**AUS DER KLINIK UND POLIKLINIK FÜR FRAUENHEILKUNDE UND GEBURTSHILFE – CAMPUS INNENSTADT**

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# Beeinflussung des Metastasierungspotentials beim Ovarial- und Mammakarzinom durch das Metastasen-assoziierte Gen MTA1 und das Leucin zipper downregulated in cancer 1-Gen (LDOC1)

Habilitationsschrift

zum Erwerb der Venia Legendi

für das Fach

Frauenheilkunde und Geburtshilfe


vorgelegt von

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Christl Friedl gewidmet,
die mich durch ihren Glauben
an mich und ihre fortwährende
Ermutigung mehr unterstützt hat, als sie denkt,
und damit diese Arbeit möglich gemacht hat.

# INHALTSVERZEICHNIS

# 1 VORWORT

Die folgende wissenschaftliche Arbeit befasst sich mit zwei in der onkologischen Gynäkologie häufigen Tumorentitäten, dem Ovarial- und Mammakarzinom. Insbesondere wird die Beeinflussung des Metastasierungspotentials durch zwei tumorrelevante Gene, dem Metastasen-assoziierten Gen 1 (MTA1) und dem Leucin zipper downregulated in cancer 1-Gen (LDOC1) bei Ovarial- und Mammakarzinomen näher untersucht.

In der Einleitung werden das MTA1-Gen und das LDOC1-Gen hinsichtlich ihres Vorkommens und ihrer Funktion beschrieben, und ihre Rolle bei der Karzinogenese und Tumorprogression beim Ovarial- und Mammakarzinom wird dargelegt.

Im nächsten Abschnitt der Arbeit werden eigene Studienergebnisse beschrieben: An Vorarbeiten führten wir unter anderem molekularbiologische Untersuchungen an Zervixkarzinom-Zelllinien durch, die zur Etablierung der Untersuchungstechniken dienten und sowohl die Bedeutung von MTA1 als auch von LDOC1 als wichtige tumorrelevante Parameter auch für andere Tumorentitäten (wie Ovarial- und Mammakarzinome) zeigten.

Anschließend wird die Fragestellung des vorliegenden Forschungsprojekts bezüglich des Ovarialkarzinoms anhand immunhistochemischer Untersuchungen an Gewebeproben und molekularbiologischer Analysen an Ovarialkarzinom-Zelllinien bearbeitet.

Nachfolgend werden unsere Untersuchungen an Mammakarzinom-Zelllinien bezüglich der Bedeutung von MTA1 und LDOC1 beim Mammakarzinom dargelegt, die epigenetischen Regulationsmechanismen von LDOC1 und schließlich die induzierte Re-Expression nach Inaktivierung dieses Gens mittels eines Expressionsplasmids beschrieben.

In einem abschließenden Ausblick auf zukünftige Forschungsprojekte wird die Möglichkeit von molekularbiologischen Untersuchungen nicht nur an Mammakarzinom-Zelllinien, sondern auch an Mammakarzinom-Gewebeproben diskutiert, sowie weitere potentielle Forschungsvorhaben wie klinische Untersuchungen zur prognostischen Bedeutung von MTA1 und LDOC1 genannt.

Ausführliche Angaben zu Methodik und Diskussion finden sich in den jeweiligen Originalarbeiten der Habilitationsschrift. Auf alle zitierten Arbeiten (eigene und fremde) wird durch Ziffernhinweise in eckigen Klammern, geordnet nach der Reihenfolge im Text, verwiesen.

# 2 EINLEITUNG

Ovarialkarzinome gehören weltweit zu den Krebsarten mit den höchsten Letalitätsraten [1-4]. Häufig werden sie erst spät diagnostiziert, was oft mit einer ungünstigen Prognose und einer durchschnittlichen 5-Jahres-Überlebensrate von nur ca. 41% verbunden ist [5]. Frühe und mehrfache Rezidive mit zunehmend geringerem Ansprechen auf die gängigen Behandlungsansätze wie Chemotherapie und zielgerichtete Therapien verschlechtern die Prognose darüber hinaus noch weiter.

Tumormarker wie beispielsweise CA-125 können bedingt zur Verlaufskontrolle und zum Monitoring für Therapieansprechen verwendet werden, sind aber weder spezifisch noch sensitiv genug, als dass sie sich zum Screening eignen würden. Da Ovarialkarzinome deshalb und wegen fehlender Frühsymptome häufig erst in fortgeschrittenen Stadien diagnostiziert werden, befasst sich die aktuelle Forschung intensiv mit der Tumorbiologie und Faktoren, die eine genauere Charakterisierung des jeweiligen Tumors und damit individuellere Therapieregime ermöglichen.

Das Mammakarzinom ist mit einer deutschlandweiten Neuerkrankungsrate von ca. 70.000 Patientinnen/Jahr der häufigste maligne Tumor der Frau, neigt zu Metastasen und Rezidivbildung und war 2015 die fünfthäufigste Todesursache von Frauen in Deutschland [6, 7]. Neben den seit Jahrzehnten bekannten Prognosefaktoren wie Tumorgröße, Lymphknotenbefall und histologisches Grading, wurden in letzter Zeit weitere, bisher allerdings noch nicht etablierte, tumorbiologische Prognosefaktoren identifiziert (z.B. Metastasen-assoziierte Gene wie MTA1 und MTA3). Durch immer genauere Charakterisierung des jeweiligen Tumors erhofft man sich im Sinne einer „maßgeschneiderten Behandlung“ (*tailored therapy*) anhand der jeweils vorhandenen oder fehlenden tumorbiologischen Faktoren den bestmöglichen Therapieansatz und damit die größtmögliche Therapieeffizienz zu erreichen.

## 2.1 Transkriptionsmodulatoren und das Metastasen-assoziierte Gen MTA1

Transkriptionsmodulatoren können in Transkriptionsvorgänge von DNA zu RNA eingreifen. Dabei interagieren sie mit weiteren Proteinen, die entweder als Co-Aktivatoren oder als Co-Repressoren wirken und damit die Expression des entsprechenden Gens fördern oder hemmen können [8]. Zu diesen Proteinen gehören die MTA-Proteine (**Abb. 1**), die 1994 erstmals

beschrieben und mit dem Metastasierungspotential von Tumorzellen in Zusammenhang gebracht wurden [9, 10]. Sie sind vorwiegend im Zellkern lokalisierte, DNA bindende Proteine und kommen in vielen Zellen unter anderem des weiblichen Genitaltrakts vor [9, 11-13].

Die physiologischen Aufgaben der MTA-Proteine als Co-Aktivatoren bzw. Co-Repressoren zur Regulation anderer Gene [14] sind unter anderem die Aufrechterhaltung des zirkadianen Rhythmus‘ [15], die Spermiogenese [16], der ovarielle Zyklus [17], Immunmodulation über Zytokine [18], und sie erfüllen bestimmte Funktionen bei der DNA-Reparatur [19].

Sie agieren über Histondeacetylasen (HDACs) als Transkriptionsrepressoren oder auch -aktivatoren [20-22]. MTA1 wurde als Bestandteil des HDAC1-Komplexes [23, 24] wie auch des NuRD- (nucleosome remodeling and histone deacetylation) Komplexes identifiziert [25-28].

Das zugrundeliegende MTA1-Gen (metastasis-associated gene 1) wurde zunächst als ein in Metastasen vermehrt exprimiertes Gen an einem Mammakarzinom-Modell der Ratte identifiziert [9], anschließend gelang auch die Sequenzierung im menschlichen Genom [28]. Weitere Studien zeigten eine Assoziation zwischen MTA1-(Über)expression und einer erhöhten Invasionsneigung sowie verstärkten Migration bei Ovarialkarzinomen [29], oralen Plattenepithelkarzinomen [30] und verschiedenen weiteren Karzinomen [31] wie Endometriumkarzinomen [32], Mammakarzinomen [33-35] und Kolonkarzinomen [36-38].

Zellbiologische Untersuchungen ergaben darüber hinaus, dass die Überexpression von MTA1 eine wichtige Voraussetzung für das sogenannte *anchorage-independent growth* ist. *Anchorage-independent growth* ist von besonderer Bedeutung für das Überleben von nicht ins Gewebe integrierten Zellen wie beispielsweise von im Blut oder in der Lymphe zirkulierenden Karzinomzellen [39].

Mittlerweile ist bekannt, dass MTA1 eine wichtige Funktion als Transkriptionsfaktor besitzt und dadurch andere Tumor-assoziierte Gene reguliert. Als eines dieser potentiellen Zielgene wurde von unserer Arbeitsgruppe das Leucin zipper downregulated in cancer 1-Gen (LDOC1) identifiziert.

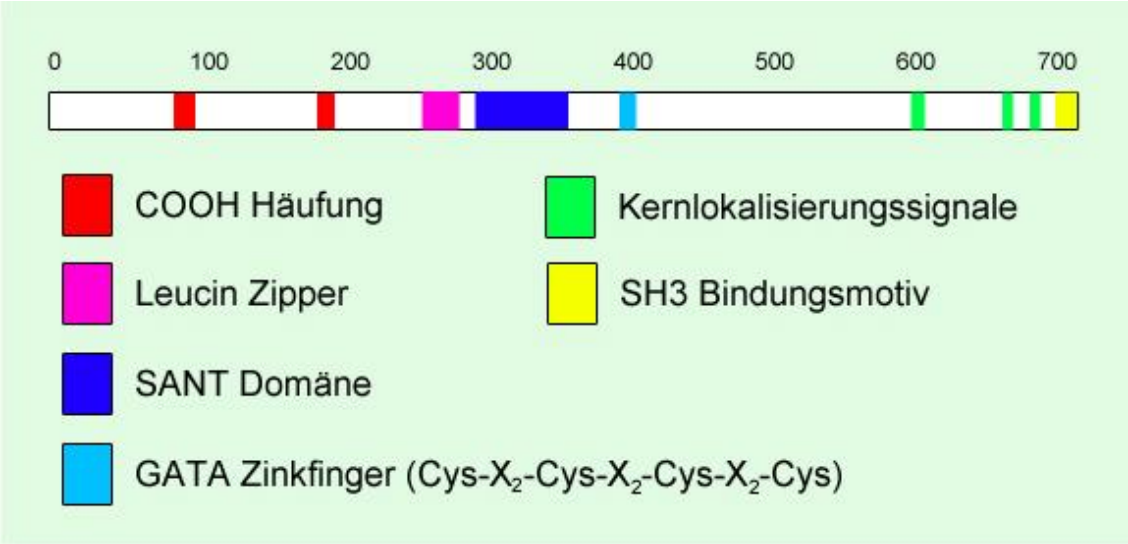


**Abb. 1: Struktur des MTA1-Proteins** *SANT: Swi3, Ada2, N-Cor, TFIIIB, codiert die Aminosäuren W (Tryptophan), F (Phenylalanin), Y (Tyrosin); SH3: Src-Homologie 3; COOH: Carboxygruppe; GATA-Sequenz: Guanin-Adenin-Thymin-Adenin-Sequenz; modifiziert nach [40]*

## 2.2 Das Leucin zipper downregulated in cancer 1-Gen (LDOC1)

Das Leucin zipper downregulated in cancer 1-Gen (LDOC1) wurde 1999 entdeckt [41], ist auf dem langen Arm des X-Chromosoms (Xq27.1) [42] lokalisiert (**Abb. 2**) und hat vermutlich die Funktion eines Tumorsuppressor-Gens. Daneben ist es mit anderen zytogenetischen Prognoseparametern assoziiert und bei chronischer lymphatischer Leukämie (CLL) ein Marker für eine ungünstige Prognose [43].

Das von LDOC1 codierte Protein war in Pankreaskarzinom-Zellen im Vergleich zu normalen pankreatischen Zellen, in Gewebeproben von Oesophaguskarzinomen und bei verschiedenen Arten von Leukämie deutlich vermindert nachweisbar [43-45].

Weiterhin ist bekannt, dass LDOC1 den nukleären Faktor Kappa B (NF-kappaB) reguliert [46-48]. Mittels NF-kappaB als Transkriptionsfaktor wiederum kann die Expression verschiedener Gene reguliert werden [49, 50], was insbesondere bei der Immunabwehr [51], dem programmierten Zelltod (Apoptose) [52] und auch bei der Kanzerogenese [53-55] eine Rolle spielt.

Über Interaktion mit dem TNF-α Signalweg [56-58] wird die Zelle zur Apoptose veranlasst und das Zellwachstum durch antiproliferative Effekte gehemmt. Sowohl NF-kappa B als auch TNF-α stellen wichtige Faktoren dar, die im Körper ubiquitär vorkommen; damit greift LDOC1 an einem sehr zentralen Mechanismus der Genregulation an [44].

LDOC1 selbst scheint durch epigenetische Modulatoren reguliert zu werden [59]. Allerdings ist die Datenlage noch sehr dünn, insbesondere die Expressionsanalyse in Tumorgeweben betreffend, was unter anderem auch der erst kürzlichen Entwicklung von geeigneten kommerziellen Antikörpern zuzuschreiben ist. Dementsprechend scheinen weitere Untersuchungen von LDOC1 sehr vielversprechend, um genauere Einblicke in tumorrelevante molekularbiologische Regulationswege zu erhalten und neue potentielle therapeutische Ansätze zu eruieren.

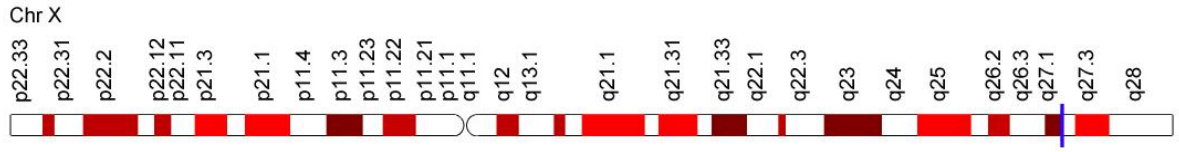


**Abb. 2: Lokalisation des LDOC1-Gens** *Der blaue Strich markiert die genaue Lokalisation des Gens. Chr X: X-Chromosom, modifiziert nach [42]*

# 3 EIGENE STUDIENERGEBINISSE

Bei unseren zuvor vorgenommenen Analysen der Zielgene des MTA1 Transkriptionsrepressors mittels sogenannter „*whole genome microarrays*“ fand sich eine größere Anzahl in ihrer Funktion stark unterdrückter Gene (Daten noch nicht publiziert), von denen allerdings nur wenige selektive Gene (unter anderem LDOC1) wegen ihrer bereits bekannten oder vermuteten Funktionen auf eine tumorrelevante Funktion schließen ließen. Aufgrund dessen, d.h. der bereits beschriebenen Repression des Gens in Karzinomen, schien das für weitere Untersuchungen interessanteste Zielgen LDOC1 zu sein, das einen Transkriptionsinhibitor darstellt und damit potentiell als Tumorsuppressorgen wirkt.

Ziel dieser Arbeit war es zum einen, den Einfluss von Regulationsmechanismen des Metastasen-assoziierten Gens MTA1 auf das Metastasierungspotential von Ovarial- und Mammakarzinomen zu untersuchen, zum anderen die Frage zu klären, ob das LDOC1-Gen von MTA1 reguliert wird, und schließlich die selektive Expression von LDOC1 mittels eines Expressionsplasmids, sowie die anschließende Identifizierung potentieller Zielgene dieses Tumorsuppressors.

## 3.1 Immunhistochemische und molekularbiologische Vorarbeiten: Untersuchungen an Zervixkarzinom-Zelllinien und Zervixkarzinom-Gewebeproben

Wir führten molekularbiologische Analysen mittels RT-PCR (Reverse Transkriptase-Polymerase-Kettenreaktion) an etablierten Zervixkarzinom-Zelllinien hinsichtlich der Expression von LDOC1 durch. Dabei zeigte sich in vier von sechs Zervixkarzinom-Zelllinien eine Inaktivierung dieses Gens.

Mit Hilfe der Bisulfit-Konvertierung [60, 61], einer Methode, bei der mittels einzelner durch Bisulfit-Behandlung ausgetauschter Basen zwischen methylierter und unmethylierter DNA unterschieden werden kann, konnten *in vitro* epigenetische Modifikationen in Form von Promotor-Methylierung [62-64] als ursächlich für die Inaktivierung des Genprodukts LDOC1 in mehreren gynäkologischen Tumor-Zelllinien [65] erkannt werden.

Die Re-Expression von LDOC1 nach Behandlung mit AdC (5-Aza-2′-Desoxycytidin; Decitabin), einem Inhibitor von DNA-Methyltransferasen [66, 67], der zur Therapie der akuten myeloischen Leukämie (AML) angewendet wird, konnte die Annahme einer epigenetischen Regulierung ebenfalls stützen: Indem die Methylierung der Promotorregion durch Decitabin verhindert wird, kann LDOC1 wieder in normalem Ausmaß exprimiert werden.

PCR-basierte epigenetische Untersuchungen und Re-Expressionsstudien zeigten somit einen signifikanten Zusammenhang zwischen der Methylierung des Promotors und einer stark reduzierten Expression von LDOC1, die durch DNA Methyltransferase-Inhibitoren aufgehoben werden konnte. Daraus konnten wir für das Zervixkarzinom schließen, dass die Inaktivierung von LDOC1 häufig vorkommt und möglicherweise als molekularer Marker für die Invasionsfähigkeit und das Metastasierungspotential dieser Tumorentität dienen kann.

Mittels eines LDOC1-Expressionsplasmids [68] konnte außerdem der Einfluss einer Überexpression von LDOC1 auf die Zellen veranschaulicht werden. Der resultierende proapoptotische Phänotyp deutet auf die tumorsuppressiven Eigenschaften von LDOC1 hin: Demnach wurde bei einer vermehrten LDOC1-Expression in vielen der malignen Zellen die Apoptose induziert, womit die weitere Tumorausbreitung verlangsamt wurde.

Wir führten zudem immunhistochemische Untersuchungen an Gewebeproben der Portio uteri durch, die im Rahmen der Primäroperation entnommen worden waren. In einzelnen Fällen konnte auch hier ein Verlust der LDOC1-Genexpression im Tumorgewebe, nicht aber im gesunden Gewebe nachgewiesen werden.

Diese Voruntersuchungen beim Zervixkarzinom bilden die Grundlage für die vorliegende Arbeit, bei der weitere immunhistochemische und molekulargenetische Analysen bezüglich MTA1 und LDOC1 für Ovarial- und Mammakarzinome durchgeführt wurden, die im Folgenden beschrieben werden.

## 3.2 Histologische Untersuchungen an Ovarialkarzinom-Gewebeproben

An der Klinik und Poliklinik für Frauenheilkunde und Geburtshilfe der LMU, Campus Innenstadt wurden 115 Ovarialkarzinomgewebe, die bezüglich des Tumorgradings (G1-G3) und des FIGO-Stadiums (FIGO I-IV) klassifiziert sind, mittels Immunhistochemie auf die Expression von MTA1, MTA3 und der Östrogenrezeptoren getestet. Dabei zeigte die

Auswertung der immunhistochemischen Reaktionen, dass beim Ovarialkarzinom die MTA1-Expression bei höherem Grading (G3) im Vergleich zu G1- oder G2-Tumoren hochsignifikant erhöht ist ($p < 0,05$), was der zunehmenden Entdifferenzierung der Tumorzellen entspricht (**Abb. 3**). MTA3 scheint beim Ovarialkarzinom dagegen eine geringere Rolle zu spielen. Auch bei vielen anderen Tumoren, einschließlich des Mammakarzinoms, konnten derartige Beobachtungen gemacht werden [20, 69].

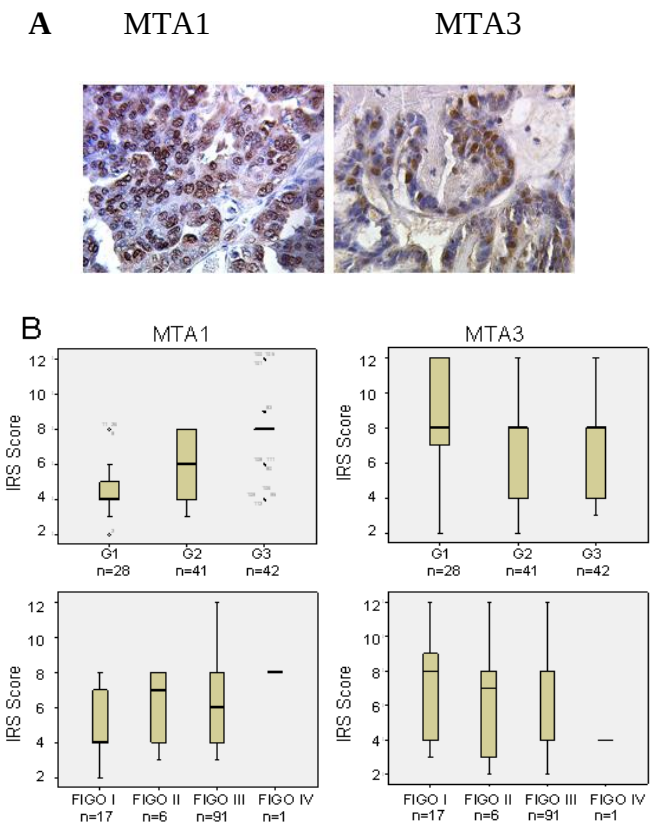


**Abb. 3: Expression von MTA1 und MTA3 bei unterschiedlichem Grading und in unterschiedlichen FIGO Stadien** *A: Immunhistochemische Anfärbung von MTA1 und MTA3, B: Boxplot-Analyse der MTA1- und MTA3-Expression (quantifiziert mittels IRS-Score) mit steigender MTA1-Expression bei gering differenzierten (G3) oder fortgeschrittenen (FIGO IV) Tumoren; aus [29], mit freundlicher Genehmigung durch Dr. A. Brüning*

## 3.3 Molekularbiologische Untersuchungen

Mittels spezifischer PCR Primer zur Amplifikation der cDNA von MTA1 und LDOC1 konnten diese beiden Gene von unserer Arbeitsgruppe jeweils in den Expressionsvektor pcDNA3.1/V5 (**Abb. 4**) kloniert werden. Dieser Vektor, der in Tumorzellen eingeschleust wird, damit sie anschließend die gewünschten Gene MTA1 und LDOC1 exprimieren, enthält daneben auch ein eingebautes Stopp-Codon. Als Promotor, der die Genexpression induziert, dient eine Sequenz, die aus dem Genom des Cytomegalievirus‘ (CMV) stammt [70-72] und zu einer starken Überexpression des klonierten MTA1- bzw. LDOC1-Gens führt. Durch das Anheften einer zusätzlichen Peptidsequenz, (V5-Tag), kann die Expression des exogenen MTA1 bzw. LDOC1 mittels eines hochspezifischen V5-Antikörpers verfolgt und vom endogenen MTA1 bzw. LDOC1 der Zelle unterschieden werden [73, 74]. Das V5-Tag erleichtert auch das Screening von stabil MTA1 (bzw. LDOC1) überexprimierenden Zellklonen, die mit diesen Expressionsvektoren gewonnen werden können.

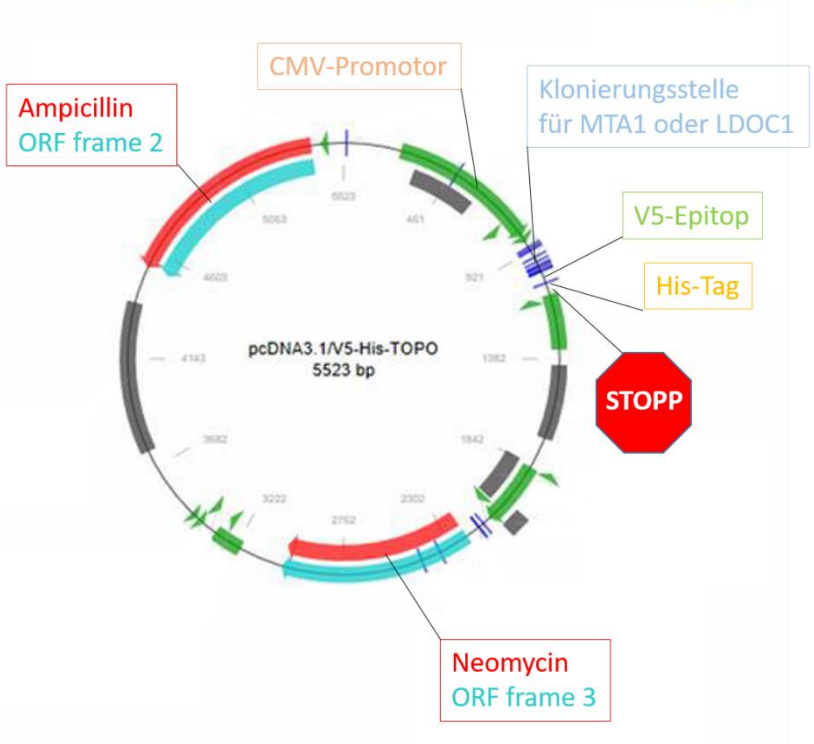


**Abb. 4: Der zur Klonierung von MTA1 und LDOC1 verwendete Expressionsvektor pcDNA3.1** *mit grafisch dargestellter Klonierungsstelle für das PCR-Produkt (MTA1 oder LDOC1), dem V5- und His-Tag, eingebautem Stopp-Codon und den Genen für die Selektionsmarker Neomycin und Ampicillin; ORF: open reading frame; modifiziert nach [75]*

## 3.4 Untersuchungen an Ovarialkarzinom-Zelllinien

Wie bei den Zervixkarzinom-Zellen beobachtet, fiel auch bei den Untersuchungen an Ovarialkarzinom-Zelllinien ein häufiger Verlust der LDOC1-Expression auf.

Insgesamt wurden bisher aus zwei Ovarialkarzinom-Zelllinien (OVCAR3 und OVMZ36) je zwei MTA1 überexprimierende Zellklone und zusätzlich zwei lediglich Neomycin-resistente Kontrollklone zum Vergleich gewonnen. **Abb. 5** zeigt die Charakterisierung dieser Klone mittels Western Blot Analyse.

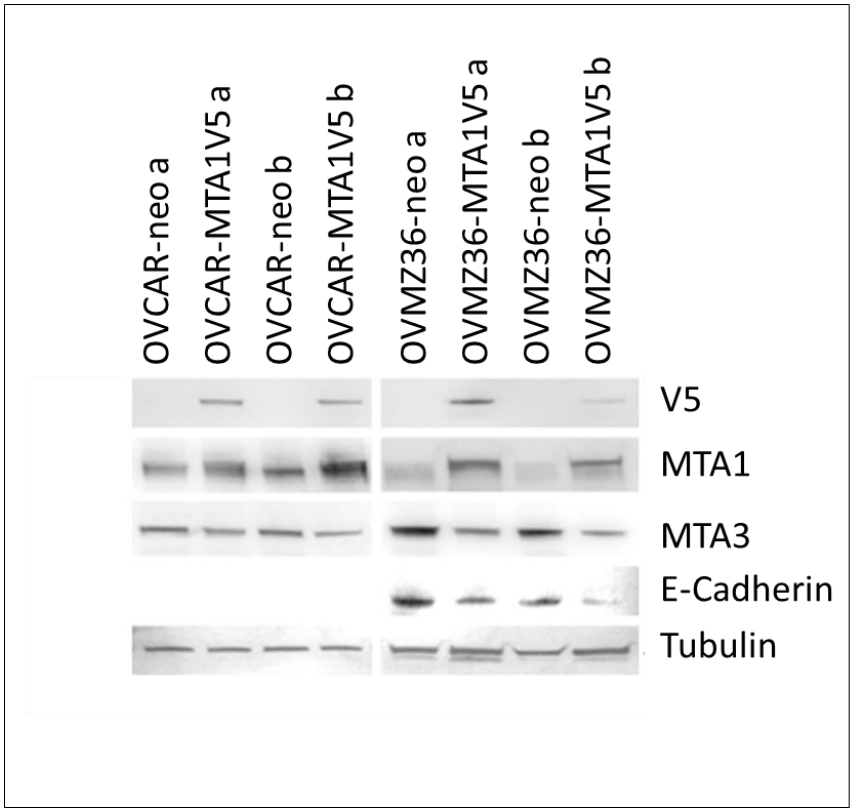


**Abb. 5: Charakterisierung MTA1 überexprimierender Ovarialkarzinom-Zellen** *Western Blot-Analyse MTA1 überexprimierender OVCAR3, bzw. OVMZ36 Zelllinien im Vergleich mit lediglich Neomycin-resistenten Kontrollzelllinien bzgl. der Expression von Metastasierungs-relevanten Genen; aus [29], mit freundlicher Genehmigung durch Dr. A. Brüning*

In einer zusätzlichen Microarray-Analyse fiel eine starke Minderexpression von LDOC1 in den MTA1 überexprimierenden Zellklonen auf. **Abb. 6** zeigt die Charakterisierung dieser Zellklone bezüglich der LDOC1-Expression mittels RT-PCR-Analyse.

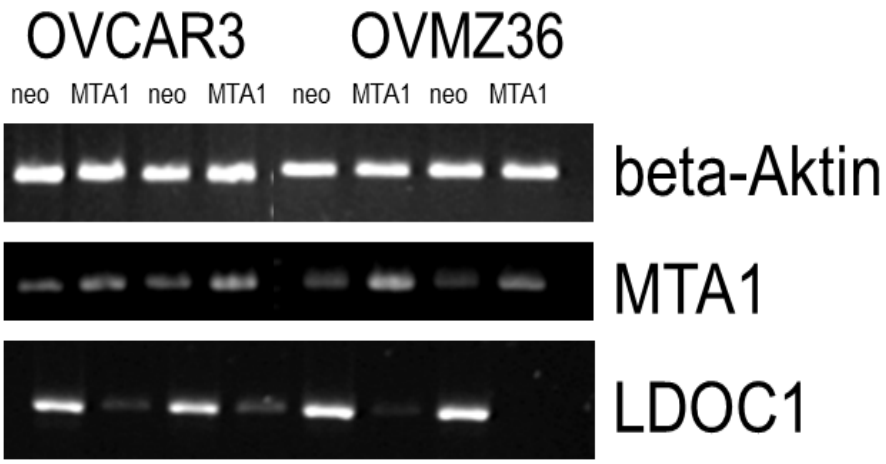


**Abb. 6: Expression von MTA1 und LDOC1 mittels RT-PCR** *Alle MTA1 überexprimierenden Zellklone zeigen eine geringere LDOC1-Expression.*

Bei Screening-Untersuchungen verschiedener Ovarialkarzinom-Zelllinien (u.a. OVMZ6, OVMZ30, OVMZ37) mittels RT-PCR auf die Expression von LDOC1 zeigte sich in vier der sieben untersuchten Ovarialkarzinom-Zelllinien ein kompletter Expressionsverlust des LDOC1-Gens.

Die Inaktivierung des Gens wurde allerdings nicht durch eine Gendeleletion ausgelöst, denn die genomische Sequenz war noch amplifizierbar (nicht dargestellt), sondern durch die Methylierung und damit Inaktivierung des betreffenden Promotors, wie sich durch Verwendung von für die Promotor-Methylierung spezifischen Primern nachweisen ließ.

Daneben gelang es uns, die Methylierung des Promotors zu verhindern und damit eine Reaktivierung des LDOC1-Gens zu erreichen: Nach Inkubation von LDOC1 negativen Ovarialkarzinom-Zelllinien mit AdC (Decitabin) war die Expression des LDOC1-Gens zu großen Teilen wieder in normalem Ausmaß vorhanden.

Wenngleich sowohl das MTA1- als auch das LDOC1-Gen wichtige Faktoren bei der Kanzerogenese und der zunehmenden Entdifferenzierung der Tumoren sind, konnten wir eine direkte Regulation von LDOC1 durch MTA1 nicht nachweisen: Die Regulation der LDOC1-Expression erfolgt durch Promotor-Methylierung und nicht durch MTA1. Es ist jedoch bekannt, dass Geninaktivierungen durch Promotor-Methylierung und Histon-Acetylierung,

(wodurch die Expression von MTA1 reguliert wird), teilweise gekoppelt sind. So konnte kürzlich eine für das Mammakarzinom wichtige Korrelation zwischen der MTA1-Expression und einer Methylierung des Östrogenrezeptor-Promotors nachgewiesen werden [76].

Durch diesen Synergismus wird vermutlich die Methylierung des LDOC1-Promotors in den OVCAR3- und OVMZ36-Zellen erleichtert, die durch Überaktivität von MTA1(-V5) in den Ovarialkarzinom-Zelllinien während des Klonierungs- und Selektionsprozesses bedingt ist. Somit wird *in vitro* ein Prozess beschleunigt, der sich in den Tumoren *in vivo* während der Kanzerogenese vollzieht. Eine direkte Inaktivierung von LDOC1 durch eine transiente MTA1-Überexpression konnte dagegen beispielsweise in HeLa-Zellen nicht beobachtet werden (nicht dargestellt).

## 3.5 Expression und Regulation von MTA1 in Mammakarzinom-Zellen

Die sogenannte epitheliale-mesenchymale Transition (EMT) [77, 78] gilt als ein typisches Charakteristikum bei der De-Differenzierung von Epithelzellen und ihrer Umwandlung zu zellkontaktunabhängigen, invasiv-malignen Zellformen. Als tumorrelevanter Transkriptionsfaktor ist MTA1 ein bedeutender Parameter auch bei dieser Umwandlung. Dabei erfolgt seine Regulation über Histondeacetylasen, und es sind bislang keine genetischen Deletionen bzw. epigenetischen Inaktivierungen von diesem Gen bekannt. Die Expressionsanalyse von MTA1 an neun Mammakarzinom-Zelllinien zeigte keinen Expressionsverlust, allerdings ein sehr unterschiedliches MTA1-Expressionsmuster (gekennzeichnet durch unterschiedliche Helligkeit der einzelnen Banden) unter diesen Zelllinien (**Abb. 7**).

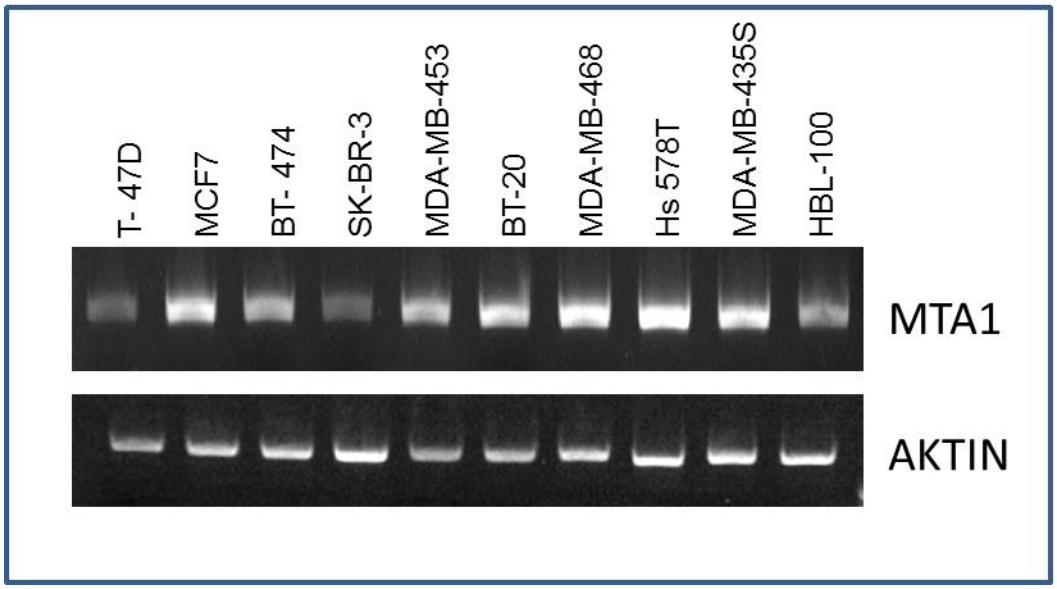


**Abb. 7: Expression von MTA1 in Mammakarzinom-Zelllinien** *Die cDNAs von 9 Mammakarzinom-Zelllinien (T-47D, MCF7, BT-474, SK-BR3, MDA-MB-453, BT-20, MDA-MB-468, Hs578T, MDA-MB-435S) und der HBL100 Zelllinie (s. auch **Abb. 9**) wurden mittels semiquantitativer Standard-PCR auf die Expression von MTA1 untersucht.*

Ein Zusammenhang zwischen einer (erhöhten) Expression des onkogenen Transkriptionsfaktors bzw. -repressors MTA1 und einer erniedrigten LDOC1-Expression konnte hierbei nicht festgestellt werden. Während sich bei Immunfluoreszenz-Analysen zum Nachweis von MTA1 auf Proteinebene in einigen Zelllinien eine für Transkriptionsfaktoren typische nukleäre Lokalisation zeigte, färbte sich das MTA1-Protein in mehreren Zelllinien auch im Zytoplasma an (**Abb. 8**, exemplarisch gezeigt für die Zelllinien MDA-MB-435 und MDA-MB-468). Dieses Phänomen war in mehreren, unabhängigen histologischen Untersuchungen vorhanden, und wurde mit unterschiedlichen Aktivitäten und Bindungspartnern von MTA1 im Zellkern bzw. im Zytoplasma erklärt [12, 79].

Je nach seiner Lokalisation in der Zelle (nukleär oder zytosolisch) übt das Protein wohl unterschiedliche Funktionen aus. So zeigten die Untersuchungen an der MDA-MB-435-Zelllinie – anders als bei der diffusen nukleoplasmatischen Fluoreszenz in Zellen der MDA-MB-468-Zelllinie – eine stark nukleäre Immunfluoreszenz, die auf eine hohe Aktivität von MTA1 in dieser Zelllinie hinweisen könnte.

Nach Behandlung mit Decitabin löste sich diese nukleäre Fluoreszenz in sich epithelial-differenzierenden MDA-MB-435 Zellen auf, und es kam zu einer verstärkt zytoplasmatischen Ansammlung von MTA1 (**Abb. 8**). Das deutet darauf hin, dass durch Decitabin induzierte

Differenzierungsprozesse Einfluss auf die Lokalisation und somit spezifische Aktivität von MTA1 haben. Somit konnte erstmals eine pharmakologisch wirksame und klinisch anwendbare Substanz identifiziert werden, mit der die Expression und Aktivität des Onkogens MTA1 reguliert werden kann.

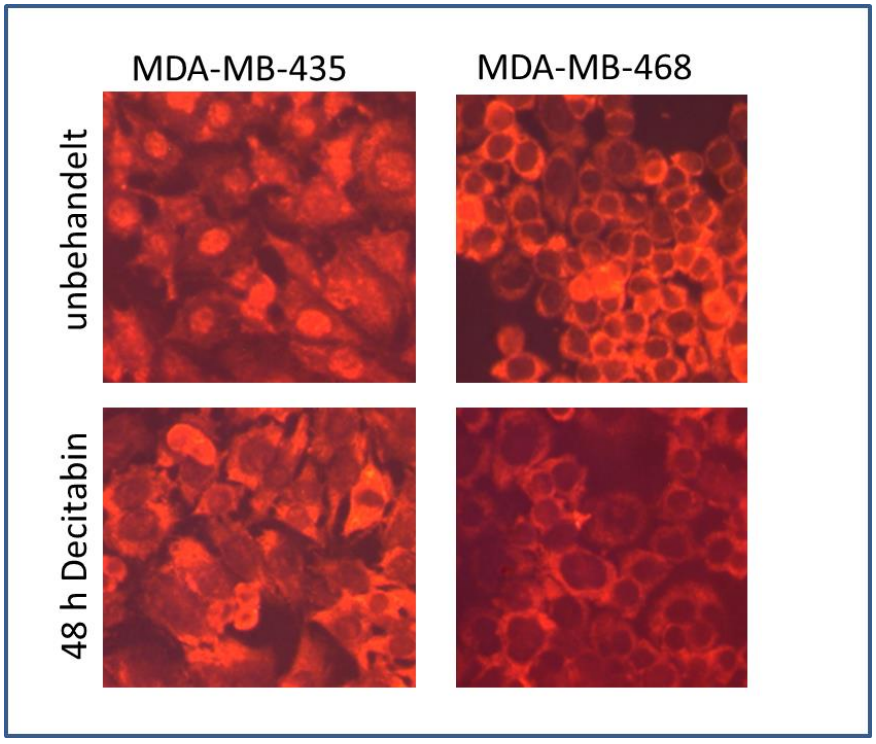


**Abb. 8: Zelluläre Lokalisation von MTA1 und durch Decitabin hervorgerufene Veränderungen** *MDA-MB-435-Zellen und MDA-MB-435-Zellen wurden teilweise mit 10 µg/ml Decitabin inkubiert und die Expression und Lokalisation von MTA1 mittels Immunfluoreszenz dargestellt. Unbehandelte MDA-MB-435-Zellen zeigen eine runde, nukleäre Immunfluoreszenz, während MDA-MB-468-Zellen bereits von vornherein eine zytoplasmatische Färbung aufweisen. Nach Decitabinzugabe zeigen MDA-MB-435-Zellen ebenfalls eine derartige zytoplasmatische Färbung. Alle Aufnahmen wurden bei gleicher Vergrößerung gemacht, d.h. die Zellen werden nach Decitabinzugabe auch insgesamt größer bzw. flacher und epithelialer.*

## 3.6 Untersuchungen der LDOC1-Expression an Mammakarzinom-Zelllinien

An Mammakarzinom-Zelllinien verschiedener Malignitätsgrade und Differenzierungsstufen konnte in ersten Analysen nachgewiesen werden, dass die Expression des potentiellen Tumorsuppressor-Gens LDOC1 beim Mammakarzinom ebenfalls inhibiert werden kann (**Abb. 9**), wenngleich in weniger Fällen als beim Zervix- bzw. Ovarialkarzinom.

Auffallend war bei den Mammakarzinom-Zelllinien jedoch, dass der LDOC1-Expressionsverlust vor allem bei den teilweise therapieresistenten, sogenannten *triple-negativen* Mammakarzinom-Zellen (keine Expression von Hormonrezeptoren, keine HER2-Überexpression) zu beobachten war.

Damit könnte LDOC1 insbesondere beim triple-negativen Mammakarzinom als diagnostischer Marker bzw. Ansatzpunkt für individualisierte Therapiekonzepte dienen.

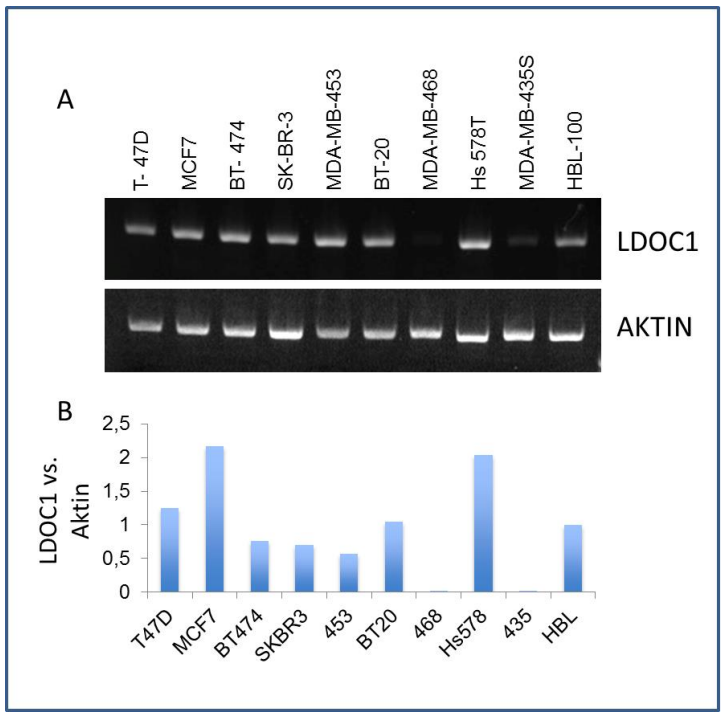


**Abb. 9: Expression von LDOC1 in Mammakarzinom-Zellen** *9 etablierte Mammakarzinom-Zelllinienwurden mittels semiquantitativer PCR* ***(A)*** *bzw. quantitativer real-time PCR* ***(B)*** *auf die Expression von LDOC1 untersucht. Zur Qualitätskontrolle der cDNA der Mammakarzinom-Zelllinien wurde die Expression von β-Aktin gemessen. T47D, MCF7 und BT474: Hormonrezeptor positiv („luminal"); SKBR3 und MDA-MB-453: HER2 überexprimierend; BT20, MDA-MB-468, Hs578 und MDA-MB-435: „triple negativ"; HBL-100: Myoepithel-Zelllinie*

### 3.6.1 Verlust der LDOC1-Expression bei triple-negativen Mammakarzinom-Zellen

Zu Beginn der Arbeiten wurden neun etablierte Mammakarzinom-Zelllinien mittels RT-PCR auf die Expression von LDOC1 untersucht (**Abb. 9**). Als Zelllinien wurden dabei jeweils typische Vertreter für die aktuell verwendeten histopathologischen Kriterien (Hormonrezeptorstatus, HER2-Überexpression) selektiert [80, 81].

Die HBL100 Myoepithel-Zelllinie wurde zusätzlich als nicht-maligne Kontrollzelllinie verwendet, da man nach gegenwärtigem Kenntnisstand diesen Zelltypus als Ausgangszelltyp für sogenannte basale Mammakarzinome ansieht, die histopathologisch wiederum meistens als *triple-negative* Mammakarzinome identifiziert werden.

Auffallend beim Screening der Mammakarzinom-Zelllinien war das Fehlen der LDOC1-Expression bei zweien dieser neun Zelllinien (MDA-MB-468 und MDA-MB-435), die der Gruppe der *triple-negativen* bzw. basalen Mammakarzinom-Zellen angehören. Auch wenn der Verlust der LDOC1-Expression nicht als typisches Kriterium des Mammakarzinoms bezeichnet werden kann, so könnte die Häufung dieses Phänomens gerade beim aggressiveren *triple negativen* Phänotyp des Mammakarzinoms eventuell von diagnostischer oder sogar therapeutischer Bedeutung sein (siehe unten).

### 3.6.2 Regulation der LDOC1-Expression durch Promotor-Methylierung

Da eine Untersuchung der genomischen DNA der Zelllinien keine Deletion des LDOC1-Gens in den Zelllinien MDA-MB-468 und MDA-MB-435 erbrachte (nicht dargestellt), musste eine sogenannte epigenetische Regulation vermutet werden. Bei Ovarial- und Zervixkarzinom-Zellen wurde von unserer Arbeitsgruppe bereits die Inaktivierung der LDOC1-Expression durch Promotor-Methylierung beschrieben [65, 82].

Promotor-Methylierungen können durch das epigenetisch wirkende Zytostatikum und Nukleosidanalogon Decitabin rückgängig gemacht werden, und die Inkubation von MDA-MB-468 und MDA-MB-435 Zellen mit Decitabin (10 µg/ml für 2 Tage) zeigte dementsprechend eine Re-Expression von LDOC1 in diesen beiden Zelllinien (**Abb. 10**).

Somit ist davon auszugehen, dass der LDOC1-Verlust in diesen Zelllinien nicht genetisch, sondern epigenetisch bedingt ist. Letzteres lässt auch die Hoffnung zu, LDOC1 als

Tumorsuppressor in *triple-negativen* Mammakarzinom-Zellen mittels Decitabin oder ähnlich wirkender Medikamente, die sich zur Zeit in der Entwicklung befinden, wieder re-exprimieren zu können, um somit den benignen Phänotyp zumindest teilweise wieder herzustellen und damit das Tumorwachstum zu hemmen.

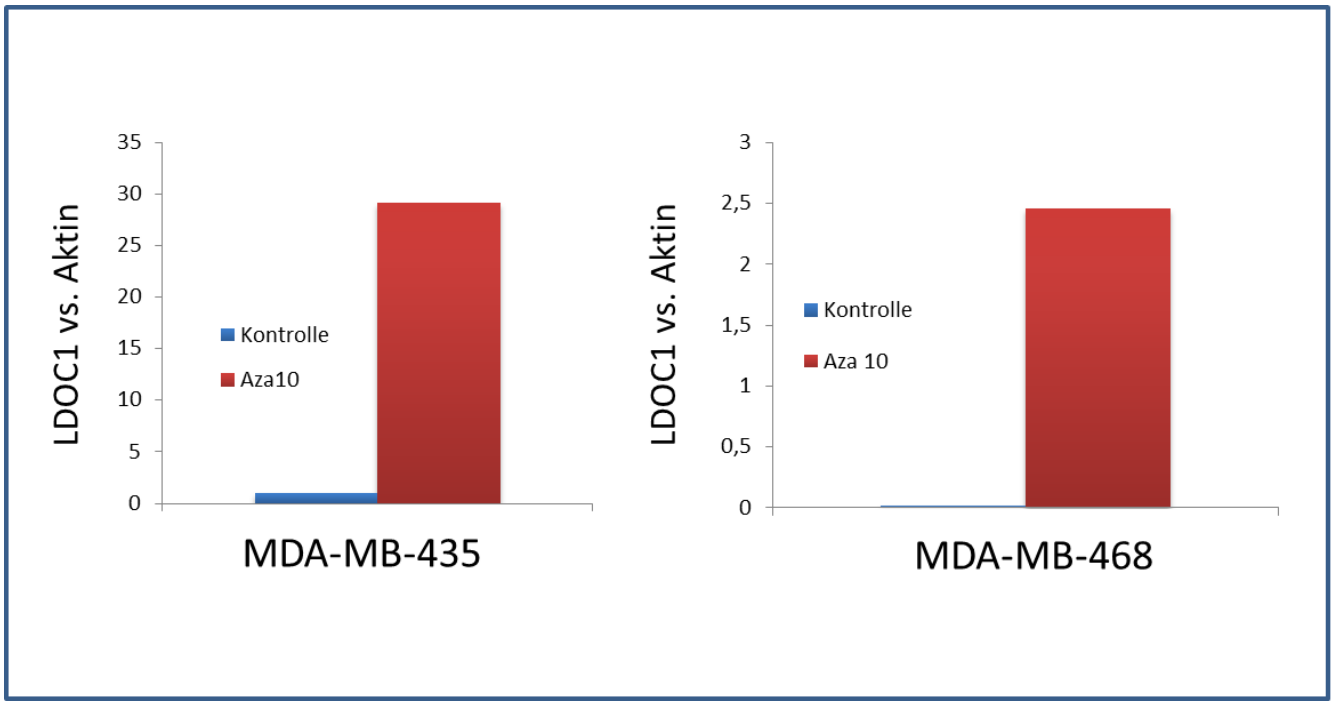


**Abb. 10: Re-Expression von LDOC1 durch Decitabin** *MDA-MB-468- und MDA-MB-435-Zellen wurden teilweise mit 10 µg/ml Decitabin inkubiert und anschließend mittels quantitativer real-time PCR auf die Expression von LDOC1 untersucht. Zur Qualitätskontrolle der cDNA der Mammakarzinom-Zelllinien wurde die Expression von β-Aktin gemessen. Unbehandelte Zellen (Kontrolle) zeigen keine LDOC1-Expression.*

Ein weiterer Effekt dieser epigenetisch wirksamen Medikamente ist die Umkehrung einer Vielzahl anderer im Verlauf der Tumorprogression entstandener Gen-Inaktivierungen, von denen LDOC1 nur einen von mehreren epigenetisch regulierten Faktoren darstellt.

Tatsächlich fiel bei den Versuchen in Zellkultur eine markante optische Veränderung auf, bei der sich unter dem Einfluss von Decitabin die Zellen von einer eher spindelförmigen (fibroblastoiden) oder kugeligen zu einer eher flachen (epithelialen) Zellform differenzieren (**Abb. 11**). Da epigenetische Medikamente wie Decitabin, anders als traditionelle Zytostatika, nicht direkt den Zelltod der Tumorzellen induzieren, werden hiermit gerade solche (Re-)Differenzierungsprozesse zu einem epithelialen Phänotyp zu erreichen versucht.

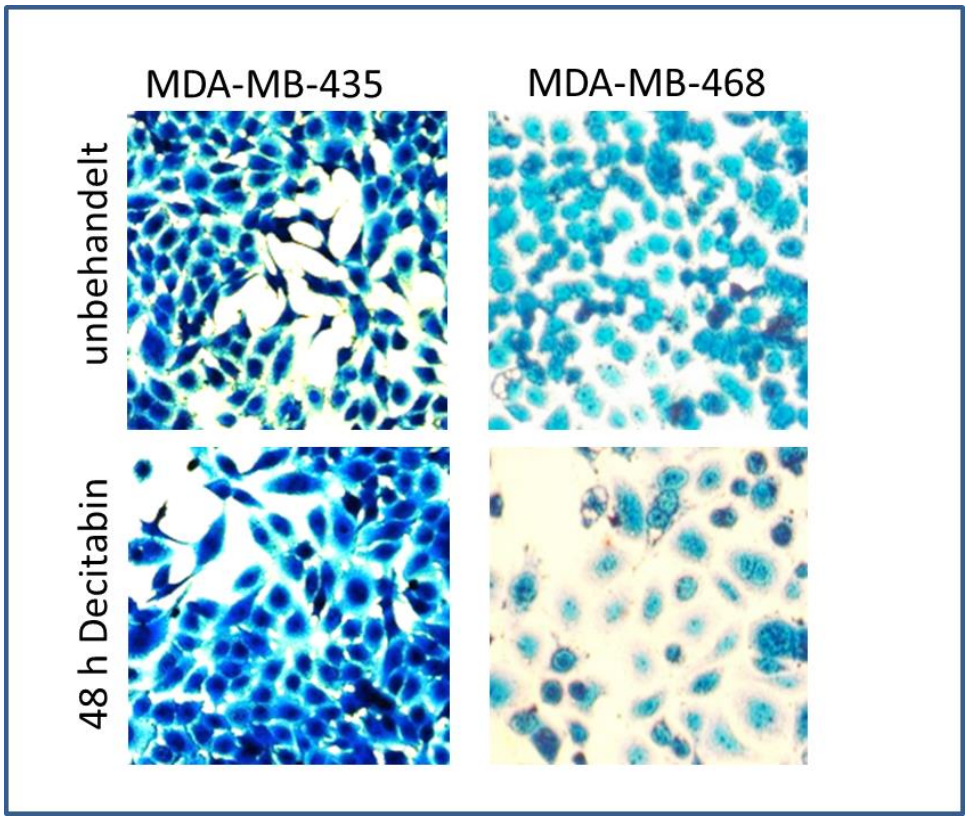


**Abb. 11:** **Morphologische Veränderungen durch Decitabin** *MDA-MB-468- und MDA-MB-435-Zellen wurden teilweise mit 10 µg/ml Decitabin inkubiert und zur besseren Sichtbarmachung mit Coomassie-Farbstoff blau gefärbt.*

### 3.6.3 Re-Expression von LDOC1 mittels Expressionsplasmid

Im Rahmen der vorliegenden Arbeit war eine der Fragestellungen bezüglich des LDOC1-Gens, ob es möglich ist, dieses Gen in als LDOC1 negativ identifizierten Mammakarzinom-Zellen mittels eines Expressionsplasmids selektiv zu re-exprimieren, um so dessen Funktion zu untersuchen und potentielle neue Zielgene zu identifizieren.

Ein LDOC1 codierendes Plasmid wurde in unserem Labor generiert, allerdings konnten keine dauerhaft LDOC1 re-exprimierenden Zellklone entwickelt werden, da diese Zellen regelmäßig apoptotisch wurden. Dadurch scheinen bisherige Erkenntnisse bestätigt zu werden, dass es sich bei LDOC1 um ein wachstumshemmendes Tumorsuppressorgen handelt, das in malignen Zellen zum programmierten Zelltod führt.

Die in den Analysen mit Decitabin gemachten Beobachtungen bestätigen diese Vermutung ebenfalls, denn nach Behandlung der Zellen mit diesem Nukleosidanalogon wurde nicht nur die Re-Expression von LDOC1 erreicht, sondern die betroffenen Zellen veränderten auch ihre Form in Richtung eines höher differenzierten Phänotyps, was auf die teilweise Wiederherstellung benigner Eigenschaften schließen lässt.

# 4 EIGENE PUBLIKATIONEN ZUM THEMA

## 4.1 Das Metastasen-assoziierte Gen MTA3 ist beim fortgeschrittenen endometrioiden Adenokarzinom herunterreguliert.

Brüning A, **Jückstock J**, Blankenstein T, Makovitzky J, Kunze S, Mylonas I

***The metastasis-associated gene MTA3 is downregulated in advanced endometrioid adenocarcinomas.***

Histol Histopathol. 2010 Nov;25(11):1447-56

**Zusammenfassung:**

Im vorliegenden Text werden Untersuchungen an Gewebeproben von Endometriumkarzinomen auf die MTA3-Expression und einen potentiellen Zusammenhang dieser Expression mit dem Östrogenrezeptor alpha und beta beschrieben. Außerdem untersuchten wir die prognostische Relevanz von MTA3 hinsichtlich progressionsfreiem, tumorbedingtem und Gesamtüberleben, die sich jedoch nicht bestätigte.

Durch immunhistochemische Reaktionen [83-89] an 200 Gewebeproben von Endometriumkarzinomen [86, 90] konnten wir zeigen, dass die Expression von MTA3 in gering differenzierten Karzinomen (G3 nach TNM) [91] signifikant niedriger ist, als in G1- oder G2-Tumoren ($p < 0,05$). Das korreliert mit dem größeren Metastasierungspotential und dem verstärkt invasiven Wachstum von G3-Karzinomen [92, 93]. Allerdings ließ sich keine Korrelation zwischen der MTA3-Expression und dem Östrogenrezeptor nachweisen.

Da MTA3 keinen unabhängigen prognostischen Faktor darstellt, eignet es sich nicht zur Identifizierung von Hochrisikopatientinnen. Trotzdem ist es, wenngleich in noch unbekannter Weise, in die Karzinogenese von Endometriumkarzinomen involviert. Nachdem die MTA3 nachgeschalteten Signalwege noch nicht im Detail bekannt sind [29, 94-96], sollten weiterreichende Untersuchungen auf diesem Gebiet zum Ziel haben, die Rolle von MTA3 beim Endometriumkarzinom vollständig aufzuklären.

Histol Histopathol (2010) 25: 1447-1456

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Histology and Histopathology

Cellular and Molecular Biology

# The metastasis-associated gene MTA3 is downregulated in advanced endometrioid adenocarcinomas

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**Summary.** The metastasis-associated gene MTA3 has an important function in invasion and metastasis of human cancer cells. Therefore, the aim of this study was to investigate the expression of this protein in endometrial adenocarcinomas and to analyse potential correlations between this nuclear transcription factor and estrogen receptors in endometrial adenocarcinomas. Additionally, we evaluated whether MTA3 might be a prognostic parameter in endometrioid adenocarcinomas. Endometrioid adenocarcinomas were obtained from 200 patients and immunohistochemically analysed for MTA3 and estrogen receptor alpha and beta (ER-alpha and ER-beta) expression. Overall, endometrioid adenocarcinomas of histological differentiation grade 3 demonstrated a significantly lower expression of MTA3 compared to carcinomas of histological grade 1 and 2 ($p<0.05$). MTA3 expression is reduced in endometrioid adenocarcinomas of poor differentiation, though without any correlation to ER-alpha and ER-beta expression. Furthermore, the expression of MTA3 did not affect progression-free, cause-specific and overall survival. Overall, MTA3 did not constitute an independent prognostic factor in this study, suggesting that MTA3 is not a useful marker to assess and identify high-risk patients with endometrial adenocarcinomas. Still, the downregulation of MTA3 predispose this cell type to be of high metastatic potential after malignant transformation, playing an essential, but as yet unknown role in human endometrial carcinogenesis.

**Key words:** MTA3, Endometrioid adenocarcinomas, Immunohistochemistry, Estrogen receptors, ER-alpha, ER-beta, Survival, Prognosis

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## Introduction

Endometrial cancer has become the most frequent gynaecologic malignancy in the Western World (Abeler and Kjorstad, 1991; Rose, 1996; Prat, 2004; Amant et al., 2005; Chan et al., 2007). An incidence of 15-20 cases/100.000 women/ year has been estimated with a life time risk to develop this type of cancer being approximately 2.5% (Gloeckler Ries et al., 2003). Meanwhile, several prognostic factors, such as histological type, histologic grade, surgical stage, pelvic lymph node involvement and myometrial invasion have been established (Abeler and Kjorstad, 1991; Rose, 1996; Prat, 2004; Amant et al., 2005; Chan et al., 2007). Although endogenous and exogenous sources of unopposed estrogen increase the risk of endometrial adenocarcinoma, the molecular pathogenesis of endometrial carcinoma remains unclear (Sherman, 2000; Prat, 2004). Furthermore, although more than 50% of patients with endometrial carcinoma are diagnosed at an early stage, as many as 20% die of their disease (Jereczek-Fossa et al., 1999). The reason for this unusual situation compared to other solid tumours is still unclear.

Major characteristics of cancer progression are thought to be invasion into connective tissues, transmigration through blood vessels and the capability of neoangiogenesis (Hanahan and Weinberg, 2000). These changes are often accompanied by the so called epithelial-mesenchymal transition (EMT) that has been described to play an essential role during cancer cell progression (Hugo et al., 2007). The EMT is accompanied by a shift in gene expression, most apparently by that of cell adhesion molecules (Vicovac and Aplin, 1996; Moustakas and Heldin, 2007). The expression of these cell adhesion proteins is predominantly regulated by nuclear transcription factors such as MTA1, MTA3, and SNAIL. These transcription regulators are nuclear proteins that mediate gene silencing by binding to histone deacetylases (Yao and

Yang, 2003).

The metastasis-associated gene 1 (MTA1) has been described to be upregulated in several types of human cancer tissues (Manavathi et al., 2007). In contrast, the expression of MTA3 has been found to be reduced in breast cancer (Fujita et al., 2003) and ovarian cancer (Dannenmann et al., 2008). We have recently demonstrated that MTA1 is upregulated in advanced ovarian cancer and regulates the expression of E-cadherin, in addition to that of angiogenic cytokines (Dannenmann et al., 2008). A significant upregulation of MTA1 in endometroid carcinomas has recently been described (Balasenthil et al., 2006) and suggests an important function of MTA proteins in endometroid cancer development, although no studies on MTA3 expression in endometrial cancer have been performed yet. Additionally, MTA3 expression in breast cancer cells is enhanced by estrogen receptor activity (Fujita et al., 2003), linking MTA3 and steroid hormone receptors. Therefore, we investigated the expression of MTA3 and the correlation between this nuclear transcription factor and estrogen receptors alpha (ER-α) and beta (ER-ß) in endometrial adenocarcinomas. Additionally we evaluated whether MTA3 might be a prognostic parameter in endometrioid adenocarcinomas.

## Materials and methods

### *Tissue samples*

Pathological and surgical records of 200 patients who had been operated in the 1st Department of Obstetrics and Gynaecology, Ludwig-Maximilians-University Munich between 1990 and 2002 were reviewed for this retrospective analysis. Only specimens with an endometrioid adenocarcinoma were included, while other histological types, including that of non-endometrioid histology, mucinous adenocarcinoma and mixed adenocarcinomas were excluded in this analysis. The evaluated patient group has been previously well characterised and an evaluation for several prognostic markers has been performed (Shabani et al., 2007a; Mylonas et al., 2009). Patients with endometrial adenocarcinoma received modified radical hysterectomy, salpingo-oophorectomy or selective pelvic lymph-adenectomy, with or without para-aortic lymph-adenectomy.

All hematoxylin and eosin–stained slides were re-reviewed by a gynaecological pathologist to verify the diagnosis, histological grade, histological type, FIGO stage, lymphangiosis, adnexal or cervical involvement as previously described (Shabani et al., 2007a; Mylonas et al., 2009). Pathological stage and histological subtype were determined for each surgical specimen according to the 1988 International Federation of Gynecology and Obstetrics (FIGO) criteria (FIGO, 1989).

Patients with endometrial carcinoma received modified radical hysterectomy, salpingo-oophorectomy or selective pelvic lymphadenectomy, with or without para-aortic lymphadenectomy. Lymph node sampling or dissection was generally performed in patients having tumours with deep myometrial invasion and/or high-grade or aggressive histological features. Obesity, advanced age and excessive comorbidity were factors against full surgical staging.

Patient data were obtained from three sources: hospital tumour registry, automated database and chart review as previously described (Shabani et al., 2007a; Mylonas et al., 2009). All cases of recurrence had radiographic evidence of disease or biopsy-proven progression of disease. Only the records of patients who died of disease were considered to be uncensored; the records of all patients who were alive at follow-up or who did not die of disease (or a related cause) were considered to be censored. Additionally, censored cases were also considered those cases where the exact cause of death was unknown but died within two years after the diagnosis of a metastatic lesion (Shabani et al., 2007a; Mylonas et al., 2009).

### *Immunohistochemistry*

Immunohistochemistry was performed using a combination of microwave-oven heating and the standard streptavidin-biotin-peroxidase complex using the mouse-IgG-Vectastain Elite ABC kit (Vector Laboratories, Burlingame, California, USA) as previously described for steroid receptors (Mylonas et al., 2004, 2005, 2007; Shabani et al., 2007a,b) and rabbit-IgG-Vectastain Elite ABC kit (Vector Laboratories, Burlingame, California, USA) for MTA3 (Brüning et al., 2009). For positive controls, sections of human breast cancer tissue and normal colon were used, while human ileum served as negative control tissue.

Briefly, paraffin-fixed tissue sections were dewaxed using xylol for 15 min, rehydrated with decreasing alcohol-water mixtures, and subjected to antigen retrieval on a high setting for 10 min in a pressure

**Table 1.** Antibodies used for immunohistochemical characterization of endometrial adenocarcinomas.

| Antibody | Clone | Isotype | Dilution | Source |
|---|---|---|---|---|
| MTA3 | | rabbit polyclonal antibody | 1:500 | Calbiochem, Darmstadt, Germany |
| ER-α | 1D5 | mouse $IgG_1$ | 1:150 | Immunotech, Hamburg, Germany |
| ER-ß | PPG5/10 | mouse $IgG_{2a}$ | 1:50 | Serotec, Oxford, United Kingdom |

ER: estrogen receptor

cooker in sodium citrate buffer (pH 6.0), containing 0.1 M citric acid and 0.1 M sodium citrate. After cooling, the slides were washed twice in PBS. Endogenous peroxidase activity was quenched by immersion in 3% hydrogen peroxide (Merck, Darmstadt, Germany) in methanol for 20 min. Non-specific binding of the primary antibodies was blocked by incubating the sections with diluted horse serum (10 ml PBS with 150 $\mu$l horse serum, provided by Vectastain Elite ABC kit) for 20 min at room temperature. Sections were then incubated at room temperature for 60 min with the primary antibodies (Table 1). ER-α and MTA3 were diluted in dilution-medium (Dako, Glostrup, Denmark) while ER-ß was diluted in PBS. After washing with PBS, the slides were incubated in diluted biotinylated anti-serum secondary antibody for a further 30 min at room temperature (10 ml PBS, 50 $\mu$l horse serum). After incubation with the avidin–biotin peroxidase complex (diluted in 10 ml PBS, provided by Vectastain Elite ABC kit) for another 30 min and a repeated washing step with PBS, visualisation was performed with ABC substrate (Vectastain Elite ABC kit) and chromogenic 3,3ß-diaminobenzidine (DAB; Dako, Glostrup, Denmark) for 8–10 min. The slides were further counterstained with Mayer's acidic haematoxylin and washed in an alcohol multiple-row (50–98%). After xylol treatment the slides were embedded. Negative controls were performed by replacing the primary antibody with normal mouse serum. Positive controls for ER-α and MTA3 include human invasive breast cancer. The ER-ß antibody was tested positive on human colon tissue. Positive cells showed a brownish colour, and negative controls, as well as unstained cells, were blue.

### *Immunohistochemical evaluation*

The intensity and distribution patterns of specific MTA3, ER-α and ER-ß immunohistochemical staining reaction was evaluated by two blinded, independent observers, including a gynaecological pathologist, using a semi-quantitative score as previously described, and used to assess the expression pattern of steroid receptors in normal and pathological endometrial tissue (Mylonas et al., 2000, 2004, 2005, 2007; Shabani et al., 2007a,b).

The immunoreactive score (IRS) score was calculated by multiplication of optical staining intensity (graded as 0 = no, 1 = weak, 2 = moderate and 3 = strong staining) and the percentage of positive stained cells (0 = no staining, 1 = <10% of the cells, 2 = 11-50% of the cells, 3 = 51-80% of the cells and 4 = >81% of the cells) as previously described (Remmele and Schicketanz, 1993). Sections were examined using a Leica (Solms, Germany) photomicroscope. Digital images were obtained with a digital camera system. The IRS-scores of MTA3, ER-α and ER-ß were compared using the non-parametric Mann-Whitney-U test. Correlations were assessed using the Spearman rank correlation test. Significance of differences was assumed at p≤0.05 at the two-sided test. The Statistical Package for the Social Sciences computer software (version 16.0; SPSS Inc., Munich, Germany) was used.

### *Statistical analysis*

For the purposes of statistical survival analysis, MTA3 expression in tumor samples was considered to be elevated if the immunoreactive score was >3 (median for MTA3=3). ER-α expression in tumour samples was considered to be elevated if >10% positive staining was

**Table 2.** Clinicopathological characteristics of the analyzed endometrial adenocarcinomas.

| Clinicopathological characteristics | | | Total (n= 200) |
|---|---|---|---|
| Age | ≤65 years | | 98 (49%) |
| | >65 years | | 102 (51%) |
| FIGO | FIGO I | FIGO Ia 25 (12,5%) | 160 (80%) |
| | | FIGO Ib 94 (47%) | |
| | | FIGO Ic 41 (20,5%) | |
| | FIGO II | FIGO 2a 2 (1%) | 15 (7,5%) |
| | | FIGO 2b 13 (6,5%) | |
| | FIGO III | FIGO 3a 8 (4%) | 18 (9%) |
| | | FIGO 3b 3 (1,5%) | |
| | | FIGO 3c 7 (3,5%) | |
| | FIGO IV | | 7 (3,5%) |
| Grading (WHO) | Grade 1 | | 122 (61%) |
| | Grade 2 | | 54 (27%) |
| | Grade 3 | | 24 (12%) |
| LN status | negative | | 133 (66,5%) |
| | positive | | 9 (4,5%) |
| | unknown | | 58 (29%) |
| LVSI | negative | | 183 (91,5%) |
| | positive | | 17 (8,5%) |
| Lymphangiosis | | negative 183 (91,5%) | |
| | | positive 17 (8,5%) | |
| Haemangiosis | | negative 194 (97%) | |
| | | positive 6 (3%) | |
| myometrial invasion | Only endometrium | | 27 (13,5%) |
| | <50% myometrial invasion | | 108 (54%) |
| | >50% myometrial invasion | | 65 (32,5%) |
| Cervical Invasion | negative | | 177 (88,5%) |
| | positive | | 23 (11,5%) |
| Ovarial invasion | negative | | 185 (92,5%) |
| | positive | | 15 (7,5%) |
| Adipositas | negative | | 125 (62,8%) |
| | positive | | 74 (37,2%) |
| Diabetes | negative | | 174 (87%) |
| | positive | | 26 (13%) |
| Hypertension | negative | | 119 (59,5%) |
| | positive | | 81 (40,5%) |
| Radiotherapy | negative | | 127 (63,5%) |
| | positive | | 73 (36,5%) |
| Anti-hormone therapy | negative | | 193 (96,5%) |
| | positive | | 7 (3,5%) |

LVSI: lymphovascular space invasion.

observed (IRS>2) (Shabani et al., 2007a; Jongen et al., 2009), while positive ER-ß expression was suggested if IRS>1 (median for ER-ß = 0) as previously suggested (Shabani et al., 2007a). For the evaluation of the MTA3 and ER-ß staining intensity the median for all tumour samples was used. Increased/positive versus not increased/negative immunostaining in tumour samples was compared using the $\chi^2$ test and the exact Fisher's test where applicable.

The outcomes analyzed were progression-free survival, cause-specific survival and overall survival. Univariate analysis was performed with Kaplan-Meier life-table curves to estimate survival (Kaplan and Meier, 1958) and were compared using the log-rank test. Prognostic models used multivariate Cox regression analysis for multivariate analyses of survival. The data were adjusted for age (≤65 years vs. >65 years), surgical stage (FIGO I/II vs. III/IV), histological grade (grade 1/2 vs. 3), lymph node status (negative vs. positive), lymphovascular space invasion (negative vs. positive), myometrial invasion (<50% vs. >50%), cervical invasion (negative vs. positive), ovarial invasion (negative vs. positive), ER-α (negative vs. positive), ER-α (negative vs. positive) and MTA3 (negative vs. positive) status. The variables were entered in a forward stepwise manner (Cox, 1972). Significance of differences was assumed at p≤0.05 (SPSS version 16.0; SPSS Inc., Chicago, IL).

## Results

### *Clinicopathological characterization*

The clinicopathological characteristics of the patients with endometrioid adenocarcinomas are summarized in Table 2. The median patient age at the

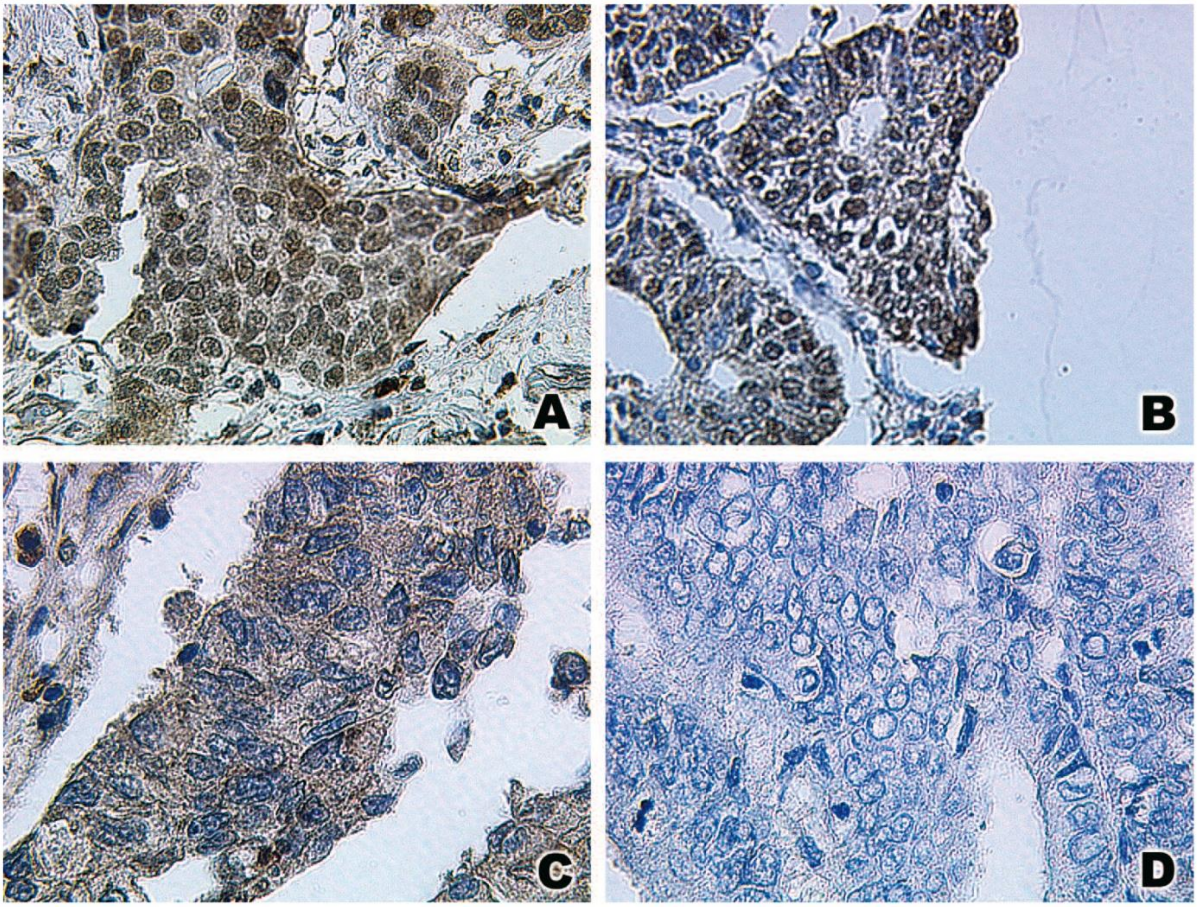


**Fig. 1.** Immunohistochemical staining reaction of MTA3 in endometrioid adenocarcinomas (**A**). Strong positive immunohistochemical staining reaction in mammary carcinoma that served as positive control. Endometrioid adenocarcinoma grade 1 expressed MTA3 with moderate to strong intensity, similar to grade 2 adenocarcinomas **(B, C)**. However, endometrioid adenocarcinoma grade 3 showed minimal to no expression of MTA3 **(D)**. A, B, x 250; C, D, x 400

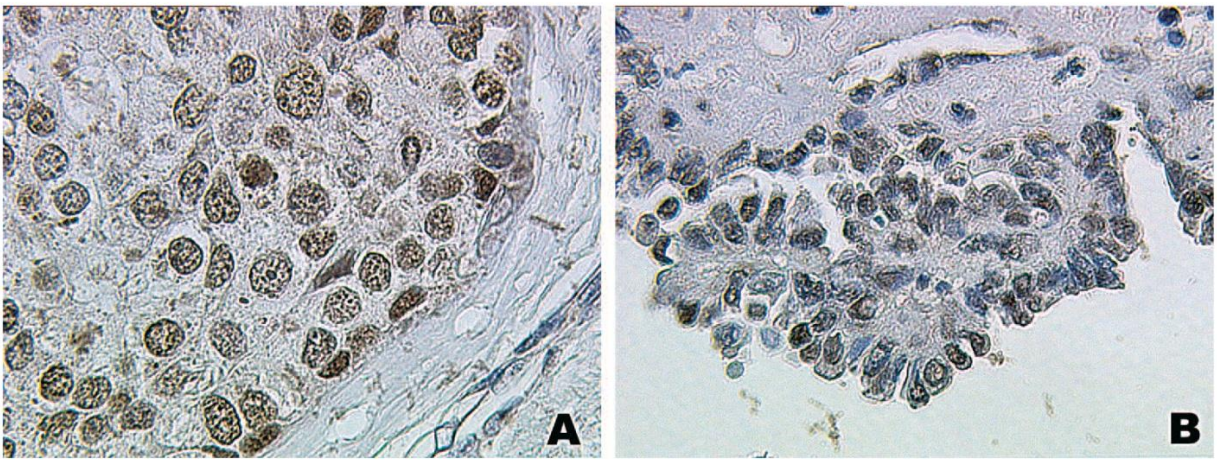


**Fig. 2.** Immunohistochemical staining reaction of ER-α and ER-ß in endometrioid adenocarcinomas. Positive immunohistochemical nuclear staining reaction in endometrioid adenocarcinoma for ER-α was observed (**A**). ER-ß however demonstrated minimal to no expression in endometrioid adenocarcinoma (**B**). x 400

**Table 3.** Univariate statistical analysis for positive MTA3, ER-α and ER-ß according to various clinicopathological features.

| | | | MTA 3 | | | ER-α | | | ER-ß | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | Total | negative | positive | P ($\chi^2$) | negative | positive | P ($\chi^2$) | negative | positive | P ($\chi^2$) |
| Age | ≤ 65 years | 98 (49%) | 53 (54.1%) | 45 (45.9%) | N.S. | 57 (58.2%) | 41 (41.8%) | N.S. | 86 (87.8%) | 12 (12.2%) | N.S. |
| | > 65 years | 102 (51%) | 59 (57.8%) | 43 (42.2%) | | 56 (54.9%) | 46 (45.1%) | | 88 (86.3%) | 14 (13.7%) | |
| FIGO | FIGO I and II | 175 (87.5%) | 97 (55.4%) | 78 (44.6%) | N.S. | 96 (54.9%) | 79 (45.1%) | N.S. | 153 (87.4%) | 22 (12.6%) | N.S. |
| | FIGO III and IV | 25 (12.5%) | 15 (60%) | 10 (40%) | | 17 (68%) | 8 (32%) | | 21 (84%) | 4 (16%) | |
| Grading (WHO) | Grade 1 and 2 | 176 (88%) | 92 (52.3%) | 84 (47.7%) | 0.004 | 95 (54%) | 81 (46%) | 0.077 | 153 (86.9%) | 23 (13.1%) | N.S. |
| | Grade 3 | 24 (12%) | 20 (83.3%) | 4 (16.7%) | | 18 (75%) | 6 (25%) | | 21 (87.5%) | 3 (12.5%) | |
| LN status | negative | 133 (66.5%) | 73 (54.9%) | 60 (45.1%) | N.S. | 72 (54.1%) | 61 (45.9%) | N.S. | 116 (87.2%) | 17 (12.8%) | N.S. |
| | positive | 9 (4.5%) | 6 (66.7%) | 3 (33.3%) | | 7 (77.8%) | 2 (22.2%) | | 8 (88.9%) | 1 (11.1%) | |
| | unknown | 58 (29%) | 33 (56.9%) | 25 (43.1%) | | 34 (58.6%) | 24 (41.4%) | | 50 (86.2%) | 8 (13.8%) | |
| LVSI | negative | 183 (91.5%) | 101 (55.2%) | 82 (44.8%) | N.S. | 103 (56.3%) | 80 (43.7%) | N.S. | 158 (86.3%) | 25 (13.7%) | N.S. |
| | positive | 17 (8.5%) | 11 (64.7%) | 6 (35.3%) | | 10 (58.8%) | 7 (41.2%) | | 16 (94.1%) | 1 (5.9%) | |
| Myometrial invasion | < 50% | 135 (67.5%) | 75 (55.6%) | 60 (44.4%) | N.S. | 74 (54.8%) | 61 (45.2%) | N.S. | 117 (86.7%) | 18 (13.3%) | N.S. |
| | > 50% | 65 (32.5%) | 37 (56.9%) | 28 (43.1%) | | 39 (60%) | 26 (40%) | | 57 (87.7%) | 8 (12.3%) | |
| Cervical Invasion | negative | 177 (88.5%) | 99 (55.9%) | 78 (44.1%) | N.S. | 100 (56.5%) | 77 (43.5%) | N.S. | 154 (87%) | 23 (13%) | N.S. |
| | positive | 23 (11.5%) | 13 (56.5%) | 10 (43.5%) | | 13 (56.5%) | 10 (43.5%) | | 20 (87%) | 3 (13%) | |
| Ovarial invasion | negative | 185 (92.5%) | 103 (55.7%) | 82 (44.3%) | N.S. | 103 (55.7%) | 82 (44.3%) | N.S. | 163 (88.1%) | 22 (11.9%) | N.S. |
| | positive | 15 (7.5%) | 9 (60%) | 6 (40%) | | 10 (66.7%) | 5 (33.3%) | | 11 (73.3%) | 4 (26.7%) | |
| Adipositas | negative | 125 (62.8%) | 70 (56%) | 55 (44%) | N.S. | 71 (56.8%) | 54 (43.2%) | N.S. | 105 (84%) | 20 (16%) | N.S. |
| | positive | 74 (37.2%) | 41 (55.4%) | 33 (44.6%) | | 41 (55.4%) | 33 (44.6%) | | 68 (91.9%) | 6 (8.1%) | |
| Diabetes | negative | 174 (87%) | 94 (54%) | 80 (46%) | N.S. | 95 (54.6%) | 79 (45.4%) | N.S. | 153 (87.9%) | 21 (12.1%) | N.S. |
| | positive | 26 (13%) | 18 (69.2%) | 8 (30.8%) | | 18 (69.2%) | 8 (30.8%) | | 21 (80.8%) | 5 (19.2%) | |
| Hypertension | negative | 119 (59.5%) | 67 (56.3%) | 52 (43.7%) | N.S. | 65 (54.6%) | 54 (45.4%) | N.S. | 103 (86.6%) | 16 (13.4%) | N.S. |
| | positive | 81 (40.5%) | 45 (55.6%) | 36 (44.4%) | | 48 (59.3%) | 33 (40.7%) | | 71 (87.7%) | 10 (12.3%) | |
| Radiotherapy | negative | 127 (63.5%) | 69 (54.3%) | 58 (45.7%) | N.S. | 68 (53.5%) | 59 (46.5%) | N.S. | 111 (87.4%) | 16 (12.6%) | N.S. |
| | positive | 73 (36.5%) | 43 (58.9%) | 30 (41.1%) | | 45 (61.6%) | 28 (38.4%) | | 63 (86.3%) | 10 (13.7%) | |
| Anti-hormone therapy | negative | 193 (96.5%) | 109 (56.5%) | 84 (43.5%) | N.S. | 109 (56.5%) | 84 (43.5%) | N.S. | 167 (86.5%) | 26 (13.5%) | N.S. |
| | positive | 7 (3.5%) | 3 (42.9%) | 4 (57.1%) | | 4 (57.1%) | 3 (42.9%) | | 7 (100%) | 0 (0%) | |

N.S.: not significant; LVSI: lymphovascular space invasion.

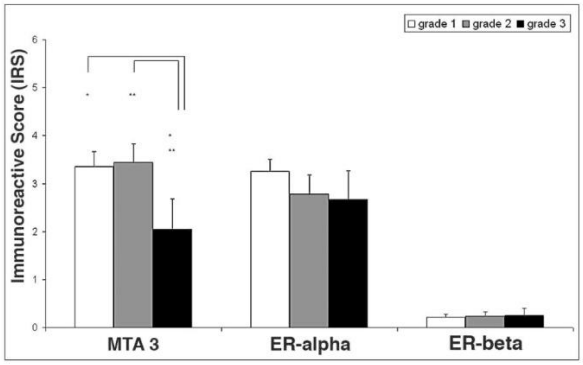


**Fig. 3.** Immunohistochemical expression analysis of MTA3, ER-α and ER-β in endometrial cancer depending on pathological differentiation. The IRS scores determined for MTA3 expression in endometrial cancer were related to the histological grading and plotted as mean +/-SEM. Statistical significant difference: *: p=0.033; **: p=0.006.

**Table 4.** Correlation of the immunohistochemical score of MTA3, ER-α and ER-β in human endometrioid adenocarcinomas.

| | MTA 3 (IRS) | ER A (IRS) | ER-beta (IRS) |
|---|---|---|---|
| MTA 3 (IRS) | | | |
| Correlation Coefficient | | -.041 | .041 |
| Significance | | N.S. | N.S. |
| ER-α (IRS) | | | |
| Correlation Coefficient | -.041 | | -.065 |
| Significance | N.S. | | N.S. |
| ER-β (IRS) | | | |
| Correlation Coefficient | .041 | -.065 | |
| Significance | N.S. | N.S. | |

N.S.: not significant.

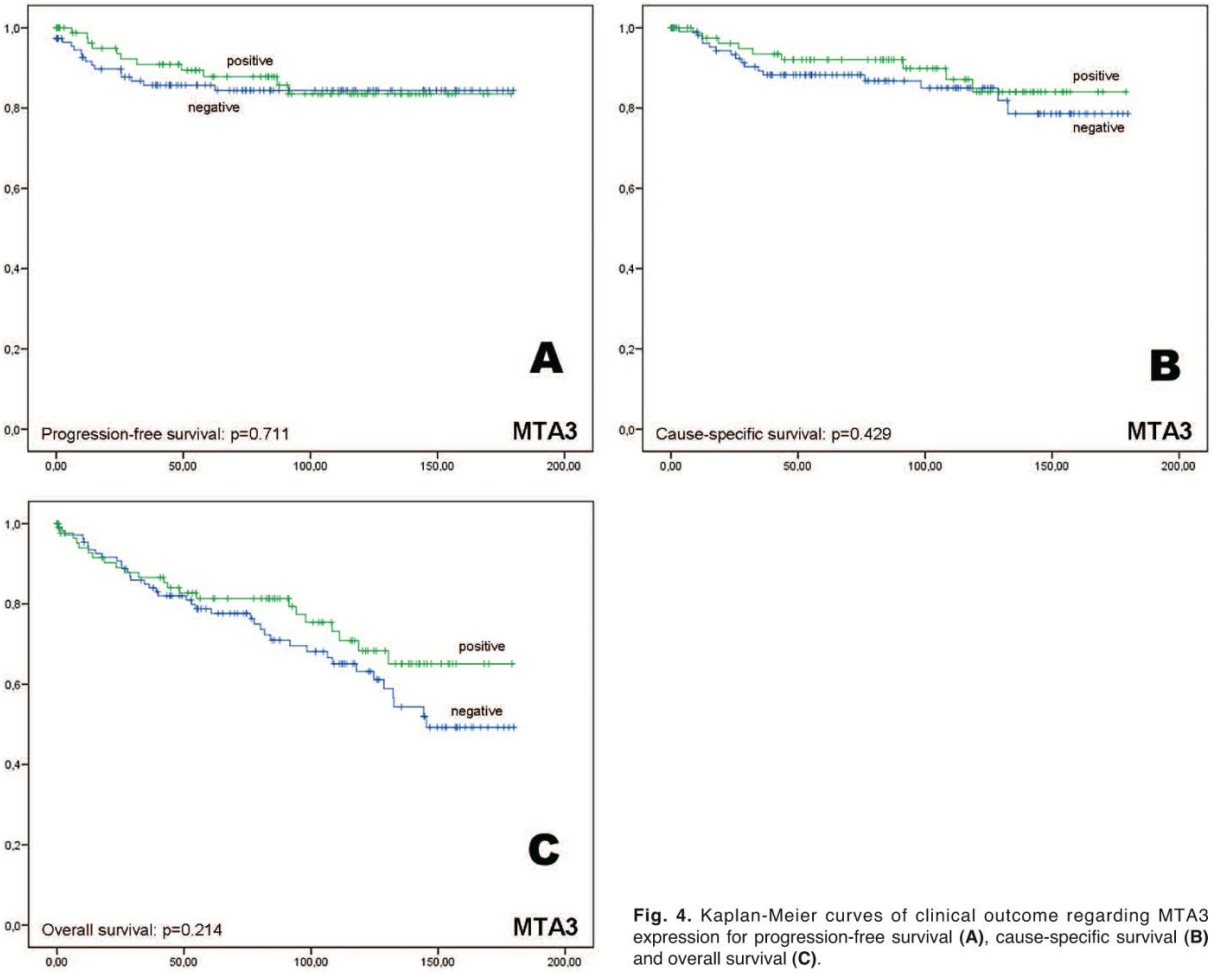


**Fig. 4.** Kaplan-Meier curves of clinical outcome regarding MTA3 expression for progression-free survival **(A)**, cause-specific survival **(B)** and overall survival **(C)**.

**Table 5.** Hazard ratios by multivariate Cox regression analysis.

| | Progression-free Survival | | | Cause-Specific Survival | | | Overall Survival | | |
|---|---|---|---|---|---|---|---|---|---|
| | RR | CI (5%-95%) | p | RR | CI (5%-95%) | p | RR | CI (5%-95%) | p |
| age (>65years) | | | | | | | 3.195 | 1.703-5.996 | <0.001 |
| WHO Grading (G1/G2 vs. G3) | 2.676 | 1.023-7.00 | 0.045 | | | | 2.086 | 1.089-3.997 | 0.027 |
| FIGO stage (I/II vs. III/IV) | 7.544 | 3.236-17.589 | <0.001 | 2.955 | 1.125-7.763 | 0.028 | 3.044 | 1.623-5.692 | <0.001 |
| cervical invasion (pos. vs. neg.) | 2.912 | 1.212-7.00 | 0.017 | 4.037 | 1.579-10.321 | 0.004 | 2.756 | 1.419-5.351 | 0.003 |
| myometrial invasion (>50% vs. <50%) | | | | 3.464 | 1.336-8.981 | 0.011 | | | |
| LN status (pos. vs. neg.) | | | | 1.615 | 1.036-2.519 | 0.035 | 1.623 | 1.703-2.139 | 0.001 |

time of diagnosis was 66.44 years (range, 36.18-89.35 years). 160 (80%) and 15 (7.5%) patients were diagnosed in FIGO stage I and II, respectively, while 18 (9%) patients had FIGO stage III and 7 patients (3.5%) presented with metastatic disease (FIGO IV). Lymph node sampling or dissection was generally performed in patients having tumours with deep myometrial invasion and/or high-grade or aggressive histological features. Pelvic and/or para-aortic lymph node sampling was performed for 142 patients (71%) while 9 patients (4.5%) demonstrated lymph node metastasis. A low FIGO stage (FIGO Ia), obesity, advanced age and excessive comorbidity were factors against a full surgical staging in 58 patients (29%). Obesity was observed in 74 (37.2%) cases, while 81 (40.5%) and 26 (13%) patients presented with high blood pressure and diabetes respectively. Of the analyzed 200 patients, 73 patients (36.5%) received radiation therapy, while seven patients (3.5%) received anti-hormone therapy. Tumour progression was observed in 27 patients (13.5%), and 61 patients (30.5%) died during the follow-up interval, of whom 25 patients (12.5%) died of their cancer disease.

### *Expression of MTA3, ER-α and ER-ß in human endometrioid adenocarcinomas*

The specificity of the MTA antibodies has previously been confirmed by us on ovarian cancer (Dannenmann et al., 2008) and placental tissues (Brüning et al., 2009). Positive MTA3 immunostaining was observed in 88 (44%) of 200 endometrial carcinoma samples respectively. Immunohistochemical staining reaction for MTA3 demonstrated immunostaining in the nuclei of malignant cells (Fig. 1a-d). Overall, endometrioid adenocarcinomas of histological grades 3 differentiation demonstrated a lower expression of MTA3 (Fig. 1d). Additionally, when using ER-α and ER-ß antibodies, 87 (43.5%) and 26 (13%) patients demonstrated a positive immunohistochemical staining reaction in the nuclei of malignant cells (Fig. 2a-b).

A significant decrease was noted from endometrioid adenocarcinomas grade 1 to grade 3 (p=0.033), as well as from grade 2 to grade 3 (p=0.006), while no significant differences could be observed between grade 1 and grade 2 endometrioid adenocarcinomas (Fig. 3). No significant difference in the MTA3 staining reaction was found among the various analysed clinicopathological characteristics, with the exception of grading in the univariate analysis ($\chi^2$) (p=0.012) (Table 3). Additionally, ER-α and ER-ß also demonstrated no significant difference among the various analysed clinicopathological characteristics, with an tendency to significance for ER-α and grading in the univariate analysis ($\chi^2$) (p=0.077) (Table 3). Moreover, no correlation was demonstrated between MTA3, ER-α and ER-ß expression (Spearman test: p>0.05 each) (Table 4).

### *Survival analysis*

Univariate survival analysis with the Kaplan-Maier test revealed no significant differences of the MTA3 immunohistochemical staining reaction for progression-free survival, cause-specific survival and overall survival (Fig. 4a-c).

Prognostic factors were also analyzed by the multivariate Cox proportional-hazard model. Forward stepwise elimination according to Cox regression results led to a model containing three independent terms that were predictive of progression-free survival: WHO grading (p=0.045, FIGO stage (p<0.001), cervical invasion (p=0.017). Independent prognostic factors for cause-specific survival were FIGO stage (p=0.028), cervical invasion (p=0.004), myometrial invasion (p=0.011) and lymph node involvement (p=0.035). Overall survival was influenced by age (p<0.001), FIGO stage (p<0.001), tumour grade (p=0.027), cervical invasion (p=0.003) and lymph node involvement (p=0.001) (Table 5).

## Discussion

Endometrial cancer is the most frequent gynaecologic malignancy in the Western World with several established prognostic factors, such as histological type, histological grade, surgical stage, pelvic lymph node involvement and myometrial invasion (Abeler and Kjorstad, 1991; Rose, 1996; Prat, 2004; Amant et al., 2005; Chan et al., 2007). However, although more than 50% of patients with endometrial carcinomas are diagnosed with FIGO stage I, as many as 20% die as a result of their disease (Jereczek-Fossa et al., 1999). This unusual situation might reflect that the

currently used diagnostic technology is insufficient to identify endometrial cancer patients with poor prognosis. Therefore, immunohistochemistry of different specific markers might be an interesting alternative to select high risk patients, leading to a more patient-specific risk profile and treatment (Oreskovic et al., 2004; Jeon et al., 2006; Shabani et al., 2007a; Jongen et al., 2009).

Because MTA3 has been shown to play an important function in invasion and metastasis of human cancer cells, the aim of our study was to investigate the expression of this protein in endometrial adenocarcinomas. We here demonstrate for the first time that MTA3 expression is reduced in endometrial adenocarcinomas of poor histological differentiation. Moreover, no association between estrogen receptors and MTA3 expression could be observed in endometrioid adenocarcinomas. Additionally, MTA3 expression was significantly associated with histological grading, although the expression of this nuclear transcriptional factor did not affect survival. Moreover, MTA3, as well as ER-α and ER-ß, did not constitute an independent prognostic factor in this study.

The function and role of MTA3 in human cancer cells, and in particular in endometrial cancer, is as yet unclear. MTA3 is part of a transcriptional regulation network and acts as a repressor of SNAIL (Fujita et al., 2003), which is associated with a lower overall survival of ovarian cancer patients (Blechschmidt et al., 2008). Moreover, the SNAIL-expressing Ishikawa estrogen receptor negative endometrial carcinoma-cell line showed a higher migration potential than Ishikawa estrogen receptor positive cell line with SNAIL expression level (Blechschmidt et al., 2007), linking SNAIL, and indirectly MTA3, to estrogen receptors in human endometrium. Moreover, MTA3 expression in breast cancer cells is enhanced by estrogen receptor activity (Fujita et al., 2003), establishing an association of MTA3 and estrogen receptor and thus to invasion and metastasis. Since estrogen receptors play several substantial roles in human diseases, including normal and pathological human endometrium (Herynk and Fuqua, 2004; Mylonas et al., 2004; Deroo and Korach, 2006; Leader et al., 2006; Shabani et al., 2007a), it might be possible that MTA3 also has important roles during endometrial carcinogenesis, especially within the view that MTA1/MTA3/SNAIL and E-cadherin are part of a transcriptional regulation network. However, we could not observe any correlation between MTA3 and ER-α and ER-ß expression, implicating that the MTA3 regulation might be independent of steroid receptors in endometrial endometrioid adenocarcinomas. Interestingly, four analysed endometrial cell lines demonstrated expression of the MTA3 protein, although no correlation with ER-α could be observed (Blechschmidt et al., 2007). Therefore, an as yet unknown regulation mechanism of MTA3 might be suggested in this tumour identity.

MTA3 expression is reduced in endometrial adenocarcinomas of poor histological differentiation, suggesting an important function in human endometrial malignant transformation. Although histological grading constitutes an important prognostic factor in endometrial cancer patients (Prat, 2004; Amant et al., 2005), the expression of MTA3 did not affect survival. Moreover, MTA3 did not constitute an independent prognostic factor in this study, suggesting that MTA3 is not a useful marker to assess and identify high-risk patients with endometrial adenocarcinomas.

The exact target genes of MTA3 in endometrial cancer remain unclear, and it can only be speculated that MTA3 is involved in the regulation of similar transcription clusters, as recently shown for other human cancer cells (Manavathi et al., 2007; Dannenmann et al., 2008). Known target genes comprise the nuclear transcription factors SNAIL and SLUG (Dannenmann et al., 2008), known to be overexpressed in human carcinoma cells (Castro Alves et al., 2007). MTA3 acts as a repressor of SNAIL, a transcriptional repressor of E-cadherin (Fujita et al., 2003) and thus links the expression of nuclear MTA3 to the expression of the metastasis-relevant cell adhesion protein E-cadherin (Beavon, 2000). However, additional gene silencing mechanisms for MTA3 are also possible, as observed for example for E-cadherin, as well as other tumour suppressor proteins and the estrogen receptor, which can be silenced by promoter hypermethylation (Auerkari, 2006; Giacinti et al., 2006). However, if and to what extent MTA3 modulates carcinogenesis in endometrioid cancer is still unclear and warrants further research.

In summary, our observations indicate that MTA3 is expressed in endometrioid adenocarcinomas of human endometrial cancer and becomes downregulated in poorly differentiated carcinomas, predisposing this cell type to be of high metastatic potential after malignant transformation. Although histological grading constitutes an important prognostic factor in endometrial cancer patients, the expression of MTA3 did not affect progression-free, cause-specific and overall survival. Moreover, MTA3 did not constitute an independent prognostic factor in this study, suggesting that MTA3 is not a useful marker to assess and identify high-risk patients with endometrial adenocarcinomas.

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## 4.2 Funktion und Regulierung von MTA1 und MTA3 bei Tumoren der weiblichen Geschlechtsorgane

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### *Function and regulation of MTA1 and MTA3 in malignancies of the female reproductive system*

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**Zusammenfassung:**

Die folgende Arbeit gibt einen Überblick über die Rolle der Metastasen-assoziierten Gene MTA1 und MTA3 [20, 97] beim Ovarial-, Zervix- und Endometriumkarzinom. Sie gehören zur Familie der nukleären Transkriptionsfaktoren [98, 99] und haben neben der Regulierung bestimmter Gene auch die Aufgabe, den Zellzusammenhalt in Geweben zu fördern und aufrechtzuerhalten [14, 100-102]. Daneben stellen sie einen wichtigen Faktor bei der lymphopoetischen Zelldifferenzierung dar. Dabei ist MTA3 für die Aufrechterhaltung des epithelialen Phänotyps verantwortlich, während MTA1 in die epithelial-mesenchymale Transition (EMT) involviert ist.

Die wesentlichen Zielstrukturen von MTA1 und MTA3 sind Snail [94], E-Cadherin [103, 104], STAT-Proteine (Signal transducers and Activators of Transcription) [105, 106] und der Östrogenrezeptor [107, 108], die mit Hilfe von in der Promotorregion der Gene ansetzenden Histon-modifizierenden Enzymen sowohl aktiviert, als auch inaktiviert werden können [109, 110].

Da es bislang keine Publikationen zur MTA-Expression im Epithel der Ovarien und Tuben gab, untersuchten wir das Vorhandensein von MTA1, MTA3, E-Cadherin, und des Östrogenrezeptors in diesen Zellen und konnten MTA1, MTA3 und den Östrogenrezeptor alpha sowohl im Ovar als auch in der Tube nachweisen, während E-Cadherin nur in der Tube nachweisbar war. Unsere Untersuchungen zeigten außerdem, dass die MTA1-Expression mit der Aggressivität des Tumors korreliert: So exprimieren fortgeschrittene und gering differenzierte Ovarialkarzinome mehr MTA1 als frühe und gut differenzierte Karzinome.

Die MTA3-Expression ist dagegen beim Ovarialkarzinom im Vergleich zu gesunden Ovarialzellen leicht reduziert.

Bei Zervixkarzinomen korreliert die Expression von MTA1 ähnlich wie beim Ovarialkarzinom mit dem Invasions- und Metastasierungspotential des Tumors. Ist MTA1 in großer Anzahl in der Zelle vorhanden, ist das ein unabhängiger Prognosefaktor für sowohl das krankheitsfreie als auch das Gesamtüberleben [111].

Beim Endometriumkarzinom spielt MTA1 im Vergleich zu MTA3 nur eine untergeordnete Rolle: Die MTA3-Expression ist in gering differenzierten Endometriumkarzinomen signifikant vermindert, allerdings ohne prognostische Relevanz, während bei Stromatumoren des Uterus eine hohe Expression von MTA3 das Überleben der Patientinnen signifikant verkürzt [112, 113].

Die klinische Bedeutung der MTA-Gene und ihrer Genprodukte liegt in der Möglichkeit, spezifische Medikamente zu entwickeln, die entweder die MTA-Expression minimieren, oder an späterer Stelle im nachgeschalteten Signalweg eingreifen. Eine vielversprechende Substanz hierfür ist Inositol [114], das allerdings in dieser Indikation bislang nur in vorklinischen Untersuchungen angewendet wurde [109].

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# Function and regulation of MTA1 and MTA3 in malignancies of the female reproductive system

**Ansgar Brüning · Thomas Blankenstein · Julia Jückstock · Ioannis Mylonas**

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**Abstract** The family of metastasis-associated (MTA) genes is a small group of transcriptional co-regulators which are involved in various physiological functions, ranging from lymphopoietic cell differentiation to the development and maintenance of epithelial cell adhesions. By recruiting histone-modifying enzymes to specific promoter sequences, MTA proteins can function both as transcriptional repressors and activators of a number of cancer-relevant proteins, including Snail, E-cadherin, signal transducer and activator of transcriptions (STATs), and the estrogen receptor. Their involvement in the epithelial-mesenchymal transition process and regulatory interactions with estrogen receptor activity has made MTA proteins highly interesting research candidates, especially in the field of hormone-sensitive breast cancer and malignancies of the female reproductive tract. This review focuses on the current knowledge about the function and regulation of MTA1 and MTA3 proteins in gynecological cancer, including ovarian, endometrial, and cervical tumors.

**Keywords** MTA1 · MTA3 · Metastasis · Ovarian cancer · Cervical cancer · Endometrial cancer

## 1 Introduction

Gynecological cancer comprises a wide variety of malignancies arising from tissues of the female reproductive tract. It includes completely different histopathological entities with different causative etiologies, ranging from papillomavirus infection-caused squamous cervical cancer to hormone receptor-driven endometrioid adenocarcinomas. Detection and treatment may lead to a good prognosis, such as in the case of endometrial cancer, or to a completely inadequate and disappointing survival rate, as in the case of ovarian cancer.

Worldwide, gynecological cancer entities represent the most frequently diagnosed cancer in females [1]. Tumor progression to metastatic disease has been recognized as one of the most important aspects for the lethal outcome of cancer. Several factors are responsible for the invasion and metastasis of gynecological cancer. Most of these factors are common to other types of cancer and include the expression of extracellular matrix degrading enzymes, such as cathepsins and matrix metalloproteinases [2]; the loss of cell adhesion proteins, such as E-cadherin [3]; the acquisition of a migratory or epithelial-mesenchymal transition (EMT)-like phenotype [4, 5]; and the ability to disseminate via the blood or the lymphatic system [6]. However, the most intriguing aspect of pathophysiology and carcinogenesis is the hormone dependency of most of the female genital tissues. Whereas the ovary is the primary source of estrogens and progesterons, the endometrium is considered to be the classical target tissue for these steroid hormones. The clinical importance of steroid hormones and their mediating receptors is also reflected by the highly effective anti-hormonal treatment of breast cancer [7–9].

Proteins of the metastasis-associated (MTA) gene family have been recently identified as key regulators of the EMT process and also as controllers of E-cadherin expression [10, 11]. MTA proteins, comprising MTA1, MTA2, and MTA3, are components of the nucleosome remodeling and histone deacetylation (NuRD) complex, in which they interact with histone deacetylases to promote chromatin compaction and thereby gene inactivation [10, 11]. Their recruitment to specific promoter regions, or specific interactions with other transcription factors, leads to transcriptional silencing of their target genes. Several of the identified MTA1 target genes are highly relevant for cancer progression. MTA1 has been shown

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to negatively interfere with the transactivation function of the estrogen receptor at its target sequences, including estrogen receptor-dependent transcription of breast cancer gene 1 (BRCA1) [12] and MTA3 [10, 11, 13]. MTA3 itself has been described as a transcriptional repressor of the E-cadherin transcription factor Snail [13], thus leading to a transcriptional regulation cascade from MTA1 to MTA3 to E-cadherin, with the final consequence of a suppressive function of MTA1 on E-cadherin expression [13].

MTA protein expression is not restricted to malignancies but can also be found in normal tissues [10, 14–17]. Much seminal work on MTA proteins has been performed in the field of breast cancer research and revealed not only a cancer-promoting interplay of MTA proteins, estrogen receptor activity, and metastasis [13, 18] but also a regulatory function of MTA proteins in normal mammary gland development and maturation [18, 19]. Since the development and physiological function of gynecologic tissues rely on female sex hormones, whose internal and external supply is also influencing the progression of some of the most widespread gynecological cancer entities, several research groups have studied the involvement and interaction of MTA proteins and female steroid hormone receptors in gynecological cancer. This review summarizes the current knowledge about the expression and regulation of MTA proteins in both normal gynecological organs and gynecological cancer.

## 2 MTA expression in ovarian cancer

The ovary primarily fulfills the function of oocyte storage and maturation. Although endowed with a high proliferative potential, the stromal oocytes and their supportive granulocytes are rarely involved in ovarian malignancies and contribute to less than 5 % of all ovarian cancers [20, 21].

Most ovarian cancers (up to 95 %) are categorized as epithelial ovarian cancers (EOC) and can be subdivided into serous, mucinous, endometrioid, and clear-cell EOC [22]. Serous EOC reflects the most common and, unfortunately, the most aggressive type of ovarian cancer [22].

EOC is the most deadly gynecological cancer worldwide (http://www.ovariancancer.org/about-ovarian-cancer/statistics/). The World Health Organization GLOBOCAN database reports a worldwide incidence of more than 190,000 cases of ovarian cancer [23]. Non-detection of specific early symptoms together with the absence of reliable screening strategies often leads to diagnosing ovarian cancer only in advanced stages, resulting in poor overall survival. Moreover, despite multimodal treatment options, high incidences of relapses occur [24–27]. Although improvements in surgical management and advances in cytotoxic therapy have been accomplished in the past decades, the overall 5-year survival rate for women with advanced disease can be as low as 13 % [24, 25, 28, 29].

The ovarian surface epithelium is a single-cell layer covering the ovary and has long been renowned as the origin of most epithelial ovarian tumors. The periodical rupture of this cell layer during ovulation, similar to a continuous process of inflammation and wound healing, has led to the "incessant ovulation" theory of the origin of ovarian cancer [30, 31]. However, based on recent histopathological analyses and marker gene expression studies, the epithelium of the oviductal fimbriae at the distal ends of the fallopian tube has also been implicated to play an important role in the genesis of epithelial ovarian cancer [22, 31–33]. According to this theory, EOC originates as a serous tubal intraepithelial carcinoma (STIC) and secondarily metastasizes to the ovarian surface and other peritoneal epithelia [22, 31–33]. The extent to which these different epithelia give rise to EOC is still under debate, but the different histological and developmental origins are of great importance when looking at the aspect of MTA expression. MTA proteins both regulate and are regulated by estrogen receptor activity and are also key regulators of E-cadherin expression (Fig. 1). Notably, fallopian tube epithelial cells (FTE cells) rely on E-cadherin expression for intercellular adhesions, whereas normal ovarian surface epithelial (OSE) cells are devoid of E-cadherin expression ([33]; see also Fig. 2).

Since no information on MTA protein expression in human FTE cells has been published, we have investigated MTA1, MTA3, E-cadherin, and estrogen receptor expression in FTE and OSE cells (Fig. 2). Expression of both MTA1 and MTA3 proteins was detectable in FTE cells and also in normal OSE cells. In contrast, E-cadherin expression was found in FTE cells only, whereas estrogen receptor alpha expression was detectable in both FTE and OSE cells.

It seems contradictory that, and initially is difficult to understand why, two transcription factors of apparent opposite function are expressed together at comparable levels in normal OSE and FTE cells. MTA3 expression is known to be important for sustaining an epithelial phenotype, whereas MTA1 expression is a promoting factor involved in EMT. It can be hypothesized that moderate levels of both MTA1 and MTA3 expression, as found in untransformed ovarian epithelial cells, are needed for an equilibrium or are involved in other as yet unrecognized vital regulatory processes. Under deregulated conditions, however, the impact of MTA protein expression becomes more obvious, as observed in various types of cancer [10, 11, 34]. In a study on 115 serous ovarian cancer samples, we found a significantly increased expression of MTA1 protein in advanced ovarian cancer tissues compared to samples of earlier tumor stages displaying either a lower differentiation grade or a larger extent of peritoneal metastasis and ascites formation [35]. In highly de-differentiated ovarian carcinomas, the expression of nuclear MTA1 protein was found to be

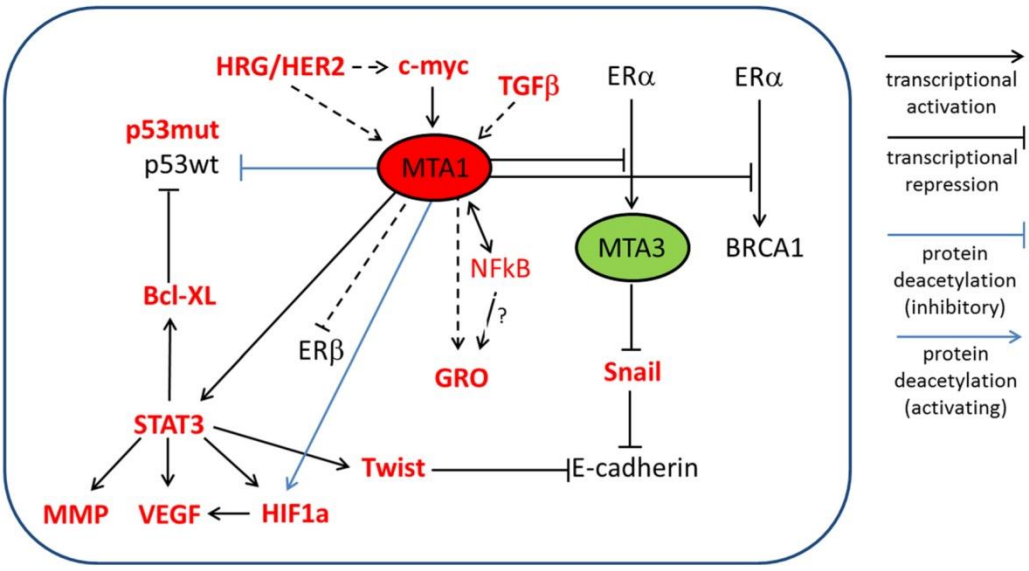


**Fig. 1** Oncogenic pathways involved in MTA expression and regulation. MTA1 expression can directly and indirectly be enhanced by several oncogenic factors, including nuclear c-myc activation and growth-promoting heregulin and TGF-beta cell membrane receptor activation. Elevated MTA1 activity can further lead to transcriptional activation of STAT3 proteins or transcriptional repression of the estrogen receptor alpha leading to reduced MTA3/E-cadherin and BRCA1 expression. Reduced BRCA1 expression, similar to reduced p53 activity as mediated by posttranslational deacetylation of p53 by MTA1, can further promote genomic instability of cancer cells. However, not all malignant transformation steps are common to all gynecological cancers. For example, up to 95 % of all high-grade serous ovarian carcinomas bear p53 mutations and around 40–50 % display direct or indirect BRCA inactivation [22]. E-cadherin expression was found to be absent in ovarian surface epithelial but not fallopian tube epithelial cells. HER2 overexpression and KRAS/BRAF mutations were identified as non-overlapping events occurring primarily in low-grade serous ovarian cancer but not in high-grade serous ovarian cancer [22]. *Red letters* activated/elevated in malignant cancer cells. *Dotted lines* indirect effects. *HRG* heregulin, *MMP* matrix metalloproteinase, *VEGF* vascular epithelial growth factor, *GRO* growth-regulated oncogene

nearly twice as high as the median MTA1 expression in well-differentiated ovarian carcinomas [35]. Similar results were obtained in an independent study on 81 ovarian cancer tissues of various histological subtypes [36]. The study also revealed that elevated MTA1 expression was associated with a shortened disease-free survival, although no significant association between MTA1 expression and overall patient survival was observed [36]. Notably, it was also found that elevated MTA1 expression was associated with a poorer response to first-line chemotherapy, in this study most frequently a platinum (plus taxane)-based treatment regimen [36].

In contrast to the pronounced upregulation of MTA1 in advanced and metastatic ovarian cancer tissues, only a slight and statistically non-relevant reduction in MTA3 expression was observed in ovarian cancer [35]. Furthermore, no statistically relevant relation between MTA1 and MTA3 expression as well as E-cadherin expression could be established among the ovarian cancer tissues tested. In single, syngeneic ovarian cancer cell lines, however, it could paradigmatically be shown and be confirmed that ectopic MTA1 overexpression leads to reduced MTA3 and E-cadherin expression in ovarian cancer cells [35]. This indicates that the known intracellular interactions and regulations of MTA proteins in ovarian cancer are probably overlapped by further, yet unidentified, factors that contribute to the expressional regulation of MTA proteins and their downstream targets in ovarian cancer. For example, gene mutation and promoter methylation have been recognized as independent mechanisms of E-cadherin silencing in ovarian cancer [3, 37]. The interaction of tumor cells with stroma cells and infiltrating immune cells has to be considered as well. Several cytokines and growth factors, secreted by fibroblasts, for example, are known to influence EMT of cancer cells. Normal OSE cells have the ability to perform EMT and respond to epithelial growth factor (EGF), transforming growth factor (TGF)-beta, and extracellular matrix proteins [38, 39].

It is also not well understood which mechanisms drive the elevated MTA1 expression observed in advanced ovarian cancer [35, 36]. The identification of MTA1 as a target gene of c-myc has linked a well-studied oncogene to the expression of proto-oncogenic MTA1 [40]. Indeed, a high frequency of c-myc gene amplification was found in epithelial ovarian cancer [41] and may account for elevated MTA1 expression in ovarian cancer cells. However, c-myc amplification is an early event in cancer progression (transformation), and MTA1 overexpression was predominantly observed in advanced cancer stages of ovarian cancer. The heregulin/human epidermal growth factor receptor 2 (HER2) tyrosine kinase receptor

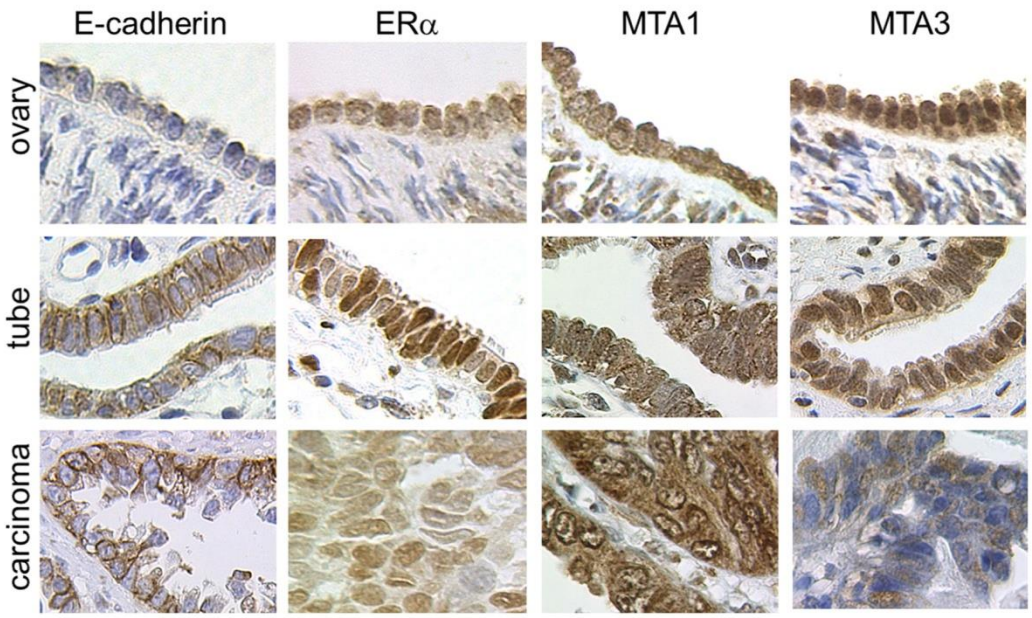


**Fig. 2** Expression of MTA1 and MTA3 in ovarian epithelial cells and cancer tissues. Non-malignant ovarian and fallopian tube tissues and ovarian cancer tissues were immunohistochemically analyzed for MTA, estrogen receptor, and E-cadherin expression as previously described [35]. Pictures of the ovary depict the single-layered cubic ovarian surface epithelium (OSE), and pictures of the adjacent fallopian tube show the ciliated columnar cells of the oviductal fallopian tube surface epithelium (FSE). Pictures of carcinoma tissues are representatives of various serous epithelial ovarian cancer tissues

pathway has also been identified as an upstream regulator of MTA1 in breast cancer [42]. Enhanced activation of the HER2 pathway occurs in ovarian cancer, but the importance to ovarian cancer progression has been questioned due to a low response of ovarian cancer to HER2-targeted therapies [43]. Therefore, future histological studies could profit from a direct comparison of MTA1 expression with putative oncogenic drivers in ovarian cancer.

Interestingly, a previous immunohistological study on MTA protein expression in ovarian cancer cells revealed an inverse correlation between estrogen receptor beta (ESR2) and MTA1 expression [35]. This supports the notion that the estrogen receptor beta form, in contrast to the growth-promoting estrogen receptor alpha (ESR1), exerts a tumor suppressive function in ovarian cancer [44, 45]. Therefore, although MTA1 has been primarily associated with invasion and metastasis, its expression has also been considered as a cancer cell survival factor during cancer progression. Overexpression of MTA1 has been shown to facilitate anchorage-independent growth of breast [42] and ovarian cancer cells [35, 46]. This has been associated with enhanced expression of the oncogenic cytokine growth-regulated oncogene (GRO) (CXCL-1) in MTA1-overexpressing ovarian cancer cell clones, although the regulation mechanism of GRO by MTA1 remains undetermined [35]. In the p53 wild-type A2780 ovarian cancer cell line, overexpression of MTA1 has recently been shown to enhance expression of the anti-apoptotic survival factor bcl-XL [46].

MTA1 has primarily been regarded as a transcriptional repressor. However, Pakala et al. recently identified MTA1 as a direct positive regulator of STAT3 transcriptional activity in breast cancer cells [47]. Activity of the JAK/STAT (Janus kinase/signal transducer and activator of transcription) signaling pathway is a frequent event in ovarian cancer and contributes to metastasis due to its regulation of pro-angiogenic vascular endothelial growth factor (VEGF), matrix metalloproteinases (MMPs), and Twist [48–50]. Since Twist has also been identified as a transcriptional repressor of E-cadherin [51], upregulation of Twist by MTA1/STAT3 interaction thus yields another link of MTA1 expression to E-cadherin regulation, invasion, and metastasis (Fig. 1).

## 3 MTA and endometrial cancer

The inner cell layer of the human uterus, the endometrium, is considered to be the classical target tissue for steroid hormones. However, it is not only a target tissue for hormones but is also an endocrine organ by itself, secreting several mediators that are thought to influence the endometrial function through autocrine and paracrine pathways. Additionally, it is the tissue that develops the most frequent gynecologic

malignancy in the Western world: endometrial cancer. The annual incidence is estimated to be 15–20 cases per 100,000 women [52–55]. The lifetime risk of developing endometrial cancer is approximately 2.5 %, while the lifetime probability of death from this cancer is estimated to be 0.52 % [1, 56].

Endometrial cancer is primarily diagnosed in postmenopausal women [57, 58]. Based on the early symptom of postmenopausal bleeding and the subsequent possibility of hysteroscopy, transvaginal ultrasound examination, and endometrial biopsy sampling, endometrial cancer is often diagnosed at early stages and usually can be eliminated by radical hysterectomy and bilateral salpingo-oophorectomy [57, 58].

Endometrial cancer has been described as consisting of two different subgroups depending on pathological and molecular parameters [55, 59–61]. Type I endometrial cancers are mostly well-differentiated endometrioid adenocarcinomas, with a more favorable outcome compared to endometrial cancer of the second group [53, 55, 56, 59, 60, 62]. Type II endometrial cancers are often of the non-endometrioid type, are poorly differentiated, and have a poor prognosis [60, 61, 63]. Most endometrial cancers (80–85 %) can be categorized as type I endometrioid endometrial cancer [57, 58]. Fortunately, this type of endometrioid adenocarcinoma has a good prognosis and accounts for only a minor portion of deaths from gynecologic malignancies. In addition to obesity, an unopposed or excessive estrogen exposure of the glandular epithelium has been identified as a high-risk factor for the origin of endometrioid carcinomas [57, 58]. The 5-year survival rate for serous-papillary histology is approximately 24–34 %, whereas the survival rate for clear-cell adenocarcinoma is estimated to be approximately 42 % [64].

These observations have led to the postulation of a dualistic model for the molecular carcinogenesis in endometrial carcinomas [61]. The carcinogenesis of type I endometrial carcinomas are thought to develop due to estrogenic risk factors [53, 55, 61] and are also characterized by genetic alterations like mutations in phosphatase and tensin homolog (PTEN) and K-ras [61]. Type II cancers more often exhibit p53 mutations [65], HER-2/neu amplification [66], and chromosomal instability [61]. Interestingly, PTEN inactivation and HER2 overexpression have been identified as further cancer-promoting factors in endometrial cancer [57, 67].

Although several prognostic factors have been established [53, 55, 68–70], it is assumed that approximately 20 % of all endometrial cancer patients die of their disease [62]. This is actually an unusual situation for a solid tumor, especially since patients with endometrial cancer are diagnosed at an early stage. Therefore, a better understanding of its pathophysiology and carcinogenesis is urgently needed to help increase life expectancy and optimize therapy for these patients.

Loss of E-cadherin expression has been found in estrogen-dependent endometrial cancers (type I) and also in the more aggressive estrogen-independent non-endometrioid endometrial cancers (type II) [71]. In both type I and type II endometrial cancers, loss of E-cadherin expression was associated with an adverse prognosis and EMT and was also found to be inversely correlated to Snail expression [71, 72]. Being involved in E-cadherin expression and modulated by estrogen receptor expression, it can be assumed that MTA proteins may have an important role in endometrial cancer progression. In fact, Balasenthil et al. [14] observed an expression of MTA1 primarily in glandular and stromal cells of the endometrium in the proliferative phase but only weak MTA1 staining in the secretory phase. In postmenopausal women, the primary risk group for endometrial cancer, a highly variable expression level of MTA1 protein in both stromal and glandular cells, was observed, although the total MTA1 protein expression in postmenopausal tissue extracts was found to be less than in tissue extracts from endometrial cancer. However, among 70 endometrial endometrioid adenocarcinomas tested, no significant association between MTA1 expression and endometrial cancer grade was observed [14].

In contrast to the findings made for MTA1, an expression analysis of MTA3 in 200 endometrioid adenocarcinomas revealed a significant reduction of MTA3 expression in grade III endometrioid adenocarcinomas. However, no statistically relevant association between the expression level of MTA3 and progression-free survival, cause-specific survival, and overall survival was observed [73]. Furthermore, no association was found between MTA3 expression and the expression of the estrogen receptors alpha and beta [73]. Therefore, although the previous immunohistochemical analyses are highly indicative of a function of MTA1 and MTA3 in endometrial cancer progression [14, 73], neither MTA1 nor MTA3 appear to be suitable single markers for a survival prognosis of endometrial cancer patients with endometrioid histology. However, by analyzing uterine non-endometrioid carcinomas (type II), MTA3 expression demonstrated a significant association with FIGO surgical stage, lymph node involvement, and lymphovascular space invasion [74], predisposing the MTA3-overexpressing cell type to high metastatic potential after malignant transformation. Moreover, MTA3 was revealed to be a significant independent prognostic parameter, demonstrating a marked association with patients' progression-free survival, cause-specific survival, and overall survival [74]. It seems that MTA3 might have a more important and selective role during tumor progression in endometrial cancer type II than observed in type I endometrial cancer and ovarian cancer.

## 4 MTA and cervical cancer

Since the implementation of screening programs, with the objective to prevent invasive cervical cancer by detecting its precursor cervical lesions, the incidence of this entity has

declined in the most developed countries. However, cervical cancer is the second most common malignant disease among women worldwide (http://report.nih.gov/nihfactsheets/viewfactsheet.aspx?csid=76), with more than 500,000 new cancer cases every year [75, 76], and more than 85 % of cases occurring in developing countries [1, 77]. The median age at diagnosis with cervical cancer is 48 years. The disease has two age peaks, one at the age of about 45 and then again at an age beyond 70. Approximately 80 % of cervical cancers arise from squamous cell dysplasias, while 15 % are adenocarcinomas and 5 % clear-cell adenocarcinomas [76, 78]. However, although the Papanicolaou smear is the most cost-effective cancer screening test ever developed, it still can be non-diagnostic or falsely negative in the presence of invasive cancer. Although several risk factors for the development of cervical cancer have been identified, including human papilloma virus (HPV) infection [77, 79–81], the precise carcinogenesis is still unclear and no effective tumor markers are available.

The border of the ecto- and endocervix, called the squamocolumnar junction, is the predominant location of an infection with high-risk HPV, allowing HPV to access the basal cells through minute lesions or abrasions in the cervical epithelium. Being a common infection, most sexually active women will temporarily experience at some time of their life a persistent HPV infection which is the precondition for a high-grade cervical dysplasia, and a previous HPV infection is detectable in 99.7 % of cervical cancers [82]. However, it is quite unclear in which way MTA proteins are involved during viral carcinogenesis. Interestingly, the hepatitis B (HB) virus transactivator protein HBx, a major regulator of cellular responses caused by the HB virus, stimulated the expression of MTA1 in hepatocellular carcinoma cells, involving HBx targeting of transcription factor nuclear factor (NF)-kappaB and the recruitment of HBx/p65 complex to the NF-kappaB consensus motif on the relaxed MTA1 gene chromatin [83]. Therefore, a similar study analyzing the effects of transforming HPV oncogenes might be of interest for our understanding of a possible involvement on MTA1 regulation in cervical cancer.

Studies concerning MTA expression in cervical carcinoma are rare. Rao et al. [84] have shown that MTA1 expression levels in a cell culture model affected migration and invasion of cervical cancer cells. MTA1 protein expression was higher in SiHa cells compared with HeLa cells, which correlated with the potential of migration and invasion. Inhibition of MTA1 expression by RNA silencing impaired cell invasion, migration, and adhesion capabilities. Furthermore, E-cadherin levels were upregulated while beta-catenin levels were downregulated. The authors suppose that an altered E-cadherin/beta-catenin complex could be responsible for the decreased migration and invasion capability.

Liu et al. [85] investigated the effect of MTA1 on survival and lymph node metastasis in cervical carcinoma tissue samples. High levels of MTA1 were shown to be an independent factor for overall survival and disease-free survival. Although normal cervical epithelia showed little or no MTA1 expression in an immunohistochemistry (IHC) assay, cervical carcinoma samples could be divided into a group with low MTA1 expression and a group with high MTA1 expression. High MTA1 expression was associated with high-histologic-grade lymph node metastasis and recurrence. The correlation with lymph node metastasis is an important observation, since cervical cancer metastasizes early through the lymphatic system, a fact that dramatically impairs patients' survival.

## 5 Further perspectives in targeting MTA1 and concluding remarks

Due to its pivotal role in cancer progression and metastasis, MTA1 represents a highly interesting target for cancer therapy. Several cell biological studies have applied an siRNA approach for specific knock-down of MTA1 expression and succeeded in reducing cancer cell transformation, proliferation, clonal growth, VEGF expression, invasiveness, and migration [40, 84, 86–89]. Furthermore, at least two microRNAs have been identified that interfere with MTA1 mRNA stabilization and could be used for MTA1 targeting [90, 91]. However, although the genetic targeting approaches revealed promising *in vitro* results, their clinical usefulness is restricted due to the still encountered limitations of human cancer gene therapy [92, 93]. Therefore, based on the current state-of-the-art, a pharmacological inhibition of MTA1 appears to be more promising.

The growth factor heregulin has been shown to enhance MTA1 protein expression by activating the HER2 pathway [42]. Targeting of HER2 activity by monoclonal antibodies or specific tyrosine kinase inhibitors has proven efficacy in breast cancer but appears less effective in gynecological cancer [43]. Natural polyphenols, such as plant-derived resveratrol and pterostilbene, have been shown to reduce the expression level of MTA1 in prostate cancer cells [94, 95]. Interestingly, this also links the cancer protective effects of a healthy diet to MTA1 expression. However, the clinical usefulness of these cancer-preventive polyphenols for effective cancer therapy is still uncertain [96], and the effects of resveratrol and pterostilbene on MTA1 expression in gynecological cancer cells remains to be evaluated.

Further to the expression regulation of MTA1, the inhibition of its functional activity might be a promising approach to interfere with the cancer-promoting effects of MTA1. Histone deacetylase inhibitors, such as vorinostat and valproic acid, have long been tested for their clinical use in a variety of

cancer entities, but they lack specificity due to their many pleiotropic effects [97].

A rational drug design to target MTA1 is therefore urgently needed. Because of the importance of MTA1 in tumor progression and metastasis, a high-resolution analysis of the three-dimensional structure of MTA1 has already insistently been suggested [47]. In fact, a recent crystallographic analysis of MTA1 bound to histone deacetylase 1 (HDAC1) revealed a close association of the ELM2 and SANT1 domain of MTA1 to HDAC1 that further employed an inositol tetraphosphate molecule when shifted to its activated form ([98]; protein database identification number=4BKX; www.rscb.org/pdb). These observations do not only provide a new function for the old inositol tetraphosphate orphan messenger but also raises completely new aspects and opportunities for pharmacological regulation of histone deacetylase activities by designing inositol derivatives to modulate MTA1 activity either directly or indirectly by modulating the specific regulatory inositol polyphosphate kinase and phosphatase activities.

With such specific inhibitors in hand, a new prospective targeted therapy against MTA1 could valuably support our limited arsenal against several types of highly aggressive, MTA1-overexpressing cancer entities, including several types of gynecological cancer.

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## 4.3 Verlust der LDOC1-Expression durch Promotor-Methylierung in Zervixkarzinom-Zellen

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***Loss of LDOC1 expression by promoter methylation in cervical cancer cells***

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### Zusammenfassung:

Das Leucin zipper downregulated in cancer 1-Gen (LDOC1) kommt ubiquitär im menschlichen Körper und damit auch in vielen Geweben des weiblichen Genitaltrakts vor [41] und ist ein potentieller Tumorsuppressor [59]. In unseren Untersuchungen an Zervixkarzinom-Zelllinien [115] und Gewebeproben von Zervixkarzinomen zum Nachweis der Expression von LDOC1 war dieses Gen in vier der sechs untersuchten Zelllinien inaktiviert. Im Karzinomgewebe stellten wir ebenfalls ein Herunterregulieren des LDOC1-Gens fest. Nicht-maligne Fibroblasten und mononukleäre Blutzellen dienten als Kontrolle, in denen die LDOC1-Expression nicht beeinträchtigt war.

Da der Mechanismus der LDOC1-Inaktivierung in den Zervixkarzinom-Zellen nicht bekannt war, führten wir PCR-basierte Untersuchungen [116, 117] hinsichtlich Gen-Deletionen, Rearrangements und epigenetischen Veränderungen durch und konnten (Hyper-)Methylierungen in der Promotorregion [62, 63] des Gens als Ursache der fehlenden LDOC1-Expression feststellen. Hierbei wird die DNA des Promotors chemisch modifiziert, indem durch spezifische Enzyme, die DNA Methyltransferasen (DNMT), an bestimmten Stellen Methylgruppen angefügt werden [118]. Der Nachweis der Promotor-Methylierung gelang mittels Methylierungs-spezifischer Primer (MSP) [59, 119-123] in allen Zelllinien mit inaktivierter LDOC1-Expression. Nach Anwendung des etablierten DNMT-Inhibitors Decitabin (AdC) [66, 67] exprimierten die Zellen LDOC1 zum größten Teil wieder. Weitere Analysen zeigten, dass eine Überexpression von LDOC1 in Zervixkarzinom-Zelllinien zur Apoptose und damit zum Zelltod führt [124].

Bezüglich der LDOC1-Expression im Zervixkarzinom-Gewebe fanden wir bei drei von insgesamt neun Patientinnen eine verminderte, bei weiteren drei Patientinnen eine normale

Expression, während die restlichen drei Patientinnen sogar leicht erhöhte Expressionslevel im Vergleich zu gesunden Zervixgewebeproben zeigten.

Unsere Untersuchungen lassen vermuten, dass die Überexpression von LDOC1 zu einem pro-apoptotischen Phänotyp der malignen Zellen führt, während das Herunterregulieren bzw. die Inaktivierung des Gens einen Überlebensvorteil für die Karzinomzellen darstellt und mit einer schlechteren Prognose für die Patientin verbunden ist.

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ORIGINAL ARTICLE

# Loss of LDOC1 Expression by Promoter Methylation in Cervical Cancer Cells

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**Cervical cancer lacks reliable prognostic factors for both progression and chemotherapeutic responsiveness. The expression of the LDOC1 tumor suppressor candidate was therefore investigated. In four of six cervical cancer cell lines tested, expression of LDOC1 was silenced. Downregulation of LDOC1 could also be shown in biopsies of cervical cancer specimens. PCR-based promoter methylation analysis revealed a significant association between promoter methylation and the loss of LDOC1 expression, which could be reverted by DNA methyltransferase inhibitors. This indicates that silencing of LDOC1 is a frequent event in cervical cancer and may be of interest as a molecular marker in cervical cancer.**

**Keywords:** LDOC1, Cervical cancer, Promoter methylation, Epigenetics, Tumor suppressor

## INTRODUCTION

Cervical cancer is the third most common cancer of the female genital tract, after endometrial and ovarian cancer, and with 275,000 deaths in 2008, it is the fourth leading cause of cancer death in females worldwide (1). Half a million estimated new cases were diagnosed worldwide in 2008, with a median age at diagnosis of 48 (2, 3). In the United States, 12,170 new cases of cancer of the uterine cervix were reported for the year 2012 (2). The incidence of cervical cancer is especially high in African-American and Hispanic women (9.8 and 11.8 cases per 100,000 individuals, respectively, compared to 8.0 for Caucasian females from the United States and 7.2 for Asian-American women). The mortality is twice as high in African-American women compared to Caucasian women (4.3 vs. 2.2 deaths per 100,000 individuals) (2). Women in developing countries account for 88% of all deaths from cervical cancer (3). These differences reflect not just various genetic factors, but also a higher exposure to potential risk factors, lower rates of screening for cervical cancer in developing countries, and differences in lifestyle and diet (4). Even though the incidence of cervical cancer has decreased from 1975 to 2008, there is still a need for early markers of premalignant lesions. Cervical cancer is strongly associated with HPV infection, which mostly occurs at a younger age, whereas the progression to an invasive carcinoma can occur over decades. This long period of cancer development offers the opportunity for early detection based on biomarkers before the cancer evolves into an invasive carcinoma (5).

Mutation, deletion, and epigenetic modulation of tumor-relevant genes are crucial events contributing to carcinogenesis. In particular, the silencing of tumor suppressor genes and the enhanced expression of oncogenes are characteristic events at early stages of tumor progression (6,7). The tumor suppressor candidate gene LDOC1 (leucine zipper downregulated in cancer) was originally identified by differential RNA display to be downregulated in pancreatic and gastric cancer cells (8). LDOC1 represents a conspicuously small transcription factor of only 146 amino acids (17 kDa) and contains an eponymous DNA-binding leucine zipper motif and a putative protein–protein interacting, proline-rich region (8). Downregulation and differential expression of LDOC1 was shown in tissue samples of esophageal cancer (9) and various types of leukemia (10). Recently, Lee et al. revealed that LDOC1 expression is epigenetically regulated in oral squamous cell carcinoma by promoter methylation (11). LDOC1 is located in the Xq27 chromosomal region, which previously attracted attention because it was identified to contain several segmental duplications with a tendency to genomic instabilities and recombination, including gene deletions, duplications, or inversions (12,13). Since no information about the expression and involvement of LDOC1 in cervical cancer exists to date, we analyzed the expression and regulation of LDOC1 in cervical cancer cells lines and tissue samples.

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## MATERIALS AND METHODS

### Cells and cell culture

The cervical adenocarcinoma cell line HeLa [American type culture collection (ATCC) CCL-2], cervical epidermoid carcinoma cell line Me180 (ATCC HTB-33), cervical carcinoma cell line C33A (ATCC HTB31), and cervical squamous cell carcinoma cell lines CaSki (ATCC CRL-1550), SW756 (ATCC CRL-10302), and SiHa (ATCC HTB-35) were all purchased from ATCC (LGC Standards, Wesel, Germany). Immortalized human foreskin fibroblasts (BJ6tert) were kindly provided by Gabriele Saretzki (Newcastle, UK). Cells were cultured in Quantum 263 medium supplemented with L-glutamine and antibiotics at 37°C in a humidified atmosphere with 5% $CO_2$. All cell culture reagents were bought from PAA (Pasching, Austria). Isolation of human peripheral mononuclear blood cells has recently been described (14).

### RNA preparation and cDNA synthesis

RNA preparation from cervical cancer cells grown as monolayers in cell culture was performed with the RNeasy Plus Mini Kit (Qiagen, Hilden, Germany) as recommended by the supplier, including the use of gDNA eliminator spin columns. Preparation of RNA from tissue samples was performed with TRIzol reagent (Sigma, Munich, Germany) as previously described (15). In brief, tissues were ground with a teflon pestle and solubilized in TRIzol, followed by chloroform extraction and alcoholic precipitation. RNA pellets were dissolved in RNAse-free water (Promega, Mannheim, Germany) and immediately converted into cDNA. Synthesis of cDNA from total RNA was performed with MMVL-reverse transcriptase (Promega) as previously described (15).

Semiquantitative PCR amplification was carried out in a 25 $\mu$L PCR reaction with PCR master mix (M7505, Promega) at 26 cycles ($\beta$-actin) or 32 cycles (LDOC1). Primer pairs used were 5′-CGCAATGGTGGATGAGTTGG-3′ and 5′-ATAATCATCCTCCTCTTCTTCGTCG-3′ for LDOC1 amplification, and 5′-GGAGAAGCTGTGCTACGTCG-3′ and 3′-CGCTCAGGAGGAGCAATGAT -5′ for $\beta$-actin amplification. Quantitative real-time PCR analysis was performed using the LDOC1-specific primers 5′-GGGTGGTGCCCTACATCGAG-3′ and 5′-ATAATCATCCTCCTCTTCTTCGTCG-3′ and the probe FAM-5′-TAGGTGATTACCGGGCCTTC-3′-TAMRA. Real-time PCR was performed with a 7500FAST System real-time PCR cycler (Applied Biosystems, Darmstadt, Germany), using iTaq Fast Supermix with ROX (BioRad, Munich, Germany). Simultaneous amplification of $\beta$-actin (5′-GGAGAAGCTGTGCTACGTCG-3′ and 3′-CGCTCAGGAGGAGCAATGAT-5′; FAM-5′-CCTTCCTGGGCATGGAGTC-3′-TAMRA) was performed as a control and reference. Relative expression analysis was calculated by the $2^{-\Delta\Delta CT}$ method (16). All primers were synthesized by biomers.net (Ulm, Germany).

### Tissue sample collection and ethical approval

Tissue samples from the cervix uteri were collected during surgery at the Department of Obstetrics and Gynecology, Ludwig Maximilians University, Munich, Germany. Samples were either derived from total mesometrial resection (TMMR), laparoscopic assisted vaginal radical hysterectomy (LAVRH), or laparoscopic radical hysterectomy (LRH). In each case, two samples of nonmalignant tissue of the portio and two samples of malignant tissue of the portio were harvested. The study was approved by the local ethics committee of the University Hospital Munich (study number 204–12) and informed consent was obtained from each patient before surgery.

### DNA preparation and bisulfite conversion

DNA isolation and purification was performed with the NucleoSpin Tissue DNA Purification Kit (Macherey-Nagel, Düren, Germany) as recommended by the supplier. Bisulfite conversion from genomic DNA was carried out with the Cells-to-CpG Bisulfite Conversion Kit (Applied Biosystems). PCR analysis on bisulfite-modified DNA was performed with LDOC1 methylation-specific primers and $\beta$-actin primers, as described by Lee et al. (11).

### Generation and transfection of LDOC1 expression plasmids

Full-length human LDOC1 was amplified from HeLa cDNA using PCR primers 5′-CGCAATGGTGGATGAGTTGG-3′ and 5′-ATAATCATCCTCCTCTTCTTCGTCG-3′. Integration of the PCR product into the pcDNA3.1/V5-His-TOPO expression vector (Invitrogen, Karlsruhe, Germany) was performed as previously been described for the MTA1-V5 expression plasmid (17). LDOC1-V5 and MTA1-V5 expression plasmids were transfected into HeLa cells grown on glass cover slips using Lipofectamine 2000 transfection reagent (Invitrogen). After 48-hr incubation, cells were fixed with ice-cold methanol, washed with PBS, and incubated for 2 hrs with a mouse anti-V5 monoclonal antibody (AbD Serotec, Martinsried, Germany), followed by a Cy3-conjugated goat anti-mouse antibody (Dianova, Hamburg, Germany). The cells were subjected to fluorescence microscopy using a Zeiss Axiophot Fluorescence Microscope (Zeiss, Jena, Germany).

### Western blot analysis

For Western blot analysis, HeLa cells were cultured in 24-well cell culture plates and transfected with LDOC1-V5 and MTA1-V5 expression plasmids using the Lipofectamine 2000 transfection reagent (Invitrogen). After the indicated periods of transfection, cell extracts were prepared by cell lysis in RIPA-buffer (Cellsignal, Frankfurt, Germany). Samples containing 20 $\mu$g protein each as determined by the BioRad Bradford Assay (BioRad) were subjected to SDS-polyacrylamide gel electrophoresis, and proteins were transferred to PVDF membranes (Carl-Roth, Karlsruhe, Germany) in a BioRad Mini Protean II Cell (BioRad) at 1 mA/cm$^2$ membrane in 10% methanol, 192 mM glycine, 25 mM Tris, and pH 8.2. The membranes were blocked with 4% nonfat milk powder in PBS-0.05% Tween for 4 hrs. Primary antibodies were applied in blocking buffer and incubated at 4°C temperature overnight. Anti-V5 tag antibody was purchased from AbD Serotec, anti-MTA1 antibody from Bethyl (Montgomery, USA), and anti-actin from Sigma. Secondary, alkaline phosphatase (AP)-coupled goat

antibodies against mouse antibodies were from Dianova. AP detection was performed by the chromogenic BCIP/NBT assay (Promega).

## RESULTS

### Expression of LDOC1 in cervical cancer cell lines

To screen for the expression of LDOC1 in cervical cancer cells, six established cervical cancer cell lines were subjected to RT-PCR analysis. In four of the six, LDOC1 mRNA was completely absent, as shown by semiquantitative RT-PCR analysis (Figure 1A) and by quantitative real-time PCR analysis (Figure 1B). RT-PCR analysis further revealed LDOC1 expression in nonmalignant cell lines, including fibroblasts and peripheral, mononuclear blood cells (Figure 1A).

### LDOC promoter methylation analysis of genomic DNA from cervical cancer cell lines

To test whether LDOC1 silencing was caused by gene deletion, gene rearrangements, or epigenetic regulation, a PCR analysis was first performed for the absence or presence of the LDOC1 coding region on genomic DNA from cervical cancer cell lines. In all six cervical cancer cell lines tested, the full LDOC1 coding region could be amplified, indicating no deletion of the LDOC1 gene in any of these cell lines (Figure 2A). Next, genomic DNA from all six cervical cancer cell lines was converted by the bisulfite reaction and analyzed with methylation-specific primers for the promoter region of LDOC1 (11). A positive reaction with the methylation-specific primers for the LDOC1 promoter was found in each of the cell lines previously shown to lack LDOC1 mRNA expression (SW756, Me180, SiHa, and C33) (Figure 2A).

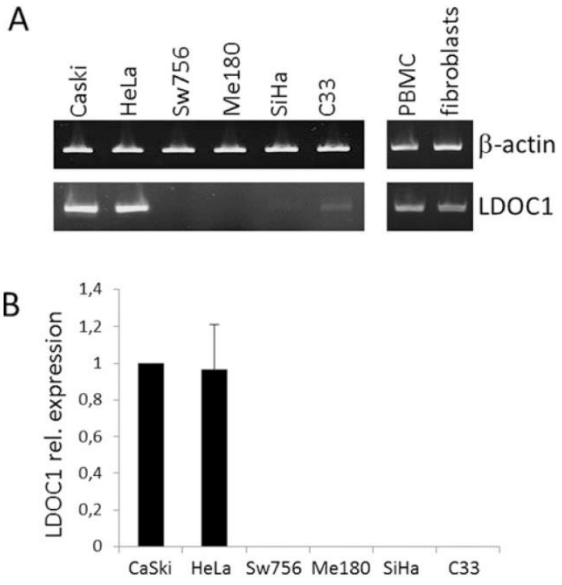


Figure 1. Expression of LDOC1 in cervical cancer cell lines. A panel of six cervical cancer cell lines, in addition to peripheral mononuclear blood cells (PBMC) and immortalized fibroblasts (BJ6 cells), were characterized by semiquantitative RT-PCR for the expression of LDOC1. Cervical cancer cell samples shown in (A) were additionally analyzed by quantitative real-time PCR analysis for LDOC1 expression (B). The expression of $\beta$-actin was used as a cDNA quality control in RT-PCR analysis (A) and as a reference for expression analysis in real-time PCR using the $2^{-\Delta\Delta CT}$ method (B).

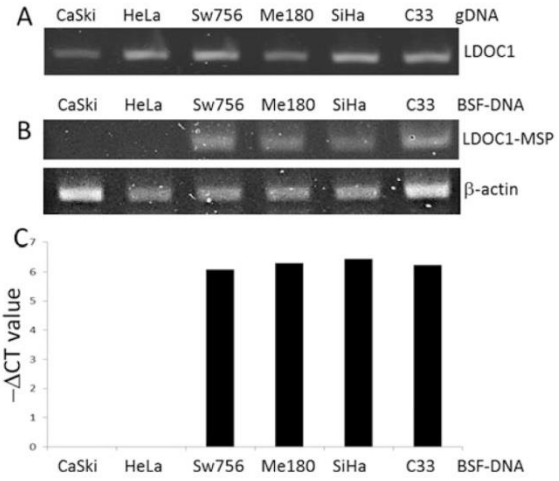


Figure 2. Qualitative PCR analysis of genomic DNA from cervical cancer cell lines. (A) Genomic DNA from cervical cancer cell lines was analyzed by PCR for the presence of the LDOC1 coding sequence. (B) PCR analysis of bisulfite-modified DNA from cervical cancer cell lines with a methylation-specific LDOC1 primer (LDOC1-MSP) and a genomic $\beta$-actin primer. (C) The samples shown in (B) were additionally characterized by a methylation-specific real-time PCR analysis. No real-time PCR signal (Ct-value = 40; $\Delta$Ct-value = 0) could be detected in CaSki or HeLa cell-derived DNA.

### Re-expression of LDOC1 after 5-aza-2′-deoxycytidine treatment

To confirm epigenetic regulation as a mechanism for LDOC1 silencing in cervical cancer cells, Me180 and SiHa cervical cancer cells were incubated with 5-aza-2′-deoxycytidine (AdC; decitabine), a clinically approved inhibitor of DNA methyltransferases. AdC markedly restored LDOC1 expression in Me180 and SiHa cells, as shown by quantitative and semiquantitative RT-PCR analysis (Figure 3).

### Expression of LDOC1 in cervical cancer tissue

To analyze the expression of LDOC1 in cervical cancer tissues, quantitative real-time PCR was performed on cervical cancer tissue samples collected from nine patients during surgery (Figure 4A). A pronounced downregulation of LDOC1 was found in three patients. Unexpectedly, three patients showed slightly elevated levels of LDOC1 compared to the corresponding nonmalignant tissue (Figure 4A).

### Overexpression of LDOC1 induces cell death in cervical cancer cells

Overexpression of LDOC1 has been associated with an apoptosis-promoting phenotype (18). To test the influence of LDOC1 overexpression in cervical cancer cells, a

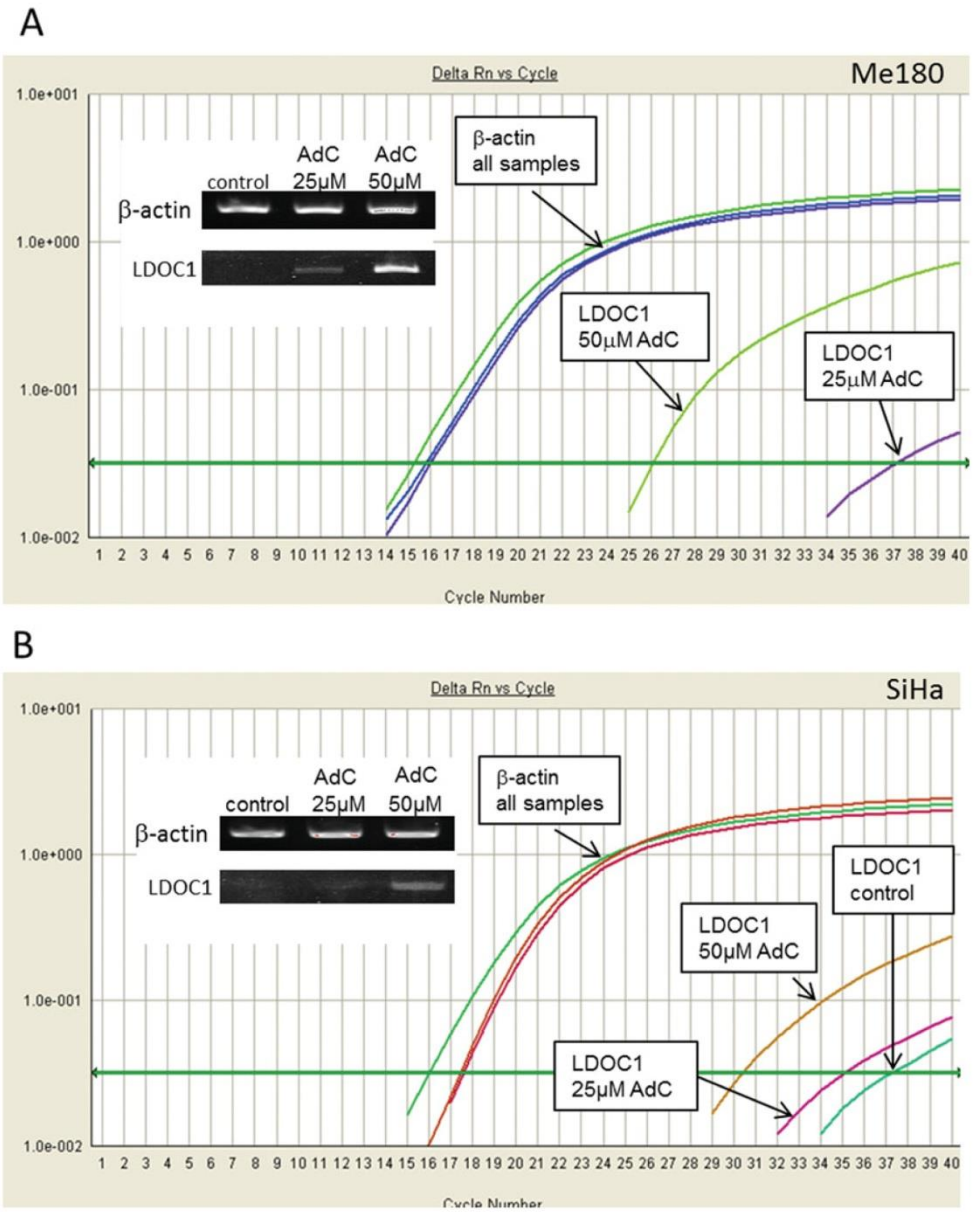


Figure 3. Re-expression of LDOC1 in cervical cancer cell lines by 5-aza-2′-deoxycytidine treatment. Me180 and SiHa cervical cancer cells were incubated for 72 hrs with 5-aza-2′-deoxycytidine and analyzed by real-time PCR and agarose-based PCR (inset) for the expression of LDOC1.

V5-tagged LDOC1 expression plasmid was generated. No stable LDOC1-expressing cell clones could be generated from HeLa, Me180, or C33 cells (data not shown), indicating a growth-inhibitory effect of LDOC1 in cervical cancer cells. Transient transfection into HeLa cells resulted in an irregular and apoptotic morphology with both nuclear and cytoplasmic staining for LDOC1 (Figure 5A). HeLa cells have been used for transfection studies because they normally display a high transfection efficacy of around 60 to 90%. However, the transfection efficacy of LDOC1-V5 appeared poor and restricted to a limited number of cells only (Figure 5A). This also indicates that further cellular mechanisms may exist that interfere with LDOC1 protein expression and stabilization. In contrast to LDOC1 transfection, transfection efficacy was much higher when the proto-oncogenic MTA1 (metastasis-associated gene 1) was transferred into HeLa cells, as documented by immunofluorescence analysis (Figure 5A) and Western blot analysis (Figure 5B). No MTA1-V5 overexpressing cells displayed morphological signs of apoptosis (Figure 5A).

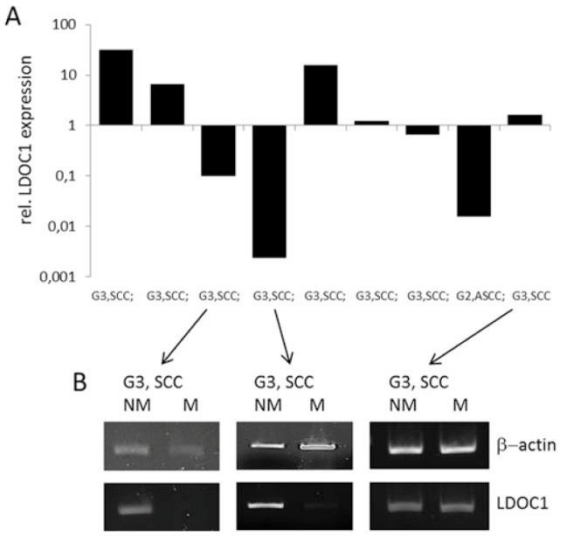


Figure 4. Analysis of LDOC1 expression in cervical cancer tissues and nonmalignant tissue samples. Tissue biopsies were taken from the portio of the cervix uteri from cervical carcinoma and nonmalignant control tissues from the same patient. LDOC1 expression was determined by real-time PCR analysis. The relative LDOC1 expression in malignant cervical cancer samples ($LDOC1_{tumor}-\beta\text{-actin}_{tumor}$) was related to the relative LDOC1 expression in nonmalignant tissue ($LDOC1_{control}-\beta\text{-actin}_{control}$). (B) Semiquantitative RT-PCR analysis of selected samples is shown in (A). NM, nonmalignant sample from the cervical portio; M, malignant sample, cervical carcinoma; SCC, squamous cell carcinoma; ASCC, adenosquamous cell carcinoma.

## DISCUSSION

During the multistep process of tumorigenesis, six biological capabilities are acquired by cancer cells—sustaining proliferative signaling, evading growth suppressors, resisting cell death, enabling replicative immortality, inducing angiogenesis, and activating invasion and metastasis (19). Epigenetic regulation contributes to several of these capabilities and is a frequent mechanism that occurs early during the process of carcinogenesis. Identification of epigenetic changes that lead to a more malignant phenotype of the tumor is therefore of great interest to oncologists. Among the epigenetic changes identified in cervical cancer, E-cadherin is frequently a target of aberrant hypermethylation in cervical carcinoma, in addition to p16, Rb (retinoblastoma protein), PTEN (phosphatase and tensin homolog), DAPK (death-associated protein kinase), VHL (von Hippel–Lindau protein), andSOCS1 (suppressor of cytokine signaling-1) (7,20,21). Our study indicates that promoter methylation of the putative tumor suppressor gene LDOC1 is also a frequent event in cervical cancer, and we present methods to analyze LDOC1 expression and its epigenetic modification in cervical cancer. LDOC1 was originally identified on the basis of its downregulation in pancreatic and gastric cancer cell lines (8), and further investigations have revealed downregulation of LDOC1 mRNA in additional types of cancer (9–11). In oral squamous cell carcinoma, it has recently been shown that LDOC1 is downregulated due to the epigenetic mechanism of promoter methylation (11). The data presented in our study additionally show epigenetic regulation of LDOC1 gene expression in cervical cancer cells.

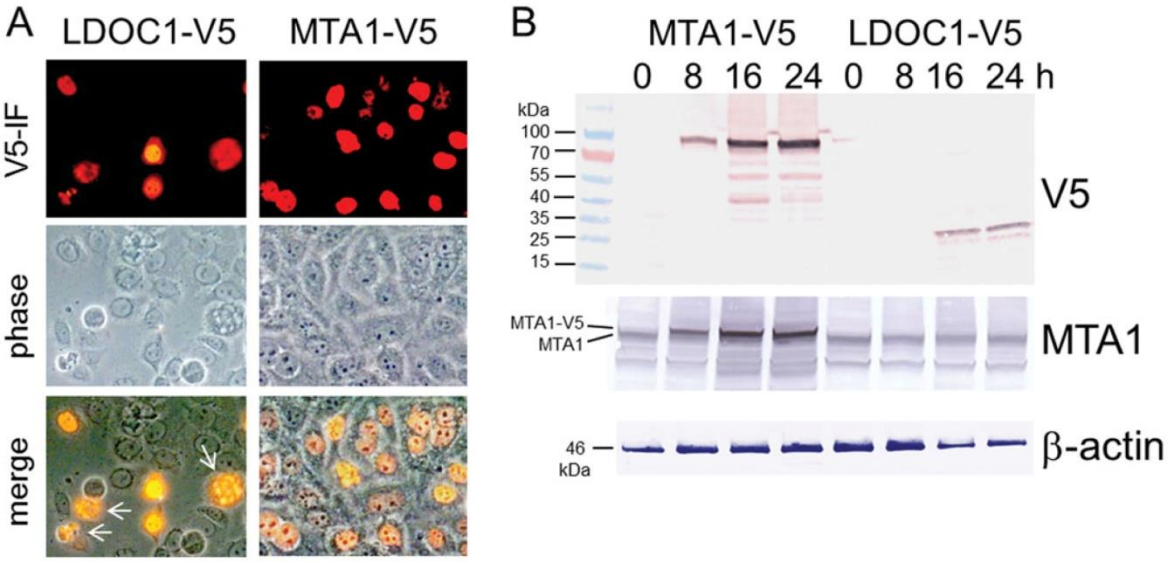


Figure 5. Ectopic overexpression of LDOC1 induces apoptosis in HeLa cells. (A) HeLa cells grown on glass cover slips were transiently transfected with an LDOC1-V5 or MTA1-V5 expression plasmid and analyzed after 48 hrs of incubation by indirect immunofluorescence with a monoclonal anti-V5 antibody followed by a Cy3-conjugated secondary antibody. Apoptotic cells are marked with an arrow. (B) HeLa cells grown in cell culture dishes were transfected for different periods with an LDOC1-V5 or MTA1-V5 expression plasmid and analyzed by Western blot analysis. Marker: prestained marker IV (Peqlab, Erlangen, Germany). LDOC1 = 17 kDa (146 AA); MTA1 = 81 kDa (715 AA); V5-HIS tag = 45 additional AA (AA = amino acids; kDa = relative molecular weight).

A marked downregulation of LDOC1 has previously been described in cases of chronic lymphatic leukemia with somatic mutation (10). However, the same study also revealed that upregulation of LDOC1 in subcases of unmutated chronic lymphatic leukemia was associated with poor prognosis (10). Overexpression of LDOC1 mRNA has also previously been noticed in blood cells of patients with both cutaneous and head and neck squamous cell carcinomas (22, 23). Since the same group also noticed elevated LDOC1 mRNA expression in blood samples of Alzheimer patients, the involvement of LDOC1 as a proapoptotic factor in neurodegenerative disease has been assumed (23). Therefore, not only silencing, but also differential expression (10) has to be considered in the analysis of LDOC1 in certain types of cancer. Notably, the tested cervical cancer tissues had elevated LDOC1 expression, indicating that a differential expression of LDOC1 in cervical cancer may also be considered. However, since LDOC1 expression has also been found in blood cells and nonmalignant cells (10), the possibility cannot be excluded that elevated LDOC1 in cervical cancer tissue samples may be derived from cancer-infiltrating immune cells or fibroblasts. Thus, screening for LDOC1 expression by real-time PCR analysis may be a practical way to identify cancer biopsies with loss of LDOC1 expression, but, for a more reliable analysis, additional or alternative testing by methylation-specific PCR is recommended. Since this technique of PCR-based promoter methylation analysis can identify epigenetic variations within a heterogeneous tissue, it may also identify small subgroups of more undifferentiated cancer cells in intraepithelial lesions or intratumoral cancer cell populations that may develop to more advanced tumor stages.

Pharmacological methylation inhibitors like 5-aza-2′-deoxycytidine (decitabine) have been shown to inhibit DNA methyltransferases, thus causing LDOC1 re-expression in oral squamous cell carcinoma cells (11). In Me180 and SiHa cervical cancer cells, which are deficient in LDOC1 expression, decitabine treatment markedly reactivated LDOC1 expression. Thus, the reversibility of LDOC1 silencing offers a possible target for tumor therapy aimed at re-expressing antiproliferative and proapoptotic genes.

Overexpression of LDOC1 has previously been shown by others to induce cell death in various transfected cell types (18,24). Interestingly, among these studies, HeLa cells were the only cell line tested that did not undergo apoptosis in response to LDOC1 overexpression within 16 hrs of transfection (18). Similar to those authors, we observed no marked apoptotic changes after short-term expression of LDOC1 in HeLa cells, but longer incubation times after LDOC transfection clearly induced apoptosis in HeLa cells (Figure 5A). This indicates that LDOC1 is not an immediate apoptotic factor but may first modulate and change cellular physiology to give rise to a proapoptotic phenotype. In addition, a threshold of LDOC1 expression within the cells seems to be important since several untransformed cell types, as well as HeLa and CaSki cells, already show endogenous LDOC1 expression. Further studies will show whether enhanced expression of LDOC1 at the protein level (e.g., by posttranscriptional or posttranslational modifications caused by apoptotic stimuli) could lead to stabilization or induced activity of the LDOC1 protein under apoptotic conditions. Unfortunately, there are currently no commercially available antibodies against LDOC1 that are suitable for staining for LDOC1 protein expression and localization. A recent study by Mizutani et al., which used a myc-tagged LDOC1 expression vector, revealed that LDOC1 can be excluded from the nucleus by interaction with WAVE3 (Wiskott–Aldrich syndrome protein family verprolin-homologous protein 3) (24). LDOC1 is assumed to function as a nuclear transcription factor or modulator, interacting with other transcription factors of the leucine zipper transcription factor family (25). Members of this family are transcriptional modulators, able to promote or to suppress transcriptional activities by interacting with other transcription factors (25). Of particular importance, LDOC1 has been shown to interact with the NF-$\kappa$B transcription factor complex and to reduce its transcriptional activity (8, 11). NF-$\kappa$B is an essential regulator of cell survival, regulating the expression of several anti-apoptotic proteins (26, 27). Thus, LDOC1 overexpression in cervical cancer cells may lead to a proapoptotic phenotype, and its epigenetic downregulation in cervical cancer, as described in this study, may provide a survival advantage for cervical cancer cells during both cancer progression and chemotherapeutic treatment. In cervical cancer, predictive epigenetic markers of chemotherapeutic response are urgently missing (28). Expression and promoter methylation analysis of LDOC1 may provide a novel biomarker not only for the detection of cervical neoplasias but also for evaluating the responsiveness of cervical cancer to chemotherapeutic regimens.

## ACKNOWLEDGMENT

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## DECLARATION OF INTEREST

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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## 4.4 Epigenetische Inaktivierung des Tumorsuppressorgens LDOC1 in Ovarialkarzinom-Zellen

Buchholtz ML, Brüning A, Mylonas I, **Jückstock J**

### *Epigenetic silencing of the LDOC1 tumor suppressor gene in ovarian cancer cells*

Arch Gynecol Obstet. 2014 Jul;290(1):149-54

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### Zusammenfassung:

Ähnlich unseren Untersuchungen zur LDOC1-Expression beim Zervixkarzinom [65], führten wir Analysen zum Nachweis dieses potentiellen Tumorsuppressorgens an Ovarialkarzinom-Zelllinien [125-127] durch: In vier von sieben Zelllinien war das LDOC1-Gen regelrecht exprimiert, während sich in den anderen Zelllinien eine komplette Inaktivierung des Gens zeigte, die wie bei unseren Versuchen an Zervixkarzinom-Zelllinien ebenfalls durch Methylierungen in der Promotorregion hervorgerufen wurde [128-131]. Die Reaktivierung der LDOC1-Expression und damit seiner physiologischen Funktion gelang, analog zum Zervixkarzinom, durch Zugabe von Decitabin [67]. Hierdurch konnte die epigenetische Regulation des Gens durch (Hyper-)Methylierung der Promotorregion indirekt bestätigt werden. Dieser Ansatz könnte in Zukunft auch zur pharmakologischen Regulation des LDOC1-Gens und damit zur Gentherapie bei Ovarialkarzinomen [132], insbesondere bei beispielsweise Platin-resistenten Rezidiven, angewendet werden: Man kann davon ausgehen, dass die Resistenz der Karzinomzellen gegen Chemotherapeutika unter anderem durch epigenetische Regulationsmechanismen im Sinne einer Inaktivierung von pro-apoptotischen Genen [133] wie dem LDOC1-Gen verursacht wird [134, 135]. Die Aufrechterhaltung der physiologischen Funktion dieses Gens als potentieller Tumorsuppressor kann mit Hilfe geeigneter Substanzen wie Decitabin erreicht werden, wodurch die (Re-)Sensibilisierung der malignen Zellen gegenüber Chemotherapeutika gewährleistet wäre.

Sollte sich in weiteren Versuchen LDOC1 außerdem als zuverlässiger Biomarker zum Nachweis von malignen Ovarialzellen erweisen, wäre seine Bestimmung im peripheren Blut eine einfache, nicht-invasive Möglichkeit zur Früherkennung von Ovarialkarzinomen.

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GYNECOLOGIC ONCOLOGY

# Epigenetic silencing of the LDOC1 tumor suppressor gene in ovarian cancer cells

**Marie-Luise Buchholtz · Ansgar Brüning · Ioannis Mylonas · Julia Jückstock**

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**Abstract**
*Purpose* Due to very unspecific symptoms ovarian cancer often is diagnosed only at a late stage of the disease. Thus, morbidity and mortality of the patients are high. Even the established tumor marker CA12-5 shows only low specificity, rising the need for alternative biomarkers capable of detecting early stages of ovarian cancer. We analyzed the expression of the tumor suppressor candidate gene LDOC1 (leucine zipper downregulated in cancer 1) as a potential early biomarker in ovarian cancer cell lines.
*Methods* A total of seven ovarian cancer cell lines were analyzed by RT-PCR (reverse transcriptase polymerase chain reaction) and real-time PCR for expression of LDOC1. Verification of promoter methylation was performed using methylation-specific primers on bisulfite-modified genomic DNA.
*Results* Three out of seven ovarian cancer cell lines showed a complete loss of LDOC1 gene expression. LDOC1 silencing was caused neither by gene deletion nor gene rearrangements, but by methylation and subsequent inactivation of the concerned promoter as proofed by methylation specific primers. Similarly, promoter methylation could be inhibited by adding AdC (5-aza-2′-deoxycytidine), an inhibitor of DNA methyltransferases. As a result, a reactivation of the LDOC1 gene was seen.
*Conclusions* The tumor suppressor gene LDOC1 in ovarian cancer cell lines is downregulated by promoter methylation and thus may serve as an early biomarker. Further investigation will show if detection of methylated LDOC1 in peripheral blood has both adequate sensitivity and specificity for a timely non-invasive detection of ovarian cancer.

**Keywords** LDOC1 · Tumor suppressor gene · Promoter · Epigenetics · Ovarian cancer

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## Introduction

Ovarian cancer is the second leading cause of death among gynecological malignancies [1]. With 224,747 new cases and 140,163 deaths per year reported worldwide the incidence of ovarian cancer rises proportionally with age. The largest number of patients present with epithelial ovarian cancer (EOC) at ages between 60 and 64 years [2], whereas germ cell tumors represent the predominant histologic type in women younger than 20 years. As ovarian cancer is associated with vague symptoms, as for example abdominal pain or discomfort, menstrual irregularities, dyspepsia and other mild digestive disturbances [2], 60 % of the patients have developed metastasized ovarian cancer at the time of diagnosis with a median 5–year survival rate of 27.3 % [3]. A stratification of patients into low-risk against high-risk disease and prognosis can be made by histologic subtype, tumor grade and disease stage. Clinically, EOCs can be divided into two risk classes: low risk (FIGO stage IA G1-2, IB-IC G1) and high risk (FIGO stages IA G3, IB - IC G2-3 and II, clear cell or undifferentiated histology). Only about 25 % of EOC are diagnosed at an early stage [4].

Risk factors involved in ovarian cancer are of genetic and reproductive origin. Early and multiple pregnancy, prolonged breastfeeding and intake of oral contraceptives are considered protective against ovarian cancer, contributing to a decline in incidence and mortality over the last decades [5–7]. According

to the former incessant ovulation theory [8], it is hypothesized that carcinogenesis of ovarian cancer can be caused by increased wound repair and inflammation after ovulation [9]. More recent approaches state that serous tubal intraepithelial carcinomas (STICS) are precursors in the onset of malignant serous cancer of the ovaries and the fallopian tubes [10, 11].

Late diagnosis and drug resistance of advanced stages [12] represent major obstacles in the treatment of ovarian cancer. Preventive measures include annual cancer checkups by a gynecologist, but both sensitive and specific screening tests for ovarian cancer do not exist yet. Even ultrasound examinations and measurement of the tumor-marker CA-125 show low predictive value [2, 5].

Progress in investigating molecular mechanisms of the tumorigenesis of ovarian cancer has led to the identification of BRCA1 and BRCA2 genes. However, hereditary factors are implicated in only 5–10 % of all cases [2]. In sporadic ovarian cancer a general multistep pathway to carcinogenesis has not yet been described. There is strong evidence that each tumor subtype is characterized by a unique molecular pattern [13]. Certain specific genetic alterations have been found to be causing the development of this cancer entity: abnormalities of growth factors (M-CSF, TGF-beta), growth factor receptors (c-fms, EGFR, Her-2/neu), genes involved in signal transduction (Ki-ras), genes involved in transcriptional regulation (c-myc, p53), and loss of heterozygosity at various chromosomal loci [14].

The tumor suppressor candidate gene LDOC1 (leucine zipper downregulated in cancer 1) was originally identified by differential RNA display to be downregulated in pancreatic and gastric cancer cells [15]. Downregulation and differential expression of LDOC1 was further shown in tissue samples of esophageal cancer and various types of leukemia [16, 17]. Recently Lee et al. [18] revealed epigenetic repression of LDOC1 expression in oral squamous cell carcinoma by promoter methylation.

Epigenetic changes are also a frequent event in ovarian cancer [19]. In a previous study we identified LDOC1 downregulation by promoter methylation in cervical cancer cell lines and in cervical cancer tissue samples as a potentially underlying mechanism of cancer development [20]. Since no information about LDOC1 expression and regulation in ovarian cancer currently exists, we have performed an analysis on the expression and regulation of LDOC1 in ovarian cancer cells.

## Materials and methods

### Cells and cell culture

The ovarian cancer cell lines OV-MZ-6, OV-MZ-26, OV-MZ-30, OV-MZ-31, OV-MZ-33, OV-MZ-37, and OV-MZ-38 were established by Volker Möbus, Frankfurt, Germany, and have previously been described [21]. Cells were cultured in RPMI-1640 medium supplemented with fetal bovine serum and antibiotics at 37 °C in a humidified atmosphere with 5 % $CO_2$. All cell culture reagents were bought from PAA (Paschling, Austria). 5-Aza-2′-deoxycytidine (AdC; decitabine) was purchased from Sigma, Munich, Germany.

### RNA preparation and cDNA synthesis

RNA preparation from ovarian cancer cells grown as monolayers in cell culture was performed with the RNeasy Plus Mini Kit (Qiagen, Hilden, Germany) as recommended by the supplier, including the use of gDNA eliminator spin columns. Synthesis of cDNA from total RNA was performed with MMVL-reverse transcriptase (Promega, Mannheim, Germany) as previously described [21].

### Semi-quantitative and quantitative PCR analysis

Semi-quantitative PCR amplification was carried out in a 25 µl PCR reaction with PCR master mix (M7505, Promega, Mannheim, Germany) at 26 cycles ($\beta$-Actin) or 32 cycles (LDOC1). Primer pairs used were 5′-CGCAATGGTGG ATGAGTTGG-3′, 5′-ATAATCATCCTCCTCTTCTTCGT CG-3′ for LDOC1 amplification, and 5′-GGAGAAGCTG TGCTACGTCG-3′, 3′-CGCTCAGGAGGAGCAATGAT-5′ for $\beta$-Actin amplification. Quantitative real time PCR analysis was performed using LDOC1-specific primers 5′-GGGTGGTGCCCTACATCGAG-3′, 5′-ATAATCATCC TCCTCTTCTTCGTCG-3′ and probe FAM-5′-TAGGTG ATTACCGGGCCTTC-3′-TAMRA. Real time PCR was performed with a 7500FAST System real time PCR cycler (Applied Biosystems, Darmstadt, Germany), using iTaq Fast Supermix with ROX (BioRad, München, Germany). Simultaneous amplification of $\beta$-Actin (5′-GGAGAAG CTGTGCTACGTCG-3′, 3′-CGCTCAGGAGGAGCAATG AT-5′; FAM-5′-CCTTCCTGGGCATGGAGTC-3′-TAMRA) was performed as a control and reference. Relative expression analysis was calculated by the $2^{-\Delta\Delta C_T}$method [22]. All primers were synthesized by biomers.net (Ulm, Germany).

### DNA preparation and bisulfite conversion

DNA isolation and purification was performed with the NucleoSpin Tissue DNA purification kit (Machery-Nagel, Düren, Germany) as recommended by the supplier. Bisulfite conversion from genomic DNA was carried out with the Cells-to-CpG Bisulfite Conversion Kit (Applied Biosystems, Darmstadt, Germany). PCR analysis on bisulfite-modified DNA was performed with LDOC1 methylation-specific primers and $\beta$-Actin primers as presented by Lee et al. [18].

## Results

### Expression of LDOC1 in ovarian cancer cell lines

To screen for the expression of LDOC1 in ovarian cancer cells, seven ovarian cancer cell lines were subjected to RT-PCR analysis. In three out of seven ovarian cancer cell lines, LDOC1 mRNA was found to be completely absent as shown by semi-quantitative RT-PCR analysis (Fig. 1).

### LDOC1 promoter methylation analysis of genomic DNA from ovarian cancer cell lines

To test whether LDOC1 silencing was caused either by gene deletion, rearrangements, or epigenetic regulation, we first performed a PCR analysis for the absence or presence of the LDOC1 coding region on genomic DNA of ovarian cancer cell lines. In all seven cell lines tested, the full LDOC1 coding region could be amplified, indicating no deletion of the LDOC1 gene (Fig. 2a). Next, genomic DNA from all seven ovarian cancer cell lines was converted by the bisulfite reaction and analyzed with methylation specific primers for the promoter region of LDOC1 [18]. A positive reaction with LDOC1 promoter methylation-specific primers was found in all of the cell lines (OVMZ6, OVMZ30, OVMZ37) shown to lack LDOC1 mRNA expression (Fig. 2c).

### Reexpression of LDOC1 after 5-aza-2′-deoxycytidine treatment

Treatment of OVMZ30 and OVMZ37 cells with 5-aza-2′-deoxycytidine (AdC; decitabine), a clinically applicable inhibitor of DNA methyltransferases, markedly restored LDOC1 expression in OVMZ30 and OVMZ37 cells, as shown by quantitative and semi-quantitative RT-PCR analysis (Fig. 3). This confirmed the hypothesis of epigenetic regulation as a mechanism of LDOC1 silencing and the opportunity of a pharmacological modulation of LDOC1 expression in ovarian cancer.

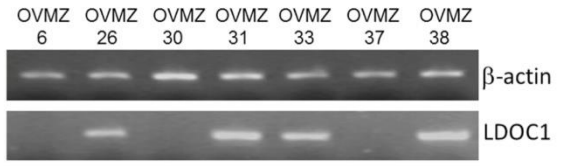


**Fig. 1** Expression of LDOC1 in ovarian cancer cells. A panel of seven ovarian cancer cell lines was characterized by semiquantitative RT-PCR for the expression of LDOC1. $\beta$-Actin expression was used as a cDNA quality control in RT-PCR analysis

## Discussion

In the present study we investigated the expression of LDOC1 in ovarian cancer cells. We found that LDOC1 was downregulated in ovarian cancer cells by the epigenetic mechanism of aberrant promoter methylation.

Several lines of evidence suggest that the process of tumorigenesis can be understood as a succession in genetic alterations leading to a progressive conversion of previously normal cells into cancer cells [23]. Not just genetic alterations but also epigenetic regulation contributes to the multistep process of carcinogenesis. Gene expression, chromosome condensation, segregation and apoptosis are affected by aberrant regulation of these epigenetic mechanisms and stress the relevance of epigenomics in the formation of cancer [23].

Epigenetic mechanisms include histone modification, promoter methylation and microRNA expression. DNA methylation is characterized by methylation of CpG-rich sequences ("CpG islands") within the promoter regions of specific genes. The methylation status of such genes is usually strictly regulated in normal cells and aberrant methylation thus leads to deregulated expression profiles. During tumorigenesis, several tumor suppressor genes have been described to be silenced by DNA hypermethylation, recruitment of repressors and resulting chromatin condensation [19]. Conversely, DNA hypomethylation is usually associated with the activation of oncogenes in cancer [19, 23]. In ovarian cancer, many tumor suppressor genes have been found to be downregulated through aberrant hypermethylation such as BRCA1, hMLH1 (human mutL homolog 1), p16, LOT1, DAPK (death associated protein kinase), TMS1/ASC, RAssF1A and APC [19, 24]. Hypermethylated and silenced BRCA1, together with the DNA mismatch repair gene hMLH1, further facilitate accumulation of mutations leading to ovarian cancer by dysfunctional DNA repair. According to the "drivers and passengers mutation theory" [25], silencing of control and repair genes can facilitate mutations by genetic and epigenetic means leading to uncontrolled proliferation [19].

The gold standard for the treatment of ovarian cancer is surgical excision followed by cytotoxic chemotherapy based on cisplatin and carboplatin [19]. Although most patients are responsive to first line chemotherapy, resistance to conventional chemotherapeutic agents frequently occurs and is associated with higher aggressiveness and metastasis to secondary target tissues [19, 26]. Several studies have indicated that epigenetic inactivation of proapoptotic genes can cause acquired resistance to chemotherapeutic drugs. Functional tumor suppressor genes controlling cell cycle, apoptosis and the DNA damage-dependent proapoptotic pathway are essential for sensitizing cancer cells to DNA-damaging chemotherapeutic agents [27].

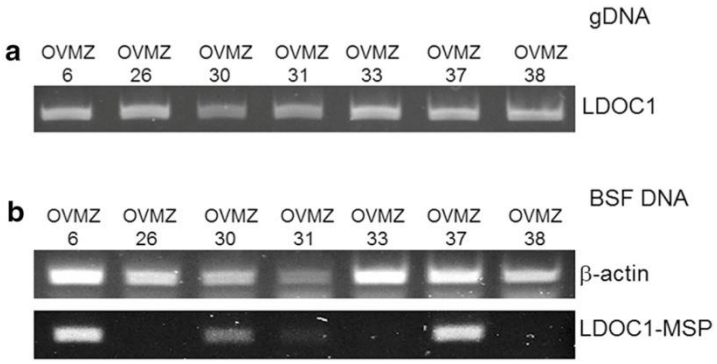


**Fig. 2** Qualitative PCR analysis of genomic DNA from ovarian cancer cell lines. **a** Genomic DNA from ovarian cancer cells was analyzed by PCR for the presence of the LDOC1 coding sequence. **b** PCR analysis on bisulfite-modified DNA of ovarian cancer cells with genomic $\beta$-actin primer and with methylation-specific LDOC1 primer (LDOC1-MSP)

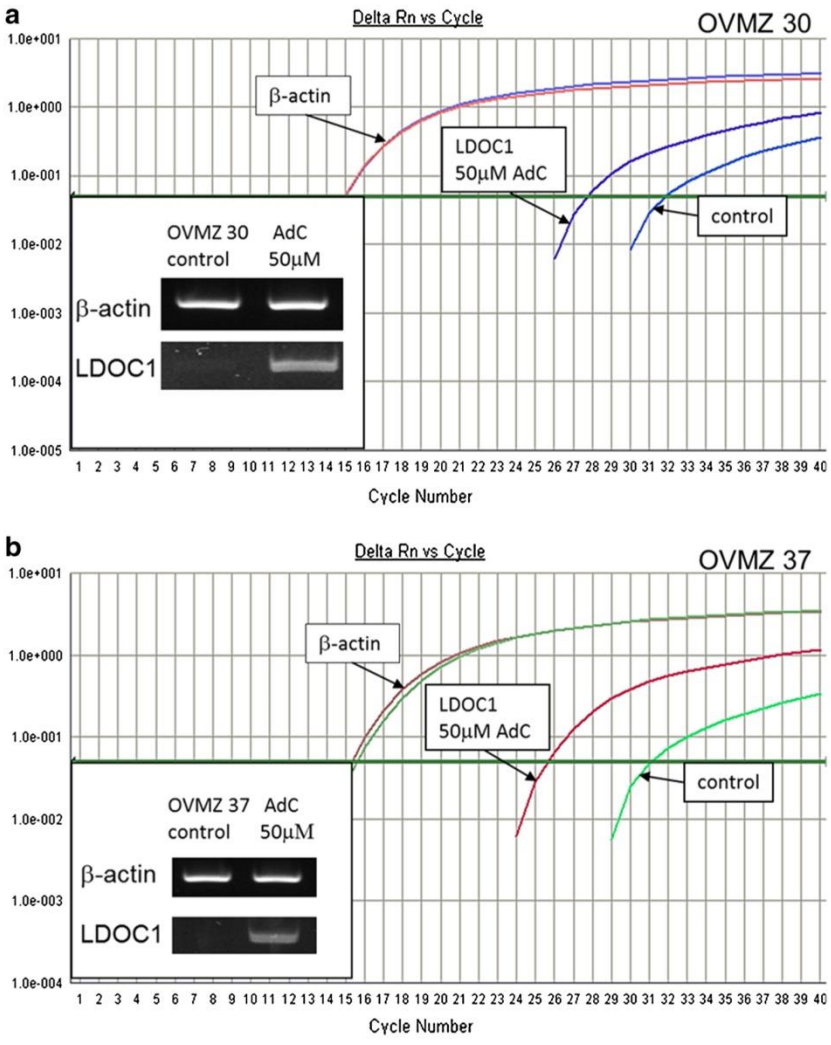


**Fig. 3** Reexpression of LDOC1 in ovarian cancer cell lines by 5-aza-2′-deoxycytidine treatment. OVMZ30 (**a**) and OVMZ37 (**b**) ovarian cancer cells were incubated for 72 h with 50 μM 5-aza-2′-deoxycytidine and analyzed by real-time PCR and agarose-based RT-PCR (inset) for the expression of LDOC1

Inhibition of DNMTs by DNA methyltransferase inhibitors (DMNTI) like 5-aza-2′-deoxycytidine (decitabine) causes reactivation of LDOC1 in oral squamous cell carcinoma [18] and in cervical cancer cell lines as shown in our previous study [20]. In OVMZ30 and OVMZ37 ovarian cancer cell lines, revealing no LDOC1 expression, we could induce a marked reactivation of LDOC1 by decitabine treatment. Thus, the reversibility of epigenetic changes offers a possible target for tumor therapy by reexpressing antiproliferative and pro-apoptotic genes. Our study indicates that promoter methylation of the putative tumor suppressor gene LDOC1 is a frequent event in ovarian cancer cells. A PCR-based LDOC1 promoter methylation analysis of peripheral blood samples that are enriched in circulating tumor cells may serve as an additional biomarker for cancer because epigenetic changes are specific alterations in malignant cells [28, 29]. In combination with other abnormal findings this might be helpful for an earlier detection of ovarian cancer.

Our previous study on LDOC1 involvement in cervical cancer [20] and previous studies by others have shown that overexpression of LDOC1 can induce cell death in various transfected cell types. [30, 31]. Transfection of LDOC1 into HeLa cervical cancer cell lines indicated that this gene is not a single-acting pro-apoptotic factor [20], but may first modulate and change cellular physiology to give rise to a pro-apoptotic phenotype. Furthermore, determination of a cut off for LDOC1 expression seems to be important, because four of the seven ovarian cancer cell lines tested still revealed endogenous LDOC1 expression. LDOC1 is assumed to represent a nuclear transcription factor, interacting with other transcription factors of the leucine zipper transcription factor family [32]. Another important finding has revealed that LDOC1 interacts with the NF-kB transcription factor complex and can reduce its transcriptional activity. NF-kB is an essential regulator of cell survival, controlling the expression of several anti-apoptotic proteins [33, 34]. Thus, LDOC1 silencing by epigenetic modulation through promoter methylation as described in this study may lead to a pro-carcinogenic phenotype in ovarian cancer cells, while overexpression, as shown in our previous study [20], may lead to a pro-apoptotic phenotype.

Accordingly, LDOC1 expression and methylomic analysis of the LDOC1 promoter in the presence of further abnormalities like suspicious ultrasound findings or elevated CA-125 levels may provide a novel biomarker for early detection of ovarian cancer and responsiveness of ovarian cancer cells to chemotherapeutic regimes.

**Conflict of interest** The authors declare that they have no conflict of interest. They also state to have full control of all primary data and they agree to allow the journal to review their data if requested.

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# 5 WEITERE GEPLANTE PROJEKTE UND AUSBLICK

Es wurden verschiedene kommerziell erhältliche Antikörper gegen LDOC1 (Sigma-Aldrich, Santa Cruz Biotech) ausgetestet, aber keiner dieser Antikörper führte zu einem eindeutigen Signal in der Immunfluoreszenz bzw. im Western Blot. Aus diesem Grund wurde ein Nachweis für LDOC1 auf RT-PCR Ebene etabliert und angewendet. Dieser Nachweis wäre grundsätzlich auch für eine Expressionsanalyse von LDOC1 an Mammakarzinom-Geweben möglich. Im Gegensatz zu immunhistologischen Arbeiten kann dabei aber aufgrund der Gewebehomogenisierung nicht zwischen verschiedenen Zelltypen unterschieden werden, was die Aussagen und Analysen eines derartigen Screenings erschweren würde.

Von besonderer Bedeutung ist aber unsere Beobachtung der LDOC1-Promotor-Methylierung in *triple-negativen* Mammakarzinom-Zellen, was mittels methylierungs-spezifischer PCR einen sehr eindeutigen Nachweis von methylierten LDOC1-Promotorregionen erlaubt. Diese Methylierungsanalyse des LDOC1-Promotors wurde von uns und anderen Arbeitsgruppen bereits etabliert [59] und hat den Vorteil eines tumorzellspezifischen Nachweises. Dadurch können selbst wenige maligne Zellen bzw. auch Krebsvorstufen mit LDOC1-Promotor-Methylierung innerhalb eines Gewebeverbands erkannt werden, was die Methode auch für kleinere Biopsien aus Brustdrüsengewebe oder Probeentnahmen aus dem Blut (sog. Flüssigkeitsbiopsien zum Nachweis zirkulierender Tumorzellen) geeignet macht.

Wenn diese vorklinischen Analysen an Mammakarzinom-Gewebeproben und zirkulierenden Tumorzellen aus dem Blut unsere Ergebnisse bestätigen, ist die Korrelation mit klinischen Daten geplant, um die Relevanz für die Klinik zu prüfen. Parallel dazu können *in vivo*-Untersuchungen im Tiermodell zu Wirkung und potentiellen Nebenwirkungen in der Anwendung von Decitabin bei soliden Tumoren erfolgen.

# 6 ZUSAMMENFASSUNG

Ovarialkarzinome sind weltweit eine der Krebsentitäten mit den kürzesten Überlebenszeiten. Da sie wegen fehlender Frühsymptome häufig erst in fortgeschrittenen Stadien diagnostiziert werden, befasst sich die aktuelle Forschung intensiv mit der Tumorbiologie und Faktoren, die eine genauere Charakterisierung des jeweiligen Tumors und damit individuellere Therapieregime ermöglichen. Dadurch wird versucht, die für die Patientin am besten geeignete Behandlung bei gleichzeitig möglichst günstigem Nebenwirkungsprofil auszuwählen.

Brustkrebs ist in Deutschland die häufigste Krebsentität und die fünfthäufigste Todesursache von Frauen. Im Frühstadium kann durch die Entfernung des Tumors und eines oder mehrerer axillärer Lymphknoten, anschließender Radiatio und adjuvanter Therapien (Chemotherapie, endokrine Therapie und zielgerichtete Therapie) manchmal komplette Tumorfreiheit erreicht werden. Wenn sich aber Metastasen gebildet haben, die teilweise auch nach über 10 Jahren auftreten können, ist die Krankheit nur noch palliativ therapierbar.
Auch beim Mammakarzinom befassen sich neuere Forschungsprojekte mit der Identifizierung tumorbiologischer Faktoren, die als Zielstrukturen für individuelle Therapiekonzepte dienen können.

Die Charakterisierung vieler dieser Parameter, die unterschiedliche Typen von Ovarial- und Mammakarzinomen kennzeichnen, ist bereits sogar auf molekularer Ebene gelungen, indem die den einzelnen Faktoren zugrundeliegenden Gene identifiziert und in Gendatenbanken erfasst wurden. Zum großen Teil noch unklar sind jedoch die Signalwege und Zielstrukturen und damit die Wirkmechanismen der jeweiligen Gene und Genprodukte.

Das Metastasen-assoziierte Gen MTA1 ist ein solcher tumorbiologischer Faktor, der im Vergleich zu gesunden Zellen des Ovars und der Mamma in Tumorzellen häufig überexprimiert ist, dadurch deren Invasionsfähigkeit fördert und das Metastasierungspotential erhöht. MTA1 hat die Funktion eines Transkriptionsfaktors und kann so das An- oder Ausschalten und damit die Expression anderer tumorrelevanter Gene regulieren.

Eines dieser Gene ist das Leucin zipper downregulated in cancer 1-Gen (LDOC1), das eine gegensätzliche Funktion hat, nämlich als Tumorsuppressor das Wachstum und Invasionspotential maligner Zellen zu hemmen.

Daher haben Patientinnen, deren Tumor eine normale Expression von LDOC1 zeigt, eine bessere Prognose als solche, in deren Tumor LDOC1 herunterreguliert bzw. komplett inaktiviert ist. Andererseits ist die Prognose von Patientinnen mit MTA1 überexprimierenden Tumoren schlechter, als bei Tumoren mit normaler MTA1-Expression.

Ziel dieses Projekts war die Untersuchung des Einflusses, den MTA1 und LDOC1 auf das Metastasierungspotential beim Ovarial- und Mammakarzinom haben, die Charakterisierung von LDOC1 als möglicherweise durch MTA1 reguliertes Gen, sowie die Identifizierung der Funktion von LDOC1 als potentieller Tumorsuppressor.

Hierzu führten wir Untersuchungen an Ovarialkarzinom-Zelllinien und Gewebeproben von Ovarialkarzinomen, sowie an Mammakarzinom-Zelllinien durch. Mit Hilfe immunhistochemischer Reaktionen konnten wir zeigen, dass beim Ovarialkarzinom die MTA1-Expression bei höherem Grading (G3) signifikant erhöht ist ($p < 0.05$), entsprechend der durch die Tumorprogression entstandenen höhergradigen Entdifferenzierung der malignen Zellen.

Unsere Versuche mittels RT-PCR an Zervixkarzinom- und Ovarialkarzinom-Zelllinien zeigten eine Unterdrückung der Expression von LDOC1 in vier der sechs untersuchten Zervixkarzinom-Zelllinien und in vier der sieben untersuchten Ovarialkarzinom-Zelllinien. Dies ist mit bekanntermaßen schnellerem Wachstum von Tumorzellen im Vergleich zu gesunden Zellen assoziiert und bestätigt die Funktion von LDOC1 als Tumorsuppressor: Nicht entartete Zellen mit normaler Expression von LDOC1 gehen bei Zellschäden bzw. drohender unkontrollierter Zellteilung, wie sie für die Tumorigenese essentiell ist, in einen pro-apoptotischen Phänotyp mit nachfolgendem programmierten Zelltod über. Bereits maligne entartete Zellen, in denen die LDOC1-Expression herunterreguliert oder ganz unterdrückt ist, sterben nicht ab, sondern teilen sich (unkontrolliert) weiter, weil die potentiell tumorsuppressive Wirkung von LDOC1 nicht zum Tragen kommt.

Unsere Untersuchungen bezüglich der Inaktivierung der LDOC1-Expression zeigten als zugrundeliegenden Mechanismus nicht eine strukturelle Veränderung des Gens, wie beispielsweise Deletionen oder Insertionen, sondern eine Methylierung am Anfang des Gens in der Promotorregion. Hierdurch kann das Gen nicht mehr exprimiert werden, und folglich seine Funktion als Tumorsuppressor nicht mehr wahrnehmen.

Durch Hemmung der Methylierungsprozesse in der Promotorregion nach Zugabe des DNA-Methyltransferaseinhibitors Decitabin wird LDOC1 wieder normal exprimiert und bewirkt, dass die Zelle bei drohender maligner Entartung apoptotisch wird und damit abstirbt.

Mit Hilfe eines von uns entwickelten Expressionsplasmids gelang die selektive Re-Expression von LDOC1 in Zervixkarzinom- und Mammakarzinom-Zellen. Allerdings konnten keine dauerhaft LDOC1 re-exprimierenden Zellklone generiert werden. Das lässt auf den tumorsupprimierenden Effekt von LDOC1 schließen, denn nach Re-Expression des Gens waren die malignen Zellen nicht (lange) lebensfähig, sondern starben durch die apoptotische Wirkung von LDOC1 ab.

Die Untersuchungen an neun Mammakarzinom-Zelllinien zeigten ebenfalls ein Herunterregulieren des LDOC1-Gens, wenn auch nicht in gleichem Ausmaß wie bei den Zervix- und Ovarialkarzinom-Zelllinien. Es war jedoch eine signifikante Korrelation des Expressionsverlusts mit der Aggressivität der malignen Zellen festzustellen: Gerade *triple-negative* Mammakarzinom-Zellen, die mit den gängigen Therapieregimen schwerer zu eliminieren sind als Brustkrebszellen anderer Subtypen, zeigten einen deutlicheren Verlust der LDOC1-Expression.

Als dem Expressionsverlust zugrunde liegenden Mechanismus konnten wir analog zu den Zervix- und Ovarialkarzinom-Zelllinien auch bei den Mammakarzinom-Zellen epigenetische Veränderungen im Sinne von Promotor-Methylierungen feststellen.
Entsprechend der Beobachtungen beim Zervix- und Ovarialkarzinom ließ sich auch bei den Mammakarzinom-Zellen die LDOC1-Expression durch Zugabe von Decitabin wiederherstellen.

Auch die im Verlauf der Tumorprogression erfolgte epitheliale-mesenchymale Transition mit Veränderung der Zellmorphologie hin zu einem nicht-epithelialen Phänotyp konnte durch Decitabin rückgängig gemacht werden, sodass die Zellen ihre epitheliale Form zurückgewannen.

Die Analysen an insgesamt neun Mammakarzinom-Zelllinien hinsichtlich MTA1 zeigten in keiner der Zelllinien einen Expressionsverlust von MTA1. Ebenso ließ sich kein Zusammenhang zwischen einer erhöhten MTA1-Expression und einer erniedrigten Expression von LDOC1 feststellen. Daraus kann man schließen, dass diese Gene zwar gegensätzliche Wirkungen hinsichtlich des Invasions- und Metastasierungspotentials maligner Zellen haben, sich aber nicht gegenseitig beeinflussen.

Die Expression des LDOC1-Gens wurde mittels einer bereits lange etablierten und von uns speziell für dieses Gen modifizierten Methode, der Polymerase-Ketten-Reaktion (PCR), untersucht. Hierbei wurde besonderes Augenmerk auf Veränderungen im Sinne von

Methylierungen im Anfangsbereich des Gens (Promotorregion) gelegt, da Genregulierungen bekanntermaßen häufig über Promotor-Methylierungen erfolgen.

In weiteren geplanten Forschungsprojekten sollen Zielgene identifiziert werden, die von LDOC1 reguliert werden und durch die das Wachstum der Tumorzellen gehemmt wird. Hierfür ist das Screening einer größeren Anzahl an Mammakarzinom-Gewebeproben mit Hilfe eines kürzlich gegen LDOC1 entwickelten kommerziellen Antikörpers geplant.

Wenn durch diese Untersuchungen nachgewiesen werden kann, dass LDOC1 beim Mammakarzinom tatsächlich das Tumorwachstum und die Ausbildung von Metastasen hemmt, kann dieses Gen oder eines seiner Zielgene zukünftig als Angriffspunkt für zielgerichtete Therapien genützt werden, wodurch eine Verbesserung der Prognose erreicht werden kann.

Zusammenfassend ist eine Überexpression von MTA1 mit einem erhöhten Invasions- und Metastasierungspotential der Tumorzellen sowohl beim Ovarial- als auch beim Mammakarzinom und mit einer geringeren Differenzierung des Tumorgewebes beim Ovarialkarzinom assoziiert.

Der Tumorsuppressor LDOC1 hat einen gegensätzlichen Wirkmechanismus und ist in vielen malignen Zellen in seiner Expression teilweise oder komplett inaktiviert. Somit beeinflussen beide Gene sowohl das Invasions- als auch das Metastasierungspotential von Tumoren, allerdings in entgegengesetzter Weise, und haben keine gegenseitigen Wechselwirkungen untereinander. Insbesondere zeigte sich, dass LDOC1 nicht wie ursprünglich angenommen durch MTA1 reguliert wird.

Unsere Untersuchungen an LDOC1 zeigten außerdem ein epigenetisches Herunterregulieren dieses Gens durch Promotor-Methylierung, was durch Zugabe des DNA-Methyltransferaseninhibitors Decitabin rückgängig gemacht werden kann.
Damit könnten neue therapeutische Ansatzpunkte und Möglichkeiten etabliert werden, wenn sich Decitabin und ähnliche Substanzen in weiteren Versuchen als verträglich und effektiv genug erweisen.

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# 8 ABKÜRZUNGSVERZEICHNIS

| | |
|---|---|
| AdC | 5-aza-2’-deoxycytidine |
| AML | akute myeloische Leukämie |
| CA-125 | Cancer-Antigen 125 |
| cDNA | complementary deoxyribonucleic acid |
| CLL | chronische lymphatische Leukämie |
| CMV | Cytomegalievirus |
| DNA | deoxyribonucleic acid (Desoxyribonukleinsäure) |
| DNMT | DNA methyltransferases |
| EMT | epithelial-mesenchymale Transition |
| FIGO | Fédération Internationale de Gynécologie et d’Obstétrique |
| HATs | Histon Acetyltransferasen |
| HBL100 | human breast lactating, donor 100 |
| HDAC | Histondeacetylasen |
| HeLa-Zellen | Henrietta Lacks-Zellen |
| HER2 | human epidermal growth factor receptor 2 |
| IRS | Immunreaktiver Score |

| | |
|---|---|
| kDa | Kilo Dalton |
| LDOC | Leucin zipper downregulated in cancer |
| MSP | methylierungsspezifische Primer |
| MTA | Metastasen-assoziiert |
| NF- kappaB | nuclear factor 'kappa-light-chain-enhancer' of activated B-cells |
| NuRD | nucleosome remodeling and histone deacetylation |
| ORF | open reading frame |
| OVCAR3 | National Institute of Health ovarian carcinoma cell line 3 |
| OVMZ36 | Ovar-Mainz-36 |
| PCR | Polymerase chain reaction (Polymerase-Kettenreaktion) |
| RNA | ribonucleic acid (Ribonukleinsäure) |
| RT-PCR | Reverse Transkriptase-Polymerase-Kettenreaktion |
| SANT | Swi3, Ada2, N-Cor, TFIIIB |
| SH3-Domäne | SRC (Sarcoma) Homologie 3-Domäne |
| STAT | Signal transducers and Activators of Transcription |
| TNM | Tumor, Nodes, Metastases |

# 9 ABBILDUNGSVERZEICHNIS

# 10 DANKSAGUNG

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# 11 PUBLIKATIONEN

## 11.1 Erst- und Letztautorenschaften

### 11.1.1 Originalarbeiten

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