

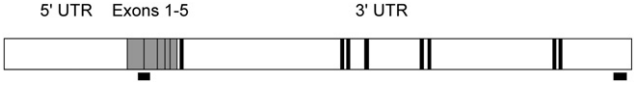

Fig. 1. Schematic representation of the full-length *HMGA2* transcript with the ORF consisting of five exons (gray boxes) and *let-7* complementary sites (LCS) in the 3' UTR (black lines) according to TargetScan [20], PicTar [21], and miRanda [22,23]. For a detailed list of the LCS, see ref. 11, Table S2. The black bars below the transcript indicate the positions of the regions amplified with two different primer sets.

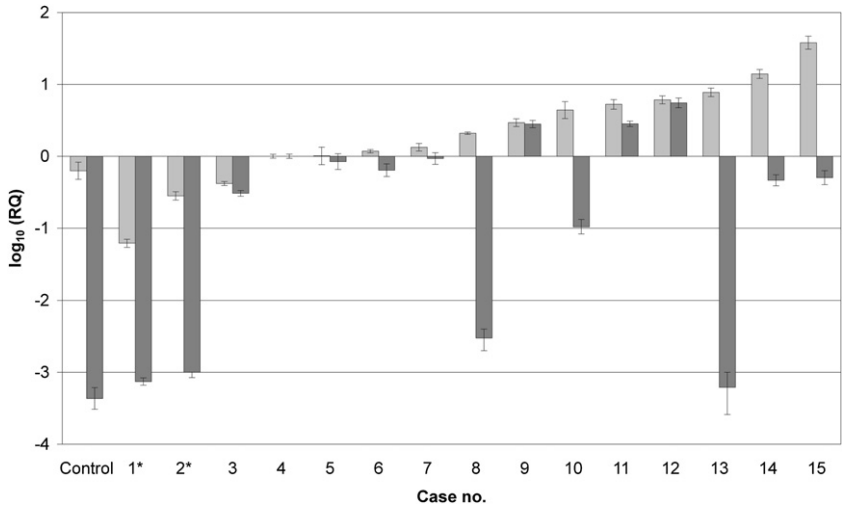

Fig. 2. Relative expression of *HMGA2* in uterine leiomyomas with chromosomal aberrations affecting region 12q14~15 and a lipoma cell line with known absence of full-length transcripts as a control. The $\log_{10}$ of the relative expression levels is shown (RQ: relative quantification). qRT-PCR was performed with primers located in exons 1 and 2 of *HMGA2* (light gray bars) and an additional set of primers located in the distal 3' UTR of the gene (dark gray bars). Case 4 was used as calibrator. Asterisks indicate cell lines of myomas with t(12;14).

hybridization (FISH) revealed split signals for *HMGA2*, indicating a hidden rearrangement of the gene locus.

With the criteria defined by Bartuma et al. [17] (i.e., an expression level that is 10 times higher for exons 1 and 2 than for the distal 3' UTR), a differential expression was observed in two myoma cell lines (cases 1 and 2), as well as in 5/13 myomas (cases 8, 10, 13, 14, and 15; Fig. 2). In contrast, no differential expression was detected in the remaining eight myomas.

In the lipoma cell line, which was used as control, the relative expression of *HMGA2* exons 1–2 was only slightly lower than in the myoma used as the calibrator, but the expression of the distal 3' UTR is clearly reduced ($\log_{10} = -3{,}36$), thus indicating the usefulness of the test procedure used.

4. Discussion

We recently reported an overexpression of *HMGA2* in uterine leiomyomas with a normal 46,XX karyotype in comparison to matching myometrial tissues [9]. Moreover, our results confirmed the previous finding, that *HMGA2* is strongly overexpressed in leiomyomas with chromosomal aberrations, affecting the locus of the gene [7–9]. However, the molecular mechanisms causing an overexpression, especially in those cases with cytogenetically detectable translocations with breakpoints in or close to chromosomal region 12q14~15, remain to be identified. Several studies [10–13] have reported the post-transcriptional regulation of *HMGA2* expression by miRNAs of the *let-7* family. Reduced expression of *let-7* family members in uterine

Table 1
Karyotypes of 13 leiomyomas and two cell lines (nos. 1 and 2) originating from myomas with chromosomal translocations affecting region 12q13~q15

Case no.	Karyotype	Age (yr)	Tumor diameter (cm)
1	46,XX,del(7)(q22q32),t(12;14)(q15;q24)[29]	–	–
2	46,X,t(X;12)(q22;q15)[8]	–	–
3	46,XX,t(12;15;14)(q15;q26;q24)[20]/46,XX[1]	49	1.5
4	46,XX,t(3;5;12)(q23~25;p13~15;q13~15)[11]/45,XX,idem,−22[10]	42	5.0
5	46,XX,der(7)del(7)(p)del(7)(q),add(8)(q2?),add(10)(q2?),t(12;14)(q15;q24)[15]	40	8.0
6	46,XX,t(12;14)(q15;q24)[9]/46,XX[3]	47	5.0
7	46,XX,r(1),t(1;12;14)(p36.3;q14;q24)[7]	40	6.0
8	46,XX,t(2;12)(q33;q13)[17]	49	10.0
9[a]	n. a.	46	3.0
10	46,XX,t(12;14)(q15;q24)[12]/ 45,XX,der(1),?t(1;2),−2,add(7)(?q36),t(12;14)(q15;q24)[2]/ 46,XX,del(4)(q31~q32),der(10),?t(10,14)(q24;q32),t(12;14)(q15;q24)[9]	47	4.0
11	46,XX,t(12;14)(q15;q24)[5]/46,XX[9]	37	7.0
12	45,XX,t(12;14)(q15;q24),der(14)t(12;14)(q15;q24),−22[8]	32	10.0
13[a]	46,XX	48	1.5
14	46,XX,t(12;14)(q15;q24)[13]/46,XX,der(1)r(1;2),t(12;14)(q15;q24)[4]	44	6.0
15	46,XX,ins(14;12)[8]/46,idem,r(1)[4]	73	2.5

Abbreviation: n.a., not available

[a] Case in which FISH revealed split signals for the *HMGA2* locus, indicating a rearrangement of the gene.

leiomyomas has been reported as well [14]. In addition to a downregulation of miRNAs responsible for the repression of *HMGA2* expression, losses of the *let-7* complementary sites (LCS) within the 3' UTR of *HMGA2* may also lead to a deficiency in *let-7*–mediated regulation [10]. Herein, we have investigated 13 leiomyomas as well as two cell lines of myomas with chromosomal aberrations affecting the *HMGA2* locus using a real-time RT-PCR approach to detect the truncation of *HMGA2* transcripts.

In 8/13 myomas, no differential expression between *HMGA2* exons 1 and 2 and the 3' UTR was observed (Fig. 2). This is in good agreement with previous studies reporting breakpoints upstream of the *HMGA2* gene in leiomyomas with rearrangements of chromosomal region 12q14~15 [18].

A lipoma with a t(3;12)(q27~q28;q14~q15) and a known disruption of the *HMGA2* gene was used as a control. The observed expression of the 3' UTR is extremely low in this sample, indicating the near absence of full-length transcripts. A complete absence should lead to negative PCR results, however, the negligible expression level is likely to be caused by a minor transcription of the unaltered allele.

Both cell lines as well as 5/13 myomas revealed a differential expression of exons 1–2 and the 3' UTR. In three of these tumors (cases 10, 14, and 15), however, a noteworthy amount of transcripts is truncated, but since full-length transcripts do not seem to be absent, the chromosomal breakpoint is unlikely to be located within the gene.

Two myomas (nos. 8 and 13) revealed expression levels of the 3' UTR, which are comparably low as the 3' UTR expression in the control, indicating the almost complete absence of full-length transcripts. Of note, the karyotype of one of these cases (no. 13) is apparently normal, but FISH performed on interphase nuclei indicated a rearrangement of *HMGA2* [9].

Overall, the test seems to be well suited to detect truncated *HMGA2* transcripts caused by cytogenetically visible genomic rearrangements as well as by those undetectable by conventional cytogenetics.

In conclusion, we were able to identify five leiomyomas with a strongly reduced expression of the full-length mRNA, which is the prevailing transcript in the remaining eight leiomyomas, also showing chromosomal rearrangements affecting 12q14~q15. Thus, a loss of *let-7* complementary sites does not account for the overexpression of *HMGA2* in the majority of UL, but seems to amplify the effects of a transcriptional de-regulation of *HMGA2* in a rather small subset of these tumors. Of note, phenotypic effects of a truncation of the *HMGA2* 3' UTR have also been described in a boy with a pericentric inversion of chromosome 12 that truncated *HMGA2* [19].

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9.8 *HMGB1* Expressionsanalyen an humanen Non-Hodgkin Lymphomen

VIII.)

Non-Hodgkin´s lymphoma expressing high levels of the danger-signalling protein HMGB1.

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- Etablierung der Methode
- Durchführung aller praktischen Arbeiten
- Verfassen der Publikation

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ORIGINAL ARTICLE: RESEARCH

Non-Hodgkin lymphoma expressing high levels of the danger-signalling protein HMGB1

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Abstract
HMGB1 is a high mobility group protein that can act either as a DNA binding protein or extracellularly as a cytokine-like danger signal. Extracellular HMGB1, either actively secreted or passively released by necrotic cells, is linked to inflammation and cancer. Herein, the results of a study to quantify the expression of *HMGB1* in lymphomas by quantitative real-time RT-PCR are presented. *HMGB1* expression was analysed in 18 non-Hodgkin lymphomas and two lymphoma cell lines. 11/18 primary lymphomas expressed *HMGB1* mRNA at a level exceeding the average of normal lymph nodes. Immunohistochemistry showed that HMGB1 positivity is confined to the lymphoma cells. No correlation between *HMGB1* expression and grading was found. However, a high percentage of lymphomas is overexpressing a danger-signalling protein. This protein can support the growth and angiogenesis of lymphoma cells in a paracrine way when released e.g. due to necrosis. Thus it constitutes an interesting therapeutic target as well.

Keywords: *Non-Hodgkin lymphoma, HMGB1, quantitative real-time RT-PCR, angiogenesis*

Introduction

Appropriate danger signalling is an important prerequisite of cells and tissues that induces a wide spectrum of responses of their surroundings. While danger signalling from normal cells thus constitutes a physiologically important process, it can be adopted by transformed cells for their own purposes. One example for a molecule causally involved in these processes is HMGB1.

The non-histone protein HMGB1 (formerly known as HMG1 or amphoterin) has a double life. It has initially been identified as a DNA-binding protein [1] but it turned out later that it has a function as an extracellular cytokine-like protein, too [2]. Cellular sources of extracellular HMGB1 are necrotic or damaged cells [3] as well as cells that can actively secrete that protein e.g. activated macrophages or monocytes [4].

While it is generally assumed that with a few exceptions *HMGB1* is overexpressed in cancer cells compared with non-malignant matching tissue, for many tumor entities this assumption has not been checked. Nevertheless, in-depth studies of this type are of high interest because extracellular HMGB1 represents one of the very promising novel target molecules in cancer therapy. In addition to its direct effects on the cancer cell population stimulating cellular proliferation and invasiveness [5], recent investigations have also clearly demonstrated that HMGB1 represents a potent angiogenetic switch molecule as well. Using recombinant protein

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Schlueter et al. were able to demonstrate that sprouting of endothelial cells (HUVEC) *in vitro* can be induced by HMGB1 [6]. These results are confirmed by another study by Mitola et al. who in addition have demonstrated that HMGB1 stimulated the outgrowth of capillaries in the chorioallantoic membrane (CAM) assay [7]. Furthermore, in an attempt to identify pro-angiogenetic proteins relevant in the development of colon cancer, Kuniyasu et al. have identified a group of five relevant proteins including HMGB1 [8]. In *in vivo* as well as *in vitro* assays they were able to reduce both the sprouting of endothelial cells and the outgrowth of capillaries in the CAM assay by using antibodies directed against HMGB1. Thus, the reduced growth of xenografted tumors in mice treated by antibodies against HMGB1 may not only depend on a direct effect on the tumor cell population itself as assumed by Taguchi et al., but in addition on an impaired neoangiogenesis [9]. However, there is ample evidence, that what constitutes a danger signal supporting e.g. wound healing in normal tissue can be misused by tumor cells. Since necrotic cells can release HMGB1 and necrosis is a common phenomenon in many tumors, necrosis can aid the tumor in proliferation and to get growth support by stimulating neoangiogenesis. This underlies a phenomenon that Zeh and Lotze have attributed to cancer cell populations by coining the term ''addicted to death'' [10]. However, these effects even can be enhanced if the cancer cells overexpress *HMGB1*. Nevertheless, for most tumor entities attempts to quantify *HMGB1* expression are lacking so far. This paper presents the results of a study analyzing the expression patterns of *HMGB1* in primary lymphomas and lymphoma cell lines. Akin to other malignant tumors, necrosis frequently occurs in lymphomas. Capillary growth is well-known to play an important role in lymphoma development and signals linking necrosis to growth and growth support of lymphomas are of potential interest as potential therapeutic targets.

Material and methods

Tissue samples: All tissue samples were removed for diagnostic purpose. Lymphoma samples were obtained from the University hospital Cologne, Cologne, Germany. The non-malignant lymph nodes were provided by the Department of General and Vascular Surgery, Clinical Center Bremen-Nord, Bremen, Germany. All tissue samples were frozen in liquid nitrogen and stored at -80°C until use.

Cell culture: All analysed cell lines used in this study were of human origin. Jurkat J08 cells represent a T-lymphocyte cell type whereas Sup-T1 cells are of a T-lymphoblast type. Cells were cultivated in culture medium (RPMI 1640 supplemented with 10% fetal bovine serum, 200 U/mL penicillin and 200 μg/mL streptomycin) and incubated at 37°C in 5% CO_2.

RNA-Isolation: Total RNA from the samples was purified according to the ''RNeasy mini protocol for isolation of total RNA from heart, muscle and skin tissue'' (QIAGEN, Hilden, Germany) homogenisation was performed with a tissue lyser (QIAGEN, Hilden, Germany) and on-column DNaseI digest was included. Cell lines were homogenised with QIAshredder columns (QIAGEN, Hilden, Germany).

The RNA from all cases was quantitated followed by a second DNaseI (6.75 U) digestion of 5 μg RNA for 12 min at room temperature and a purification according to the RNeasy mini protocol to remove possible contaminating DNA as completely as possible.

Reverse Transcription: 250 ng of total RNA were reverse transcribed with 200 units of M-MLV Reverse Transcriptase and 150 ng random hexamers according to the manufacturer's instructions (Invitrogen, Karlsruhe, Germany). The resulting cDNA was stored at -20°C. As a control, each reaction was also carried out with water instead of the M-MLV enzyme and is further referred to as – RT.

Real-time quantitative RT-PCR: RT-PCR amplification was performed using the ABI Prism 7300 Sequence Detection System (Applied Biosystems, Darmstadt, Germany). Relative quantification based on Taqman principle with 18S rRNA used as endogenous control was performed. The expression of 18S rRNA in all lymphoma tissue samples showed only a low variation, showing a mean Ct value of 10.16 ± 0.46. *HMGB1* and 18S rRNA expression analyses were performed in triplicate in a total volume of 20 μL using 2 μL of each cDNA corresponding to 25 ng of total RNA. In every case the Mastermix with Uracil N-Glycosylase was taken to ensure reproducible constant results without contaminations. For the measurement of *HMGB1* expression the following sequences were used:

forward primer: $^{5'}$AATTTCACATAGCCCACTT ACATTTACA$^{3'}$;
reverse primer: $^{5'}$TTGATTCTAATAATCCCAT GCTTTGA$^{3'}$

Taqman probe: 6-FAM-ACTGAAGAGTAAT CAATCTA-MGB resulting in a PCR product of 76 bp. The *HMGB1* primer and probes were designed using the Primer Express Software v2.0 (Applied Biosystems, Darmstadt, Germany) using the reference sequence (Accession number NM002128). The sequences of the 18S rRNA primers for an amplicon of 65 bp and the probe were described previously [11]. Because of the higher

expression of 18S rRNA the cDNAs hired for this analysis were diluted 1:10. For each sample non-template controls (NTC) and reactions without reverse transcriptase (-RT) were included. PCR conditions were as follows: 2 min at 50°C and 10 min at 95°C, followed by 50 cycles with 15 s at 95°C and 1 min at 60°C.

Analysis of gene expression: The relative gene expression was calculated by the $\Delta\Delta C_t$ method. For the efficiency test, the absolute value of the slope of log input amount vs. ΔCt was plotted, and passed the test with −0.0035 (accomplishing the Applied Biosystems guideline, which accepts a value of 0.1 to be valid). For this study, a non-malignant lymph node acts as the calibrator sample.

Immunohistochemistry: Immunohistochemical analysis was performed using standard protocols. The formalin-fixed paraffin-embedded 4 μm sections were cut and deparaffinised twice in Xylene. After rehydration in graded alcohol and washing in water slides were boiled in 10 mM citrate buffer for antigene retrival. The peroxidases were blocked with H_2O_2. The slides were incubated with a 1:100 diluted primary polyclonal antibody HMG-1 (T-16): sc-12523 (Santa Cruz Biotechnology, Santa Cruz). Negative controls were run without primary antibody. After washing with PBS the sections were incubated with biotinylated secondary anti-goat antibody (DAKO Deutschland GmbH, Hamburg, Germany). Antibody reactions were detected with Avidin/Streptavidin and 3,3′-diaminobenzidine chromogen solution (DAKO Deutschland GmbH, Hamburg, Germany). Subsequently, slides were counterstained with hemalaun (Merck, Darmstadt, Germany) and coversliped with Kaisers Glyceringelantine (Merck, Darmstadt, Germany).

Results

The expression of *HMGB1* in human lymphomas has been determined by quantitative RT-PCR in samples from 18 lymph nodes from lymphoma patients and two lymphoma cell lines. Details of the samples are summarised in Table I. To check the *HMGB1* level in normal lymph nodes the *HMGB1* mRNA level was determined in six lymph nodes from patients who underwent surgery because of a benign thyroid gland nodule or carotid arteries dysfunctions.

The human genome contains numerous retroposed copies of *HMGB1* [12,13]. Thus, a DNA contamination of the samples subjected to the qRT-PCR analyses can lead to an overestimation of *HMGB1* expression. To detect DNA contamination,

Table I. Grading and data of cases analysed for *HMGB1* expression.

Lab code	Age	Sex	Size (cm)*	Grading
nln0701	–	f	–	Non-malignant lymph node
nln 6	–	f	–	Non-malignant lymph node
nln 7	–	m	–	Non-malignant lymph node
nln 8	71	m	–	Non-malignant lymph node
nln 9	63	m	–	Non-malignant lymph node
nln 10	61	f	–	Non-malignant lymph node
K-NHL 1	64	f	3.0	Diffuse large B-cell lymphoma
K-NHL 2	40	f	3.2 × 2.5 × 1.7	Diffuse large B-cell lymphoma
K-NHL 3	24	m	–	Anaplastic large cell lymphoma (T)
K-NHL 4	57	m	5.0 × 3.0 × 2.5	Diffuse large B cell lymphoma
K-NHL 5	60	f	2.5 × 1.0 × 1.0	Diffuse large B-cell lymphoma
K-NHL 6	17	m	3.5 × 3.0 × 0.5	Diffuse large B-cell lymphoma
K-NHL 7	55	m	5.5 × 3.0 × 2.5	Follicle centre lymphoma, grade 1
K-NHL 9	53	m	5.5 × 3.0 × 3.0	Follicle centre lymphoma, grade 1
K-NHL 10	71	f	3.5 × 2.5 × 3.0	Follicle centre lymphoma, grade 1/2
K-NHL 11	76	m	2.0 × 1.5 × 1.4	Small lymphocytic lymphoma
K-NHL 12	46	m	3.0 × 1.0 × 1.0	Follicle centre lymphoma, grade 2
K-NHL 13	46	f	3.2	Diffuse large B-cell lymphoma
K-NHL 14	47	–	1.5 × 1.0 × 0.9	Follicle centre lymphoma, grade 1
K-NHL 15	28	–	3.5 × 3.0 × 1.8	Diffuse large B-cell lymphoma
K-NHL 16	76	f	0.8 × 0.5 × 0.5	Nodal marginal zone B-cell lymphoma
K-NHL 17	87	m	4.5 × 3.0 × 3.0	Follicle centre lymphoma, grade 1
K-NHL 18	55	–	1.0 × 0.3 × 0.4	Small lymphocytic lymphoma
K-NHL 19	65	–	1.5 × 1.0 × 1.0	Diffuse large B-cell lymphoma
supT1	8	m	–	Cell line
Jurkat J08	14	m	–	Cell line

*Maximum dimension.

for all samples, controls prepared without adding reverse transcriptase were run. Due to a DNaseI double digest as described above none of the samples showed a signal for the *HMGB1* gene within the detection limit.

The difference between the signal for the endogenous control and the – RT control was about 24 cycles indicating that these signals resulting from contaminations account for less than 0.0001% of the total signal and can be ignored.

To compare the expression levels of *HMGB1* in lymphomas the expression in a lymph node was used as a calibrator (expression level = 1) (Figure 1).

The expression of the non-Hodgkin lymphomas varies from a 3-fold decrease up to an increase up to 8-fold (0.3–8.0) compared with the calibrator. On the basis of the results of quantitative RT-PCR 11/18 primary lymphomas expressed HMGB1 mRNA at a level exceeding the average value obtained for six apparently normal lymph nodes. The ranges of expression for 18 primary malignant lymphomas, the to cell lines, and the normal lymph nodes are shown in Figure 1.

In addition, it was shown by immunohistochemistry that the protein expression correlates with the data obtained by quantitative RT-PCR. Furthermore, HMGB1 positivity turned out to be restricted to the lymphoma cells or subpopulations thereof (Figure 2).

Both NHL-derived cell lines did also not significantly differ from what was detected in the primary lymphomas. There is no evidence for an additional stimulation of *HMGB1* expression resulting from the cell culturing e.g. due to the use of fetal calf serum.

Discussion

One relevant mechanism by which tumors can support their own growth seems to be the release of ''danger signals'' from necrotic or otherwise damaged areas. Recently Zeh and Lotze have addressed this point by coining the term ''cancer cells addicted to death'' [10].

Necrotic areas are a frequent finding in lymphomas as well. Among the proteins that can be released from these areas and involved in the stimulation of tumor growth and its angiogenesis the high mobility group protein HMGB1 is one promising target molecule for novel treatment strategies. HMGB1 is known to be released in general from necrotic cells

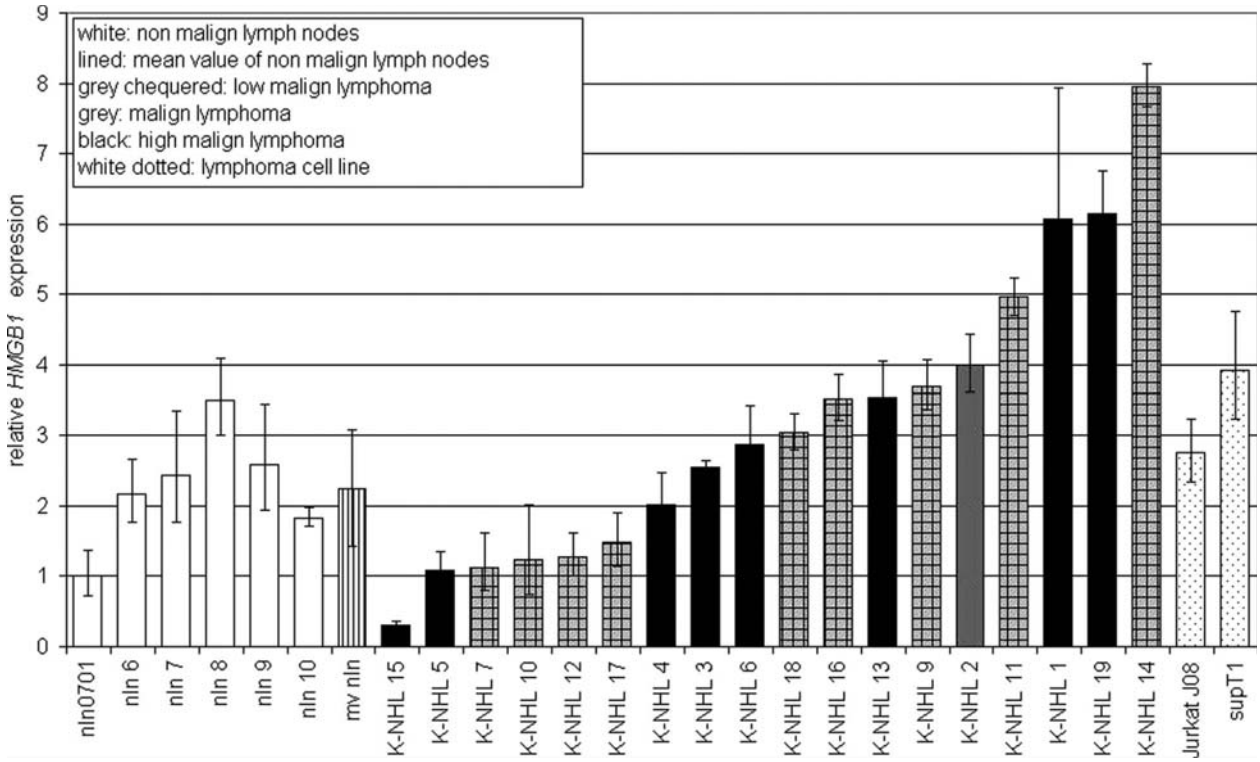

Figure 1. qRT-PCR results of the *HMGB1* gene expression analyses. The average expression in a control lymph node (nln0701) (calibrator) and further non-malign lymph nodes is presented in white while the mean value (mv) is a white lined dyed column. Whereas the human non-Hodgkin lymphomas were low malign was grey chequered malign grey and high malign black bars. The analysed T-cell lymphoma cell lines were presented white spotted.

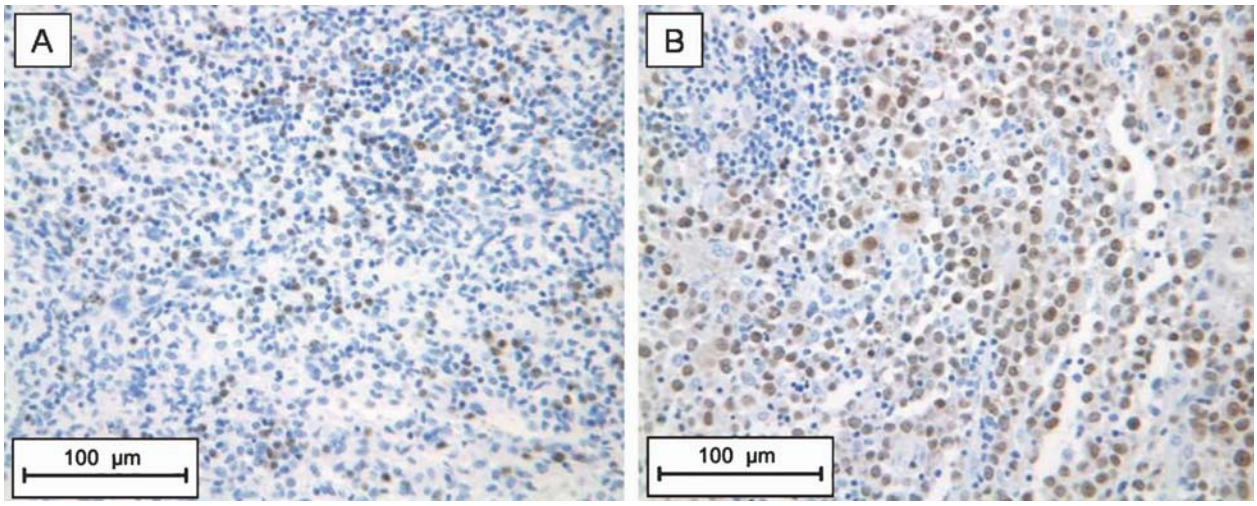

Figure 2. Histomorphology of lymph node sections from patients with diffuse large B-cell lymphoma. Photographs (Magnification × 250) of immunohistochemical staining with HMGB1 (polyclonal antibody HMG-1 (T-16): sc-12523 (Santa Cruz Biotechnology, Santa Cruz, USA)) are shown. One out of three comparable cases with respectively low (A) or high (B) RNA levels for HMGB1 are depicted. (A) 10–15% of the tumor cells present a strong nuclear staining. The majority of the tumor cells as well as the bystander cells and endothelial cells remain unstained. (B) In this case almost all tumor cells here present a strong nuclear staining. The smaller reactive bystander cells are negative for HMGB1.

and can act as both a paracrine angiogenic and proliferation factor [14]. While it is well known that HMGB1 has both functions and this protein generally seems to be overexpressed in certain types of malignancies such as colon cancer [15–17], gastric cancer [18,19], breast cancer [20,21], hepatocellular carcinomas [22], prostate cancer [23] and melanomas [24] little is known about hematologic malignancies.

To the best of the author's knowledge this is the first study aimed at the quantification of *HMGB1* expression in malignant lymphomas. The expression of *HMGB1* mRNA was quantified by qRT-PCR in a variety of primary human lymphomas and two lymphoma cell lines. The results clearly show that lymphomas often overexpress *HMGB1* compared with normal samples. Most likely, due to the presence of the bystander cells expressing low levels of HMGB1 the degree of overexpression in the lymphoma cells as determined by quantitative RT-PCR is even underestimated. Thus, these tumors may be ''addicted to death'' as well because once necrosis occurs the dead tumor cells can release much higher amounts of the danger signal than do normal cells. As proved by immunohistochemistry the lymphoma cells were identified as the source of *HMGB1* expression. A similar situation is described in colon cancer where *HMGB1* is also known to be overexpressed in roughly 75% of the analysed cases [16].

Interestingly, Taguchi et al. have reported a strongly decreased growth of xenografted as well as induced tumors in mice that received an antibody against HMGB1 when compared with control mice [9]. While they have shown that this effect can be due to a direct HMGB1-medulated stimulation of proliferation of the tumor cell population antagonised by the antibody they have excluded effects of the antibody on tumor angiogenesis. Nevertheless, as recently discussed [6] it is assumed that the conclusion by Taguchi is rather due to the selection of their angiogenesis assay that generally does not allow excluding HMGB1 as an angiogenetic switch molecule. Three recent papers addressing the possible pro-angiogenetic effects of HMGB1 [6,10,15] clearly show that it is a strong pro-angiogenetic switch molecule. Moreover, this effect can be antagonised by antibodies directly against those proteins in vitro as well as in vivo assays [15].

In addition to its pro-angiogenetic activity on endothelial cells HMGB1 is also linked to direct effects on the tumor cell population as e.g. enhancing local invasiveness and inducing anti-apoptotic activity [6,10,25,26]. As to the growth of malignant lymphomas all three factors are known to play an important role.

Tumor cell populations do not only have the opportunity to use this type of signal for their own purpose but, as shown herein for malignant lymphomas, apparently can even send much stronger signals than the normal cells the tumors are derived from. Accordingly, therapeutic strategies preventing ''danger signalling'' of tumor cell populations may open interesting new avenues for the treatment of

tumors. As shown herein one interesting target molecule for these approaches not only in epithelial cancer but also in non-Hodgkin lymphomas is HMGB1.

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9.9 HMGB1 Proteinanalysen an caninen Lymphomen

IX.)

High Mobility Group B1 (HMGB1) proteins in dogs with lymphoma: Analysis of serum levels and association to outcome following combination chemotherapy.

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- Planung, Durchführung und Auswertung der Experimente
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Original Article

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High-mobility group B1 proteins in canine lymphoma: prognostic value of initial and sequential serum levels in treatment outcome following combination chemotherapy

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Abstract

Elevated high-mobility group box 1 (HMGB1) levels have been demonstrated in different human neoplasias. Information on serum HMGB1 before and during chemotherapy is lacking, as is data pertaining to its prognostic significance. The aim of this study was to characterize serum HMGB1 level in dogs with lymphoma and to assess its influence on the outcome following chemotherapy. Serum HMGB1 concentrations were measured in 16 dogs with lymphoma before treatment (W1) and on weeks 2 (W2), 6 (W6) and 12 (W12) of treatment with chemotherapy. Initial serum HMGB1 levels were significantly higher than HMGB1concentrations in control dogs and the levels in W2, W6 and W12. HMGB1-W1 concentrations were lower in dogs achieving complete remission than that in the single dog with partial remission. The ratio W12/W6 exhibited significant influence on remission duration. In these dogs with lymphoma, serum HMGB1 was elevated in comparison with that in controls. Initial serum HMGB1 level and its modulation during treatment may possess prognostic value.

Keywords
dog, HMGB1, prognosis, remission, survival, tumour

Introduction

HMGB1 (also known as amphoterin or HMG1) belongs to the group of the so-called high-mobility group proteins. Proteins of the HMGB family, comprising HMGB1, HMGB2 and HMGB3, are characterized by two DNA-binding domains called 'HMG-boxes'.[1] Currently, the member of this group analysed best is HMGB1. HMGB1 is an intracellular protein, secreted by activated monocytes, macrophages and astrocytes. In addition, this protein can be released by necrotic or damaged cells.[2,3] Extracellular HMGB1 signals via the receptor for advanced glycation end products (RAGE) and the Toll-like receptors TLR2 and TLR4.[4–8] Activation of these receptors enables NF-kB signalling pathways linked to angiogenesis and inflammatory processes.[6,8–10] An over-expression of *HMGB1* has been detected in a number of human tumour types such as mammary carcinoma tumours of the gastrointestinal tract, hepatocellular carcinoma and malignant lymphoma.[11–14] Increased levels of extracellular HMGB1 have been associated with increased proliferation and migration of cancer cells, increased stem cell self-renewal and inhibition of tumour cell apoptosis.[9,15,16] In addition, angiogenic properties of HMGB1 can lead to an improved supply of tumour vasculature, resulting in accelerated tumour growth.[6,7,17] However, extracellular HMGB1 not only appears to exert

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pro-neoplastic effects, but there is also evidence that it can amplify the effects of chemotherapy. The release of HMGB1 to the extracellular space seems to constitute a crucial step in the activation of antigen-presenting cells. Neutralization or knockdown of HMGB1 or TLR4 has been shown to abolish the capacity of dying tumour cells to elicit anti-cancer immune responses.[18–20] In summary, the complex functions of released HMGB1 lead to direct as well as indirect promotion of tumour growth. On the contrary, HMGB1 has the properties of an immune alarmin, suggesting that elevated release of HMGB1 to the extracellular space can exert adverse effects on the growth of tumours.[21]

Canine lymphoma represents a model for human non-Hodgkin lymphoma (NHL) and is hence suited to study the prognostic significance of initial serum HMGB1 levels and the modulation of serum HMGB1 levels during the course of chemotherapy. Malignant lymphoma is one of the most common neoplastic diseases in dogs, with an annual incidence of up to 114 cases per 100 000 dogs.[22–25] Treatment with combination chemotherapy has resulted in high remission rates as well as durable median remission and survival times.[26–32] However, clinical outcome varies significantly among individual patients, and a number of studies investigating the prognostic significance of clinical, histological and molecular parameters have been performed. Factors such as WHO substage, immunophenotype and anatomic type have been shown to possess prognostic significance.[26–32] For parameters including clinical stage, histological grade, Ki-67, argyrophilic nucleolar organizer regions (AgNOR), potential doubling time (Tpot) and proliferating cell nuclear antigen (PCNA), different results have been reported.[26–35] The prognostic relevance of additional molecular parameters such as P-glycoprotein and survivin expression, glutathione-S-transferase, thymidine kinase, vascular endothelial growth factor (VEGF) and matrix metalloproteinases (MMP) also remains unclear to date.[36–39] The apoptotic index has been described to possess prognostic value.[35] A high apoptotic index was associated with short relapse-free interval (RFI), whereas a low apoptotic index was associated with increased duration of first RFI, coinciding with results found for human NHL.[35,40,41] Because apoptotic cells are known to release HMGB1, it is tempting to speculate that the pro-neoplastic effects of HMGB1 may dominate at least in the case of lymphoma. However, this issue has not been addressed to date. Therefore, the aim of this study was to measure the pre-treatment serum level of HMGB1 and its changes during the course of chemotherapy in dogs with lymphoma. Additionally, the study was aimed to determine whether initial serum levels of HMGB1 or particular alterations during treatment would be associated with clinical outcome.

Materials and methods

Patients

Dogs presenting with histologically or cytologically confirmed diagnosis of lymphoma and for which blood samples at the time points described below had been obtained were eligible for this study.

Signed owner consent for blood sampling was obtained prior to the study. The study protocol was reviewed and accepted by the governmental animal protection committee.

Control group

A control group consisting of 16 healthy dogs belonging to hospital staff was also sampled for serum HMGB1 measurement. The dogs were defined as healthy and included into the control group in case of unremarkable physical examination and complete blood count (CBC) and serum biochemistry results.

Pre-treatment evaluation

Pre-treatment patient evaluation consisted of physical examination, CBC (including platelet count); serum biochemistry; urinalysis; thoracic and abdominal radiographs; abdominal ultrasound; electrocardiogram (ECG); echocardiogram if indicated by abnormalities on cardiac auscultation, ECG or radiographs; bone marrow aspiration cytology if indicated by abnormalities in the CBC and documentation of measurable disease. Tumours were measured either directly or via radiographic or ultrasonographic imaging. Clinical

staging was performed according to the WHO clinical staging system for lymphoma in domestic animals.[42] Immunophenotyping was performed via FACS-analysis (FACS Calibur, Becton Dickinson, San Jose, CA, USA) of lymph node aspiration material.[43]

Chemotherapy regimen and patient monitoring

Patients were treated with a 12-week combination chemotherapy protocol with weekly treatment intervals and monitored as described previously elsewhere.[31] This protocol consisted of four cycles of alternating weakly treatment with vincristine (0.7 mg m^{-2} IV), cyclophosphamide (200 mg m^{-2} IV) and doxorubicin (30 mg m^{-2} slow IV infusion) combined with oral prednisolone. The first treatment was a combination of vincristine and L-asparaginase (400 IU kg^{-1} SC). In case of relapse, the patients were either re-treated with the initial protocol or a different rescue protocol, depending on the owners' consent.

Serum sampling for HMGB1 measurement

Serum samples for HMGB1 measurement were taken at four time points in relation to chemotherapy treatment: before treatment (W1), at the time of the 2nd (W2), the 6th (W6) and the 12th treatment (W12). Blood samples were obtained by venipuncture of the jugular vein, centrifuged and serum was stored at −80 °C until batch measurement was performed.

HMGB1 measurement

A two-step sandwich ELISA was performed using the HMGB1 ELISA Kit II (Shino-Test Corporation, Tokyo, Japan) following the manufacturer's instructions. Internal controls (standard and positive control using porcine protein) were performed. All samples were measured in triplicates, and the results were averaged.

Assessment of response to treatment

The following criteria were used to evaluate response to therapy: complete remission (CR), 100% reduction in size of all measurable disease; partial remission (PR), ≥50% but <100% reduction in size of all measurable disease; stable disease (SD), <50% reduction in size of all measurable disease, no change in size or <25% increase in size of all measurable disease and progressive disease (PD), >25% increase in size of all measurable disease or the appearance of new lesions. When measuring lymph nodes directly or via ultrasonographic imaging, volume was calculated by measuring in three dimensions. Two-dimensional measurement was performed when evaluating masses radiographically. All responses were required to last for at least 21 days. Remissions of shorter duration were classified as SD.

Statistical analysis

Comparison of HMGB1 concentrations in the control and lymphoma groups was performed using the Mann–Whitney Test (non-normal distribution on Kolgomorov Test). In the lymphoma dogs, complete and partial response rates were defined as the number of dogs achieving CR or PR compared with the total number of dogs treated. CR duration was defined as the time from documentation of CR to relapse. Dogs not reaching CR were defined as having CR duration of 0 days. Kaplan–Meier product limit analysis was used for remission and survival analysis. A complete event was defined as lymphoma relapse or death. The dogs were censored if they were still in remission at the time of death or data accrual closure (remission analysis), or alive at the time of data accrual closure. Univariate Cox regression analysis was used to evaluate the following variables for their influence on the duration of CR: measurements of serum HMGB1-W1, HMGB1-W2, HMGB1-W6 and HMGB1-W12; the difference between the HMGB1 measurements at different time points (ΔW2-W1, ΔW6-W1, ΔW12-W1, ΔW6-W2, ΔW12-W2 and ΔW12-W6) and the per cent values of the ratio of HMGB1 measurements at the different time points (W2/W1, W6/W1, W12/W1, W6/W2, W12/W2 and W12/W6). In case of significance of a continuous variable, the mean value was used to categorize the data and depict the effect using the Kaplan–Meier analysis and the pertaining curve. The influence of the above

factors on whether a dog reached CR was analysed by univariate logistic regression analysis. The association between initial HMGB1 (W1) level and clinical parameters was analysed using Spearman correlation (age and weight), the Mann–Whitney test (gender, tumour immunophenotype and clinical substage) and the Kruskal–Wallis test (clinical stage).

A P value of <0.05 was considered significant. All statistical analyses were performed using SPSS 16 statistics software (SPSS 16, SPSS, Chicago, IL, USA).

Results

Patient population

A total of 16 dogs with lymphoma were included in this study. The dogs included belonged to eleven breeds. Breeds represented with more than one dog were as follows: Mixed-breed ($n = 5$) and Bernese mountain dog ($n = 2$). The remaining nine dogs were represented by nine breeds. Median age was 7 years (minimum, 3 years; maximum, 13 years) and median body weight was 30.9 kg (minimum, 8.4 kg; maximum, 44.5 kg). In total, 10 dogs were female (8 spayed females) and 6 were male (3 castrated males). Three dogs (19%) were classified as clinical stage 3, nine (56%) as stage 4 and four (25%) as stage 5. A total of 10 (63%) individuals were substage a and six (38%) were substage b. Immunophenotyping was performed in all dogs, of which 13 (81%) were diagnosed with B-cell and three (19%) with T-cell lymphoma.

The median age of the control dogs ($n = 16$) was 4 years (minimum, 2 years; maximum, 15 years) and the median weight was 24.5 kg (minimum, 10 kg; maximum, 35 kg). Of all the control dogs, 10 were females (6 spayed females) and 6 were males (5 castrated males).

Serum HMGB1 levels

The median serum HMGB1 level in the control group ($n = 16$) was 3.63 ng mL^{-1} [mean, 3.82 ng mL^{-1}; 95% confidence interval (CI), 2.86–4.78; minimum, 1.74 ng mL^{-1}; maximum, 7.93 ng mL^{-1}]. Median serum HMGB1 levels in the 16 dogs with lymphoma at W1, W2, W6 and W12 were 8.14 ng mL^{-1} (mean, 7.56 ng mL^{-1}; 95% CI, 5.41–9.72; minimum, 2.15 ng mL^{-1}; maximum, 15.58 ng mL^{-1}), 3.67 ng mL^{-1} (mean, 5.05 ng mL^{-1}; 95% CI, 2.86–7.24; minimum, 2.14 ng mL^{-1}; maximum, 13.89 ng mL^{-1}), 1.95 ng mL^{-1} (mean, 2.39 ng mL^{-1}; 95% CI, 1.48–3.30; minimum, 0; maximum, 6.76 ng mL^{-1}) and 2.56 ng mL^{-1} (mean, 2.64 ng mL^{-1}; 95% CI, 2.06–3.23; minimum, 0.67 ng mL^{-1}; maximum, 4.62 ng mL^{-1}), respectively.

The initial HMGB1 levels of the lymphoma dogs (W1) were significantly higher than those of the control group ($P = 0.005$, Fig. 1). In comparison with W1, the median serum HMGB1 concentrations in W2 ($P = 0.046$), W6 ($P < 0.001$) and W12 ($P < 0.001$, Fig. 2) were significantly lower. There was no statistical difference in serum HMGB1 concentration between the control group and the lymphoma dogs in W2 ($P = 0.49$) and W12 ($P = 0.6$), whereas in W6, the HMGB1 concentration was even lower than that in the control dogs ($P = 0.004$).

Different types of HMGB1 alterations could be demonstrated during the first 2 weeks after the beginning of chemotherapy. In four patients with the initially lowest HMGB1 concentrations, the HMGB1 levels increased; in two dogs, the levels remained unchanged and in the remaining 10 dogs, the levels decreased. After 6 weeks, only one case showed a HMGB1 level exceeding that before chemotherapy.

No significant association of initial serum HMGB1 level (W1) with any of the patient parameters such as age, weight, gender, tumour stage, substage and immunophenotype was found.

Treatment outcome

Median time from diagnosis to induction of chemotherapy was 4 days (minimum, 1 day; maximum, 18 days). Response distribution in the 16 dogs was 15 (94%) CR and 1 (6 %) PR. Median time from the first day of chemotherapy to documentation of CR was 7 days (minimum, 7 days; maximum, 21 days). As only one dog did not achieve CR, no statistical evaluation was performed for the association of HMGB1 values with the likelihood to reach remission. However, pre-treatment

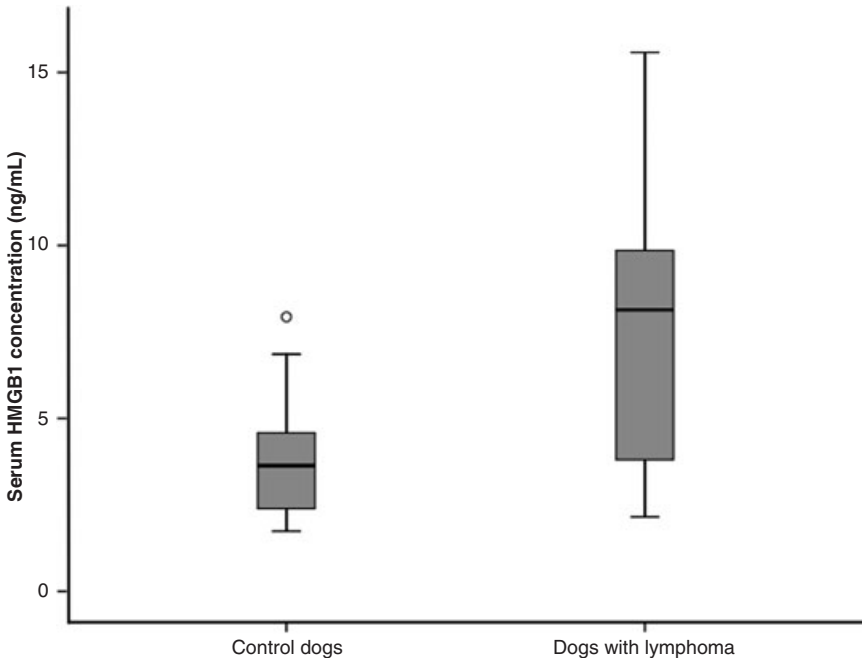

Figure 1. Boxplot figures depicting median serum HMGB1 levels of healthy dogs ($n = 16$; median, 3.63 ng mL^{-1}; minimum, 1.74 ng mL^{-1}; maximum, 7.93 ng mL^{-1}) and dogs with lymphoma before treatment ($n = 16$; median, 8.14 ng mL^{-1}; minimum, 2.15 ng mL^{-1}; maximum, 15.58 ng mL^{-1}). The HMGB1 levels differed significantly [$P < 0.005$; o, outliers (>/<1.5 × interquartile range)].

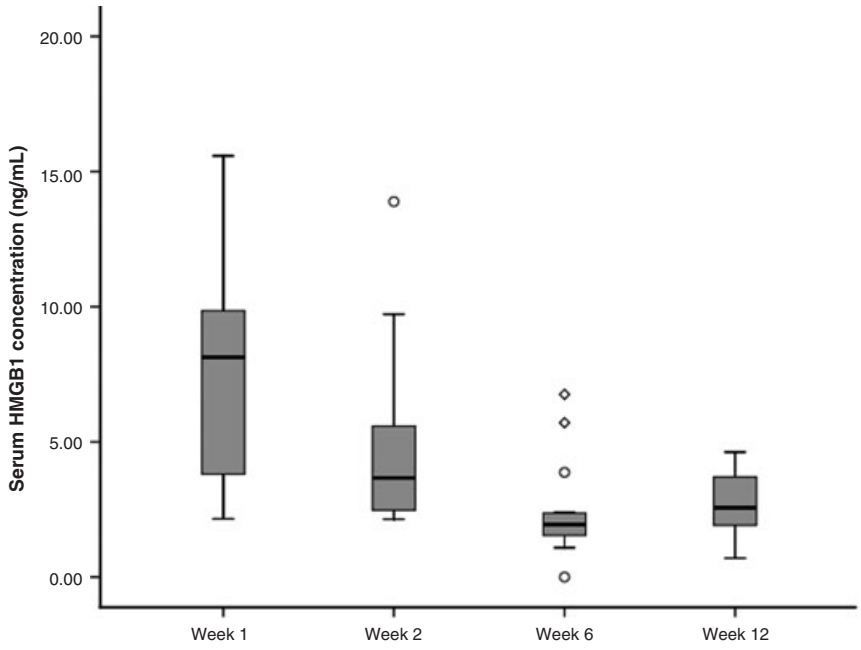

Figure 2. Boxplot figures depicting median serum HMGB1 levels of dogs with lymphoma during the course of combination chemotherapy ($n = 16$). Serum levels before treatment (week 1), at the time of the 2nd (week 2), 6th (week 6) and 12th treatment (week 12). The HMGB1 levels differed significantly between W1 and W2 ($P = 0.046$), W1 and W6 ($P \leq 0.001$) and W1 and W12 [$P < 0.001$; o: outliers (>/<1.5 × interquartile range); ◇: extremes (>/<3 × interquartile range)].

serum HMGB1 values in the dog with PR was considerably higher (15.58 ng mL^{-1}) than the values in dogs achieving CR (median, 7.54 ng mL^{-1}), suggesting that initial HMGB1 concentration may predict response.

Median follow-up period from date of diagnosis was 398 days (minimum, 177 days; maximum, 685 days). Median CR duration was 348 days (minimum, 0; maximum, 526 days; 95% CI, 267–428 days). Median survival time in the 16 dogs was 644 days (minimum, 177 days; maximum, 765 days; 95% CI, 105–1183 days).

In the univariate Cox regression analysis, the only factor with a significant association with the duration of CR was the HMGB1-W12/W6 ratio ($P = 0.027$). Dogs with HMGB1-W12/W6 ratio <122% exhibited significantly longer remission times ($n = 10$; median not reached) than those with HMGB1-W12/W6 >122% ($n = 6$; median, 231 days, Fig. 3). None of the analysed factors concerning HMGB1 measurements showed a statistically significant influence on survival time in these dogs.

Discussion

The aim of this study was to characterize the serum HMGB1 level in dogs with lymphoma in comparison with that in the control population, and to verify its prognostic significance in this neoplastic entity. Because of a paradoxical dual effect, the release of HMGB1 and its subsequent action as an extracellular danger signal can be expected to have both positive and negative effects on tumour progression.[6,8,17,44]

In the dog, the *HMGB1* gene has been assigned to chromosome 25, with a 95.4% identity in the encoded amino acid sequence to its human counterpart as well as a 100% homology of the human and canine antibody-bound epitopes.[45,46]

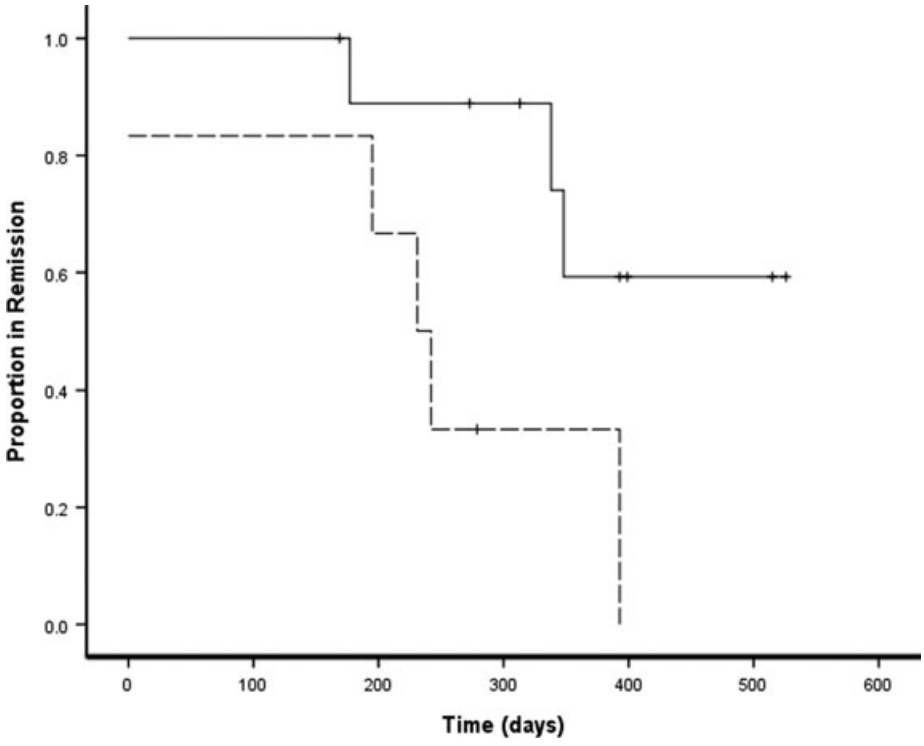

Figure 3. Kaplan–Meier curve depicting CR duration of dogs with lymphoma treated with a 12-week combination chemotherapy protocol with HMGB1-W12/W6 <122% [$n = 10$, continuous line, median remission not reached (minimum, 177 days; maximum, 526 days; 6-month and 1-year remission rates, 89 and 59%, respectively)] or HMGB1 W12/W6 >122% [$n = 6$, interrupted line, median remission 231 days (minimum, 0; maximum, 393 days; 6-month and 1-year remission rates, 83 and 33%, respectively; Log rank test, $P = 0.026$).

Additionally, no cross-reactivity to the similar protein HMGB2 could be demonstrated.[46] This suggests that the assay is valid for the measurement of canine HMGB1. Furthermore, the manufacturer is marketing this assay for use in dogs. However, limitations of an ELISA test in general must be considered. These include the possible occurrence of a hook effect leading to false low values, target saturation and the respective test's measuring accuracy. In the employed kit, the dynamic range goes up to 80 ng mL^{-1} and the measuring accuracy amounts to 1 ng mL^{-1}; therefore, these constraints should not play a relevant role in this study.

Varying expression patterns of HMGB1 have been described in canine osteosarcoma.[45,47] No study has so far analysed the development of serum HMGB1 values in human or canine lymphoma or other tumour patients before and during 'therapeutic stress' by chemotherapy or irradiation. However, it has been supposed that HMGB1 release can amplify the effects of these treatments via stimulation of the immune response against the tumour cells.[18–20]

In this study, the median serum HMGB1 levels in a population of dogs with lymphoma were significantly elevated in comparison with those in the healthy canine control group. This finding corroborates previous observations in other human malignancies and offers further support for the hypothesis that the level of HMGB1 released by cell necrosis and the cellular expression of *HMGB1* is increased in neoplastic entities.[11–14] On the contrary, a certain overlap of the HMGB1 values of the two groups was observed. Dogs with lymphoma in this study had elevated HMGB1 serum levels at diagnosis; however, the individual values vary over a broad range. Although the reasons for these observed variations may possibly lie in metabolic differences, they remain unclear until further clarification.

High HMGB1 levels may be as a result of over-expression of HMGB1 by the tumour cell, a high tumour load, a high rate of cells becoming necrotic or apoptotic and an increased ability of the cells to secrete HMGB1 actively. The increased initial serum HMGB1 level in this group of dogs with lymphoma can be seen as an important finding as this molecule represents a possible target molecule for novel anti-cancer therapy. Decreased growth of xenografted and induced tumours has been demonstrated in mice receiving antibodies against HMGB1, and further studies addressing this aspect of targeted therapy are warranted in the future.[9]

The level of serum HMGB1 at diagnosis may have an implication on prognosis. The study population encompassed only one dog with PR and there were no dogs with an SD or PD. Therefore, a true statistical association between initial HMGB1 levels and the likelihood for a patient to reach CR could not be evaluated. However, pre-treatment serum HMGB1 values in the dog with PR was considerably higher than the values in dogs achieving CR, suggesting that initial HMGB1 concentration may predict response. Whether this finding reflects higher tumour aggressiveness or whether this simply is a sign of higher tumour burden and subsequently a greater number of necrotic cells remains unresolved to date.[48] A comparison of HMGB1 levels between patients reaching remission and those not responding should be addressed in future studies to verify the observation of this study.

This study demonstrated that during the course of treatment with a combination chemotherapy protocol, median serum HMGB1 levels decreased from initially elevated levels to low levels (W1 versus W6). This may be assumed to be as a result of the cytotoxic effect of chemotherapy on the lymphoma cells and therefore may represent an objective measure of remission. Most dogs attained remission between week 1 and 2, and therefore the highest death rate of tumour cells can be expected during this treatment phase. Thus, it cannot be excluded that HMGB1 values may reach a short peak during this period, reflecting an augmented release of this molecule by dying tumour cells. Whether an increase of HMGB1 during this early treatment period has clinical importance concerning response to treatment and prognosis remains unclear to date and may be investigated in future studies.

Additionally, it remains unclear whether the alterations in HMGB1 reflect only tumour burden or whether the administered drugs could also have an impact on HMGB1 expression and/or release. There has been no study to date investigating the impact of administered drugs on HMGB1 release or expression in dogs; however,

as there is one description of corticosteroid-related down-regulation of HMGB1 expression in humans, prednisolone administration may have played a certain role in the presented canine patients. Further investigations must address this important aspect.[49]

Generally, the individual patients showed varying courses of their HMGB1 levels during the 12-week treatment period, which may reflect different responses to chemotherapy. Most interestingly, the relationship between HMGB1 in week 12 and treatment at week 6 is associated with the duration of remission in this study population. Dogs with an increase in serum HMGB1 level in week 12 compared with that at week 6 experienced significantly shorter remission durations than dogs in which HMGB1 levels remained stable or decreased.

The data suggest a clinical use of serum HMGB1 that may not be restricted to veterinary medicine. Certainly, the small number of patients and the small size of the control group must be considered a limitation of this study, and further investigations incorporating a larger patient number must be performed to confirm the presented observations. Additionally, results of the univariate analysis must be verified using multivariate statistics to validate whether initial HMGB1 and its development during therapy exert an independent influence on outcome, or whether the effects are overridden by other confounding prognostic factors with stronger influential effect.

The control group in this study was slightly younger than the lymphoma group, which may be considered as a constraint. On the contrary, HMGB1 is an ubiquitous protein for which no age-dependency has been demonstrated so far; therefore, this aspect should not be influencing the results of the comparison. However, the control group consisted of only healthy dogs, allowing a comparison with normal HMGB1 release but not with release in other disease states. Therefore, studies encompassing dogs with non-neoplastic diseases such as inflammatory or immune-mediated entities are needed to better characterize HMGB1 release in dogs.

Furthermore, a possible bias could be based on patient accrual as these were not consecutive patients in every case. This is because of the inclusion of patients only when serum samples had been acquired at the pertaining time points. However, failure to sample serum was not because of patients' lack of response but because of logistical reasons in the clinical routine or owner preferences, and in the majority also encompassed patients responding to treatment. Nevertheless, a prospective study including all consecutive cases is warranted to verify the presented findings.

In summary, this study offers first insights elucidating the role of HMGB1 in lymphoma progression and response to treatment. The results indicate that initial high HMGB1 levels and secondary increases of the levels during chemotherapy may both represent adverse prognostic parameters in dogs with lymphoma. Additional prospective studies with larger patient numbers and multivariate statistics are warranted to verify the presented observations. Future investigations may also focus on whether HMGB1 levels may bear value as an early marker of lymphoma relapse or may help identify patients in need of treatment modulation, such as continuation or consolidation. Finally, investigations including additional tumour types such as carcinomas and leukaemia could add to an improved understanding of the dual effects of HMGB1 in neoplasias.

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9.10 HMGB1 Proteinanalysen an humanen Exsudaten

X.)

Elevated levels of HMGB1 in cancerous and inflammatory effusions.

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Elevated Levels of HMGB1 in Cancerous and Inflammatory Effusions

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Abstract. *Background: Recently, it has become obvious that the HMGB1 protein can act as a proinflammatory and proangiogenic mediator when actively secreted by macrophages or passively released from necrotic cells playing an important role in the pathogenesis of several diseases including cancer. Materials and Methods: The absolute and relative amount of HMGB1 was measured with an ELISA in different effusion types. Results: The amount of HMGB1 protein in the samples differed between 0.0004% and 0.0025% of the total sample protein. The mean values of transudates were significantly ($p<0.001$) lower than the mean values of exudates. Conclusion: HMGB1, a so-called danger signalling protein, was found to be highly expressed in human pleural and peritoneal effusions due to cancer and inflammation. Compared to transudates the average level of HMGB1 was significantly higher in exudates. These results underline the characteristics of HMGB1 as a possible target for treatment in advanced cancer as well.*

High mobility group box 1 (HMGB1), also known as amphoterin or HMG1, belongs to a group of chromatin-associated non-histone proteins characterized by low molecular weight, acidic solubility and high content of charged amino acids (1, 2). Proteins of the HMGB family, comprising HMGB1, HMGB2, and HMGB3, are characterized by two DNA-binding domains called HMG boxes (3). Currently the best analyzed member of this group is HMGB1. HMGB1 is an intracellular protein, which can be secreted for example by activated monocytes, macrophages, and astrocytes and can be released by necrotic or damaged cells (4-6). Extracellular HMGB1 can signal through the receptor for advanced glycation end products (RAGE) (7, 8) and the toll-like receptors TLR2 and TLR4 (9-11). Activation of these receptors can enable nuclear factor kappa B (NF-κB) signalling pathways linked to *e.g.* angiogenesis and/or inflammatory processes (9, 10, 12-15). In cancer, these angiogenic properties of HMGB1 can also lead to a better vascular supply resulting in a faster and greater growth of the tumor (11, 16). Generally, overexpression of *HMGB1* has been detected in subsets of carcinomas of the breast (17, 18), tumors of the gastrointestinal tract (19), hepatocellular carcinomas (20), and malignant lymphomas (21).

The aim of this study was to measure the level of HMGB1 in effusions of various origin and to determine if effusions due to cancer or inflammatory disease show elevated levels of HMGB1 protein compared to theses from transudates. Furthermore, it was investigated whether the amount of HMGB1 in effusions due to cancer is consistently increased or if it is not a general phenomenon in effusions of malignant tumors.

Patients and Methods

A total of 36 samples were collected from patients at the General Hospital Bremen Mitte, Bremen, Germany: 30 samples from pleural and 6 from ascitic effusions were obtained (Table I). As a control group patients with transudative effusions were included in this study as well; the most common cause for transudates is congestive heart failure or liver cirrhosis (22, 23), and as a rule no malignant or inflammatory background exists. Samples were subdivided into three groups, namely transudative effusions, infectious or inflammatory exudative effusions, and malignant exudative effusions.

Sample preparation. Samples of 50-100 ml pleural or peritoneal fluid were collected in 50 ml tubes and immediately frozen at –20˚C for no more than 15 hours. After thawing, 2 ml of each sample were

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Key Words: HMGB1, angiogenesis, ELISA, exudates, transudates.

Table I. *Samples from patients with various effusions and their total and relative HMGB1 concentration (T: transudate; E: exudate; P: pleural effusion; A: ascitic effusion).*

Case no.	Type	Location	Death of patient	Observation time (days)	Diagnosis	Age (years)	HMGB1 total (ng/ml)	HMGB1 relative (%x1000)
1	T	P		25	Cardiac insufficiency	82	9.43	0.13
2	T	P		25	Cardiac insufficiency	82	2.83	0.04
3	T	P		1	Cardiac insufficiency	65	23.58	0.62
4	T	P		24	Cardiac insufficiency	69	51.42	0.81
5	T	P		6	Cirrhosis of the liver	60	4.62	0.06
6	T	A		32	Cirrhosis of the liver	49	7.60	0.23
7	T	P		77	Cardiac insufficiency	68	5.98	0.09
8	E	P		20	Pneumonia	91	36.57	0.80
9	T	A		600	Cardiac insufficiency, liver dysfunction	74	70.93	0.71
10	E	P		136	Lymphocytic chronic effusion	76	116.35	1.83
11	E	A		30	Peritonitis	58	57.60	1.10
12	E	A	†	757	Kidney transplantation/peritonitis	61	49.06	2.10
13	E	A		173	Pneumonia	71	35.22	0.24
14	E	P		23	Pneumonia	76	300.00	2.20
15	E	P		651	Post infectious	63	34.26	1.24
16	E	P		1	Suspicion of hyperergy (allergy)	60	261.13	2.33
17	E	P	†	35	Mycobacteriosis	75	129.59	1.64
18	E	P	†	208	Bacterial pleurisy	69	51.29	0.44
19	E	P		8	Pleurapneumonia	74	153.09	2.24
20	E	P		1	Hemorrhagic pulmonary infarction	58	113.25	1.58
21	E	P	†	62	Bronchial carcinoma	56	216.67	2.16
22	E	P	†	47	Mamma carcinoma	63	225.47	2.51
23	E	P	†	23	Carcinoma of the esophagus	48	58.49	0.56
24	E	P		807	Malignancy with unknown primary tumor	48	44.90	0.96
25	E	P	†	18	Mesothelioma	79	151.91	1.80
26	T	P		2	Cardiac insufficiency	47	82.96	0.74
27	E	P		742	Malignancy with unknown primary tumor	48	29.56	0.79
28	T	P		1	Renal insufficiency	82	19.07	0.39
29	E	P		1	Rendu-Osler disease, mesothelioma	62	92.06	1.65
30	E	P		105	Dedifferentiated adenocarcinoma	75	120.90	1.33
31	E	A	†	55	Mamma carcinoma	57	134.95	1.08
32	T	P	†	1	Cardiac insufficiency	66	124.36	2.23
33	E	P	†	26	Mesothelioma	58	136.67	1.57
34	E	P	†	30	Mamma carcinoma	63	118.33	1.36
35	E	P	†	22	Mamma carcinoma	63	156.57	1.43
36	E	P	†	1	Mamma carcinoma	53	47.57	0.57

taken and stored at –80˚C until further use. A total of 200 μl of each sample were used for the enzyme-linked immunosorbent assay (ELISA).

Protein preparation. Total proteins were isolated with RIPA-buffer (containing: 50 mM Tris-HCl, 150 mM NaCl, 1% (v/v) NP-40, 0.5% (w/v) sodium deoxycholate, 1 mM EDTA). Protein concentration was measured with the Pierce® BCA Protein Assay Kit (Thermo Fisher Scientific, Rockford, USA) following the manufacturer's instructions. All measurements were carried out in triplicate and compared to bovine serum albumin (BSA) and bovine gamma globulin (BGG) standard curves.

ELISA. A 2-step sandwich ELISA was performed using the HMGB1 ELISA Kit (Shino-Test Corporation, Tokyo, Japan) following the manufacturer's instructions.

Analysis of results. All samples were measured at least in duplicate or triplicate, and the results were averaged. Statistical analysis was performed using Student's *t*-test, with a significance level of $p<0.05$. In addition the percentage of HMGB1 in comparison to the total protein amount was calculated.

Results

The effusions (Table I) were subdivided into the three groups: transudates (n=11), effusions due to inflammation or infectious diseases (n=12), and malignant effusions (n=13). Whereas the average total protein concentration within the groups showed only slight variation (transudates 6.83 mg/ml; inflammatory effusions 7.83 mg/ml; malignant effusions 8.74 mg/ml), the amount of HMGB1 strongly varied between 2.83 ng/ml (case

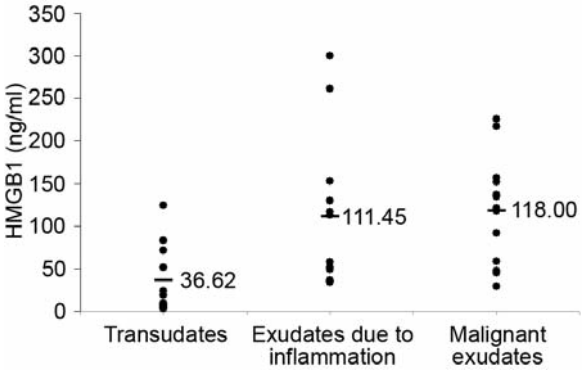

Figure 1. *Average protein concentration of HMGB1 in effusion samples quantified with the ELISA.*

no. 2, cardiac insufficiency) and 300.00 ng/ml (case no. 14, pneumonia) (Figure 1). The average HMGB1 concentration of the three groups was 36.62 ng/ml for the transudates, 118.00 ng/ml for malignant effusions, and 111.45 ng/ml for inflammatory effusions (Figure 1).

A highly significant association (*t*-test, $p<0.001$) was found between the HMGB1 concentration and the existence of an exudate. Higher HMGB1 protein concentrations did not simply depend on the different cell densities varying in transudates and exudates (Figure 2). With regard to the percentage of HMGB1 present relative to the total protein amount, the increase in exudative effusions was also statistically significant ($p<0.001$). The total HMGB1 protein level as well as HMGB1 relative to the total protein amount was three-fold higher in exudates than in transudates: 0.0005% in transudative effusions compared to 0.0015% from both groups of exudates.

Eleven patients died from their disease within two months after sampling, among these, one patient with a transudative effusion was affected by cardiac insufficiency, one by an inflammatory effusion and the others died from cancer. The patient dying from cardiac insufficiency showed the highest percentage of HMGB1 by far within this group. Within the exudative samples, no correlation between the HMGB1 concentration and survival of the patients was detected.

Discussion

HMGB1 plays an important role in tumorigenesis and acts as a proinflammatory mediator in the immune response (24, 25). This suggests that HMGB1 can serve as an important target in the diagnosis and management of exudative effusions. In the present study, most of the patients with transudative effusions had cardiac insufficiencies or renal dysfunctions (Table I). The exudative effusions were mainly caused by carcinoma or inflammatory diseases.

Recently several studies were carried out addressing 'newer' biomarkers for peritoneal and pleural effusions. A cytological test was performed in which D2-40, X-linked inhibitor of apoptosis (XIAP), MOC-31, and Wilms tumor 1 (WT1) were tested (26), describing the markers MOC-31 and D2-40 as very sensitive and specific markers of epithelial and mesothelial cells, respectively, compared to the established calretinin. In addition to these newer markers, the classification with Light *et al*.'s criteria is amongst others the most common to differentiate between transudates and exudates (27). In our investigation, only slight differences of total protein amount were detected in the groups of effusions. Nevertheless, the total protein amount was lower for transudates than for exudates (6.83 mg/ml *vs*. 8.29 mg/ml).

The results of the present study demonstrate that the relative amount of HMGB1 was approximately three-fold higher in exudative effusions than in transudates. This significantly higher protein level was detected with the HMGB1 ELISA alone and also with regard to the total amount of protein (percentage of HMGB1). This definitively proves that the lower HMGB1 protein value is not caused by the lower cell densities in transudate samples.

A high HMGB1 protein level in the effusion may indicate highly active NF-κB or mitogen-activated protein kinase (MAPK) signalling pathways mediated by receptors such as *e.g*. RAGE or TLR (9, 10, 15). This could serve as an indicator for transformed cells (28). Malignant effusions due to metastatic spread represent a frequent problem in advanced cancer. Herein, it has been shown that malignant effusions are often characterized by high levels of the danger signaling protein HMGB1 that are in the same range as in effusions due to inflammatory processes. Extracellular HMGB1 has been linked to both inflammation and cancer. In the latter group of diseases HMGB1 can promote tumor growth by the stimulation of cell proliferation and by inducing neo-angiogenesis (9). On the other hand, HMGB1 is also able to stimulate dendritic cells and thus may support an immune response against tumor cells (29, 30). Antibodies against HMGB1 are able to reduce inflammation and suppress cell migration, tumor growth and metastasis formation (6, 15). The therapeutic use *e.g*. of HMGB1 antibodies instilled locally in case of malignant effusions remains to be investigated. While such therapy will reduce the proliferative potential of the cancer cells as well as their further spread, it might also repress a proper response of the immune system against the cancer cells (15, 31).

This is to the best of our knowledge the first report aimed at determining the concentration of HMGB1 in effusions and suggests interesting new approaches for the treatment of patients with malignant effusions.

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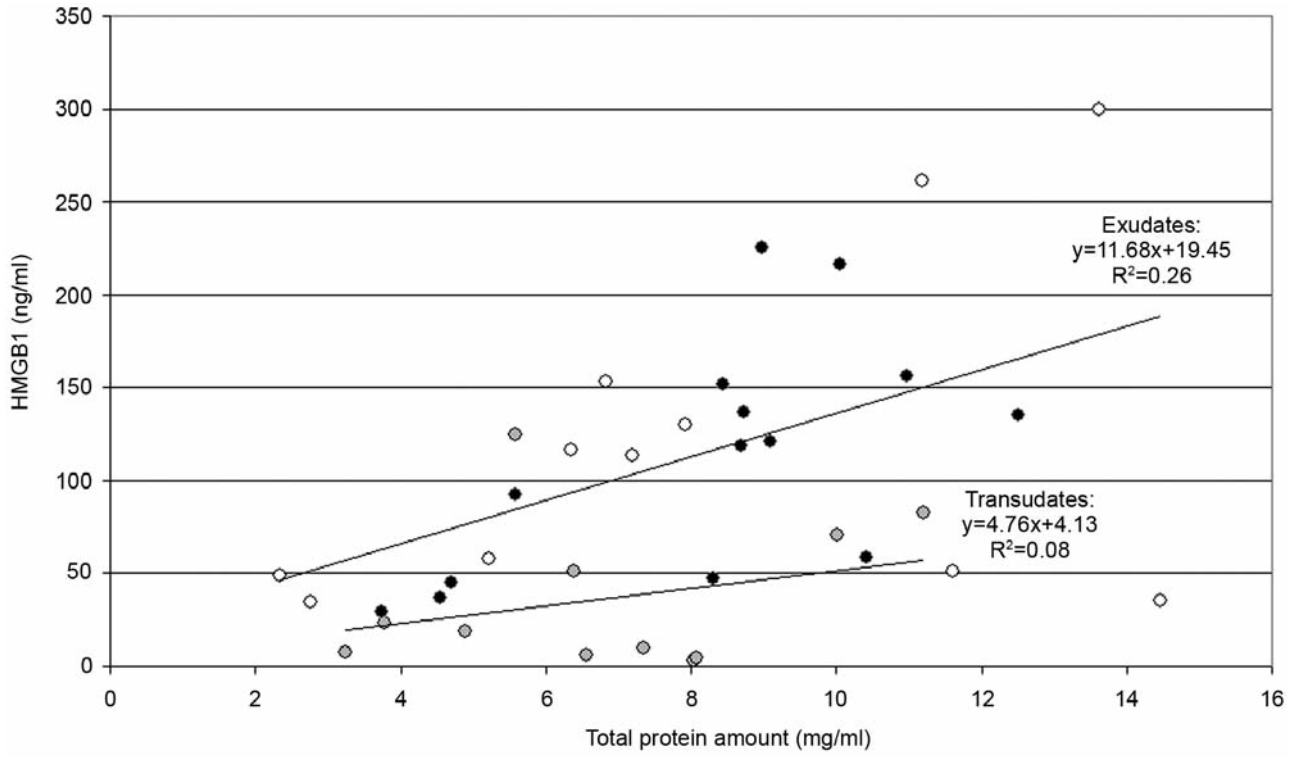

Figure 2. *Concentration of HMGB1 in effusion samples quantified with the ELISA in correlation to total protein amount (light grey: transudates; black: malignant exudates; white: exudates due to inflammation).*

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10 Anhang

10.1 Patientendaten

Nachfolgend ist eine Auflistung der in dieser Arbeit untersuchten Patientendaten aufgeführt, wobei alle Patienten neu nummeriert wurden, so dass es nach den Richtlinien des Daten- und Patientenschutzes nicht zu Problemen kommt. Es gilt durchgehend in den folgenden Tabellen:

- 1 = +; etwas ist vorhanden; ein Ereignis bzw. ein Signal ist positiv
- 0 = -; etwas fehlt; ein Ereignis tritt nicht ein bzw. ein Signal ist negativ
- -1 = x; die Informationen sind unbekannt
- x = die Information ist unbekannt
- Werte mit einem IHC Score von 1 oder darunter wurden als negativ gewertet, alle Werte darüber als positiv für das jeweilige Protein.

10.1.1 Lungengewebe

Probe	Histologie	Alter	Geschlecht	Grad	Stadium	TNM	*HMGA2* Transkripte im Tumor	Standardabweichung Tumorgewebe	*HMGA2* Transkripte im Umgebungsgewebe	Standardabweichung unauffälliges Umgebungsgewebe
A1	AC	61	m	G1	1B	pT2 N0	385.069	61.775	7.519	1.055
A2	AC	73	m	G3	3A	pT2 N2	7.153	1.499	289	51
A3	AC	70	m	G3	3A	pT2 N2	8.320.000	1.660.000	525.947	79.351
A4	AC	62	m	G2	2B	pT2 N1	616.236	68.660	69.511	12.150
A7	AC	63	f	G3	3A	pT2 N2	5.422	1.441	1.664	513
A8	AC	x	f	G2	x	x	123.801	13.178	8.203	2.476
A9	AC	57	m	G3	2A	pT1 N1	127.197	4.752	2.331	558
A10	AC	61	m	G2	4	pT1 N1 M1	16.470	2.774	2.542	308
A11	AC	61	f	G3	3A	pT3 N1	5.584.977	194.186	192.350	16.100
L3	AC	65	m	G2	1B	pT2 N0 Mx	31.314	3.247	40.932	5.256
L9	AC	71	m	G2	2B	pT2 N1 Mx	1.889.122	50.890	41.741	2.719
L11	AC	60	m	G3	3B	pT4 N1 Mx	11.487.392	910.283	9.075	436
L13	AC	52	f	G3	1B	pT2 N0 Mx	592.257	5.904	38.706	233
L15	AC	56	m	G3	3A	pT3 N1 Mx	3.758.594	191.048	4.126	81
L16	AC	62	m	G2	1B	pT2 N0 Mx	11.084.326	169.586	130.432	1.914
L17	AC	66	m	G3	3B	pT2 N3 Mx	4.665.494	61.455	29.208	6.171
L18	AC	64	f	G1	1B	pT2 N0 Mx	402.597	32.762	395.087	34.994
L8	AC	57	m	G3	2A	pT2 N3 M1	58.171	4.127	26.660	1.808
P1	SSC	70	m	G2	2B	pT2 N1	3.528.705	34.060	337.270	15.409
P2	SSC	x	m	G2	2B	pT3 N1 M0	7.778.535	92.751	8.954	3.222
P3	SSC	64	m	G3	1A	pT1 N0	362.274	54.854	8.611	1.318
P4	SSC	62	f	G1	3B	pT4 N1	13.749.450	910.048	55.266	6.079
P5	SSC	62	m	G2	2B	pT3 N0	164.355	13.654	15.855	2.089
P6	SSC	74	m	G1	3B	pT4 N1	1.321.792	229.894	11.754	1.866
P7	SSC	69	m	G1	1B	pT2 N0	11.478.720	381.008	14.884	1.569
P8	SSC	62	f	G3	2B	pT4 N0	31.432	594	5.802	1.948
P9	SSC	59	m	G1	2B	pT2 N1	1.243.528	68.927	4.423	727
L2	SSC	68	m	G1	1B	pT2 N0 Mx	209.373	3.197	1.008	425
L4	SSC	60	m	G1	1B	pT2 N0 Mx	7.429.843	347.040	69.940	7.898
L5	SSC	63	m	G3	3A	pT2 N2 Mx	8.305.805	296.192	22.256	1.379
L6	SSC	75	m	G3	1B	pT2 N0 Mx	16.991.545	249.715	6.787	143
L7	SSC	68	m	G2	1B	pT2 N0 Mx	3.324.433	143.336	33.487	3.886
L10	SSC	64	m	G3	1A	pT2 N2 Mx	1.171.467	51.296	269.841	4.309
L12	SSC	73	m	G2	3A	pT2 N2 Mx	187.488	1.352	4.492	157
L14	SSC	50	m	G2	2B	pT2 N1 Mx R1	5.008.100	332.309	169.524	5.855

10.1.2 Schilddrüsengewebe

Probe	Alter	Ge-schlecht	Histo-logie	Größe [cm]	TNM	Grad	relative *HMGA2* Menge
1	58	f	Thyroiditis	x	x	x	0,2
2	40	m	Adenom	7,5	x	x	0,2
3	47	m	Adenom	6,8	x	x	0,1
4	25	f	Adenom	2,5	x	x	0,5
5	85	m	PTC	4,0	pT3a	x	0,5
6	53	f	Adenom	6,5	x	x	0,6
7	44	m	Adenom	4,0	x	x	0,8
8	48	f	Adenom	5,0	x	x	0,9
UG	x	x	unauffälliges Gewebe	x	x	x	1,0
9	63	f	SCN mit Thyrioditis	x	x	x	1,1
10	29	f	Adenom	7,0	x	x	1,1
11	54	f	Adenom	1,0	x	x	1,2
12	71	f	Adenom	1,7	x	x	1,3
13	35	m	Adenom	6,5	x	x	1,5
14	51	f	Adenom	6,0	x	x	1,5
15	42	m	Adenom	3,5	x	x	1,6
16	42	f	Adenom	1,0	x	x	1,8
17	34	f	FTC	2,2	x	1	0,9
18	61	f	Adenom	4,0	x	x	2,5
19	44	m	Adenom	1,0	x	x	4,0
20	39	f	Adenom	1,5	x	x	5,6
21	30	f	Adenom	0,3	x	x	6,8
22	40	f	Adenom	3,8	x	x	9,6
23	42	f	Adenom	4,0	x	x	11,5
24	35	f	MI-FTC (t)	2,1	pT2 N0 MX	x	9,3
25	61	m	FTC (o)	8,0	pT4	x	11,6
26	72	f	FV-PTC	1,0	pT1	x	7,0
27	84	f	FV-PTC	6,0	pT3 pNX	x	14,5
28	38	f	PTC	0,6	pT1	x	3,1
29	50	f	PTC	2,2	pT2	x	15,1
30	21	f	FV-PTC	1,0	pT1 pNX pMX	x	3,8

Probe	Alter	Ge-schlecht	Histo-logie	Größe [cm]	TNM	Grad	relative *HMGA2* Menge
31	31	M	PTC	2,5	pT2 pN0	x	25,6
32	46	F	PTC	1,5	pT1	2	20,1
33	65	F	ATC	2,0	pT4 No	4	27,3
34	54	F	PTC	0,6	pT1 pNX pMX	x	4,1
35	85	F	PTC	1,5	x	2	23,7
36	85	F	PTC	14,0	pT3	3	28,6
37	42	M	FV-PTC	0,7	pT1 N0 MX	x	9,1
38	66	F	MI-FTC	2,0	pT1	x	46,3
39	72	F	FV-PTC	1,0	pT3	x	46,7
40	38	M	PTC	0,8	pT1	1	11,9
41	76	F	ATC	3,8	pT4b	x	51,4
42	33	F	PTC	2,5	pT2	2	42,4
43	69	M	FV-PTC	0,5	pT1 NX MX	x	6,3
44	42	F	FV-PTC	1,4	pT2a	2	25,9
45	30	F	PTC	2,5	pT2 NX	x	72,9
46	49	F	PTC	1,2	pT2	x	25,8
47	25	F	FV-PTC	2,3	pT2 pN0	x	73,9
48	48	F	PTC	0,8	pT1	1-2	43,7
49	76	F	ATC	1,7	pT4b	x	96,0
50	67	M	FTC (t)	5,5	pT3 pNX pM1	x	68,6
51	59	F	FTC	1,2	pT1	2	22,1
52	34	F	PTC	2,3	pT2 pN1 pMX	x	96,0
53	57	F	PTC	0,9	pT1	x	11,7
54	31	M	PTC	2,0	pT3 pN1	x	136,7
55	27	M	FV-PTC	2,5	pT2	x	65,4
56	66	F	PTC	2,0	pT3	2	137,2
57	41	M	PTC	2,0	pT1	2	105,5
58	37	F	FTC	2,8	pT2 N0	x	95,0
59	69	M	FTC	3,0	pT4	x	304,0
60	79	F	PTC	2,0	pT3 pNX pM1	1	398,1
61	71	F	FTC (o,i)	9,0	pT4 NX MX	x	432,6

10.1.3 Herzgewebe

Pat.-Nr.	Probe	Geschlecht	Alter	Histologie	relative *HMGA2* Menge
1	H01	m	62	AG ohne AAD	4,8
2	H02	m	55	AG ohne AAD	4,1
3	H03	m	41	AG ohne AAD	7,1
4	H04	m	65	AG ohne AAD	7,0
5	H05	m	38	AG ohne AAD	2,4
6	H06	x	x	AG ohne AAD	0,9
7	H07	m	65	AG ohne AAD	49,8
8	H08	m	71	AG ohne AAD	0,8
9	H09	m	65	AG ohne AAD	3,9
10	H10	m	77	AG ohne AAD	5,2
11	H11	m	68	AG ohne AAD	5,1
13	H13	m	75	AG ohne AAD	11,2
14	H14	x	x	AG ohne AAD	18,0
15	H15	m	34	AG ohne AAD	2,6
16	H16	x	x	AG ohne AAD	3,7
17	H17	m	49	AG ohne AAD	6,5
18	H18	m	39	AG ohne AAD	5,6
19	H19	m	79	AG ohne AAD	7,3
20	H20	f	48	AG ohne AAD	11,1
21	H21	m	63	AG ohne AAD	7,1
22	H22	m	50	AG ohne AAD	2,2
23	H23	m	45	AG ohne AAD	11,3
24	H24	m	58	AG ohne AAD	4,5
29	H29	m	59	AG ohne AAD	4,9
30	H30	m	63	AG ohne AAD	6,9
31	H31	m	63	AG ohne AAD	9,3
32	H32	m	46	AG ohne AAD	11,5
33	H33	m	22	AD (alle Schichten)	290,6
34	H34	m	36	AG ohne AAD	3,8
35	H35	m	55	AD (alle Schichten)	5,2
36	H36	m	63	AD (alle Schichten)	186,1
37	H37	f	63	AD (alle Schichten)	149,5
38	H38	m	27	AG ohne AAD	12,0
39	H39	f	77	AG ohne AAD	5,2
40	H40	m	57	AG ohne AAD	15,5
41	H41	x	x	AG ohne AAD	6,4

Pat.-Nr.	Probe	Geschlecht	Alter	Histologie	relative *HMGA2* Menge
42	H42	m	77	AG ohne AAD	1,0
44	H44	x	x	AG ohne AAD	4,4
45	H45	m	53	AG ohne AAD	23,9
46	H46	x	x	AG ohne AAD	10,4
47	H47	m	48	AG ohne AAD	2,5
48	HB48	f	84	AD (alle Schichten)	194,6
49	HB49	m	23	AD (alle Schichten)	41,8
50	HB50	m	65	AD (alle Schichten)	17,5
51	HB51	m	75	AD (alle Schichten)	12,2
	HB52			AD (alle Schichten)	179,0
	HB53			AD (Endothel)	451,6
52	HB54	f	68	AD (Media)	48,0
53	HB55	m	52	AD (alle Schichten)	32,8
54	HB56	m	51	AD (alle Schichten)	1212,4
55	HB57	f	65	AD (alle Schichten)	243,4
	HB58			AD (alle Schichten)	249,3
	HB59			AD (Endothel)	927,8
56	HB60	m	53	AD (Media)	563,7
	HB61			AD (alle Schichten)	116,6
	HB62			AD (Endothel)	150,5
57	HB63	m	79	AD (Media)	108,4
	HB64			AD (alle Schichten)	65,2
	HB65			AD (Endothel)	131,8
58	HB66	m	76	AD (Media)	61,2
	HB67			AD (alle Schichten)	8,8
	HB68			AD (Endothel)	278,5
59	HB69	m	46	AD (Media)	555,5
	HB70			AD (alle Schichten)	50,8
	HB71			AD (Endothel)	126,6
60	HB72	m	54	AD (Media)	201,7
	HB73			AD (alle Schichten)	436,6
	HB74			AD (Endothel)	268,5
61	HB75	m	54	AD (Media)	791,9
	HB76			AD (alle Schichten)	175,8
62	HB77	f	64	AD (alle Schichten)	160,8

10.1.4 Speicheldrüsengewebe

Probe	Alter	Geschlecht	Grad	Fusions-transkript	relative *HMGA2* Menge
1	60	f	1	ja	199,0
2	80	f	1	ja	139,7
3	52	m	1	ja	85,4
4	71	m	3	ja	22,6
5	72	f	1	ja	7,0
6	77	f	1	ja	7,6
7	79	f	2	ja	83,3
8	74	f	1	ja	20,8
9	49	f	1	nein	57,6
10	63	m	3	nein	1273,4
11	17	m	1	ja	7,3
12	79	f	2	ja	149,0
13	38	m	1	ja	10,9
14	72	m	2	ja	337,7
15	42	m	1	ja	41,8
16	42	m	3	nein	66,2
17	75	f	1	ja	2,9
18	49	f	2	ja	55,9
19	15	m	1	nein	24,9
20	65	m	3	nein	1343,0
21	58	f	1	ja	36,3
22	40	m	1	ja	3,2
23	25	f	2	nein	44,4
24	61	f	1	ja	25,1
25	62	m	2	ja	81,2
26	47	m	1	ja	3,9
27	48	f	1	nein	88,5

Probe	Alter	Geschlecht	Grad	Fusions-transkript	relative *HMGA2* Menge
28	37	m	2	nein	101,8
29	61	f	1	ja	67,4
30	54	m	2	nein	36,1
31	55	m	3	nein	16,4
32	65	m	2	nein	402,4
33	46	m	3	ja	60,1
34	51	m	3	ja	47,0
35	45	m	1	ja	7,8
36	34	f	2	ja	140,0
37	45	m	2	ja	6,3
38	75	f	2	ja	36,4
39	76	f	3	ja	24,9
40	65	m	3	ja	139,4
41	71	f	2	ja	17,8
42	x	m	3	nein	693,0
43	49	m	3	nein	37,1
44	44	m	3	ja	15,2
45	49	f	2	ja	281,8
46	58	f	2	ja	256,1
47	62	m	1	ja	44,4
48	22	m	2	ja	41,4
49	29	f	2	ja	74,0
50	69	m	2	ja	5,6
51	73	f	3	ja	4,1
52	66	m	1	ja	35,1
53	80	f	3	nein	53,8

10.1.5 Brustgewebe

Probe	Alter	Nodalstatus	ER	PR	Her2	Größe [cm]	Grading	Stadium	Metastasen	Rezidiv freies Überleben [Monate]	Rezidiv	Tod	Überlebenszeitraum [mind. Monate]	Hormontherapie	Strahlentherapie	Chemotherapie	Aromatasehemmer	Antikörpertherapie	Schwangerschaft [Anzahl]	familiäre Belastung	*HMGA2*	*uPA*	*PAI*	*ESR1*	*PGR*	*Her2neu*
MK001	59	0	12	12	3	1,4	2	1	1	20	1	1	23	0	0	1	0	0	-1	-1	7,68	1,12	2,67	0,53	0,15	1,49
MK002	37	0	9	0	-1	5,5	2	1	1	22	1	0	90	1	0	0	0	0	-1	-1	7,14	0,93	2,86	0,07	0,09	0,29
MK003	66	0	12	8	-1	1,1	2	1	0	85	0	0	85	1	0	0	0	0	-1	-1	x	0,57	0,05	0,07	0,05	0,37
MK004	55	0	12	8	2	1,9	2	1	0	10	0	0	10	1	1	1	0	0	-1	-1	9,47	1,21	1,16	0,18	0,65	1,85
MK005	61	0	12	12	1	1,0	2	1	0	71	0	0	71	1	0	0	1	0	-1	-1	1,35	1,35	1,04	0,16	2,25	0,23
MK006	64	0	8	9	2	1,8	2	1	-1	-1	-1	-1	-1	0	0	0	1	0	-1	-1	2,08	0,53	0,46	0,01	0,06	0,08
MK007	84	0	6	6	2	1,4	2	1	0	33	0	0	33	1	0	0	0	0	5	-1	5,20	1,89	1,94	0,35	1,71	0,39
MK008	84	0	9	4	3	0,9	1	1	0	33	0	0	33	1	0	0	0	0	5	-1	0,72	4,77	11,06	1,32	1,36	1,69
MK009	40	0	8	6	3	1,2	2	1	-1	-1	-1	-1	-1	1	1	1	0	0	-1	1	3,77	0,92	0,34	0,24	0,56	0,52
MK011	61	0	12	6	2	1,2	2	1	0	61	0	0	64	0	1	0	1	0	-1	-1	1,87	1,12	0,33	0,55	0,11	0,45
MK013	64	0	12	0	3	1,5	2	1	-1	-1	-1	0	1	1	0	0	0	0	-1	1	1,38	1,15	2,55	0,22	0,01	0,86
MK016	52	0	8	8	0	0,7	3	1	0	37	0	0	37	1	0	1	0	0	-1	-1	3,73	2,54	4,24	0,08	1,36	x
MK017	62	0	9	9	2	3,0	2	2	0	59	0	0	59	1	0	1	1	0	-1	-1	1,24	3,22	5,80	0,08	0,05	0,48
MK018	71	0	12	12	1	0,8	2	1	0	60	0	0	60	1	1	0	0	0	-1	0	0,35	0,45	0,90	0,04	0,02	0,22
MK019	55	0	12	12	-1	0,7	2	1a	0	73	0	0	85	1	0	0	0	0	-1	-1	1,27	0,66	0,14	0,02	0,08	0,35
MK020	53	0	12	2	0	0,7	2	1b	0	34	0	0	69	1	1	0	0	0	-1	-1	11,43	3,98	0,82	0,17	0,16	0,99
MK021	63	0	12	1	2	0,6	2	1b	0	65	0	0	84	0	1	1	0	0	-1	-1	9,33	2,25	2,10	0,82	0,02	0,95
MK023	55	0	12	6	1	-1	1	1b	0	80	0	0	80	1	0	0	1	0	4	-1	3,38	1,93	1,65	0,06	0,64	1,81
MK024	67	0	9	4	1	0,4	2	1b	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	13,08	3,32	2,58	0,39	0,42	1,76
MK025	81	0	12	4	0	0,5	1	1b	0	37	0	0	37	1	1	0	0	0	-1	-1	1,46	0,30	0,11	0,14	0,07	0,18
MK026	68	0	9	3	-1	1,3	2	1c	0	74	0	0	74	1	0	0	0	0	-1	-1	1,37	0,54	0,89	0,92	0,10	0,69
MK027	42	0	12	2	2	2,5	2	2	0	13	0	0	13	1	1	0	0	0	-1	-1	4,70	1,28	1,17	0,32	0,19	5,93
MK028	56	0	9	6	2	2,5	2	2	0	0	0	0	20	1	0	0	1	0	-1	-1	7,21	1,88	1,63	0,03	0,06	0,50
MK029	53	0	12	6	1	1,5	2	1c	0	69	0	0	69	0	0	0	1	0	-1	-1	10,05	2,25	1,62	0,12	0,28	0,84

Probe	Alter	Nodalstatus	ER	PR	Her2	Größe [cm]	Grading	Stadium	Metastasen	Rezidiv freies Überleben [Monate]	Rezidiv	Tod	Überlebenszeitraum [mind. Monate]	Hormontherapie	Strahlentherapie	Chemotherapie	Aromatasehemmer	Antikörpertherapie	Schwangerschaft [Anzahl]	familiäre Belastung	*HMGA2*	*uPA*	*PAI*	*ESR1*	*PGR*	*Her2neu*
MK030	60	0	12	4	1	0,9	1	1b	0	61	0	0	61	0	0	0	1	0	-1	-1	7,16	1,15	3,46	0,23	0,09	1,23
MK031	62	0	12	6	-1	1,3	2	1c	0	13	0	0	60	1	1	0	0	0	-1	-1	2,43	1,21	0,26	0,04	0,12	0,82
MK032	51	0	6	6	2	0,6	1	1b	0	66	0	0	74	0	1	0	1	0	-1	-1	1,14	0,51	0,34	0,07	0,06	0,38
MK033	62	0	9	4	1	1,2	2	1c	-1	-1	-1	0	0	1	0	0	0	0	-1	-1	x	1,53	x	0,02	0,03	0,27
MK034	68	0	12	0	-1	1,8	2	1c	0	17	0	0	18	1	1	1	0	0	-1	-1	5,11	2,45	2,54	0,12	0,07	0,62
MK035	48	0	12	6	-1	1,5	3	1c	0	71	0	0	71	1	1	1	0	0	-1	-1	3,51	1,55	1,79	0,14	0,49	0,82
MK036	65	0	12	2	-1	1,2	1	1c	0	77	0	0	77	1	0	0	0	0	-1	-1	1,02	0,75	0,49	0,06	0,03	0,19
MK037	40	0	12	0	1	2,3	3	2	0	77	0	0	78	1	0	1	0	0	-1	-1	3,56	0,67	0,10	0,12	0,66	0,26
MK038	62	0	12	9	1	0,9	2	1b	0	82	0	0	82	1	1	0	0	0	-1	-1	1,68	1,33	1,09	0,10	0,15	0,86
MK039	76	0	12	12	0	0,7	1	1b	0	2	0	0	2	1	1	0	0	0	-1	-1	4,57	4,97	13,36	0,05	0,09	0,13
MK040	79	0	9	4	-1	1,7	2	1c	-1	-1	-1	-1	-1	1	1	0	0	0	-1	-1	0,69	0,58	0,66	0,06	0,25	1,78
MK042	56	0	12	12	1	0,9	2	1b	0	72	0	0	72	0	1	0	1	0	-1	-1	1,20	0,89	0,34	0,04	0,28	0,17
MK043	48	0	12	6	1	3,0	2	2	0	70	0	0	76	1	0	0	0	0	-1	-1	1,47	1,53	0,82	0,27	0,04	0,97
MK044	52	0	12	2	3	0,8	2	1b	0	67	0	0	67	1	1	0	0	0	-1	-1	0,85	1,28	1,35	0,14	0,02	0,18
MK045	55	0	12	6	2	0,6	2	1b	0	38	0	0	38	1	0	0	0	0	-1	-1	2,12	1,09	0,76	0,09	0,32	0,45
MK046	61	0	12	12	3	1,2	2	1c	0	18	0	0	18	0	1	0	1	0	-1	-1	3,18	1,17	1,67	0,70	0,75	2,72
MK047	47	0	6	2	0	1,1	2	1c	0	63	0	0	75	0	1	0	1	0	-1	-1	0,64	1,28	0,25	0,13	0,03	0,37
MK048	65	0	1	4	3	1,2	2	1c	1	24	1	0	44	1	1	1	1	1	-1	-1	0,69	1,73	0,97	0,01	0,00	1,43
MK049	61	0	12	6	1	-1	2	1b	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	1,92	3,19	6,83	0,01	0,01	0,10
MK050	60	0	8	6	2	1,3	2	1c	0	62	0	0	62	1	0	1	0	0	-1	-1	6,12	2,04	3,94	0,16	0,34	1,23
MK051	57	0	2	2	-1	1,3	2	1c	1	16	1	1	39	1	1	1	0	0	-1	1	12,63	3,10	1,66	0,00	0,01	0,16
MK053	65	0	12	12	2	1,4	2	1c	0	52	0	0	52	1	0	0	0	0	-1	-1	1,14	0,07	1,23	0,04	0,00	0,30
MK054	60	0	12	6	3	1,2	2	1c	-1	-1	-1	0	61	-1	-1	-1	-1	-1	-1	-1	2,84	2,42	0,28	0,52	0,04	1,02
MK055	51	0	12	9	2	1,6	3	1c	0	37	1	0	69	0	1	1	1	0	-1	-1	1,42	3,92	1,12	0,33	0,45	0,36
MK056	53	0	8	4	-1	1,6	2	1c	0	13	0	0	13	0	1	0	1	0	-1	-1	24,76	6,00	1,51	0,12	0,27	0,90
MK057	56	0	6	4	3	1,2	2	1c	0	10	0	0	78	1	1	1	0	0	-1	-1	7,83	5,96	9,97	0,10	0,11	0,63
MK058	63	0	12	2	2	2,1	2	2	0	57	0	0	57	1	0	0	1	1	-1	1	2,89	2,66	3,07	0,20	0,06	0,74

Probe	Alter	Nodalstatus	ER	PR	Her2	Größe [cm]	Grading	Stadium	Metastasen	Rezidiv freies Überleben [Monate]	Rezidiv	Tod	Überlebenszeitraum [mind. Monate]	Hormontherapie	Strahlentherapie	Chemotherapie	Aromatasehemmer	Antikörpertherapie	Schwangerschaft [Anzahl]	familiäre Belastung	*HMGA2*	*uPA*	*PAI*	*ESR1*	*PGR*	*Her2neu*
MK059	68	0	1	4	2	1,5	3	1c	0	60	0	0	60	1	1	1	0	0	-1	-1	3,22	5,91	6,65	0,01	0,03	0,15
MK060	42	0	0	2	0	5,3	3	3	x	41	0	0	64	0	1	1	0	0	-1	1	0,65	1,21	0,92	0,00	0,01	0,05
MK062	52	0	12	8	2	1,6	2	1c	0	17	0	0	22	1	1	1	0	0	-1	-1	4,05	1,67	0,60	0,32	0,06	0,23
MK065	50	0	12	12	0	2,3	2	2	0	74	0	0	74	1	1	0	0	0	-1	1	1,45	0,85	0,65	0,07	0,20	0,38
MK066	82	0	12	9	2	2,2	2	2	-1	-1	-1	0	5	-1	-1	-1	-1	-1	-1	-1	1,27	5,13	0,60	0,28	0,24	0,40
MK067	51	0	8	0	0	0,6	2	1b	0	62	0	0	62	1	0	1	1	0	-1	-1	2,48	0,82	0,18	0,07	0,04	0,41
MK069	56	0	2	0	2	2,4	3	2	0	62	0	0	62	0	1	0	0	0	-1	-1	14,35	4,39	1,05	0,01	0,00	0,29
MK070	63	0	12	6	3	1,5	2	1c	0	31	0	0	31	1	0	1	0	0	-1	-1	1,25	0,45	0,33	0,56	0,04	0,37
MK071	55	0	12	6	1	1,1	2	1c	0	67	0	0	67	1	1	1	0	0	-1	-1	x	1,19	0,29	0,18	0,29	0,36
MK072	79	0	12	2	1	3,8	2	4b	-1	-1	-1	0	53	1	1	0	0	0	-1	-1	0,90	2,02	2,15	0,23	0,06	0,21
MK073	65	0	8	0	1	1,5	2	1c	0	73	0	0	73	1	1	1	0	0	1	-1	2,92	5,81	0,42	0,31	0,11	0,32
MK074	56	0	12	12	2	1,5	3	1c	-1	-1	-1	0	75	1	1	1	0	0	-1	1	0,88	4,37	0,93	0,58	1,05	0,12
MK076	59	0	1	0	3	1,5	3	1c	0	80	0	0	80	0	1	0	1	0	-1	-1	2,89	0,98	0,96	0,12	0,11	0,37
MK077	51	0	12	6	3	1,1	2	1c	0	61	0	0	61	1	1	1	0	0	-1	-1	4,95	1,89	3,66	0,39	0,13	1,10
MK078	54	0	12	6	0	3,5	2	2	0	63	0	0	63	1	0	1	1	0	-1	-1	0,15	0,17	0,52	0,02	0,01	0,08
MK079	60	0	9	6	2	1,3	2	1b	0	60	0	0	60	0	1	0	1	0	-1	-1	8,01	3,07	4,29	0,10	0,06	0,21
MK080	60	0	9	6	-1	5,0	2	1c	-1	-1	-1	0	83	-1	-1	-1	-1	-1	-1	-1	2,65	2,08	4,38	0,07	0,25	0,29
MK081	61	0	12	6	-1	0,4	2	Ta	-1	-1	-1	0	57	1	0	0	0	0	-1	-1	4,53	0,58	0,19	0,48	0,17	0,42
MK082	75	0	6	0	3	1,2	3	1c	-1	-1	-1	0	26	0	1	0	1	0	-1	-1	2,49	2,65	2,99	0,06	0,02	11,69
MK083	42	0	8	12	-1	1,2	3	1c	0	65	0	0	65	1	1	1	0	0	-1	1	1,22	1,96	0,74	0,09	0,36	0,35
MK084	65	0	12	12	2	1,1	2	1c	-1	-1	-1	0	15	1	0	1	0	0	-1	-1	1,85	1,72	2,03	0,26	0,51	0,37
MK085	63	0	12	12	2	2,1	2	2	0	60	0	0	60	0	1	1	1	0	-1	-1	7,27	1,93	1,92	0,43	0,41	1,03
MK086	56	0	12	6	1	3,0	2	2	0	19	0	0	74	1	1	1	0	0	-1	-1	1,51	1,14	1,15	0,11	0,18	0,22
MK087	51	0	12	0	0	1,1	2	1c	-1	-1	-1	0	19	1	1	1	0	0	-1	-1	x	0,91	0,32	0,09	0,03	0,14
MK088	63	0	8	6	3	1,4	3	1c	-1	-1	-1	0	3	-1	-1	-1	-1	-1	-1	-1	6,23	5,69	0,52	0,09	0,95	0,53
MK090	60	0	2	2	3	3,0	2	2	-1	-1	-1	0	76	0	1	1	0	0	-1	-1	1,65	1,90	3,51	0,01	0,01	10,76
MK091	60	0	12	4	3	3,5	2	2	0	29	0	0	62	1	1	1	0	0	-1	-1	0,90	0,55	2,58	0,11	0,05	0,29

Probe	Alter	Nodalstatus	ER	PR	Her2	Größe [cm]	Grading	Stadium	Metastasen	Rezidiv freies Überleben [Monate]	Rezidiv	Tod	Überlebenszeitraum [mind. Monate]	Hormontherapie	Strahlentherapie	Chemotherapie	Aromatasehemmer	Antikörpertherapie	Schwangerschaft [Anzahl]	familiäre Belastung	*HMGA2*	*uPA*	*PAI*	*ESR1*	*PGR*	*Her2neu*
MK093	66	0	8	4	0	1,6	2	1c	0	35	0	0	35	1	0	1	0	0	-1	1	0,41	1,19	0,32	0,08	0,28	0,49
MK094	47	0	12	9	2	2,1	3	2	0	23	0	0	67	1	1	1	0	0	-1	-1	3,79	1,16	1,13	0,14	0,32	0,61
MK095	55	0	12	12	2	1,5	3	1c	0	9	0	0	9	1	1	1	0	0	-1	1	3,36	1,69	0,63	0,33	0,53	0,77
MK097	48	0	12	12	3	2,5	2	2	0	5	1	0	80	1	0	1	0	0	-1	1	3,82	x	x	0,84	6,12	0,70
MK098	68	0	12	9	1	1,9	2	1c	0	66	0	0	66	0	1	0	1	0	-1	-1	1,79	0,63	1,16	0,65	2,60	0,99
MK099	64	0	12	6	0	0,8	2	1a	-1	-1	-1	0	68	1	1	0	1	0	-1	-1	3,78	2,17	1,32	0,02	0,02	0,08
MK100	54	0	6	1	1	1,7	2	1c	-1	-1	-1	0	21	-1	-1	-1	-1	-1	-1	-1	4,52	3,69	2,21	0,53	0,15	0,68
MK101	31	0	12	6	0	0,8	3	1b	0	51	0	0	51	1	1	1	0	0	-1	-1	0,14	1,41	0,99	0,04	0,10	0,38
MK103	55	0	4	6	3	1,3	3	1c	0	77	0	0	77	0	0	0	1	0	-1	-1	16,92	4,42	4,48	0,05	0,10	1,92
MK104	77	0	12	6	0	1,8	2	1b	0	57	0	0	57	1	0	0	1	0	-1	-1	2,72	3,72	3,23	0,52	0,37	0,55
MK106	60	0	9	4	2	1,5	-1	1b	0	31	0	0	81	1	1	0	1	0	-1	-1	2,21	2,10	1,63	0,09	0,05	1,70
MK107	57	0	12	2	0	1,4	2	1c	0	72	0	0	72	1	1	1	1	0	-1	-1	15,90	3,52	1,31	0,18	0,38	1,00
MK108	46	0	12	6	2	1,6	2	1c	0	16	0	0	63	1	1	1	0	0	-1	-1	9,67	3,59	1,98	0,18	1,01	0,90
MK109	66	0	12	1	1	0,9	2	1b	0	74	0	0	74	0	1	0	1	0	-1	-1	6,36	3,01	2,50	0,11	0,09	0,54
MK111	53	0	12	6	-1	1,3	2	1c	0	71	0	0	71	1	0	1	0	0	-1	-1	2,17	1,35	0,37	0,12	0,20	0,51
MK113	50	0	12	2	1	1,2	2	1b	0	4	0	0	73	1	0	1	0	0	-1	-1	6,13	4,20	2,54	0,09	0,07	1,08
MK115	71	0	12	3	0	1,5	2	-1	-1	-1	-1	0	0	-1	-1	-1	-1	-1	-1	-1	26,11	26,72	18,92	0,04	x	0,72
MK116	80	0	9	12	1	1,3	2	1c	1	-1	-1	0	58	1	1	0	0	0	-1	0	1,17	1,59	0,98	0,12	0,83	0,39
MK118	61	0	12	6	2	2,3	2	2	1	7	1	0	7	1	0	0	0	0	-1	-1	15,53	5,33	4,81	0,37	0,21	0,69
MK119	57	0	12	2	3	1,3	3	1c	-1	-1	-1	0	11	0	1	0	1	0	-1	-1	3,37	5,17	3,59	0,99	0,04	4,33
MK120	46	0	12	12	3	2,0	2	1c	0	26	0	0	27	1	0	1	0	0	-1	1	146,94	0,24	x	x	x	0,88
MK121	76	0	12	8	0	0,9	2	1b	0	5	0	0	73	0	1	0	1	0	-1	-1	x	1,69	x	0,09	x	0,41
MK122	32	0	0	0	3	1,5	2	1c	-1	4	1	0	12	-1	-1	-1	-1	-1	-1	1	11,57	x	x	x	x	x
M21 P	36	0	+	(+)	-1	x	3	2	-1	20	1	0	66	-1	-1	-1	-1	-1	-1	-1	20,38	3,80	2,86	x	x	x
M22 P	75	0	+	+	-1	x	2	2	-1	0	0	0	66	-1	-1	-1	-1	-1	-1	-1	1,00	1,00	1,00	1,00	1,00	1,00
M28 P	42	0	0	0	-1	x	3	2	-1	4	1	1	21	-1	-1	-1	-1	-1	-1	-1	42,00	1,74	1,98	x	x	x

Probe	Alter	Nodalstatus	ER	PR	Her2	Größe [cm]	Grading	Stadium	Metastasen	Rezidiv freies Überleben [Monate]	Rezidiv	Tod	Überlebenszeitraum [mind. Monate]	Hormontherapie	Strahlentherapie	Chemotherapie	Aromatasehemmer	Antikörpertherapie	Schwangerschaft [Anzahl]	familiäre Belastung	*HMGA2*	*uPA*	*PAI*	*ESR1*	*PGR*	*Her2neu*
M31 P	38	0	+	+	-1	x	1	1	-1	115	1	0	121	-1	-1	-1	-1	-1	-1	-1	0,49	0,31	0,34	x	x	x
M75 P	59	0	+	0	-1	x	3	3	-1	40	1	0	57	-1	-1	-1	-1	-1	-1	-1	3,38	2,99	0,84	x	x	x
U2	40	0	+	+	-1	x	2	2	-1	0	0	0	144	-1	-1	-1	-1	-1	-1	-1	1,55	1,02	2,84	x	x	x
U3	66	0	+	+	-1	x	2	2	-1	0	0	0	107	-1	-1	-1	-1	-1	-1	-1	1,76	0,2	x	x	x	x
U6	52	0	+	0	-1	x	2	2	-1	0	0	0	143	-1	-1	-1	-1	-1	-1	-1	5,27	1,37	0,26	x	x	x
U69	55	0	+	+	-1	x	2	2	-1	0	0	0	136	-1	-1	-1	-1	-1	-1	-1	2,56	0,56	0,19	x	x	x
U78	76	0	+	+	-1	x	2	2	-1	0	0	0	68	-1	-1	-1	-1	-1	-1	-1	2,51	4,99	3,25	x	x	x
U82	68	0	+	+	-1	x	2	1	-1	0	0	0	136	-1	-1	-1	-1	-1	-1	-1	2,05	1,46	0,23	x	x	x
U115	72	0	+	+	-1	x	3	2	-1	0	0	0	73	-1	-1	-1	-1	-1	-1	-1	0,59	1,32	0,5	x	x	x
U173	63	0	0	0	-1	x	3	2	-1	0	0	0	125	-1	-1	-1	-1	-1	-1	-1	6,55	1,37	0,73	x	x	x
U187	56	0	0	0	-1	x	3	2	-1	10	1	1	11	-1	-1	-1	-1	-1	-1	-1	1,07	1,24	0,78	x	x	x
U462	32	0	+	+	-1	x	3	1	-1	75	1	1	76	-1	-1	-1	-1	-1	-1	-1	5,72	3,73	0,88	x	x	x
U508	38	0	0	+	-1	x	3	2	-1	37	1	1	54	-1	-1	-1	-1	-1	-1	-1	2,91	1,76	0,26	x	x	x
U509	58	0	+	+	-1	x	3	3	-1	5	1	1	48	-1	-1	-1	-1	-1	-1	-1	4,77	3,97	1,39	x	x	x
M20	56	0	+	+	-1	x	3	-1	-1	14	1	1	26	-1	-1	-1	-1	-1	-1	-1	15,32	6,46	4,94	x	x	x
M21	59	0	0	0	0	x	3	2	0	0	0	0	69	-1	-1	-1	-1	-1	-1	-1	5,64	5,71	1,06	x	x	x
M22	48	0	1	1	1	x	3	3	0	0	0	0	55	-1	-1	-1	-1	-1	-1	-1	7,14	1,59	2,02	x	x	x
M23	63	0	2	2	1	x	2	1	2	55	1	1	55	-1	-1	-1	-1	-1	-1	-1	13,20	1,82	0,57	x	x	x
M24	42	0	1	2	1	x	1	2	2	9	1	0	9	-1	-1	-1	-1	-1	-1	-1	10,56	1,66	2,42	x	x	x
M25	60	0	3	2	1	x	2	1	2	39	1	0	48	-1	-1	-1	-1	-1	-1	-1	3,19	2,14	2,20	x	x	x
M26	48	0	1	1	2	x	3	2	1	12	1	0	22	-1	-1	-1	-1	-1	-1	-1	6,55	4,06	0,92	x	x	x
M27	74	0	2	1	1	x	3	2	2	0	0	0	63	-1	-1	-1	-1	-1	-1	-1	3,48	0,28	0,13	x	x	x
M28	68	0	4	4	1	x	2	1	0	0	0	0	38	-1	-1	-1	-1	-1	-1	-1	29,07	0,58	x	x	x	x
M29	55	0	1	1	1	x	3	1	0	0	0	0	1	-1	-1	-1	-1	-1	-1	-1	27,11	3,87	2,81	x	x	x
M30	54	1	0	0	0	x	2	1	0	8	1	0	9	-1	-1	-1	-1	-1	-1	-1	1,97	15,54	1,84	x	x	x
M31	40	x	1	1	1	x	3	-1	0	28	1	1	28	-1	-1	-1	-1	-1	-1	-1	x	2,03	1,45	x	x	x

Probe	Alter	Nodalstatus	ER	PR	Her2	Größe [cm]	Grading	Stadium	Metastasen	Rezidiv freies Überleben [Monate]	Rezidiv	Tod	Überlebenszeitraum [mind. Monate]	Hormontherapie	Strahlentherapie	Chemotherapie	Aromatasehemmer	Antikörpertherapie	Schwangerschaft [Anzahl]	familiäre Belastung	*HMGA2*	*uPA*	*PAI*	*ESR1*	*PGR*	*Her2neu*
M32	53	0	0	0	0	x	3	1	2	47	1	0	48	-1	-1	-1	-1	-1	-1	-1	3,12	0,60	0,56	x	x	x
M33	57	0	1	1	4	x	3	2	2	13	1	0	61	-1	-1	-1	-1	-1	-1	-1	1,74	1,32	0,55	x	x	x
M34	53	0	1	1	4	x	3	1	1	10	1	1	22	-1	-1	-1	-1	-1	-1	-1	6,35	3,22	3,64	x	x	x
M35	42	0	3	4	2	x	2	1	0	0	0	0	62	-1	-1	-1	-1	-1	-1	-1	0,72	0,65	2,72	x	x	x
M36	59	0	4	2	3	x	2	2	0	0	0	0	32	-1	-1	-1	-1	-1	-1	-1	13,79	3,68	1,16	x	x	x
M37	40	0	1	1	1	x	3	1	0	0	0	0	56	-1	-1	-1	-1	-1	-1	-1	14,62	3,11	1,90	x	x	x
M38	70	0	1	1	1	x	3	4	2	17	1	1	21	-1	-1	-1	-1	-1	-1	-1	12,96	2,38	0,25	x	x	x
M39	72	0	1	1	1	x	2	2	1	14	1	0	32	-1	-1	-1	-1	-1	-1	-1	1,57	0,25	0,09	x	x	x
M40	53	0	1	1	1	x	2	2	0	23	1	1	23	-1	-1	-1	-1	-1	-1	-1	0,71	1,22	1,95	x	x	x
M41	52	0	1	3	1	x	3	1	0	37	1	0	37	-1	-1	-1	-1	-1	-1	-1	24,36	3,06	1,78	x	x	x
M42	44	0	3	1	1	x	1	1	1	33	1	1	44	-1	-1	-1	-1	-1	-1	-1	4,85	0,43	0,62	x	x	x
M43	47	0	2	2	1	x	1	1	x	0	0	0	28	-1	-1	-1	-1	-1	-1	-1	4,48	1,46	1,02	x	x	x
M45	67	0	4	3	1	x	2	2	0	47	1	1	47	-1	-1	-1	-1	-1	-1	-1	4,66	1,27	1,10	x	x	x
M46	51	0	4	2	2	x	2	2	0	0	0	0	12	-1	-1	-1	-1	-1	-1	-1	11,25	3,47	1,19	x	x	x
M47	42	0	1	1	0	x	2	2	0	0	0	0	71	-1	-1	-1	-1	-1	-1	-1	1,08	0,72	0,08	x	x	x
M48	62	0	4	2	2	x	2	1	2	30	1	1	30	-1	-1	-1	-1	-1	-1	-1	x	0,46	0,21	x	x	x
M49	58	0	3	1	2	x	2	2	0	0	0	0	52	-1	-1	-1	-1	-1	-1	-1	6,52	0,81	0,49	x	x	x
M50	50	0	2	2	1	x	1	1	0	26	1	0	40	-1	-1	-1	-1	-1	-1	-1	1,32	2,41	0,86	x	x	x
M51	31	0	3	4	1	x	2	1	0	0	0	0	34	-1	-1	-1	-1	-1	-1	-1	2,25	1,84	0,72	x	x	x
M52	79	0	4	4	1	x	1	x	x	x	x	0	62	-1	-1	-1	-1	-1	-1	-1	75,95	4,34	1,62	x	x	x
M53	45	0	1	1	1	x	2	1	0	0	0	0	14	-1	-1	-1	-1	-1	-1	-1	3,76	1,28	0,36	x	x	x
M54	65	0	1	2	1	x	1	1	0	0	0	0	38	-1	-1	-1	-1	-1	-1	-1	60,85	11,47	1,59	x	x	x
M55	58	0	4	2	1	x	1	1	0	0	0	0	29	-1	-1	-1	-1	-1	-1	-1	16,23	1,42	1,28	x	x	x

Probe	Alter	Nodalstatus	ER	PR	Her2	Größe [cm]	Grading	Stadium	Metastasen	Rezidiv freies Überleben [Monate]	Rezidiv	Tod	Überlebenszeitraum [mind. Monate]	Hormontherapie	Strahlentherapie	Chemotherapie	Aromatasehemmer	Antikörpertherapie	Schwangerschaft [Anzahl]	familiäre Belastung	*HMGA2*	*uPA*	*PAI*	*ESR1*	*PGR*	*Her2neu*
M56	73	0	1	1	1	x	2	1	0	0	0	0	27	-1	-1	-1	-1	-1	-1	-1	9,45	3,81	0,63	x	x	x
M57	50	0	4	3	1	x	2	1	0	0	0	0	61	-1	-1	-1	-1	-1	-1	-1	4,50	1,30	0,72	x	x	x
M58	53	0	4	3	1	x	2	2	0	0	0	0	11	-1	-1	-1	-1	-1	-1	-1	13,33	0,23	0,08	x	x	x
M59	62	0	1	0	2	x	2	2	2	7	1	1	7	-1	-1	-1	-1	-1	-1	-1	8,58	2,45	1,24	x	x	x
M60	69	0	3	2	1	x	2	2	0	0	0	0	69	-1	-1	-1	-1	-1	-1	-1	4,91	1,38	0,86	x	x	x
M61	79	0	2	2	1	x	2	1	0	0	0	0	24	-1	-1	-1	-1	-1	-1	-1	81,41	2,10	0,53	x	x	x
M62	62	0	4	4	1	x	1	4	0	0	0	0	24	-1	-1	-1	-1	-1	-1	-1	4,84	3,17	0,25	x	x	x
M63	61	0	4	1	1	x	2	3	0	0	0	0	62	-1	-1	-1	-1	-1	-1	-1	17,26	0,73	0,51	x	x	x
M64	59	0	0	0	0	x	1	4	2	0	0	0	22	-1	-1	-1	-1	-1	-1	-1	x	0,41	2,33	x	x	x
M65	53	0	1	1	1	x	2	1	2	6	1	1	9	-1	-1	-1	-1	-1	-1	-1	11,86	1,80	1,78	x	x	x
M66	51	0	1	1	1	x	2	1	0	0	0	0	26	-1	-1	-1	-1	-1	-1	-1	4,30	1,98	0,40	x	x	x
M67	63	0	1	1	1	x	2	2	0	0	0	0	33	-1	-1	-1	-1	-1	-1	-1	9,42	13,49	2,13	x	x	x
M68	46	0	1	1	1	x	2	2	0	0	0	0	23	-1	-1	-1	-1	-1	-1	-1	x	0,72	0,18	x	x	x
M69	57	0	0	0	0	x	2	2	0	0	0	0	20	-1	-1	-1	-1	-1	-1	-1	7,00	0,51	0,33	x	x	x
M70	79	0	4	1	1	x	3	1	0	0	0	0	18	-1	-1	-1	-1	-1	-1	-1	2,01	0,90	0,36	x	x	x

Die Proben M21-M70 wurden bezüglich der Färbung nicht nach dem Remmle Score System klassifiziert.

10.1.6 Myomgewebe

Probe	Karyotyp	Größe [cm]	Alter	relative *HMGA2* Menge im Myom	relative *HMGA2* Menge im Myometrium
Myom 003	46,XX	10,0x8,0x6,0	48	894,53	x
Myom 005	46,XX	2,0x2,0x2,0	43	3,73	0,62
Myom 006	46,XX,add(5),t(12;14)	4,0x4,0x4,0	40	8,60	1,18
Myom 007A	46,XX	4,0-5,0	x	3,08	x
Myom 007B	46,XX	2,0		8,38	
Myom 008	46,XX	2,0	x	1,10	x
Myom 009	46,XX	5,0	41	4,41	x
Myom 010A	46,XX	1,6	x	9,10	x
Myom 011	46,XX	x	55	23,47	x
Myom 012A	46,XX; 47,XX,+8 [1]	<3,0	41	65,45	1,71
Myom 012B	46,XX	x		4,14	
Myom 013A	46,XX	1,0x4,0	24	6,85	x
Myom 013B	46,XX	1,0x1,5		4,12	
Myom 014A	46,XX	6,0	46	1,67	5,91
Myom 014B	46,XX	x		6,51	
Myom 015	46,XX del(7)(q22q31)	4,0x5,0	42	0,88	1,11
Myom 016	46,XX	x	43	338,41	x
Myom 017A	t(12;14)(q15;q24)	3,0	46	993,25	0,66
Myom 020	46,XX	x	36	1,06	x
Myom 021	46,XX	x	42	743,20	x
Myom 022	46,XX	4,0x5,0	38	2404,97	x
Myom 024	46,XX	1,0	44	0,79	4,05
Myom 025A	45,XX, t(12;14)(q13;q22) or t(12;14)(q15;q24) der(14)t(12;14)(q13;q22) or t(12;14)(q15;q24),-22	x	33	7760,34	x
Myom 025B	45,XX, t(12;14),der(14)t(12;14),-22	x		11539,75	
Myom 026	46,XX, del(7)(q22q34)	x	40	10,00	x
Myom 027A	46,XX	5,0	40	4,74	0,70
Myom 027B	46,XX	8,0		9,13	
Myom 028	46,XX	6,0	37	6,12	0,52
Myom 029	46,XX	3,5	48	1,96	1,30
Myom 030B	46,XX	6,0x4,0	44	6,49	1,01
Myom 030C	46,XX	7,0x5,0		9,20	
Myom 031A	46,XX	2,5	47	5,03	1,00
Myom 031B	46,XX	3,5		15,73	
Myom 031E	46,XX	4,5		5,68	
Myom 032	45,XX,rob(13;14)(q10,q10)c	3,0	42	12,04	x
Myom 033	46,XX; 46,XX,t(5;12)(p15.1;q15) [1]	1,0	34	x	x
Myom 034A	46,XX	2,0	39	2,32	x
Myom 034B	46,XX, inv(1)(p13q32)	5,0		18,65	
Myom 034C	46,XX, inv(1)(p13q32)	x		36,31	
Myom 035	46,XX,r(1), t(1;12;14)(p36.3;q14;q24)	6,0	41	1722,51	x
Myom 036	46,XX	4,0x6,0	39	18,35	5,80

Probe	Karyotyp	Größe [cm]	Alter	relative *HMGA2* Menge im Myom	relative *HMGA2* Menge im Myometrium
Myom 037A	47,XX,+10 [2] /46, XX [7]	5,0	43	2,77	0,79
Myom 037B	46,XX,t(8;11)(p23;q13.1) / 47,XX,+12/ 46,XX	4,0		2,92	
Myom 037C	46,XX	3,0		9,58	
Myom 037D	46,XX	2,0		22,00	
Myom 037E	46,XX, del(7)(q11.2?) [2]/46,xx [12]	3,0		3,06	
Myom 038A	46,XX, del(7)(q22q31.2)	6,0	46	46,05	0,55
Myom 038C	46,XX, del(7)(q21.2q31.2)	3,0		27,15	
Myom 038D	46,XX	2,0		18,39	
Myom 039A	46,XX	5,0	59	20,51	0,56
Myom 040A	46,XX	3,0	36	21,55	2,20
Myom 040D	46,XX	3,0		57,27	
Myom 040F	46,XX	8,0		4,89	
Myom 040G	46,XX	9,0		3,37	
Myom 041A	46,XX	3,0	46	7,59	1,86
Myom 041B	46,XX	4,0		0,55	
Myom 041C	46,XX	4,0		8,63	
Myom 041D	46,XX	3,0		14,41	
Myom 042A	46,XX	4,0	49	9,49	0,87
Myom 042B	46,XX	x		1,14	
Myom 042C	46,XX	x		1,63	
Myom 043	46,XX,t(12;14)(q15q24)/ 46,XX	7,0	37	5906,14	0,78
Myom 044A	46,XX,inv(7)(p21q22)/ 46,XX	7,0	46	4,62	0,22
Myom 044B	46,XX	4,0		4,21	
Myom 044C	46,XX	5,0		2,52	
Myom 044D	46,XX	2,0		1,69	
Myom 044E	46,XX	1,0		0,64	
Myom 046A	46,XX	6,0	49	4,20	0,80
Myom 046B	46,XX,del(7)(q22q32)/ 46,XX	4,0		4,70	
Myom 046C	46,XX	2,5		12,74	
Myom 047	46,XX,t(12;14)(q15q24)/ 46,XX	5,0	47	1327,31	x
Myom 048	47,XX,+12/ 46,XX	6,0	45	1,97	0,18
Myom 049A	46,XX,der(12),der(14)?ins(14;12)/46,idem ,r(1)	2,5	73	3444,31	2,41
Myom 049B	46,XX	x		1,54	
Myom 050	46,XX	10,0	49	1,83	x
Myom 051A	46,XX	7,0x4,0	49	6,12	0,26
Myom 051B	46,XX	3,5		4,12	
Myom 051C	46,XX	4,0		3,71	
Myom 051D	48,+der(6),-8,+11,+mar mar: inv (8) or t(6;8) mit der (6)	6,0		0,46	
Myom 051E	46,XX	2,0		1,16	
Myom 052B	48,XX,+4,+12/ 48,XX,+4, del(7)(q22q31.2),+12; t(2;8) [1]; t(1;3) [1]	5,0		18,79	0,63
Myom 053	46,XX, add(1)(p),der(1)t(1;3?)(q;q), der(3)del(3)(p)or add(3)(p)t(1;3)(q;q),der(4)t(3;4)(p;q) [17]	x	30	0,95	x
Myom 054A	46,XX	2,0x3,0	49	2,91	x
Myom 054B	46, XX, t(2;12)(q33;q13)	10,0		2381,63	

Probe	Karyotyp	Größe [cm]	Alter	relative *HMGA2* Menge im Myom	relative *HMGA2* Menge im Myometrium
Myom 055	46,XX	x	51	3,25	3,77
Myom 056A	46,XX	2,5	41	1,95	x
Myom 057	46, XX, t(3;5;12)/ 45, XX, t(3;5;12) -22 Bruchpunkte unsicher, mehrere Möglichkeiten: -3q23;5p13;12q13 oder 3q25;5p14;12q15	5,0	42	4450,72	1,25
Myom 058A	46,XX	4,0	46	15,12	x
Myom 058B	46,XX	3,0		4,75	
Myom 058C	46,XX	3,0		2,99	
Myom 059	46,XX	1,0	34	86,36	x
Myom 060A	46,XX	1,0	38	54,36	0,67
Myom 060B	46,XX	3,0		68,96	
Myom 060C	46,XX	2,0		13,21	
Myom 060D	46,XX	4,0		22,69	
Myom 061A	46,XX	5,0	73	0,65	0,36
Myom 061B	46,XX, der(7) del(7)(q11.2q31) inv(7)(q11.2q36)/46,XX	10,0		3,97	
Myom 062A	46,XX	6,0	47	2,08	1,90
Myom 062B	46,XX	3,0		3,32	
Myom 062C	46,XX	3,0		9,56	
Myom 062D	46,XX	2,0		4,39	
Myom 062E	46,XX	1,5		3,84	
Myom 065A	46,XX	1,5	51	5,39	x
Myom 065B	46,XX	4,0		12,38	
Myom 067A	46,XX	6,0x7,0	65	18,80	1,17
Myom 067B	46,XX,del(7)(q21.2q31.2)/ 46,XX,t(1;3)(q25;q26),del(7)(q21.2q31.2)	3,0		49,41	
Myom 067C	46,XX	2,0x3,0		6,73	
Myom 068A	46,XX	10,0	42	77,71	0,65
Myom 068B	46,XX	6,0x4,0		4,49	
Myom 069C	46,XX	2,5	38	6,04	x
Myom 069D	46,XX	4,0		8,19	
Myom 069E	46,XX	4,0		6,40	
Myom 070	46,XX	3,0	40	8,36	x
Myom 071B	46,XX	1,5	60	2,05	11,72
Myom 072A	46,XX	2,0	46	4,46	0,38
Myom 072B	46,XX	3,0		2,14	
Myom 073	46,XX	>10	42	35,38	x
Myom 074A	46,XX	4,0	38	10,24	17,02
Myom 074B	46,XX	4,0		3,38	
Myom 074C	46,XX	4,0		4,63	
Myom 074D	46,XX	3,0		1,09	
Myom 075	46,XX	4,0	50	5,76	1,13
Myom 076	46,X,t(X;2)(p11.4;p25),del(7)(q11.2q22),inv(9)(p11q13)c/46,XX,inv(9)(p11q13)c [1x46,XX,del(7),inv(9)(p11q13)c]	5,0	37	7,53	x
Myom 077A	46,XX	3,5	44	9,67	x
Myom 077B	46,XX	3,0		1,47	
Myom 078A	46,XX	2,0x3,0	35	8,46	x
Myom 078B	46,XX	>15,0		13,57	

Probe	Karyotyp	Größe [cm]	Alter	relative *HMGA2* Menge im Myom	relative *HMGA2* Menge im Myometrium
Myom 079C	46,XX	8,0	49	12,79	0,91
Myom 079D	46,XX	6,0		11,78	
Myom 079E	46,XX, del(7)(q22q32)r(16)(pterqter)/ 46,XX [1xdel(7),+12,-16]	6,0		17,56	
Myom 079F	46,XX	5,0		13,77	
Myom 080B	46,XX	3,0	45	10,42	0,44
Myom 082	46,XX,t(12;15;14)(q15;q26;q24)/46,XX	1,5	49	302,34	2,77
Myom 083	46,xx,der(7)del(7)(p)del(7)(q),t(12;14)(q15;24)[3]/46xx,idem,der(8)add(8)(q),der(10)add(10)(q)[16]	8,0	40	670,91	x
Myom 084B	46,XX	2,5x1,5	51	10,43	0,62
Myom 084C	46,XX	1,5		17,34	
Myom 086	46,XX	5,5	40	61,55	0,77
Myom 087	46,XXt(6;14)(p23;q24)[6]/ 46,XX, idem, tas (14;21)(pter;qter)[11]/ 46,XX, [2], nicht clonal: 47,XX, +12 [2]	x	63	1,06	0,39
Myom 088	46,XX	3,0	45	3,60	0,90
Myom 089C	46,XX	2,0	31	6,32	x
Myom 089D	46,XX	3,0		6,35	
Myom 091	46,XX	x		0,81	x
Myom 092	46,XX	20,0	41	8,56	0,61
Myom 093A	46,XX	2,0	48	13,95	1,05
Myom 093B	46,XX	2,0		5,00	
Myom 095A	46,XX	0,5	44	3,87	x
Myom 095B	46,XX	0,5		1,63	
Myom 096	47,XX,+12/46,XX	1,0	44	1,41	1,10
Myom 097	46,XX	x	43	0,48	x
Myom 098A	46,XX, del(7)(q22q32)/46,XX/47,XX,+X	3,0	50	5,51	1,97
Myom 098B	46,XX	2,0		4,26	
Myom 100B	46,XX,del (7)(q11.2q21)(ev.2.Linie mit del(7),aber anderenBP[2])	x	37	3,15	x
Myom 101	46,XX	2,0-3,0	47	15,05	x
Myom 102	46,XX	x	52	0,96	x
Myom 103	46,XX	10,0	44	1,01	x
Myom 104	45,XX,del(1)(p3?),-16/46,XX	5,0	40	0,41	x
Myom 105	46,XX	6,0x10,0	32	10,13	x
Myom 106A	46,XX	1,5x1,0	35	3,74	1,32
Myom 107B	46,XX	2,0	33	4,70	x
Myom 108	46,XX	x	45	5,95	x
Myom 109A	46,XX	4,0	50	10,86	0,10
Myom 109B	46,XX	2,5		13,75	
Myom 109C	46,XX	6,0		14,04	
Myom 109D	46,XX	4,5		1,54	
Myom 111	46,XX	5,0	46	1,11	x
Myom 112	46,XX, der (3), t(3;6?)(p23:q?), der (6)(q), der(8)(p)[13]/46,XX [4] (evt. T (3;8;6))	4,0	33	9,29	x

Probe	Karyotyp	Größe [cm]	Alter	relative *HMGA2* Menge im Myom	relative *HMGA2* Menge im Myometrium
Myom 113A	46,XX	6,0	53	3,19	x
Myom 113B	46,XX	4,5		4,36	
Myom 113C	46,XX,t(6;10=(p23;q23)[5]/ 46,XX [7]	3,5		19,42	
Myom 113D	46,XX	3,5		3,21	
Myom 113E	46,XX	3,5		6,36	
Myom 113F	46,XX	3,0		4,42	
Myom 114	46,XX	x	40	8,68	x
Myom 115	46,XX, der (1)r(1;2),t(12;14)(q15;q24)[4]/46,XX,t (12;14)(q15q24)[13]	6,0	44	1047,76	x
Myom 116A	46,XX, del(7)(q22q32)[2]/46,XX [21], nicht clonal: t(1;1)[1],der(1)[1]	2,0	56	1,02	0,59
Myom 116B	46,XX	1,5		1,89	
Myom 117B	46,XX	4,5x7,0	39	24,27	x
Myom 117C	46,XX	7,0		20,14	
Myom 117D	46,XX, t(6;11)(p23;q21)/46,XX	4,5		44,54	
Myom 117E	46,XX	8,0		39,42	
Myom 118A	46,XX, del(6)(q15 or q16)	5,0	47	2,04	x
Myom 118B	46,XX	3,0		8,13	
Myom 136	46,XX,t(12;14)(q15;q24)[12]/46,XX,del(4)(q31orq32),der(10),?t(10;14)(q24;q32),t(12;14)(q15;q24)[9]/45,XX,der(1),?t(1;2),-2,add(7)(?q36),t(12;14)(q15;q24)]	4,0	47	1826,31	x

10.1.7 Humane Non-Hodgkin Lymphome und unveränderte Lymphknoten

Probe	Histologie	Alter	Größe [cm]	Geschlecht	relative *HMGB1* Menge
nln0701	unveränderter Lymphknoten	x	0,4	f	**1,00**
nln 2	unveränderter Lymphknoten	x	0,4	f	**0,92**
nln 7	unveränderter Lymphknoten	x	0,3	m	**2,43**
nln 8	unveränderter Lymphknoten	71	0,2	m	**3,50**
nln 9	unveränderter Lymphknoten	63	0,3	m	**2,59**
nln 10	unveränderter Lymphknoten	61	0,2	f	**1,83**
K-NHL 1	hoch malignes Non-Hodgkin Lymphom der B-Zellreihe	64	3	f	**6,07**
K-NHL 2	follikuläres, teils diffus wachsendes malignes Non-Hodgkin Lymphom	40	3,2x2,5x1,7	f	**4,00**
K-NHL 3	großzellig-anaplastisches Non-Hodgkin Lymphom	24	x	m	**2,55**
K-NHL 4	hoch malignes Non-Hodgkin Lymphom der B-Zellreihe	57	5,0x3,0x2,5	m	**2,01**
K-NHL 5	hoch malignes Non-Hodgkin Lymphom der B-Zellreihe	60	2,5x1,0x1,0	f	**1,09**
K-NHL 6	hoch malignes Non-Hodgkin Lymphom der B-Zellreihe	17	3,5x3,0x0,5	m	**2,87**
K-NHL 7	niedrig malignes Non-Hodgkin Lymphom	55	5,5x3,0x2,5	m	**1,13**
K-NHL 9	niedrig malignes Non-Hodgkin Lymphom der B-Zellreihe	53	5,5x3,0x3,0	m	**3,70**
K-NHL 10	niedrig malignes Non-Hodgkin Lymphom der B-Zellreihe	71	3,5x2,5x3,0	f	**1,23**
K-NHL 11	niedrig malignes Non-Hodgkin Lymphom der B-Zellreihe	76	2,0x1,5x1,4	m	**4,96**
K-NHL 12	niedrig malignes Non-Hodgkin Lymphom mit leichter Fibrose	46	3,0x1,0x1,0	m	**1,27**
K-NHL 13	hoch malignes Non-Hodgkin-Lymphom der B-Zellreihe	46	3,2	f	**3,55**
K-NHL 14	niedrig malignes Non-Hodgkin Lymphom der B-Zellreihe	47	1,5x1,0x0,9	x	**7,96**
K-NHL 15	großzellig-anaplastisches Non-Hodgkin Lymphom vom 0-Typ	28	3,5x3,0x1,8	x	**0,30**
K-NHL 16	niedrig malignes Non-Hodgkin Lymphom der B-Zellreihe	76	0,8x0,5x0,5	f	**3,52**
K-NHL 17	niedrig malignes Non-Hodgkin Lymphom der B-Zellreihe	87	4,5x3,0x3,0	m	**1,48**
K-NHL 18	niedrig malignes Non-Hodgkin Lymphom der B-Zellreihe	55	1,0x0,3x0,4	x	**3,05**
K-NHL 19	hoch malignes Non-Hodgkin-Lymphom der B-Zellreihe	65	1,5x1,0x1,0	x	**6,14**

10.1.8 Zelllinien

Name	Ursprungsgewebe	Karyotyp	Alter	Geschlecht
ARO	humane Schilddrüse	62~69<3n>,XX,-X [3]; komplexer Karyotyp	x	f
Jurkat J08	humanes Lymphom	87(78-91)<4n>XX,-Y,-Y,-5,-16,-17,-22,add(2)(p21)/del(2)(p23)x2; der(5)t(5;10)(q11;p15), del(9)(p11)	14	m
LM168.1	humanes Uterusleiomyom	46,X,t(X;12)(q22;q15)[8]	x	f
LM220	humanes Uterusleiomyom	46,XX,del(7)(q22q32),t(12;14)(q15;q24)[29]	x	f
S121/TSV40	humane Schilddrüse	46,XX,t(5;19)(q13;q13) [52]	20	f
S211/TSV40	humane Schilddrüse	46,XX,inv(4)(p15.2;q12),t(5;19)(p14 or p15.1;q12 or 13),t(9;18)(q12;q22) [25]	29	f
S325/TSV40	humane Schilddrüse	46,XX,t(2;20;3)(p21;q11.1;p25) [17]	49	f
S40.2/TSV40	humane Schilddrüse	46,XX,t(1;19)(p35 or 36.1;q13) [19]	35	f
S731	humane Schilddrüse	46,XX	x	f
S734.1	humane Schilddrüse	46,XY	x	m
supT1	humanes Lymphom	85<4n>XXX/XXX?Y,-8,-8,-9,-12,inv(2)(p22q11)x2,t(2;?20)(p13;?p11),del(4)(q31q35),del(6)(q25)x2,add(7)(q32),add(9)(q34)x2,inv(14)(q11q32)x2;	8	m
WRO	humane Schilddrüse	66~76,XXX,+del(2)(q14),-6,+7,+9,-10,+i(12)(p),+i(12)(p),-15,-17,+mar[cp6]	x	f

10.1.9 Canine Lymphomseren

Die Daten aus den Untersuchungen und zu den Patienten liegen in der Tierärztlichen Hochschule in Hannover vor, wo auch die statistischen Analysen durchgeführt wurden.

10.1.10 Humane Exsudate

Probe	Art	Lokalisation	Tod	Beobachtungs-zeit [Tage]	Diagnose	Alter	HMGB1 total [ng/ml]	HMGB1 relativ [%*1000]
1	T	P	0	25	Herzinsuffizienz	82	9,43	0,13
2	T	P	0	25	Herzinsuffizienz	82	2,83	0,04
3	T	P	0	1	Herzinsuffizienz	65	23,58	0,62
4	T	P	0	24	Herzinsuffizienz	69	51,42	0,81
5	T	P	0	6	Leberzirrhose	60	4,62	0,06
6	T	A	0	32	Leberzirrhose	49	7,60	0,23
7	T	P	0	77	Herzinsuffizienz	68	5,98	0,09
8	E	P	0	20	Stauungserguss	91	36,57	0,80
9	T	A	0	600	Herzinsuffizienz, Leberfehlfunktion	74	70,93	0,71
10	E	P	0	136	lymphozytärer chronischer Erguss	76	116,35	1,83
11	E	A	0	30	Peritonitis	58	57,60	1,10
12	E	A	1	757	Peritonitis, Nierentransplantation	61	49,06	2,10
13	E	A	0	173	Stauungserguss	71	35,22	0,24
14	E	P	0	23	Stauungserguss	76	300,00	2,20
15	E	P	0	651	postinfektiös	63	34,26	1,24
16	E	P	0	1	allergische Reaktion	60	261,13	2,33
17	E	P	1	35	Mykobakteriose	75	129,59	1,64
18	E	P	1	208	bakterielle Pleuritis	69	51,29	0,44
19	E	P	0	8	Pleurapneumonie	74	153,09	2,24
20	E	P	0	1	Hämorrhagischer Lungeninfarkt	58	113,25	1,58
21	E	P	1	62	Bronchialkarzinom	56	216,67	2,16
22	E	P	1	47	Mammakarzinom	63	225,47	2,51
23	E	P	1	23	Espophaguskarzinom	48	58,49	0,56
24	E	P	0	807	malignitätsverdächtig; unbekannter Primärtumor	48	44,90	0,96
25	E	P	1	18	Mesotheliom	79	151,91	1,80
26	T	P	0	2	Herzinsuffizienz	47	82,96	0,74
27	E	P	0	742	malignitätsverdächtig; unbekannter Primärtumor	48	29,56	0,79
28	T	P	0	1	Niereninsuffizienz	82	19,07	0,39
29	E	P	0	1	Rendu-Osler Krankheit, Mesotheliom	62	92,06	1,65
30	E	P	0	105	Adenokarzinom	75	120,90	1,33
31	E	A	1	55	Mammakarzinom	57	134,95	1,08
32	T	P	1	1	Herzinsuffizienz	66	124,36	2,23
33	E	P	1	26	Mesotheliom	58	136,67	1,57
35	E	P	1	30	Mammakarzinom	63	118,33	1,36
36	E	P	1	22	Mammakarzinom	63	156,57	1,43
37	E	P	1	1	Mammakarzinom	53	47,57	0,57