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Subtypen des Ovarialkarzinoms - Vorläuferläsionen sowie prognostische und prädiktive Biomarker

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Inhaltsverzeichnis

1. Zusammenfassung

Die histologischen Subtypen des Ovarialkarzinoms zeichnen sich durch jeweils bestimmte molekulare Alterationen aus, welche die Tumorentstehung und Progression entscheidend mitbestimmen und den klinischen Verlauf der Patientinnen beeinflussen. Einige charakteristische molekulare Alterationen dienen in der Diagnostik als immunhistochemische Marker, die in der Zuordnung des jeweiligen Subtyps hilfreich sind. Darüber hinaus können die unterschiedlichen molekularpathologischen Pathogenesewege Hinweise für die Prognose der einzelnen Patientin liefern und potentielle Ansätze für individualisierte Therapieziele darstellen. Die Forschungsarbeiten dieses Habilitationsprojektes sollen zum tumorbiologischen Verständnis der unterschiedlichen histologischen Subtypen des Ovarialkarzinoms beitragen, mit dem Ziel hieraus prognostisch relevante Biomarker zu abzuleiten. Weiterhin sollen die Untersuchungen Anregung für weiterführende Forschungen zu individualisierten Therapieoptionen beim Ovarialkarzinom geben.

Zu Beginn beschäftigten sich unsere Forschungsarbeiten mit der Pathogenese des high-grade serösen Ovarialkarzinoms. Studien der letzten Jahre konnten zeigen, dass ein Großteil der high-grade serösen Ovarialkarzinome sich aus dem Epithel der Tubenmukosa entwickelt und erst sekundär mit dominierendem Tumoranteil im Ovar klinisch manifest wird. Dementsprechend können in diesen Fällen Vorläuferläsionen dieses Subtyps im Epithel der Tuben gefunden werden [33, 43, 57]. Wir hatten kanzeröse und potentiell präkanzeröse Läsionen des Tubenepithels bezüglich ihrer Lokalisation in der Tube untersucht und auf die Expression von Stammzellmarkern in den Tubenläsionen sowie in der Übergangszone zwischen Tubenmukosa und Peritoneum (sogenannte tubar-peritoneale Junktionszone) analysiert. Unsere Untersuchungen unterstützten die Annahme, dass das seröse tubare intraepitheliale Karzinom (STIC) vornehmlich im Fimbrienkranz lokalisiert ist und in Nähe der tubar-peritonealen Junktionszone entsteht. Darüber hinaus konnten wir zeigen, dass sich STICs sowie weitere intraepitheliale Läsionen der Tubenmukosa, wie auch die p53-Signatur durch eine verstärkte Expression des Lymphoid enhancer-binding factor 1 (LEF1) auszeichnen. LEF1, ein Marker des Wnt-Signalweges, war dabei weiterhin in high-grade serösen Ovarialkarzinomen mit einem schlechteren Überleben assoziiert.

Ein Schwerpunkt dieses Habilitationsprojektes stellen die Untersuchungen zu prognostisch relevanten Biomarkern in den histologischen Subtypen des Ovarialkarzinoms dar. Endometrioide und klarzellige Karzinome sind seltene Subtypen des Ovarialkarzinoms, die sich bei unterschiedlicher Morphologie teils auf gleichartige Pathogenesewege begründen und zum Teil dieselben molekularen Alterationen aufweisen. Neben anderen Faktoren ist AT-rich interaction domain 1A (ARID1A), ein nukleäres Protein mit Funktion als Tumorsuppressor, sowohl in endometrioiden als auch in klarzelligen Karzinomen häufig inaktiviert [72]. Durch die Zusammenführung von endometrioiden und klarzelligen Karzinomen des Ovars sowie des Endometriums konnten wir an einem großem Kollektiv demonstrieren, dass die Marker ARID1A, p53 und β-Catenin prognostisch relevante Faktoren für diese beiden Subtypen darstellen.

In zwei weiteren Forschungsprojekten untersuchten wir die Expression von Galektinen und Thyroidhormonrezeptoren (THR) in Ovarialkarzinomen hinsichtlich ihrer prognostischer Bedeutung. In diesen beiden Arbeiten konnten wir demonstrieren, dass sich die untersuchten Galektine und THRs als prognostische Biomarker für Ovarialkarzinome eignen können. Galektine sind Galaktosid-bindende Proteine, die vielfältige Wirkungen haben und auch in der Entstehung und Metastasierung von malignen Tumoren involviert sind [20, 47]. Wir untersuchten die Expression von Galektin-8 und -9 in den histologischen Subtypen des Ovarialkarzinoms und konnten zeigen, dass diese in Abhängigkeit des Expressionsmusters prognostisch relevante Faktoren darstellen. THRs vermitteln als nukleäre Rezeptoren die Transkription bestimmter Gene und können auf diese Weise auch Signalwege der Karzinogenese beeinflussen [37]. Aufgrund ihrer funktionellen Ähnlichkeiten zu Hormonrezeptoren und der Hormonabhängigkeit der Ovarien könnten THRs auch in der Pathogenese von Ovarialkarzinomen von Bedeutung sein und prognostisch relevante Faktoren darstellen. Wir analysierten die Expression von THRα und dessen Isoformen -1 und -2 und konnten zeigen, dass diese für Ovarialkarzinome prognostisch relevante Faktoren darstellen können, die sich jedoch in ihrer prognostischen Bedeutung in den verschiedenen histologischen Subtypen teilweise unterscheiden.

Die extrakapsuläre Ausbreitung einer Lymphknotenmetastase stellt für viele Karzinome, wie beispielsweise des HNO- und des Gastrointestinal-Traktes ein

wichtiges prognostisches Kriterium dar [1, 13]. Auch Ovarialkarzinome weisen bei Erstdiagnose häufig Lymphknotenmetastasen auf, jedoch war die prognostische Bedeutung der extrakapsulären Ausbreitung zu Beginn unserer Untersuchungen noch weitgehend unklar. An einem großen Kollektiv nodal metastasierter Ovarialkarzinome konnten wir zeigen, dass die extrakapsuläre Ausbreitung von Lymphknotenmetastasen auch für Ovarialkarzinome als Indikator eines aggressiven Tumorverhalten gesehen werden kann. Eine extrakapsuläre Ausbreitungen von Lymphknotenmetastasen war weiterhin häufiger in endometrioiden und muzinösen als in serösen Subtypen festzustellen.

Target-basierte Therapiestrategien, deren Wirksamkeit über prädiktive Biomarker in der Primärdiagnostik analysiert werden, bieten inzwischen für viele Tumorpatienten eine aussichtsreiche Behandlungsoption. Zu den mit am häufigsten eingesetzten prädiktiven Biomarkern, die in verschiedenen Tumorentitäten zur Anwendung kommen können, gehören unter anderem der Status von HER2 und PD-L1 sowie das Vorhandensein einer Mikrosatelliteninstabilität (MSI) [6, 55, 63, 69]. Demgegenüber gibt es für Ovarialkarzinome bislang vergleichsweise nur wenige personalisierte Therapiemöglichkeiten. Wir beschäftigten uns in einer Studie mit der Frage, ob sich bereits aus anderen Tumorerkrankungen bekannte therapeutische Zielstrukturen möglicherweise auch in Ovarialkarzinomen finden und sich hieraus relevante prädiktive und prognostische Biomarker ableiten lassen. Wir konnten zeigten, dass ein großer Anteil der high-grade serösen Ovarialkarzinome eine Expression von PD-L1 aufweist. Inzwischen konnte eine potentielle Wirksamkeit von Checkpoint-Inhibitoren von vielen Forschungsgruppen für das high-grade seröse Ovarialkarzinom gezeigt werden, welche im Rahmen von Studien derzeit klinisch geprüft wird [28, 38]. Die PD-L1-Positivität war weiterhin in unserem Studienkollektiv mit einer schlechteren Prognose assoziiert, jedoch ist nach aktueller Wissenslage die prognostische Bedeutung des PD-L1-Status für Ovarialkarzinome bislang unklar [16, 71, 75]. In der Gruppe der high-grade serösen Ovarialkarzinome konnten wir weiterhin einzelne Fälle mit HER2-Amplifikation, MSI und ALK/EML4-Fusion nachweisen. Obgleich diese molekularen Alterationen in Ovarialkarzinomen selten sind, könnten sie jedoch für Einzelfälle eine therapeutische Option beinhalten.

Im Rahmen dieses Habilitationsprojektes gingen wir darüber hinaus auf die differenzialdiagnostische Abgrenzung von muzinösen Neoplasien des Ovars und ovariellen Metastasen der Appendix vermiformis ein. Low-grade muzinöse Neoplasien der Appendix (LAMN) können sich makroskopisch und histologisch wie ein muzinöser Borderline-Tumor oder ein muzinöses Ovarialkarzinom darstellen und dadurch problematische Verwechselungen bedingen [61, 74]. An einer Fallserie ovarieller Metastasen der LAMN konnten wir demonstrieren, wie unter zusätzlicher Anwendung des immunhistochemischen Markers Special AT-rich sequence-binding protein 2 (SATB2) die Abgrenzung zwischen primär muzinösen Neoplasien des Ovars und ovariellen Metastasen der LAMN gelingen kann.

Die Untersuchungen zu den verschiedenen prognostischen und prädiktiven Biomarkern dieses Habilitationsprojektes tragen zum Verständnis der Pathogenese sowie des biologischen Verhaltens der unterschiedlichen Subtypen des Ovarialkarzinoms bei.

2. Einleitung

Ovarialkarzinome stellten 2018 weltweit die achthäufigste malignen Tumore sowie auch die achthäufigste zum Tode führende Tumorentität der Frau dar [9]. Aufgrund weitgehend fehlender Möglichkeiten der Früherkennung sind bei Diagnosestellung ca. 70% der Ovarialkarzinome bereits peritoneal metastasiert, so dass auch nach chirurgischer Resektion und adjuvanter Chemotherapie die Prognose ungünstig ist [3, 34]. Dennoch weisen Ovarialkarzinome im klinischen Verlauf sowie in der Prognose deutliche Unterschiede auf. Wesentliche Erkenntnisse der letzten Jahrzehnte konnten zeigen, dass das Ovarialkarzinom einen Überbegriff für eine sehr heterogene Tumorgruppe darstellt, welche hinsichtlich unterschiedlicher Histomorphologie und molekularpathologischer Pathogenesewege verschiedene Subtypen unterscheiden lässt. Zu den wichtigsten prognostischen Faktoren gehören das Tumorstadium sowie der postoperative Tumorrest [19]. Darüber hinaus stellen für den klinischen Verlauf und die Prognose neben Alter und Allgemeinzustand der Patientin auch die Tumorgraduierung und der histologische Subtyp einen entscheidenden Parameter dar (Abbildung 1).

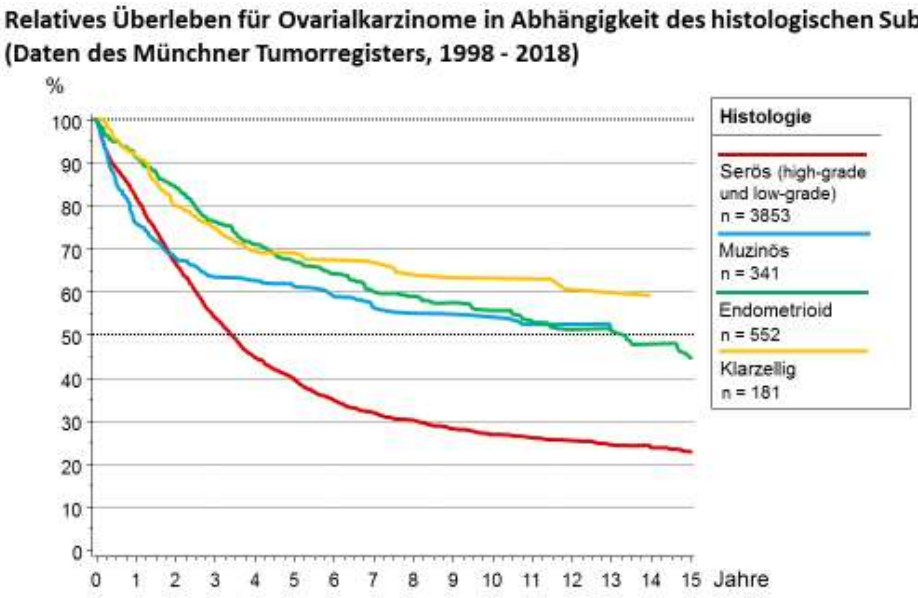


Abbildung 1: Daten des Münchner Tumorregisters für das relative Überleben von Ovarialkarzinomen in Abhängigkeit des histologischen Subtyps (1998–2018; Gesamtanzahl = 4927). Das relative Überleben berücksichtigt die Lebenserwartung der Normalbevölkerung und stellt so einen Schätzer des tumorabhängigen Überlebens dar. Unberücksichtigt in dieser Darstellung sind weitere prognostisch relevante Faktoren, wie beispielsweise das Tumorstadium. Es handelt sich um teils ältere Daten, bei denen eine Untergliederung des serösen Subtyps in high-grade und low-grade Karzinome noch nicht vorgenommen wurde. Mit freundlicher Genehmigung des Münchner Tumorregisters [31].

Aufgrund molekularer Analysen gelang es, die Tumorbiologie der bekannten histomorphologischen Subtypen besser zu verstehen, so dass neue Klassifikationen bzw. Subklassifikationen entwickelt wurden. So weiß man heute, dass der häufigste Subtyp, das high-grade seröse Karzinom eine p53-Mutation aufweist und in diese Gruppe auch die meisten genetisch verursachten Ovarialkarzinome mit somatischer oder hereditärer Mutation im BRCA1 oder -2-Gen fallen [11, 40]. Im Rahmen prophylaktischer Adnexektomien bei Patientinnen mit BRCA1/-2-Keimbahnmutation wurde weiterhin entdeckt, dass ein Großteil der high-grade serösen Karzinome nicht im Ovar, sondern in der Tube über ein seröses tubares intraepitheliales Karzinom entsteht [10, 45]. Demgegenüber entwickeln sich low-grade seröse und muzinöse Karzinome schrittweise über Zystadenome und Borderline-Tumoren [23, 48]. Low-grade seröse Karzinome weisen häufig eine KRAS- oder BRAF-Mutation auf [66]. KRAS-Mutationen werden auch bei muzinösen Karzinomen als ursächlich gesehen [15, 23]. Die selteneren Subtypen, wie die endometrioiden und klarzelligen Karzinome sind zumindest in einem Teil der Fälle auf eine atypische Endometriose zurückzuführen. Sie zeigen weiterhin häufig eine Dysregulation im Wnt-Signalweg sowie Mutationen in PIK3CA, PTEN, ARID1A und KRAS [44, 62, 72]. Ein Teil der endometrioiden und klarzelligen Ovarialkarzinome weist darüber hinaus eine Mikrosatelliteninstabilität auf und ist mit einem Lynch-Syndrom assoziiert [12, 36].
Trotz dieses enormen Kenntnisstandes in der Tumorbiologie sind spezielle, Target-orientierte Therapien und prognostische Marker in der Diagnostik des Ovarialkarzinoms im Vergleich zu Karzinomen des Gastrointestinal-Traktes oder der Lunge deutlich weniger etabliert. Einen großen Erfolg in der Target-basierten Therapie des high-grade serösen Ovarialkarzinoms sind jedoch PARP-Inhibitoren, die über ein Zusammenwirken mit BRCA1/-2-Mutationen DNS-Schäden der Tumorzellen bewirken [25, 60]. Weiterhin konnte gezeigt werden, dass eine anti-hormonelle Therapie den Krankheitsverlauf von low-grade serösen Ovarialkarzinome günstig beeinflussen kann [24]. In Studien wird derzeit ferner untersucht, ob PD-L1 Inhibitoren eine mögliche Therapieoption für high-grade seröse Ovarialkarzinome darstellen [28, 38]. Charakteristische Parameter der häufigsten Subtypen des Ovarialkarzinoms sind in Abbildung 2 dargestellt.

Charakteristische Parameter der häufigsten histologischen Subtypen des Ovarialkarzinoms

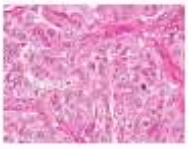
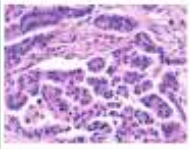
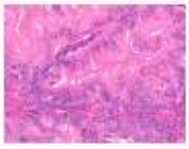
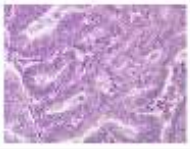
	High-grade serös	Low-grade serös	Muzinös	Endometrioid	Klarzellig
					
Häufigkeit	70%	<5%	3-7%	10-15%	5-10%
Risikofaktoren	BRCA1/2 Keimbahn-mutation	-	-	Lynch Syndrom	Lynch Syndrom
Vorläuferläsionen	STIC	BOT	BOT	Endometriose, BOT	Endometriose
Häufige Mutationen	p53, BRCA1/2	KRAS, NRAS, BRAF	KRAS, HER2	PTEN, CTNNB1, ARID1A	ARID1A, CTNNB1
Spezielle Therapieansätze	PARP-Inhibitoren	Anti-hormonelle Therapie	-	Anti-hormonelle Therapie (PARP-Inhibitoren bei geringer Differenzierung)	-

Abbildung 2: Charakteristische Parameter der häufigsten histologischen Subtypen des Ovarialkarzinoms (ohne maligne Brennertumore). Modifiziert nach *Lheureux et al, CA - A Cancer Journal for Clinicians, 2019* und *Singh et al, Frontiers in Cell and Developmental Biology, 2019* [46, 67].

3. Zielsetzung und Fragestellung

Für viele Tumorerkrankungen sind molekulare Biomarker etabliert, die neben den klinisch-pathologischen Parametern eine weitere Einschätzung der Prognose und des Krankheitsverlaufs ermöglichen und Target-basierte Therapieoptionen aufzeigen können. Im Rahmen dieses Habilitationsprojektes wurde untersucht, ob sich histologische Subtypen des Ovarialkarzinoms durch spezielle molekularpathologische Alterationen hinsichtlich prognostischer und prädiktiver Biomarker weiter charakterisieren lassen. Mit dem Ziel zu einer Verbesserung und Individualisierung in der Therapie von Patientinnen mit Ovarialkarzinom beizutragen, wurden Kollektive mit unterschiedlichen histologischen Subtypen des Ovarialkarzinoms auf prognostische Biomarker untersucht. Darüber hinaus können möglicherweise auch bereits bekannte Zielstrukturen mit etablierten Therapieoptionen anderer Tumorerkrankungen auch für Ovarialkarzinome einen vielversprechenden Ansatz darstellen, deren Eignung als prädiktive Biomarker analysiert wurden.

Im Speziellen wurde in den Forschungsarbeiten auf folgende Fragestellungen eingegangen:

- Welche molekularen Alterationen finden sich in den unterschiedlichen histologischen Subtypen und Vorläuferläsionen des Ovarialkarzinoms?

- Ergeben sich aus den jeweiligen molekularen Alterationen prognostische Informationen für die verschiedenen histologischen Subtypen des Ovarialkarzinoms?

- Können bereits in anderen Tumorerkrankungen etablierte und therapeutisch nutzbare Biomarker auch für Ovarialkarzinome potentielle Therapieansätze darstellen, und weisen diese eine signifikante Häufung in bestimmten histologischen Subtypen auf?

In Bezug auf diese Fragestellungen wurde im Rahmen des Habilitationsprojektes Tumormaterial aus Patientenkollektiven mit gut dokumentiertem Krankheitsverlauf

untersucht und die gewonnen Befunde mit klinisch-pathologischen Parametern korreliert. Durch Analysen der histologischen Subtypen wurden Rückschlüsse auf eine mögliche prognostische und prädiktive Wertigkeit der analysierten Biomarker gezogen.

4. Zusammenfassung und Diskussion themenbezogener eigener Arbeiten

4.1. LEF1 is preferentially expressed in the tubal-peritoneal junctions and is a reliable marker of tubal intraepithelial lesions.

Schmoeckel E, Odai-Afotey AA, Schleißheimer M, Rottmann M, Flesken-Nikitin A, Ellenson LH, Kirchner T, Mayr D, Nikitin AY.
Mod Pathol. 2017 Sep;30(9):1241-1250. doi: 10.1038/modpathol.2017.53.

Studien der letzten Jahre konnten zeigen, dass ein Großteil der high-grade serösen Ovarialkarzinome, welche den häufigsten Subtyp darstellen, aus dem Epithel der Tubenmukosa hervorgehen. Nach diesem Modell führen seröse tubare intraepitheliale Karzinome (STIC) des Tubenepithels zu peritonealen Metastasen, die mit Hauptmanifestation im Ovar klinisch auffällig werden [33, 57]. Diese Theorie geht zum einen auf den Nachweis dysplastischer Vorläuferläsionen des Tubenepithels zurück, die in prophylaktischen Adnexektomien bei BRCA1/2 Keimbahnmutationsträgerinnen gefunden wurden [59]. Weiterhin konnte die identische p53-Mutation in STICs und high-grade serösen Karzinomen jeweils derselben Patientin nachgewiesen werden [39]. Vorangegangene Studien zeigten, dass STICs meistens in der Nähe der tubar-peritonealen Junktionszone lokalisiert sind [43, 64]. Um zu untersuchen, ob weitere Läsionen des Tubenepithels, wie die p53-Signatur und die Proliferation sekretorischer Zellen (secretory cell outgrowth, SCOUT) ebenfalls in Nähe der Junktionszone auftreten und damit möglichweise Vorläuferläsionen des STIC darstellen, untersuchten wir Tuben aus prophylaktischen Adnexektomien von BRCA1/2-Keimbahnträgerinnen sowie Tuben aus Adnexektomien sporadischer high-grade seröser Ovarialkarzinome.

Wir konnten in dieser Studie zeigen, dass auch in unseren Kollektiven STICs nahe der Junktionszone lokalisiert sind. p53-Signaturen fanden sich ebenfalls am häufigsten in der distalen Tube. Hingegen waren SCOUTs hauptsächlich im proximalen Tubenabschnitt lokalisiert. Weiterhin konnten wir zeigen, dass STICs signifikant größer waren als p53-Signaturen und SCOUTs.

Da epitheliale Transitionszonen, wie beispielweise an der Cervix uteri, des gastro-ösophagealen - und ano-rectalen Überganges Merkmale von Stammzellen aufweisen können [21, 52], untersuchten wir die Expression von Stammzellmarkern in der Junktionszone von Tuben mit und ohne tubare intraepitheliale Läsionen im Vergleich. Unter den untersuchten Markern, wurde LEF1 in der tubar-peritonealen Junktionszone

sowie in den tubaren intraepithelialen Läsionen exprimiert, unabhängig des p53-Status. SCOUTs wiesen zusätzlich eine starke nukleäre Expression von β-Catenin auf, welches zusammen mit LEF1 im WNT-Signalweg interagiert [14]. STICs und p53-Signaturen zeigten hingegen eine zytoplasmatische β-Catenin-Expression, welches auf einen WNT-unabhängige Funktion von LEF1 in diesen beiden Läsionen deuten lässt. In high-grade serösen Karzinomen korrelierten sowohl die Expression von LEF1 als auch die nukleäre β-Catenin-Expression mit einem schlechteren 5-Jahres Überleben.

Unsere Ergebnisse unterstützen damit die Hypothese einer sogenannten Stammzellnische in der tubar-peritonealen Junktionzone, von der ausgehend, sich möglicherweise Vorläuferläsionen des serösen high-grade Karzinoms entwickeln können. Weiterhin lassen die Befunde annehmen, dass sich die Pathogenese der SCOUTs von STICs und p53-Signaturen unterscheidet. Darüber hinaus könnten LEF1 und β-Catenin als ergänzende Faktoren in der Diagnostik von tubaren intraepithelialen Läsionen hilfreich sein.

4.2 The ARID1A, p53 and ß-Catenin statuses are strong prognosticators in clear cell and endometrioid carcinoma of the ovary and the endometrium.

Heckl M, Schmoeckel E, Hertlein L, Rottmann M, Jeschke U, Mayr D.
PLOS One 2018 Feb 16;13(2):e0192881. doi: 10.1371/journal.pone.0192881.
Geteilte Erst-Autorenschaft mit Heckel M

Endometrioide und klarzellige Karzinome des Ovars und des Endometriums weisen bei unterschiedlicher Histomorphologie tumorbiologische Gemeinsamkeiten auf, welche beispielsweise in einer Endometriose als gemeinsame Vorläuferläsionen zum Ausdruck kommen [56]. Weiterhin zeigen beide Tumorentitäten teilweise gleichartige molekulare Alterationen, wie Mutationen in den Tumorsuppressorgenen ARID1A und p53 und eine Dysregulation des β-Catenin-vermittelten WNT-Signalwegs [44, 62, 72]. In dieser Studie untersuchten wir die Expression von ARID1A, p53, p16 und β-Catenin hinsichtlich ihrer prognostischen Bedeutung in insgesamt 80 endometrioiden und 17 klarzelligen Karzinomen des Ovars und Endometriums an einem tissue microarray (TMA).

Die beiden histologischen Subtypen zeigten zunächst signifikante Unterschiede in der Expression von ARID1A, p53, p16 und β-Catenin: Klarzellige Karzinome zeigten häufiger einen Expressionsverlust von ARID1A sowie eine aberrante Expression (Überexpression oder vollständiger Expressionsverlust) von p53. Weiterhin wies dieser Subtyp etwas häufiger eine Überexpression oder vollständig fehlende Expression von p16 auf. Hingegen war eine nukleäre Expression von β-Catenin häufiger in endometrioiden Karzinomen zu finden. In Bezug auf das Gesamtkollektiv war der Expressionsverlust von ARID1A mit einem höheren FIGO Stadium (III-IV) assoziiert. Die nukleäre Expression sowie auch der vollständige Expressionsverlust von β-Catenin waren mit einer schlechteren Tumorgraduierung (G2 und G3) korreliert. In den multivariaten Überlebensanalysen stellten der Expressionsverlust von ARID1A, die aberrante p53 Expression, eine starke Expression von p16 sowie die nukleäre Expression und der vollständige Expressionsverlust von β-Catenin jeweils unabhängige prognostische Faktoren dar, die mit einem kürzeren Gesamtüberleben assoziiert waren.

4.3 Overall Survival of Ovarian Cancer Patients Is Determined by Expression of Galectins-8 and -9.

Schulz H, Kuhn C, Hofmann S, Mayr D, Mahner S, Jeschke U, **Schmoeckel E**.
Int J Mol Sci. 2018 Jan 22;19(1). pii: E323. doi: 10.3390/ijms19010323.

Galektine sind eine Familie der Karbohydrat-bindenden Proteine, die über die Bindung zu β-Galaktosiden Zell–Zell- und Zell–Matrix-Interaktionen sowie auch intrazelluläre Signalwege beeinflussen [4]. In der Pathogenese maligner Tumoren haben Galektine vielfältige Wirkungsweisen und können in Zellproliferation, Resistenz gegenüber dem Zelltod sowie Invasion, Angiogenese und Metastasierung involviert sein [20, 47]. Die Galektine-8 und -9 stellten sich unter anderem in triple-negativen Mammakarzinomen und in Magenkarzinomen als prognostisch relevante Faktoren heraus [26, 35]. Wir untersuchten in dieser Studie die Expression von Galektin-8 und -9 an einem TMA (tissue microarray) mit 156 Ovarialkarzinomen hinsichtlich deren prognostischer Bedeutung. In Bezug auf das Gesamtüberleben sowie das erkrankungsfreie Überleben stellte die Expression von Galektin-8 einen positiven prognostischen Faktor dar. Die prognostische Bedeutung von Galektin-9 erwies sich hingegen abhängig von

der Expressionsstärke: Bei moderater Expression zeigte sich eine Korrelation mit einem schlechteren Verlauf während eine starke Expression mit einer günstigeren Prognose assoziiert war. Die gegensätzlich prognostische Bedeutung der Galektin-9 Expression kann möglicherweise auf die Funktion von Galektin-9 zum einen in der Tumorimmunität sowie auf dessen Funktion in der Apoptose, Adhäsion der Tumorzellen und Metastasierung zurückzuführen sein [29, 65]. Hinsichtlich des histologischen Subtyps fand sich eine Korrelation mit Galektin-9, jedoch nicht mit Galektin-8.

Dieser Studie zeigt, dass sich die Galektine-8 und -9 als prognostische Biomarker für Ovarialkarzinome eignen können, wobei deren prognostische Bedeutung auch von dem jeweiligen Expressionsmuster abhängig zu sein scheint.

4.4 Cytoplasmic versus nuclear THR alpha expression determines survival of ovarian cancer patients

Ditsch N, Heublein S, Jeschke U, Sattler C, Kuhn C, Hester A, Czogalla B, Trillsch F, Mahner S, Engel J, Mayr D, Schmoeckel E

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Die Thyroidhormon-Rezeptoren (THR) sind nukleäre Rezeptoren, die als ligandenabhängige Transkriptionsfaktoren die Transkription bestimmter Gene bewirken und hierdurch auch in der Karzinogenese verschiedener Tumoren involviert sein können [17, 37]. Aufgrund ihrer funktionellen Ähnlichkeiten zu Hormonrezeptoren sowie der Hormonabhängigkeit der Ovarien könnten THRs auch in der Pathogenese von Ovarialkarzinomen von Bedeutung sein und prognostische relevante Faktoren darstellen, wie dies zuvor für Mammakarzinome gezeigt werden konnte [17, 70]. In dieser Studie untersuchten wir die Expression von THRα und dessen Isoformen -1 und -2 in Bezug auf deren prognostische Bedeutung in einem Kollektiv von 156 Ovarialkarzinomen.

In Abhängigkeit des Expressionsmusters (nukleär oder zytoplasmatisch) konnten wir für THRα und dessen Isoformen -1 und -2 unterschiedliche Auswirkungen für die Prognose der verschiedenen Subtypen des Ovarialkarzinoms feststellen: Unter Berücksichtigung aller Subtypen stellte die nukleäre Expression von THRα1 einen positiven prognostischen Faktor für das Gesamtüberleben dar. Die nukleäre Expression von THRα2 korrelierte mit einem längeren Überleben in serösen Subtypen,

wohingegen die zytoplasmatische Expression von THRα2 unter Berücksichtigung aller Subtypen mit einem kürzeren Überleben assoziiert war. Die zytoplasmatische Expression von THRα1 war in muzinösen Ovarialkarzinomen ebenfalls mit einem kürzeren Überleben korreliert. Unter allen Subtypen zeigte sich die stärkste THRα-Expression in klarzelligen Ovarialkarzinomen, wobei die nukleäre Expression in diesem Subtyp mit einem kürzeren Gesamtüberleben einherging.

4.5 Extracapsular Lymph Node Involvement in Ovarian Carcinoma

Heublein S, Schulz H, Marmé F, Angele M, Czogalla B, Burges A, Mahner S, Mayr D, Jeschke U, Schmoeckel E.
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Die extrakapsuläre Ausbreitung von Lymphknotenmetastasen stellt für Karzinome des HNO- und des Gastrointestinal-Traktes sowie auch der Mamma, der Cervix uteri und der Vulva ein entscheidendes prognostisches Kriterium dar [1, 13, 22, 30, 49], welches als prognostischer Faktor in die Tumorklassifikationen von HNO-Karzinomen und Vulvakarzinomen mit eingeht. Ovarialkarzinome weisen bei Erstdiagnose häufig eine Metastasierung in retro-peritoneale Lymphknoten auf [7, 54], jedoch war zum Zeitpunkt des Beginns dieser Studie noch weitgehend unklar, ob die extrakapsuläre Ausbreitung von Lymphknotenmetasten auch in dieser Tumorentität von prognostischer Bedeutung ist.
An einem Kollektiv mit 143 Fällen nodal metastasierter Ovarialkarzinome untersuchten wir die Häufigkeit von Lymphknotenmetasen mit extranodaler Ausbreitung und deren prognostische Bedeutung. Daneben analysierten wir die Expression von immunhistochemischen Biomarkern im Primärtumor mit der Frage, ob diese Indikatoren für das Vorliegen von Lymphknotenmetastasen mit extrakapsulärer Ausbreitung darstellen können.
In unserem Kollektiv lag eine extrakaspuläre Ausbreitung von Lymphknotenmetastasen in 24.5% der untersuchten Fälle vor. Diese Fälle waren mit einer höheren Gesamtanzahl an Lymphknotenmetasen sowie auch mit einer schlechteren Tumorgraduierung assoziiert. Interessanterweise war eine extrakapsuläre Ausbreitung häufiger in den nicht serösen Subtypen (high-grade sowie low-grade) zu finden. Sowohl die extrakapsuläre Ausbreitung einer

Lymphknotenmetase als auch eine hohe Anzahl an Lymphknotenmetasen waren mit einem kürzeren Gesamtüberleben assoziiert.
In der Arbeit stellten wir darüber hinaus eine Signatur mit vier Markern vor, deren Expression im Primärtumor mit einer extrakapsulären Ausbreitung von Lymphknotenmetastasen korrelierte. Die immunhistochemische Expression von VU4H5 (Mucin-1) korrelierte positiv, die Expression von Galektin-3, Galektin-8 und G protein-coupled estrogen receptor 1 (GPER) hingegen negativ mit dem Vorkommen einer extrakapsulären Ausbreitung der Lymphknotenmetastasen.
In dieser Studie zeigten wir, dass die extrakapsuläre Ausbreitung von Lymphknotenmetastasen in einem großen Teil der nodal metastasierten Ovarialkarzinome vorliegt und einen Indikator für ein aggressives Tumorverhalten darstellen kann.

4.6 Comprehensive analysis of PD-L1 expression, HER2 amplification, ALK/EML4 fusion, and mismatch repair deficiency as putative predictive and prognostic factors in ovarian carcinoma.

Schmoeckel E, Hofmann S, Fromberger D, Rottmann M, Luthardt B, Burges A, Jeschke U, Kirchner T, Lax SF, Mayr D.
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Inzwischen sind für viele maligne Tumorerkrankungen prädiktive Biomarker etabliert, die über den Einsatz von zielgerichteten therapeutischen Targets entscheiden. Zu den derzeit am häufigsten verwendeten Biomarkern gehören die HER2-Amplifikation, der MSI-Status, die ALK/EML4-Translokation und die Expression von PD-L1, welche insbesondere in Mammakarzinomen [55, 63], gastro-intestinalen Karzinomen [6, 55, 73] und in Bronchialkarzinomen [41, 69] zur Anwendung kommen. Wir beschäftigten uns mit der Frage, ob sich diese therapeutischen Zielstrukturen möglicherweise auch in Ovarialkarzinomen finden und relevante prädiktive und prognostische Biomarker für diese Tumorerkrankung darstellen können. Wir erstellten für dieses Projekt ein Kollektiv von 288 Fällen, welches alle primären Ovarialkarzinome umfasste, die innerhalb von fünf Jahre an der LMU diagnostiziert worden sind. Anhand eines TMA untersuchten wir die Expression von PD-L1, der Mismatchreparatur-Proteine MLH1, PMS2, MSH2 und MSH6, HER2 und ALK. Für den Status von HER2 und ALK/EML4 erfolgten zusätzlich Fluoreszenz-in-situ Hybridisierungen.

Das häufigste Ereignis mit 19.5% (57 Fälle) war eine PD-L1 Positivität (Expression ≥ 1 % der Tumorzellen), die mit einem längeren Gesamtüberleben korrelierte. Eine signifikante Korrelation zu einem histologischen Subtyp ergab sich nicht, jedoch konnte eine starke Expression (≥ 25% der Tumorzellen) nur in high-grade serösen Karzinomen nachgewiesen werden. Eine HER2-Amplifikation fand sich in 4% (11 Fälle). HER2-Amplikationen sind bislang vorwiegend für muzinöse Ovarialkarzinome bekannt [2, 50], jedoch waren die von uns nachgewiesenen Fälle alle dem high-grade serösen Subtyp zuzuordnen. In einem high-grade serösen sowie einem endometrioiden Ovarialkarzinom lag eine Fusion von ALK/EML4 vor (<1%). Neben unserer Studie gibt es auch einzelne Fallbericht, die Hinweise auf einen potentiell wirksamen Therapieansatz von Tyrosinkinase-Inhibitoren bei Ovarialkarzinomen mit ALK/EML4-Translokation geben [11, 32]. Eine Mikrosatelliteninstabilität mit Expressionsverlust von MSH2 und MSH6 war in einem high-grade serösen Karzinom nachzuweisen (< 1%). Diese Patientin verstarb 8.3 Monate nach Diagnosestellung. Die MSI wird als Indikator für die Wirksamkeit von immunmodulatorischen Therapieansätzen gesehen und ist in colorektalen Karzinomen mit einer besseren Prognose assoziiert [27, 42]. Nach den derzeitigen Kenntnissen weisen hauptsächlich endometrioide und klarzellige Ovarialkarzinome im Rahmen eines Lynch-Syndroms eine MSI auf [12, 36]. Der von uns detektierte Fall eines high-grade serösen Ovarialkarzinoms wurde entsprechend sorgfältig bezüglich des histologischen Subtyps überprüft.

Das Expressionsverhalten von PD-L1 in Ovarialkarzinomen wurde inzwischen von vielen Forschungsgruppen untersucht, mit teilweise gegensätzlichen Ergebnissen bezüglich der prognostischen Bedeutung [16, 71, 75]. Die Untersuchungen, wie auch die von uns vorliegende Arbeit, lassen weiterhin vermuten, dass ein Teil der high-grade serösen Ovarialkarzinome einer Therapie mit Checkpoint-Inhibitoren zugänglich sein kann. Ob sich Checkpoint-Inhibitoren in der Therapie des Ovarialkarzinoms eignen, wird derzeit in Studien untersucht [8, 28, 38]. Molekulare Alterationen von HER2, ALK/EM4 und MSI sind hingegen in Ovarialkarzinomen selten, könnten jedoch für Einzelfälle eine therapeutische Option beinhalten.

4.7 SATB2 is a supportive marker for the differentiation of a primary mucinous tumor of the ovary and an ovarian metastasis of a low-grade appendiceal mucinous neoplasm (LAMN): A series of seven cases

Schmoeckel E, Kirchner T, Mayr D.
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Muzinöse Neoplasien des Ovars können in der differentialdiagnostischen Abgrenzung zu ovariellen Metastasen aus dem Gastrointestinaltrakt Schwierigkeiten bereiten. Insbesondere low-grade muzinöse Neoplasien der Appendix (LAMN) können Metastasen im Ovar verursachen, die makroskopisch und histologisch das Bild eines muzinösen Borderline-Tumors oder auch eines muzinösen Ovarialkarzinoms imitieren [61, 74]. Im Gegensatz zu einer metastasierten LAMN weisen muzinöse Borderline-Tumoren und auf das Ovar begrenzte muzinöse Ovarialkarzinome jedoch in der Regel eher eine gute Prognose auf, so dass die differentialdiagnostische Abgrenzung für die weitere Behandlung der Patientin wesentlich ist. Aufgrund überlappender Expressionsmuster muzinöser Neoplasien des Ovars und gastrointestinaler Karzinome können immunhistochemische Untersuchungen von CK7, CK20 und CDX2 oftmals nicht zu einer sicheren Differenzierung beitragen [51]. Der zum Zeitpunkt dieser Studie noch weniger bekannte immunhistochemische Marker SATB2 wird in Epithelzellen des unteren Gastrointestinal-Traktes exprimiert [18], zeigt jedoch zumeist keine Expression in muzinösen Neoplasien des Ovars [53, 58, 68].

An sieben Fällen ovarieller Metastasen einer LAMN konnten wir demonstrieren, wie unter zusätzlicher Anwendung des immunhistochemischen Markers SATB2 die Abgrenzung zu primär muzinösen Neoplasien des Ovars gelang. Die untersuchten Fälle fielen klinisch jeweils durch eine dominierende Tumormanifestation im Ovar auf und wurden zunächst unter dem Verdacht eines primären Ovarialtumors operiert. In Zusammenschau der SATB2-Positivität sowie des Nachweises einer LAMN in der Appendix vermiformis konnten die Fälle jeweils einer ovariellen Metastase der LAMN zugeordnet werden. Nach dem derzeitigen Kenntnistand sind muzinöse Ovarialkarzinome SATB2 negativ, ausgenommen von Fällen, die auf dem Boden eines Teratom entstehen [68]. Auch in unserem Kontrollkollektiv konnten wir die SATB2-Negativität in muzinösen Ovarialkarzinomen und muzinösen Borderline-Tumoren belegen.

In dieser Studie konnten wir demonstrieren, wie sich primäre muzinöse Neoplasien des Ovars gegenüber ovariellen Metastasen einer LAMN mit Hilfe des immunhistochemischen Markers SATB2 unterscheiden lassen.

5. Ausblick

Im vorliegenden Habilitationsprojekt lag der Schwerpunkt auf der Untersuchung von prognostischen und prädiktiven Biomarker in histologischen Subtypen des Ovarialkarzinoms. Dabei konnte insbesondere auf die high-grade serösen, endometrioiden und klarzelligen Karzinome näher eingegangen werden. Aufgrund ihrer Seltenheit waren in den meisten unserer Arbeiten low-grade seröse und muzinöse Karzinomen hingegen geringer repräsentiert. Die Untersuchung dieser beiden seltenen Subtypen erfordert separate Patientenkollektive, über welche wir weitere Untersuchungen zu prognostischen und prädiktiven Biomarkern ermöglichen möchten. Darüber hinaus zeichnet die Pathogenese der low-grade serösen und muzinösen sowie auch der endometrioiden Karzinome eine stufenartige Entwicklung über Zystadenome und Borderline-Tumore aus [5, 48]. In den künftigen Projekten möchten wir uns daher auch mit prognostischen Faktoren in den Vorstufen der low-grade serösen, muzinösen und endometrioiden Karzinome beschäftigen. Weiterführende Arbeiten sollen hier beitragen prognostische Biomarker für Borderline-Tumore zu entwickeln, die Hinweise auf das biologische Verhalten und das Risiko eines Rezidivs oder Progression der Erkrankung geben können.

6. Abkürzungen

ALK/EML4, Anaplastic lymphoma kinase/echinoderm microtubule associated protein-like 4

ARID1A, AT-rich interaction domain 1A

CTNNB1, Catenin (cadherin-associated protein), beta 1

BOT, Borderline-Tumor

BRAF, B-Raf protooncogene,serine/threonine kinase

BRCA1/2, Breast cancer type 1 susceptibility protein ½

GPER, G protein-coupled estrogen receptor 1

HER2, Human epidermal growth factor 2

KRAS, Kirsten rat sarcoma viral oncogene homolog

LAMN, Low-grade muzinöse Neoplasie der Appendix

LEF1, Lymphoid enhancer-binding factor 1

MLH1, MutL homolog 1

MSH2, MutS homolog 2

MSH6, MutS homolog 6

MSI, Mikrosatelliteninstabilität

p53, Tumor protein p53

PARP, Poly (ADP-ribose) polymerase

PD-L1, Programmed death-ligand 1

PMS2, (Mismatch repair endonuclease) PMS2

PTEN, Phosphatase and tensin homolog

SATB2, Special AT-rich sequence-binding protein 2

STIC, Seröses tubares intraepitheliales Karzinom

THR, Thyroidhormon-Rezeptoren

TMA, Tissue microarray

7. Literaturverzeichnis

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8.3 Buchbeiträge

Tumormanuale München:

Manual Mammakarzinome
16. Auflage 2017 Zuckschwerdt Verlag
Kapitel: Pathologie der Mammakarzinome und der intraepithelialen Proliferationen der Mamma
Autoren: D. Mayr, M. Beer, **E. Schmoeckel**
Kapitel: Sonderfälle
Autoren: P. Stadler, M. Beer, A. Lück, **E. Schmoeckel**

Manual Mammakarzinome
15. Auflage 2015 Zuckschwerdt Verlag
Kapitel: Pathologie der Mammakarzinome und der intraepithelialen Proliferationen der Mamma
Autoren: D. Mayr, **E. Schmoeckel**

Manual Maligne Ovarialtumoren
10. Auflage 2014 Zuckschwerdt Verlag
Kapitel: Histologische Klassifikation maligner und potentieller maligner Ovarialtumoren, Stadieneinteilung und Prognosefaktoren
Autoren: D. Mayr, P. Dettmar, J. Dorn, H. Schmalzried, **E. Schmoeckel**

Manual Zervixkarzinom
4. Auflage 2020 Zuckschwerdt Verlag
Kapitel: Histopathologie
Autoren: D. Mayr, **E. Schmoeckel**

8.4 Sonstige Publikationen

Pathologie: Aktuelle Standards und weitere Optionen in der Diagnostik des Mammakarzinoms
Elisa Schmoeckel, Veronika Kanitz, Doris Mayr
Onkologie heute, Deutsche Krebsgesellschaft 05/2019

9. Lebenslauf

10. Danksagung

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11. Eidesstattliche Erklärung

Hiermit erkläre ich, dass

- die schriftliche Habilitationsleistung selbständig verfasst wurde und die Herkunft des verwendeten oder zitierten Materials ordnungsgemäß kenntlich gemacht wurde

- mir bisher kein akademischer Grad entzogen wurde und kein Verfahren gegen mich anhängig ist, welches die Entziehung eines akademischen Grades zur Folge haben könnte

- ich noch kein Habilitationsverfahren im gleichen Fach erfolglos beendet habe.

München, den 04.05.2021 Dr. med. Elisa Schmoeckel

12. Abdrucke der zugrundeliegenden Originalarbeiten

Modern Pathology (2017), 1–10

LEF1 is preferentially expressed in the tubal-peritoneal junctions and is a reliable marker of tubal intraepithelial lesions

Elisa Schmoeckel[1], Ashley A Odai-Afotey[2], Michael Schleißheimer[1], Miriam Rottmann[3], Andrea Flesken-Nikitin[2], Lora H Ellenson[4], Thomas Kirchner[1], Doris Mayr[1] and Alexander Yu Nikitin[2]

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Recently it has been reported that serous tubal intraepithelial carcinoma (STIC), the likely precursor of ovarian/extra-uterine high-grade serous carcinoma, are frequently located in the vicinity of tubal-peritoneal junctions, consistent with the cancer-prone features of many epithelial transitional regions. To test if p53 (aka TP53)-signatures and secretory cell outgrowths (SCOUTs) also localize to tubal-peritoneal junctions, we examined these lesions in the fallopian tubes of patients undergoing salpingo-oophorectomy for sporadic high-grade serous carcinomas or as a prophylactic procedure for carriers of familial *BRCA1* or *2* mutations. STICs were located closest to the tubal-peritoneal junctions with an average distance of 1.31 mm, while SCOUTs were not detected in the fimbriated end of the fallopian tube. As many epithelial transitional regions contain stem cells, we also determined the expression of stem cell markers in the normal fallopian tube, tubal intraepithelial lesions and high-grade serous carcinomas. Of those, LEF1 was consistently expressed in the tubal-peritoneal junctions and all lesions, independent of p53 status. All SCOUTs demonstrated strong nuclear expression of β-catenin consistent with the LEF1 participation in the canonical WNT pathway. However, β-catenin was preferentially located in the cytoplasm of cells comprising STICs and p53 signatures, suggesting WNT-independent function of LEF1 in those lesions. Both frequency of LEF1 expression and β-catenin nuclear expression correlated with the worst 5-year patient survival, supporting important role of both proteins in high-grade serous carcinoma. Taken together, our findings suggest the existence of stem cell niche within the tubal-peritoneal junctions. Furthermore, they support the notion that the pathogenesis of SCOUTs is distinct from that of STICs and p53 signatures. The location and discrete patterns of LEF1 and β-catenin expression may serve as highly sensitive and reliable ancillary markers for the detection and differential diagnosis of tubal intraepithelial lesions.
Modern Pathology advance online publication, 30 June 2017; doi:10.1038/modpathol.2017.53

Ovarian carcinoma is the most lethal gynecological malignancy and is fifth among all cancer related deaths of women in the US.[1] During the past decade a number of studies have shown that about half of the most malignant ovarian/extra-uterine cancers, high-grade serous carcinomas, may arise from the fallopian tube epithelium.[2] In this situation, neoplastic tubal lesions lead to metastatic disease with the main manifestation in the ovary. This possibility is supported by the finding of dysplastic lesions in prophylactically removed fallopian tubes from high-risk carriers of BRCA1/2 germline mutations,[3] detection of identical *p53* mutations in early tubal lesions and high-grade serous carcinomas of the same patients,[4,5] and experimental demonstration of high-grade serous carcinoma induction by conditional mutations in the secretory (PAX8 positive) cells of mouse tubal epithelium.[6] Considering that ovarian cancer can be successfully treated if diagnosed at an early stage, with 90% of such patients

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having over a 5-year survival, its early detection is of particular importance.

Early dysplastic lesions associated with high-grade serous carcinoma include p53 (aka TP53) signatures, serous tubal intraepithelial carcinomas (STICs) and potentially secretory cell outgrowth (SCOUTs). p53 signatures are characterized by areas of a single layer of consecutive PAX8-positive secretory cells that contain aberrant *p53* expression, commonly associated with p53 mutations, but lack cellular atypia and show a low proliferative index.[4] STICs also show aberrant p53 expression and express PAX8, but are characterized by dysplastic epithelial cells with the loss of cell polarity and high proliferative index.[7–10] A large fraction of STICs (30–50%) completely lack p53 expression, largely due to p53 null mutation in the *p53* gene, and their detection by immunohistochemistry may be more challenging.[4,5,9] SCOUTs consist of a row of at least 30 almost exclusively PAX8-positive secretory epithelial cells with a pseudostratified benign appearance and low proliferative index.[11–13]

p53 signatures and STICs are preferentially located in the distal, fimbriated region of the fallopian tube,[4] whereas SCOUTs are reported to be evenly distributed between fimbriated and more proximal areas of the fallopian tube.[13] However, it remains elusive if SCOUTs, p53 signatures and STICs represent independent or continuous stages of neoplastic progression.[11,13,14]

A recent study demonstrated that the majority STICs are located in the vicinity of the tubal-peritoneal junction.[15] The tubal-peritoneal junctions connects the columnar epithelium of the fimbriated end of the fallopian tube to the flat mesothelial layer of the peritoneum. The finding of STICs near the tubal-peritoneal junctions is consistent with previous observations that transitional epithelial zones, such as the corneal limbus and squamo-columnar junctions in the uterine cervix, gastro-esophageal, and ano-rectal areas are frequently cancer prone.[16–18] Furthermore, it may offer an equivalent to the recently discovered cancer-prone stem cell niche in the mouse hilum area, which connects the ovarian surface epithelium, tubal epithelium, and mesothelium.[19] The location of p53 signatures and distal SCOUTs with respect to tubal-peritoneal junctions remains unknown. Such information could provide an important clue to their role as precursor lesions to STICs.

One consistent marker of hilum stem cells is lymphoid enhancer-binding factor-1 (LEF1). LEF1 is a transcription factor that interacts with β-catenin in the nucleus as a component of canonical WNT signaling.[20] LEF1 expression has been reported in human STICs and SCOUTs.[21] However, the frequency and extent of this expression were not described. Furthermore, expression of LEF1 in normal tubal-peritoneal junctions and p53 signatures remains unknown.

In this study we compared the distance from the tubal-peritoneal junctions to STICs, p53 signatures and SCOUTs in the fallopian tubes of patients undergoing salpingo-oophorectomy for sporadic high-grade serous carcinomas or as prophylactic procedures for carriers of familial *BRCA1* or *2* mutations. Furthermore, we have determined expression of LEF1 and other stem cell markers in the tubal-peritoneal junctions, tubal intraepithelial lesions and high-grade serous carcinomas. We have also compared survival of patients with tumors containing high and low number of LEF1-expressing cells.

Materials and methods

Study Groups and Clinical Data

Material in these studies was submitted to the Institute of Pathology, Ludwig Maximilians University, Munich, Germany between 2009 and 2014. Cases included fallopian tubes from 42 patients with sporadic high-grade serous carcinoma without *BRCA1/2* germline mutation (further denoted as 'high-grade serous carcinoma group'), from 44 patients tested positive for *BRCA1* or *2* germline mutations (further denoted as 'BRCA group'), and 31 control cases.

In the high-grade serous carcinoma group the status of somatic *BRCA* mutation of tumor was unknown. In order to avoid that a bulky tumor obscured the potential tubal intraepithelial lesions only cases with complete right and left tube and presentable fimbria were included. Cases with advanced tumor mass in the tube and invasion of one or both tubes were excluded. Patient's age ranged from 42 to 85 years, with an average of 65 years. 37 patients were of advanced tumor-stage (FIGO $\geq$ IIIA). Two patients presented with a FIGO stage I (one of IA and one of IC). Three patients were of FIGO stage II. A representative paraffin block of the invasive cancer was obtained for immunohistochemical stainings.

In the BRCA group 1 patient presented with an early invasive high-grade serous carcinoma of the fallopian tube (FIGO IC). All other cases were from prophylactic salpingo-oophorectomy and encompassed 28 cases of BRCA1- and 16 cases of BRCA2-mutation. Patients' age ranged from 33 to 79 years, with an average of 52 years.

Control cases of fallopian tubes were from 31 patients who underwent hysterectomy for leiomyoma of the uteri or carcinoma of the cervix. Patients' age ranged from 40 to 74 years, with an average of 56 years.

Location and size of STICs and their distance to tubal-peritoneal junctions were additionally evaluated in 10 cases (5 high-grade serous carcinoma, 2 BRCA and 3 without either high-grade serous carcinoma or BRCA) from Weill Cornell Medicine, New York, New York.

Histotechnology and Immunohistochemistry

The fallopian tubes were fixed in buffered formalin and prepared for paraffin embedding according to

the SEE-FIM-protocol.[8] Serial 4 μm sections were prepared from each paraffin block and mounted on SuperFrost Plus microscope slides (Menzel Gläser, Braunschweig, Germany). After deparaffinization and rehydration step-wise slides were stained with hematoxylin and eosin, while parallel slides were subjected immunohistochemical stainings for p53, Ki67, PAX2, PAX8, CD44, CD49f, CD133, LEF1, and β-catenin (Supplementary Table 1). For β-catenin two different antibodies were used. All cases were first stained automatically. Cases of unsecure nuclear reaction were stained additionally by manual staining.

Stainings for β-catenin, CD133, Ki67, p53, and PAX8 were subjected to heat-induced epitope unmasking with pressure cooker heating followed by staining using a Ventana Benchmark XT autostainer (Ventana Medical Systems, Oro Valley, AZ, USA) with the XT UltraView diaminobenzidine kit (Vector Laboratories, Burlingame, CA, USA) and hematoxylin counterstaning (Vector Laboratories, Burlingame, CA, USA).

For CD44, LEF1, β-catenin, CD49f, and PAX2 manual staining slides were exposed to heat pretreatment, followed by incubation with primary antibodies for 1 h at room temperature, secondary anti-species antibodies (Vectastain, ABC kit universal, PK 6200, Vector Laboratories, Burlingame, CA, USA for LEF1, β-catenin BD and CD44; ImmPRESS reagent kit, anti-rabbit IgG, MP -7401, Vector Laboratories, Burlingame, CA, USA for CD49f and PAX2) and detection with ABC Elite kit (Vector Laboratories, Burlingame, CA, USA). The DAB+ system (Dako, Hamburg, Germany) was used as chromogen and the slides were then counterstained with hematoxylin (Vector Laboratories, Burlingame, CA, USA). System controls were included.

Analysis of Lesions

STIC is defined by a proliferation of dysplastic epithelial cells with loss of cell polarity and absence of ciliated cells. PAX8 is expressed continuously. STIC is marked by increased proliferation (Ki67 > 10% of cells) and aberrant status of p53 immunostaining, which includes either strong diffuse staining of p53 or complete absence of p53 staining, as compared with weak nuclear staining in neighboring stromal cells.[7–9,22–25]

p53 signatures are morphologically inconspicuous and consists of at least 12 consecutive PAX8-positive secretory cells, that are p53 positive, but lack any cellular atypia and show a low proliferative index (Ki67 < 10%).[4,7,23,24]

SCOUTs consist of at least 30 almost exclusively secretory PAX8-positive epithelial cells with a pseudostratified appearance. Immunohistochemistry for p53 is negative and the proliferative index is low (Ki67 < 10%).[11–13] The cells of these lesions usually contain wild-type p53 manifested as weak nuclear staining. Such staining is also typically observed in stromal cells.

All lesions were independently analyzed by several pathologists (ES, LHE, DM, and AYN). All histological sections were digitized at giga-pixel resolution, 24-bit color, and 40× magnification using Aperio ScanScope CS2 (Leica Biosystems, Buffalo Grove, IL, USA). Image analysis was performed using ImageJ software (National Institute of Health, Bethesda, MD, USA). Size of STICs, p53 signatures and SCOUTs was determined in parallel sections followed by 3D reconstruction of lesions. The distance to the tubal-peritoneal junctions was measured for all STICs, p53 signatures and SCOUTs located in the same tissue section as the tubal-peritoneal junctions.

For the immunohistochemical analysis of LEF1 expression in the tubal-peritoneal junctions 15 mesothelial cells and 60 tubal epithelial cells were evaluated. Together a total length of 400–550 μm (in most cases 475 μm) was counted for each junction. The number of junctions found per analyzed case ranged from 1 to 5 and the average expression was calculated for each case. The referring distant regions were chosen and defined based on them having overall representative staining composition of the distal tubal epithelium in each case. The average of two distant regions, each consisting of 60 cells was calculated.

Expression of LEF1 in STICs, p53 signatures, SCOUTs and high-grade serous carcinoma was quantified as the percentage of positive stained cells in each lesion using ImageJ cell counter in digital images. Due to injured tubal mucosa or missing tubal-peritoneal junctions on parallel slides used for the immunohistochemical stainings, subsets of 19 high-grade serous carcinoma cases, 20 BRCA cases, and 12 controls were used for detailed analysis of immunohistochemical stainings. Among those, 21 STICs, 16 p53 signatures, and 15 SCOUTs were stained for LEF1, β-catenin, CD44, and CD133. High-grade serous carcinoma were considered to be positive for LEF1 and nuclear β-catenin in cases with over 30% stained neoplastic cells.

Statistical Analysis

Statistical analysis was performed using SPSS version 19.0 (PASW Statistics; SPSS Inc., IBM, Chicago, IL, USA), GraphPad Prism version 6 (GraphPad, La Jolla, USA) and MedCalc version 17 (Ostend, Belgium). An average percent positivity and standard deviation was taken for each region for each independent uterine tube case. Average percent positivity was used in all statistical analysis. Unpaired *t*-tests with Welch's correction (Gaussian distribution) or Mann–Whitney tests (nonparametric test) were used to determine if within a given region differences in means between markers were statistically significant. Overall survival was analyzed

using Kaplan–Meier method with log rank test and Cox proportional hazards regression. *P*-values of < 0.05 were considered statistically significant.

Results

Incidence and Size of Tubal Intraepithelial Lesions

Among 19 STIC cases in the high-grade serous carcinoma group (Table 1), 7 cases presented with a small solid tumor mass in the ovary (≤3.5 cm in diameter) and an ipsilateral manifestation of STIC. The remaining 12 cases presented with a larger solid and cystic tumor mass in the ovaries. In 9 out of 19 STIC cases in the high-grade serous carcinoma group a microscopic invasive tumor in the fimbria was bordering the STIC. In the BRCA group one case included an early high-grade serous carcinoma of the fallopian tube (FIGO IC) and a contralateral STIC as well as SCOUT on the side of the invasive cancer.

10 out of 19 (52.63%) STICs completely lacked p53 immunostaining, as typical for *p53* null mutations, in the high-grade serous carcinoma group. In all cases the p53 immunostaining status (aberrant accumulation *vs* complete lack of staining) in STICs was identical in the high-grade serous carcinoma of the same patient. In the BRCA group 2 out of 4 (50%) STICs had complete lack of p53 staining. One case of the BRCA group contained a STIC with complete lack of p53 staining and a p53 signature in the same tube.

The average size of STICs (2.73 mm) was significantly larger than p53 signatures (1.12 mm, $P = 0.0004$) and SCOUTs (1.04 mm, $P = 0.0001$; Table 2). Sizes of SCOUTs and p53 signatures were not significantly different ($P = 0.6974$).

Table 1 Distribution of tubal intraepithelial lesions in study groups

Lesion/group	*HGSC*, n = *42*[a]	*BRCA*, n = *44*[a,b]	*Control*, n = *31*[a]
STIC	19 (45%)	4 (9%)	0
p53 signature	10 (24%)	6 (14%)	5 (16%)
SCOUT	11 (26%)	4 (9%)	3 (10%)
No lesions	14 (33%)	34 (77%)	26 (84%)

[a]Some cases include several types of lesions. [b]BRCA group includes BRCA1 ($n = 29$) and BRCA2 ($n = 15$) cases with STIC/p53 signature/SCOUT distribution 3/5/3 and 1/1/1, respectively.

Distance from Tubal Intraepithelial Lesions to Tubal-Peritoneal Junctions

Consistent with previous reports,[2,4] the majority of STICs (85%) and p53 signatures (62%) were located in the fimbriated region (Table 2; Figure 1a). Furthermore, no STICs were located more proximally than the infundibulum (15%), while 19% of p53 signatures were found in the ampulla region. Importantly, the majority of SCOUTs were observed in the infundibulum (65%), followed by the ampulla (26%).

Among lesions located in the fimbriated region, STICs tend to be closer to the tubal-peritoneal junctions (mean distance 1.34 mm) than p53 signatures (1.96 mm); however this difference is not statistically significant ($P = 0.1217$). In three cases, STICs were located directly at the tubal-peritoneal junctions and were flanked by the mesothelial layer at the one side and columnar tubal epithelium at the other (Figure 1b).

Increased Frequency of LEF1-Expressing Cells in the Tubal-Peritoneal Junctions and all Tubal Intraepithelial Lesions

Since epithelial transitional regions may contain stem cell niches, we have evaluated a panel of markers characteristic for stem/progenitor cell, such as LEF1, CD44, CD49f, CD133, and β-catenin in the morphologically normal fallopian tube epithelium at the tubal-peritoneal junctions and in the proximal tubal epithelium of the same section at distance of 4 mm. Among those markers the frequency of LEF1 positive cells was significantly higher in the area of tubal-peritoneal junctions. LEF1-positive cells were also more frequent in morphologically normal epithelium of high-grade serous carcinoma and BRCA groups (Figure 2). There were no preferential distribution of CD44, CD49f, and β-catenin stained cells, while CD133 was mainly detected in the proximal region of the fallopian tube.

The average number of LEF1-positive cells was significantly higher in STIC, p53 signature, and SCOUT compared with the bordering normal tubal epithelium (Figure 3). Importantly, STICs completely

Table 2 Size, location and distance to the TPJ among tubal intraepithelial lesions

Lesion/group	*Size, mm ± s.d.*	*Location*				*Distance to TPJ, mm ± s.d.*[a]
		Fimbriae	*Infundibulum*	*Ampulla*	*Isthmus*	
STIC, $n = 33$[b]	2.73 ± 1.55	28 (85%)	5 (15%)	0 (0%)	0 (0%)	1.34 ± 1.15
p53 signature, $n = 16$	1.12 ± 0.90[c]	10 (62%)	3 (19%)	3 (19%)	0 (0%)	1.96 ± 1.55
SCOUT, $n = 15$	1.04 ± 0.33[d]	1 (4%)	15 (65%)	6 (26%)	1 (4%)	NA

[a]For lesions located in the fimbria. [b]Includes 10 cases from Weill Cornell Medicine, New York. [c]$P = 0.0004$ *vs* STIC. [d]$P = 0.0001$ *vs* STIC.

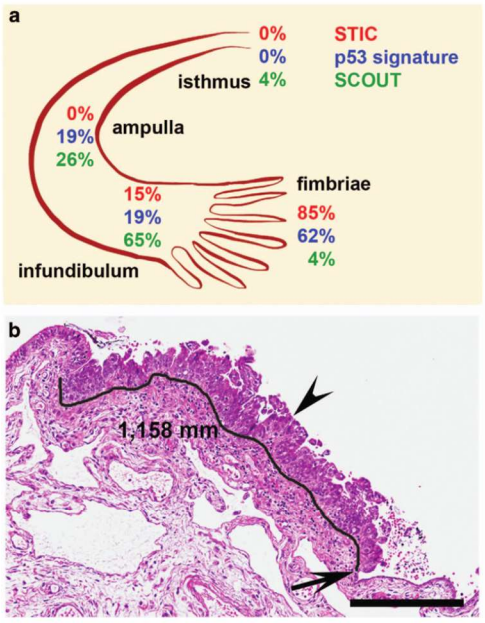

Figure 1 Lesions in the fallopian tube. (**a**) Distribution of tubal lesions. (**b**) STIC (arrowhead) at the tubal-peritoneal junction (arrow), flanked by the mesothelium and the tubal epithelium at the right and the left sides, respectively. Line shows the length of STIC. Hematoxylin and eosin. Scale bar, 300 μm.

lacking p53 expression also expressed LEF1, thereby simplifying detection of such lesions. (Figures 3c and d).

Divergent β-catenin Cellular Location Differentiates STICs, Signatures, and High-Grade Serous Carcinoma from SCOUTs

In all SCOUTs the expression of LEF1 was associated with a marked nuclear expression of β-catenin (Figure 3). STICs and p53 signatures showed only minimal nuclear expression of β-catenin in single cells, whereas the majority of cells in these lesions presented with a distinct membranous expression of β-catenin.

To establish if divergent expression of LEF1 and β-catenin is also present in advanced malignancies, immunostaining was additionally performed in 35 cases of sporadic high-grade serous carcinoma (Figure 4). Nuclear expression was similar to that of intraepithelial lesions, but the frequency of LEF1-ranged from 0 to 75% (mean ± s.d., 18 ± 16.7%). Similar to STICs and p53 signatures, β-catenin showed a distinct membranous expression in the cancers with only minimal nuclear expression in the majority of cases. However, focal nuclear expression was detected in all high-grade serous carcinoma cases studied, ranging from 2 to 55%. As compared with LEF1, expression of β-catenin was observed more uniformly in all high-grade serous carcinoma cases. Patient's survival correlated significantly with frequency of LEF1 and nuclear β-catenin positive cells in the cancer (Figure 4).

Discussion

Recent studies of risk-reducing salpingo-oophorectomies in women with BRCA1/2 germline mutations showed that STICs are identified in 2–12% of the patients.[2,14,26] In context of high-grade serous carcinoma the incidence of STIC ranges rather up to 19–60%.[15,27–29] Our studies have found the frequency of STICs to be 9% and 45% in the above groups, respectively, thereby confirming the earlier observations. It should be noted that all of these studies cannot exclude that some STICs may represent metastatic implants from the associated high-grade serous carcinoma.[30,31] Due to its proximity to the ovary, the location of the majority of STICs in the fimbriae may be the preferential site of implantation from extra-tubal carcinomas. In such a scenario, it would be more likely to observe random distribution of tumor implants within the tubal fimbria. However, consistent with the recent study by Seidman,[15] we have observed preferential location of STICs at or in the immediate vicinity to the tubal-peritoneal junctions. Furthermore, we have observed similar distribution of STICs in the BRCA1/2 cases, where no overt tumor was observed. This is consistent with the notion that a large fraction of lesions described as STICs are of primary, not metastatic implantation, origin.

Consistent with previous observations,[11,13,14] we have shown that STICs and p53 signatures are preferentially located within the tubal fimbria, while SCOUTs are detected in the more proximal regions of the tube. Both p53 signatures and SCOUTs have approximately the same size (about 1.1 mm on average), while STICs are 2.5 times larger (2.73 mm). All STICs and p53 signatures but not SCOUTs contain aberrant p53 immunostaining. In high-grade serous carcinoma cases, the type of p53 immunostaining, either aberrant accumulation or aberrant lack of staining, were identical to the carcinoma of the same patient. Further supporting the common pathogenesis of STICs and p53 signatures, in contrast to that of SCOUTs, only SCOUTs have marked nuclear location of β-catenin, a key component of the canonical WNT pathway. STICs, p53 signatures as well as high-grade serous carcinomas were characterized by preferential membrane and cytoplasmic staining with only minimal nuclear location of β-catenin. In sum, based on the distinct location and different patterns of p53 and β-catenin expression in STICs/p53 signatures, p53 signatures

but not SCOUTs are likely to represent the steps in the continuous progression towards STICs and overt high-grade serous carcinoma. Consistent with this notion, the frequency of p53 signatures is two times higher than that of STICs in fallopian tubes from women in the BRCA group (14% *vs* 9%). However, in high-grade serous carcinoma group, these frequencies are inverse (24% *vs* 45%) suggesting that either all STIC's arise from p53 signatures or that some STICs may in fact represent implants of high-grade serous carcinoma. Consistent with this possibility, according to recent next-generation sequencing, a STIC represented a micro-metastasis in 25% of cases.[31,32] As in our study SCOUTs are defined by an accumulation of at least 30 secretory cells, we cannot rule out that some smaller groups of secretory cells might exist in the distal tube and progress to p53 signatures and/or STICs. More extensive results based on targeting tubal epithelium stem cells and their lineages in combination with single cell genome sequencing will better inform us in future.

Previously, we have reported the existence of a cancer-prone stem cell niche in the junction/transitional zone between ovarian surface epithelium, tubal epithelium and mesothelium located within the mouse hilum of the ovary.[19] In humans, the same hilum-based transitional zone is not maintained. However, the tubal-peritoneal junctions of the tubal fimbria may represent a counterpart to the mouse junction zone. Consistent with the possibility of stem cell niche located in the tubal-peritoneal junctions we have observed expression of LEF1 in that area. LEF1 is expressed in a number of normal stem cells, such as hematopoietic[33] and ovarian surface epithelium stem cells.[19] Moreover, deregulated LEF1 expression has been implicated in leukemic transformation.[33] Further studies testing functional properties of tubal-peritoneal junction cells should specifically evaluate the key parameters of stem cells, such as their ability to self-renew and to give rise to more differentiated progeny. These studies will also test if putative stem cells previously identified in the distal end of the fallopian tube[34] are identical or similar to LEF1 positive cells found in the tubal-peritoneal junctions area. We have noted that other tested stem/progenitor cell markers did not have preferential association with cells located near the tubal-peritoneal junctions. Some of these markers, such as CD44 and CD49f (aka integrin α6) have been reported to be expressed in putative stem cells of the human tubal epithelium.[34] Thus it is possible that the tubal epithelium may have several

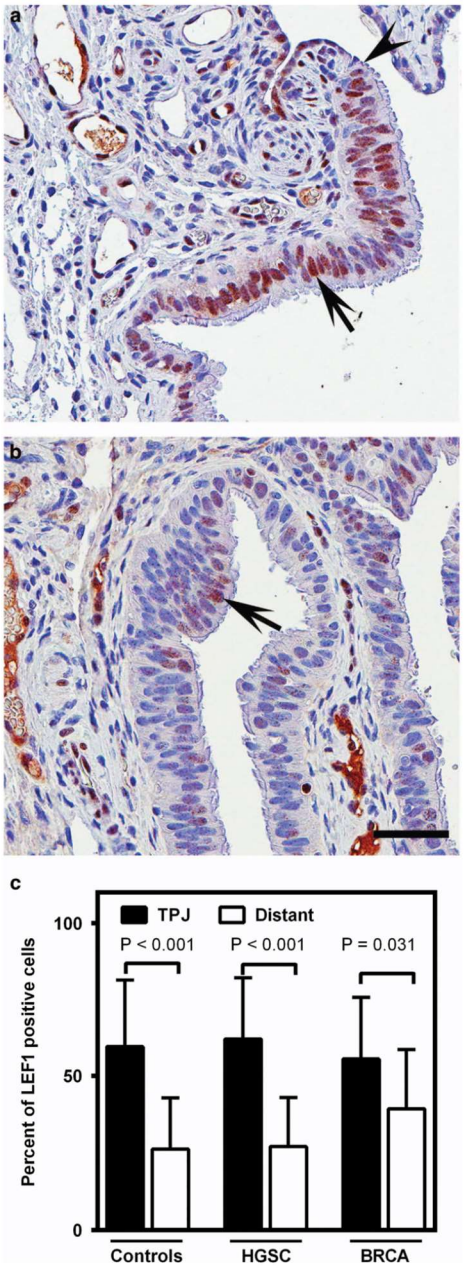

Figure 2 LEF1 expression in the morphologically normal tubal epithelium. (**a**) LEF1 expression in a large fraction of cells (arrow) in the tubal epithelium near the tubal-peritoneal junctions region (arrowhead) of the control group. (**b**) LEF1 in few cells (arrow) of the tubal epithelium of the proximal region of the control group. ABC Elite method, hematoxylin counterstaining. Scale bar, 50 μm. (**c**) Quantitative analysis of LEF1 expression in control ($n=12$), high-grade serous carcinoma ($n=19$) and BRCA ($n=16$) groups. Error bars denote s.d.

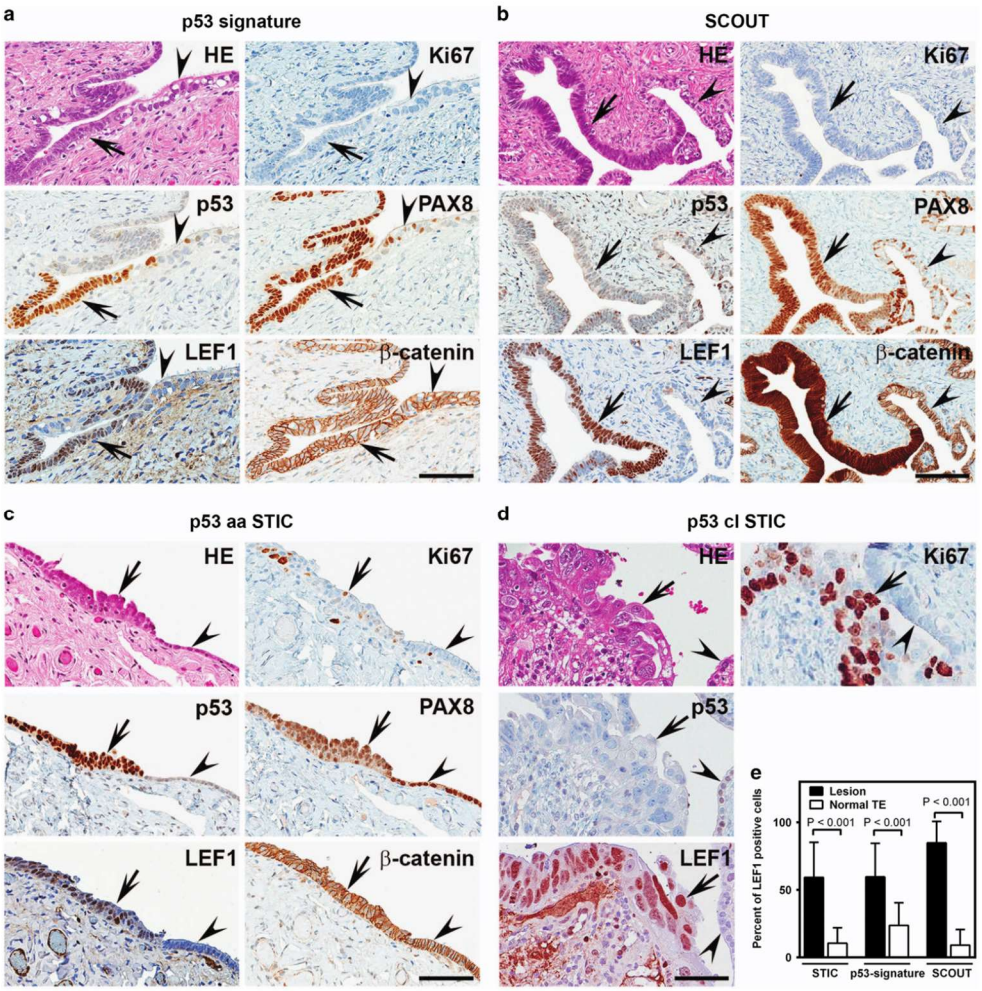

Figure 3 Immunohistochemical characterization of tubal lesions. (**a–d**) Expression of p53, LEF1, Ki67, PAX8, and *β*-catenin in the altered (arrow) and morphologically normal (arrowhead) epithelium in p53 signature (**a**), SCOUT (**b**), and STIC cases with p53 aberrant accumulation (aa), (**c**) and p53 complete lack (cl) of staining. (**d**) Note mainly membrane staining of *β*-catenin in p53 signatures and STICs. ABC Elite method, hematoxylin counterstaining, HE, hematoxylin and eosin. Scale bars, 70 *μ*m for all images. (**e**) Quantitative analysis of LEF1 expression in STIC ($n = 21$), p53 signature ($n = 16$), SCOUT ($n = 15$) and adjacent morphologically normal tubal epithelium (TE). Error bars denote s.d.

pools of stem cells. At the same time, such markers are not always entirely specific and may label non-stem cells in some tissues.

LEF1 is frequently found to be an important component of WNT pathway.[20] However, according to our studies, LEF1 expression in p53 signatures, SCOUTs and high-grade serous carcinomas does not correlate with the nuclear location of *β*-catenin, a property of active WNT signaling. Thus it is likely that the LEF1 function in these lesions are WNT pathway independent. WNT-independent functions of LEF1 were previously described in granulocyte progenitor cells[35] and murine T-cell lymphomas.[36] At the same time, we observed a strong coincidence of nuclear accumulation of *β*-catenin and LEF1 expression in SCOUTs, supporting a distinct pathogenesis of SCOUTs, as compared with other tubal lesions. We have also shown that both high LEF1

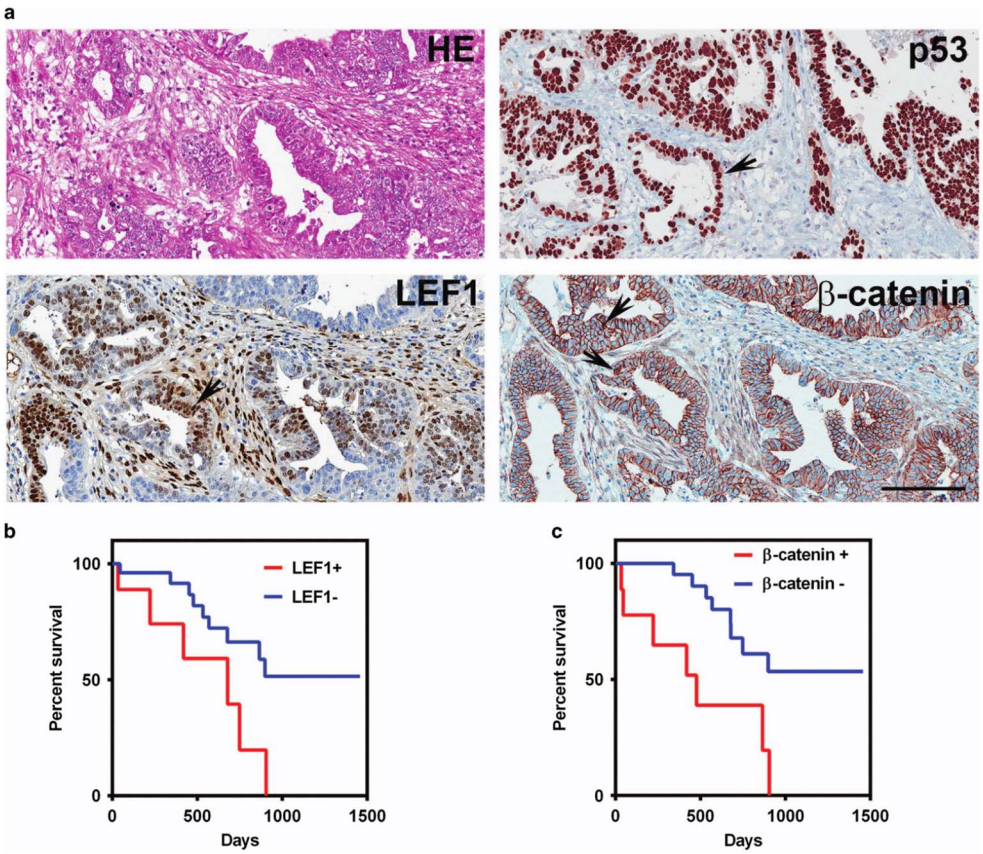

Figure 4 LEF1 and *β*-catenin in high-grade serous carcinoma. (**a**) Expression of p53, LEF1 and *β*-catenin. Arrows indicated nuclear staining. Note mainly membrane staining of *β*-catenin in neoplastic cells. Parallel sections of the same block. ABC Elite method, hematoxylin counterstaining, HE, hematoxylin and eosin. Scale bar, 150 *μ*m for all images. (**b**,**c**) Survival of patients with high-grade serous carcinoma stratified according to LEF1 (**b**) and nuclear *β*-catenin (**c**) expression. Kaplan–Meier survival analysis, $n = 35$, $P = 0.0306$ (**a**) and $P = 0.0029$ (**b**). Cox proportional hazards regression analysis $P = 0.0394$ (**a**) and $P = 0.0060$ (**b**).

expression and nuclear location of *β*-catenin correlate with decreased survival of high-grade serous carcinoma patients. Further studies should elucidate specific mechanisms by which LEF1 regulates the function of stem and neoplastic cells in tubal-peritoneal junctions, tubal lesions and high-grade serous carcinoma. They also should address the extent to which both WNT pathway dependent and independent functions contribute to the high-grade serous carcinoma pathogenesis.

Our study has shown that LEF1 expression together with strong nuclear location of *β*-catenin is indicative of SCOUTs, while LEF1 expression together with largely membranous and cytoplasmic *β*-catenin is typical for p53 signatures and STICs. Thus LEF1 may be used as an auxiliary marker for detection of tubal lesions, particularly in conjunction with *β*-catenin detection. This could be a particularly useful tool for the detection of TP53 with aberrant lack of immunostaining in STICs and p53 signatures. It has been previously reported that high LEF1 expression correlates with poor prognosis of acral melanoma[37] and colorectal cancer,[38] B-precursor acute lymphoblastic leukemia[39] and in chronic lymphocytic leukemia.[40,41] Our studies indicate that LEF1 expression may be also a useful prognostic marker in ovarian cancer. As both LEF1 and strong nuclear location of *β*-catenin correlate with a worse prognosis, further studies on a larger cohort of patients are needed to establish if correlation between these parameters has additional prognostic value.

In sum, our findings support the existence of cancer-prone stem cell niche within the tubal-peritoneal junctions. They also suggest that the pathogenesis of STICs and p53 signatures is distinct from that of SCOUTs. Furthermore, detection of LEF1 expression, especially in combination with β-catenin intracellular distribution, may be used as a highly sensitive and reliable tool for the diagnosis of tubal intraepithelial lesions, particularly well-suited for detection of STICs with aberrant lack of p53 immunostaining.

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Disclosure/conflict of interest

The authors declare no conflict of interest.

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PLOS ONE

RESEARCH ARTICLE

The ARID1A, p53 and ß-Catenin statuses are strong prognosticators in clear cell and endometrioid carcinoma of the ovary and the endometrium

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Abstract

Aim

The objective of this study was to evaluate the prognostic value of ARID1A, p53, p21, p16 and ß-Catenin in endometrioid and clear cell ovarian and endometrial carcinomas.

Materials and methods

97 tumors were available for analysis of ARID1A, p53, p21, p16 and ß-Catenin with the techniques of tissue microarray and immunohistochemistry. 32 were ovarian carcinomas and 65 were endometrial carcinomas.

Results

Endometrioid ovarian carcinomas showed negative staining for ARID1A (a) and p21 (b), aberrant expression of p53 (c) and p16 (d) and ß-Catenin positive nuclear expression (e) respectively in 19% (a), 100% (b), 28.6% (c), 52.4% (d) and 4.8% (e) of all cases. In the group of clear cell ovarian carcinomas it was 63.6% (a), 100% (b), 81.8% (c), 54.5% (d) and 0% (e). For endometrioid uterine carcinomas it was 75.7% (a), 94.9% (b), 30.5% (c), 52.1% (d) and 6.8% (e) and for clear cell uterine carcinomas it was 8.6% (a), 100% (b), 50% (c), 100% (d) and 0% (e). Survival analysis showed that negative expression of ARID1A, p53 aberrant expression and ß-Catenin nuclear positive staining are independent negative prognosticators in both, clear cell and endometrioid carcinoma, regardless of ovarian or uterine origin. Cox-Regression analysis showed them again as negative prognostic factors. Furthermore, we found a significant correlation between ARID1A and ß-Catenin expression in endometrioid uterine tumors.

data collection and analysis, decision to publish, or preparation of the manuscript.

Competing interests: The authors have declared that no competing interests exist.

Conclusion

The analyzed gynaecological carcinoma showed a distinct expression scheme of proteins that are associated with tumor suppression. We may conclude that ARID1A, p53 and ß-Catenin are the strongest prognostic factors by analyzing a subgroup of tumor suppressor genes in clear cell and endometrioid subtypes of ovarian and endometrial cancer and may be used along with traditional morphological and clinical characteristics for prognosis.

Introduction

The traditional histopathological classification of endometrial epithelial cancer, which was first proposed by Bokhman, includes type I tumors that are usually estrogen-dependent low-grade endometrioid cancers and type II tumors which are usually estrogen-independent high-grade serous or clear cell carcinomas [1]. While the first pathogenetic type has a frequency of 80–90% and is associated with highly or moderately differentiated tumors with a favorable prognosis, the second type has a frequency of only 10–20% and includes poorly differentiated tumors with a doubtful prognosis [1].

For ovarian epithelial cancer pathogenesis a less accepted paradigm exists suggesting to differ between type I and type II molecular profiles [2]. Type I tumors contain endometrioid, clear cell and low-grade serous carcinoma and mostly arise from atypical endometriosis or from borderline serous tumors [2,3]. Type II carcinomas include high-grade serous, which show typically a p53 mutation and, at least for patients with a BRCA mutation frequently arise from the fimbriated end of the fallopian tube via serous tubar intraepithelial carcinoma (STIC) [4,5].

Many studies have aimed to understand the cell origin and pathogenesis of these cancer subtypes in order to better diagnose and treat patients. Recently several studies suggested that the origin of clear cell and endometrioid carcinomas might derive from atypical endometriosis, which is believed to originate from the endometrium by retrograde menstruation [6–8]. The three-staged tumor grading system of endometrioid ovarian carcinoma is equivalent to the grading of endometrioid endometrial cancer and considers growth patterns and nuclear aplasia while there is no validated grading system for clear cell ovarian cancer, which are still classified as high-grade carcinoma [9].

Considerable interest has not only been generated in understanding the pathogenesis but also in the identification of factors that influence the prognosis of these tumors. Early diagnosis of epithelial ovarian and uterine cancer is critical for patient survival. Ovarian cancer has the highest mortality rate of the three main malignant tumors of the female reproductive system, with an overall 5-year survival rate of 45% [10]. Known prognostic factors of ovarian and endometrial carcinoma are histological subtype, tumor grading, International Federation of Gynecology and Obstetrics (FIGO) staging as well as estrogen receptor positivity for endometrial carcinoma and age, general condition and residual tumor for ovarian cancer [9,11]. Previous studies have explored molecular alterations in clear cell and endometrioid ovarian and endometrial tumors as additional prognostic factors, including changes in expression of ARID1A, p53, p21, p16 and ß-Catenin carcinoma.

ARID1A is a recently identified tumor suppressor participating in forming SWI/SNF chromatin complexes [12]. Somatic inactivating mutations of ARID1A and loss of ARID1A expression appear to be an early event in the development of most ovarian clear cell and endometrioid carcinomas as well as atypical endometriosis [13,14]. ARID1A is also frequently

mutated and plays an important role in tumor progression in uterine endometrioid carcinoma [15,16].

p53 is a well-studied tumor suppressor gene that plays a key role in regulating the cell cycle. It is a principal mediator of growth arrest, senescence and apoptosis in response to a broad array of cellular damage [17]. The p53 wild-type protein directly induces the expression of the p21 protein which binds to a variety of cyclin-dependent kinases and inhibits their activity as well as regulates the repair of DNA and blocks its replication by inhibiting cell-cycle progression [18,19].

The p16 protein is also a tumor suppressor gene that, in response to various stresses, inhibits cyclin-dependent kinases and causes the arrest of the cell-cycle in G1 phase [20].

ß-Catenin however is the effector of the Wnt signaling pathway [21]. It accumulates in cell-cell junctions in cells not receiving the Wnt signal bound by a complex referred to as the destruction complex. When cells receive the Wnt signal it is stabilized, enters the nucleus and activates Wnt target genes [21]. Inappropriate activation of the Wnt pathway underlies many cancers including ovarian and uterine carcinoma, mostly endometrioid [22].

In this study the expression of ARID1A, p53, p21, p16 and ß-Catenin was determined in 97 tumors by performing immunohistochemistry to evaluate their prognostic value. Human tissue samples of the ovary and the uterus, obtained after surgical resection, were used to investigate the correlation between their expression and clinical parameters, including overall survival.

Materials and methods

Tumors and patients

This study assessed 97 patients who all underwent primary surgery between January 1, 1990 and December 31, 2001 in the Department of Gynaecology, Ludwig-Maximilians-University, Munich, Germany. A total of 59 cases were endometrioid uterine carcinoma, 6 clear cell uterine carcinoma, 21 endometrioid ovarian carcinoma and 11 clear cell ovarian carcinoma. The analyzed tissue samples were taken from the hospital archive of the Department of Pathology, Ludwig-Maximilians-University, Munich, Germany. In cooperation with the tumor register of Munich necessary data about the patients' survival was available. The patients were staged and the tumors graded according to 1988 International Federation of Gynaecology and Obstetrics (FIGO) criteria [23]. Patients' characteristics, *e.g.* age, FIGO stage, histological subtype and FIGO grade are shown in Table 1. Survival was taken from the date of confirmed histological diagnosis after primary surgery to the date of recurrence or last visit.

Ethical approval

All patients´ data were fully anonymized, and the study was performed according to the standards set in the Declaration of Helsinki 1975. All tumor tissue used was leftover material that had initially been collected for histopathological diagnostics. All diagnostic procedures had already been fully completed when samples were retrieved for the study. The current study was approved in writing by the Ethics Committee of the Ludwig Maximilians University, Munich, Germany (approval number 449–14). Authors were blinded for clinical information during experimental analysis.

Sampling and tissue microarray construction of ovarian and uterine cancer tissue

New samples from the original slides of tumors were taken and representative areas of tumor tissues were selected. Three core biopsies from each specimen were removed and attached on

Table 1. Patients characteristics (N = 97).

Age (median)	61.0 (range 35–82)
Histopathology	
Clear cell uterine carcinoma	6 (6.2)
Endometrioid uterine carcinoma	59 (60.8)
Clear cell ovarian carcinoma	11 (11.3)
Endometrioid ovarian carcinoma	21 (21.6)
Tumor grading	
Grade 1	27 (27.8)
Grade 2	29 (29.9)
Grade 3	41 (42.3)
FIGO-staging	
I	24 (24.7)
II	20 (20.6)
III	24 (24.7)
IV	29 (29.9)

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tissue microarrays. The presence of tumor tissue on the arrayed samples was verified by a pathologist.

Immunohistochemistry and interpretation

Serial sections of the recipient tissue microarray paraffin blocks were cut at 2–3 μm, deparaffinized with xylene, and rehydrated through a series of graded alcohols. The immunostaining procedure was performed using an automated stainer (Benchmark® XT, Ventana). The following monoclonal primary antibodies were used: ARID1A/BAF250a Rabbit mAb (New England Biolabs GmbH) directed against ARID1A protein, p53 Ab-5 (Thermo Scientific) directed against 53, p16-Arc (p16INK4a, CINtec® Histology) directed against p16, p21 Cip (CDKN1A) directed against p21 and ß-Catenin Mouse IgG-1 (Roche, Ventana, ready to use) directed against ß-Catenin. All staining's were performed at the Department of Pathology, Ludwig-Maximilians-University, Munich.

The IHC stains were evaluated by the authors (MH and DM) in a double-blind process using the immunoreactive Remmele score (IRS) [24]. The quantity of cells stained was scored as: 0 = no staining, 1 = 1–10%, 2 = 11–50%, 3 = 51–80% and 4 = >81% staining. The IRS was rendered as a product of the scores obtained for staining intensity (0 = no expression, 1 = weak expression, 2 = moderate expression, 3 = strong expression) and quantity. A total score of 0–2 was considered negative, 3–5: weak, 6–8: moderate and 9–12: strong immunoreactivity. ARID1A and p21 were dichotomized into negative (0–2 points in IRS) and positive cases (3–12 points in IRS) and p53 and p16 were dichotomized into no expression/overexpression (= aberrant) and normal expression (= regulated). Weak to moderate immunoreactivity (3–8 points in IRS) was considered p53/p16 normal expression. Strong and diffuse nuclear p53/p16 immunoexpression (9–12 points in IRS) or complete absence of p53/p16 staining (0–2 points in IRS) was interpreted as likely indicating a p53/p16 gene mutation. The presence of rare weakly positive nuclear staining is a pattern that is commonly associated with wild type p53 and can be found in normal ovarian and uterine tissues [25]. ß-Catenin was classified in 3 groups of ß-Catenin nuclear negative (0–2 points in IRS) and membrane positive (n-m+) staining (3–12 points in IRS), nuclear negative (0–2 points in IRS) and membrane weak (3–6 points in IRS) or negative staining (n-m-) (0–2 points in IRS) and ß-Catenin nuclear and membrane/cytoplasm positive (n+m+) staining (3–12 points in IRS) (Fig 1).

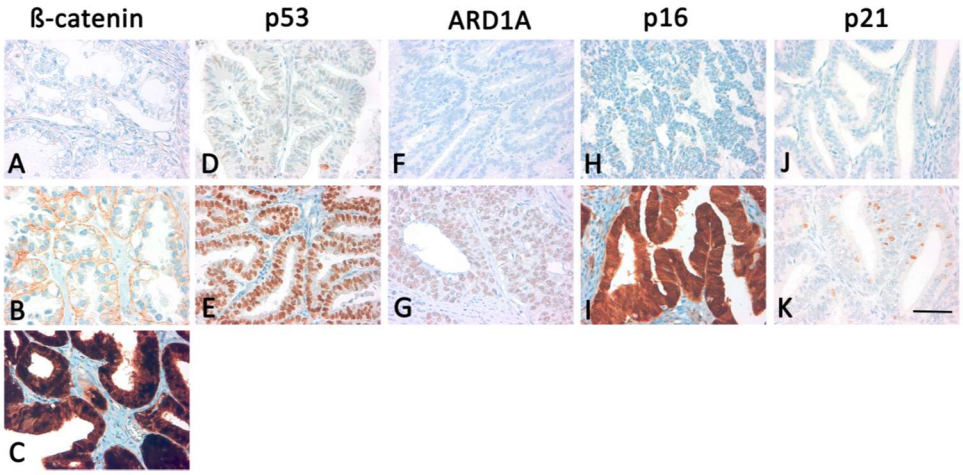

Fig 1. Immunohistochemical stainings for ß-Catenin, p53, ARID1A, p16 and p21 in clear-cell (A and B) and endometrioid (C—K) carcinomas. Focal, very weak membranous (n-m-) (A), membranous (n-m+) (B) and nuclear (n+m+) (C) expression of β-catenin. Regulated (D) and aberrant (E) expression of p53. Preserved expression of ARID1A (F) and loss (G) of ARID1A expression. Negative staining (H) and positive (I) staining for p16. Negative (J) and positive staining (K) of p21. 400 × magnification was used for all pictures; scale bar (K) refers to 60 μm for all images.

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Statistical analysis

For statistical analysis the SPSS Statistics Version 23 (SPSS Inc., Chicago, IL, USA) was used. For testing proportional differences in univariate analysis the Pearson's Chi-square test or Fisher's exact test for qualitative variables and unpaired t-test for quantitative normally distributed variables was applied. For normally distributed variables in several groups the single-factor variance analysis was used. The survival curves were generated by using the Kaplan Meier technique and differences between these curves were tested by the log-rank test. All tests were two-sided and the level of statistical significance was accepted at $p \leq 0.05$. For multivariate analyses the Cox regression model was used with tumor-related death as the endpoint.

Results

Results from immunohistochemistry

ARID1A. ARID1A nuclear positive staining was observed in 27 (27.8) out of 97 tumors. The ARID1A status was significantly ($p < 0.001$) related to histological subtype (Table 2). Positive nuclear staining seen more frequently in endometrioid tumors and it was infrequent in clear cell carcinoma. The ARID1A status alone was not associated with tumor grade ($p = 0.581$) or age ($p = 0.369$), but it was related statistically significantly to FIGO stage ($p < 0.001$). Positive nuclear staining for ARID1A was seen more frequently in FIGO I tumors while FIGO III and IV tumors were mostly ARID1A negative (S1 Fig). Survival analysis (Fig 2) demonstrated significant ($p = 0.014$) differences for patients according to the ARID1A status. Patients with ARID1A negative tumors had a 5-year survival of 60.2% compared with 84.9% survival for those with ARID1A positive tumors. Significantly better survival in the subgroup of ARID1A positive tumors could also be observed for sole analysis of all endometrioid ($p = 0.039$) and all ovarian carcinoma ($p = 0.008$) as well as tumors of grading G3 ($p = 0.028$).

Table 2. Status of ARID1A, p53, p21, p16 and ß-Catenin according to histological subtypes (N = 97).

Expression N (%)	ARID1A –	ARID1A +	P53 -/+++	P53 +	P16 -/+++	P16 +	P21 –	P21 +	ß-Catenin n-m+	ß-Catenin n-m-	ß-Catenin n+m+
	70 (72.2)	27 (27.8)	36 (37.1)	61 (62.9)	48 (49.5)	49 (50.5)	94 (96.9)	3 (3.1)	64 (66.0)	28 (28.9)	5 (5.2)
Histology											
Clear cell uterine	6 (8.6)	0 (0)	3 (8.3)	3 (4.9)	6 (12.5)	0 (0)	6 (6.4)	0 (0)	1 (1.6)	5 (17.9)	0 (0)
Endometrioid uterine	53 (75.7)	6 (22.2)	18 (50)	41 (67.2)	25 (52.1)	34 (69.4)	56 (58.3)	3 (3.1)	42 (65.6)	13 (46.4)	4 (80)
Clear cell ovarian	7 (10.0)	4 (14.8)	9 (25)	2 (3.3)	6 (12.5)	5 (10.2)	11 (11.5)	0 (0)	6 (9.4)	5 (17.9)	0 (0)
Endometrioid ovarian	4 (5.7)	17 (63.0)	6 (16.7)	15 (24.6)	11 (22.9)	10 (20.4)	21 (21.9)	0 (0)	15 (23.4)	5 (17.9)	1 (20)
P-value	< 0.001	< 0.001	0.008	0.008	0.049	0.049	0.756	0.756	0.07	0.07	0.07

https://doi.org/10.1371/journal.pone.0192881.t002

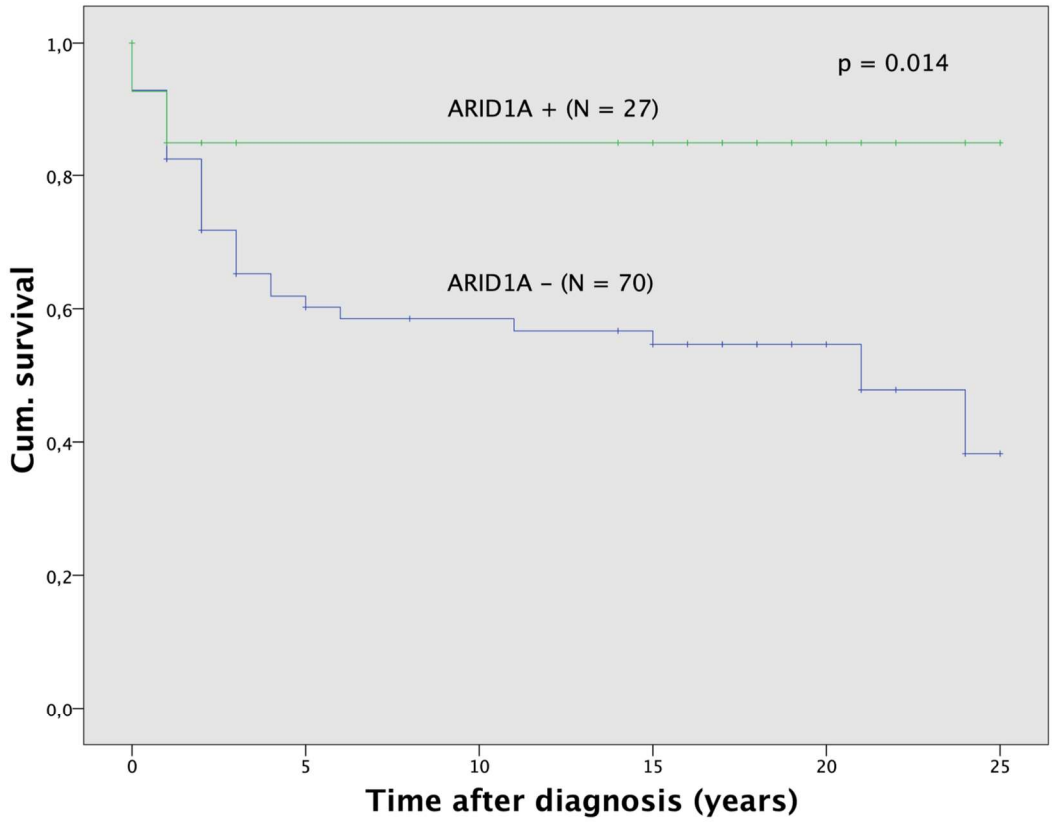

Fig 2. Survival analysis for ARID1A. Survival was better for the subgroup of patients with ARID1A positive tumors (N = 27) compared to the subgroup with ARID1A negative tumors (N = 70).

https://doi.org/10.1371/journal.pone.0192881.g002

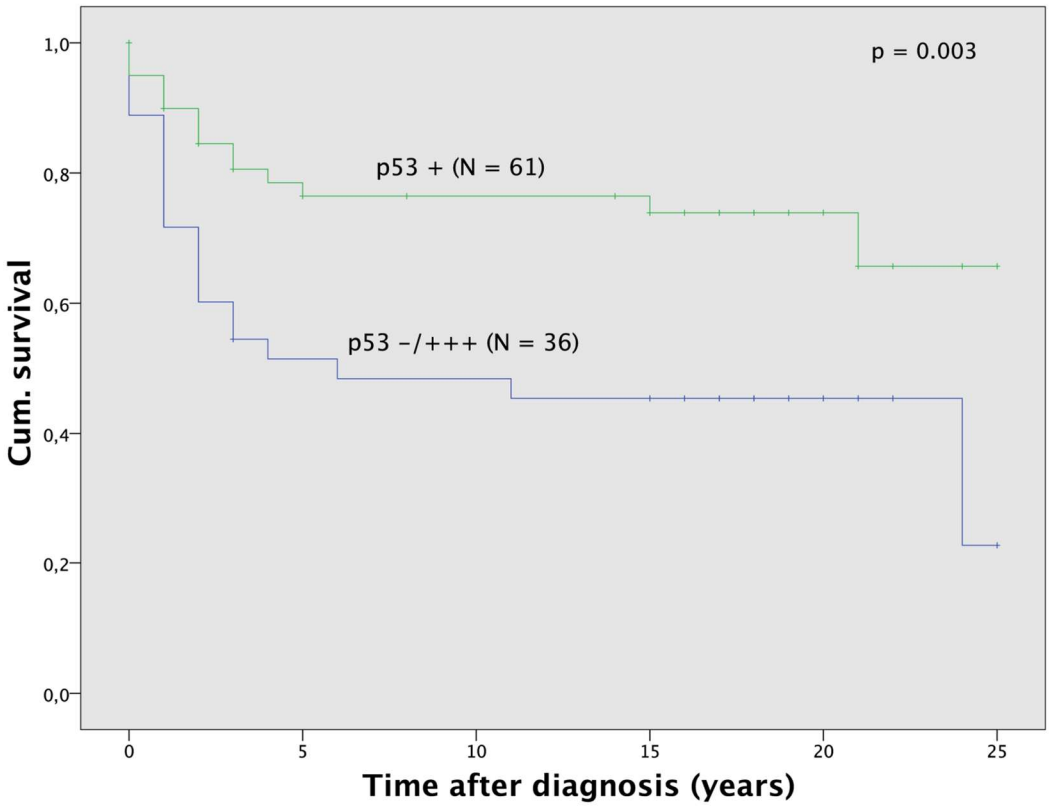

Fig 3. Survival analysis for p53. Survival was better for the subgroup of patients with p53 positive tumors (N = 61) compared to the subgroup with p53 negative/overexpression tumors (N = 36).

https://doi.org/10.1371/journal.pone.0192881.g003

p53. p53 overexpression or complete absence was seen in 36 (37.1) out of 97 carcinoma. The status of p53 was significantly (p = 0.008) associated with histological subtype (Table 2). p53 overexpression or complete absence was seen mostly in clear cell tumors, but infrequent in endometrioid carcinoma. The p53 status was not related to age (p = 0.487) or FIGO stage (p = 0.081), but it was associated significantly ($p < 0.001$) with tumor grade. Overexpression or no expression of p53 was most frequently seen in poorly differentiated (G3) clear cell and endometrioid tumors and infrequent in G1 and G2 endometrioid tumors (S1 Table). Survival analysis (Fig 3) showed significant (p = 0.003) differences for patients according to p53 status. Patients with overexpression or complete absence of p53 in their tumors had a 5-year survival rate of 51.5% compared to 76.5% survival for those with positive p53 expression. Significantly better survival in the subgroup of p53 regulated tumors could also be observed for sole analysis of all clear cell (p = 0.020) and all uterine carcinoma (p = 0.012) as well as tumors of grading G1 and G2 (p = 0.036) and G3 (p = 0.011) and tumors with FIGO staging 0, I and II (p = 0.012).

p16. p16 overexpression or complete absence was seen in 48 (49.5) out of 97 tumors. The p16 status was significantly (p = 0.049) related to histological subtype (Table 2). p16 overexpression or no expression was seen more frequently in clear cell tumors and it was infrequent in endometrioid carcinoma. The p16 status alone was not associated with tumor grade (p = 0.749), age (p = 0.359), FIGO stage (p = 0.645) or survival (p = 0.436).

p21. p21 nuclear positive staining was confined to the nucleus and observed in 3 (5.1%) out of 97 carcinoma, which were all endometrioid uterine tumors. The p21 status was not related to histological subtype (p = 0.756), grading (p = 0.114), age (p = 0.673), FIGO stage (p = 0.433) or survival (p = 0.153).

ß-Catenin. ß-Catenin nuclear negative and positive membrane staining (n-m+) was observed in 64 (66.0), ß-Catenin nuclear and membrane negative (n-m-) staining in 28 (28.8) and ß-Catenin positive nuclear and membrane/cytoplasm staining (n+m+) in 5 (5.2) out of 97 tumors. The ß-Catenin status was significantly (p = 0.07) related to histological subtype (Table 2). ß-Catenin n-m+ staining was more frequently seen in endometrioid tumors and it was infrequent in clear cell carcinoma. ß-Catenin n+m+ staining was only observed in endometrioid tumors, mostly in endometrial cancer. The ß-Catenin status was not related to age (p = 0.483) or FIGO stage (p = 0.750). It was significantly (p = 0.046) associated with tumor grade. Thus ß-Catenin n+m+ staining and n-m- staining was mostly seen in G2 and G3 tumors whereas positive membrane staining alone was almost evenly spread among all grading. Survival analysis (Fig 4) demonstrated significant (p = 0.028) differences for patients according to ß-Catenin status.

Patients with n-m- staining for ß-Catenin in their tumors had a 5-year survival rate of 44.4% and patients with n+m+ staining had a 5-year survival rate of 40,0% compared to 78.5% survival for those with n-m+ expression. Significantly better survival in the subgroup of ß-Catenin n-m+ stained tumors could also be observed for sole analysis of all endometrioid tumors (p = 0.031) and uterine carcinoma (p = 0.014) as well as tumors of grading G3 (p = 0.036) and tumors with FIGO staging III and IV (p = 0.021).

Relationship between ARID1A/ß-Catenin status, and ß-Catenin/p16 status and their association to clinico-pathological data

Correlation between the expressions of the five different proteins was tested by using Pearsons chi-square test (Table 3). A low significant correlation was found between ARID1A and ß-Catenin with a corrected coefficient of contingency of C_{cor} = 0.282 (p = 0.045) and ß-Catenin and p16 with a corrected coefficient of contingency of C_{cor} = 0.282 (p = 0.045). Further the differences between the expressions of ARID1A/ß-Catenin and ß-Catenin/p16 were investigated based on different clinical parameters, including age, histological subtype, grading and FIGO staging. While the ARID1A/ß-Catenin status was not significantly related to age (p = 0.697), a significant correlation to histological subtype ($p < 0.001$), grading (p = 0.018) and FIGO staging (p = 0.014) was observed (Table 4). ARID1A negative staining and simultaneous ß-Catenin n+m+ staining was only seen in endometrioid uterine tumors. ARID1A negative staining and simultaneous ß-Catenin n-m- staining was also observed mostly in endometrioid endometrial cancer. This staining combination was found to be mostly in tumors with grading G3 and FIGO staging IV. ARID1A positive staining and simultaneous ß-Catenin n-m+ staining were mostly seen in endometrioid ovarian carcinoma and the tumors belonged mostly to FIGO staging I.

The ß-Catenin/p16-status showed no significant correlation to the parameters mentioned above (age (p = 0.750), histological subtype (p = 0.189), grading (p = 0.379) and FIGO staging (p = 0.613)).

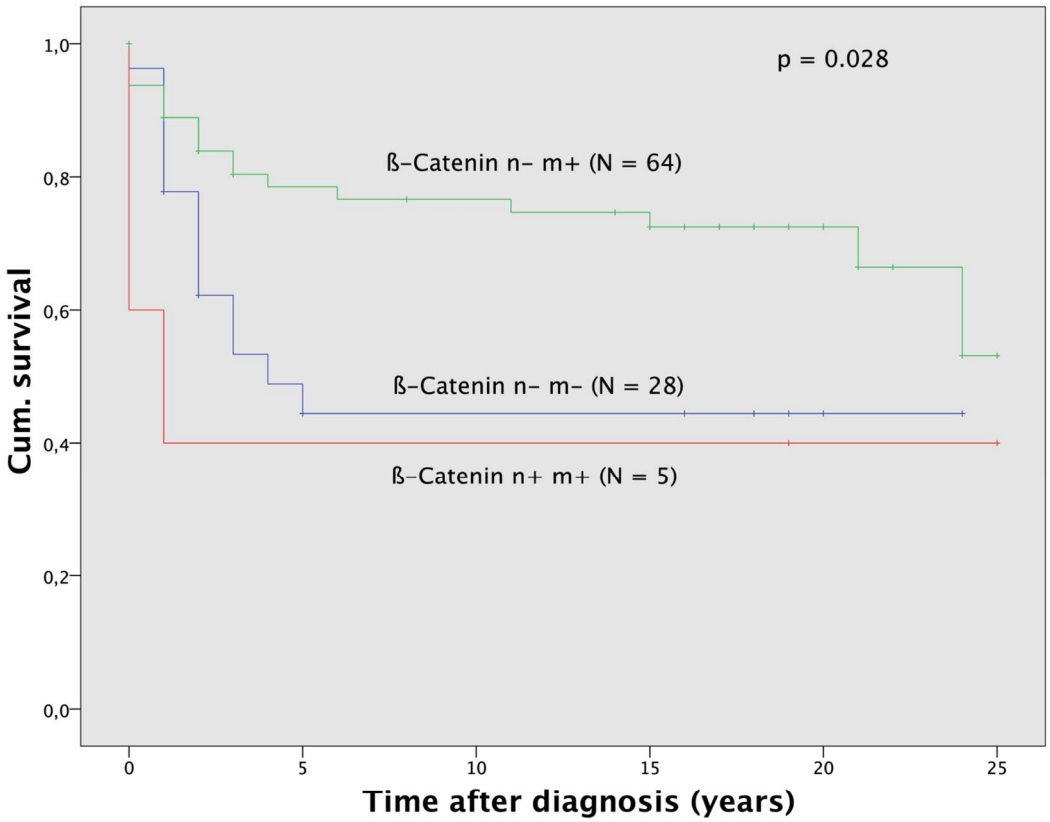

Fig 4. Survival analysis for ß-Catenin. Survival analysis for ß-Catenin. Survival was better for the subgroup of patients with ß-Catenin n-m+ tumors (N = 64) compared to the subgroup ß-Catenin n-m- (N = 28) and ß-Catenin n+m+ tumors (N = 5).

https://doi.org/10.1371/journal.pone.0192881.g004

Table 3. Correlation among ARID1A/ß-Catenin and ß-Catenin/p16 in endometrioid and clear cell ovarian and uterine tissues.

	ß-Catenin n-m-/n+m+	ß-Catenin n-m+	
N (%)	33 (34.0)	64 (66.0)	N (%)
ARID1A -	28	42	70 (72.2)
ARID1A +	5	22	27 (27.8)
C_{corr} = 0.282, p-value: 0.045			97
P16 –o +++	21	27	48 (49.5)
P16 +	12	37	49 (50.5)
C_{corr} = 0.282, p-value: 0.045			97

C_{corr}, corrected coefficient of contingency

https://doi.org/10.1371/journal.pone.0192881.t003

Table 4. Status of ARID1A/ß-Catenin according to histological subtype, grading and FIGO staging.

Expression N (%)	ARID1A +/ ß-Catenin n-m+	ARID1A +/ ß-Catenin n+m+	ARID1A -/ ß-Catenin n-m-	ARID1A -/ ß-Catenin n-m+	ARID1A -/ ß-Catenin n+m+
	25 (25.8)	2 (2.1)	25 (25.8)	42 (43.3)	3 (3.1)
Histological subtype					
Clear cell uterine	0 (0)	0 (0)	5 (20)	1 (2.4)	0 (0)
Endometrioid uterine	5 (20)	1 (50)	13 (52)	37 (88.1)	3 (100)
Clear cell ovarian	4 (16)	0 (0)	5 (20)	2 (4.8)	0 (0)
Endometrioid ovarian	16 (64)	1 (50)	2 (8)	2 (4.8)	0 (0)
P-value < 0.001					
Grading					
G1	7 (28)	1 (50)	3 (12)	16 (38.1)	0 (0)
G2	5 (20)	1 (50)	6 (24)	16 (38.1)	1 (33.3)
G3	13 (52)	0 (0)	16 (64)	10 (23.8)	2 (67.7)
P-value = 0.018					
FIGO staging					
FIGO I	13 (52)	1 (50)	4 (16)	6 (14.3)	0 (0)
FIGO II	6 (24)	1 (50)	4 (16)	9 (21.4)	0 (0)
FIGO III	4 (16)	0 (0)	7 (28)	12 (28.6)	1 (33.3)
FIGO IV	2 (8)	0 (0)	10 (40)	15 (35.7)	2 (67.7)
P-value = 0.014					

https://doi.org/10.1371/journal.pone.0192881.t004

Multivariate analysis

In a multivariate Cox regression analysis with tumor-related death as the end point significant and independent prognostic factors were ARID1A status with hazard ratio (HR) of 3.359, p53 status with HR of 3.408, ß-Catenin status with HR of 2.251 and ARID1A/ß-Catenin status with HR of 2.209 (Tables 5–8). An HR of 2.209 for concomitant ARID1A negativity and ß-Catenin n-m-/n+m+ staining of tumors versus other combinations of ARID1A/ß-Catenin meant a 2.209 fold increased risk for tumor-related death for patients who belonged to the first subgroup. Similarly, an HR of 3.359 for ARID1A negative tumors versus ARID1A positive tumors, an HR of 3.408 for p53 aberrant expression versus p53 regulated expression and an HR of 2.251 for ß-Catenin weak/negative membranous and positive nuclear expression versus ß-Catenin positive membranous expression meant a 3.359, 3.408 respectively 2.251 fold increased risk for tumor-related death for patients who belonged to the first subgroups.

Table 5. Multivariate Cox regression analysis with tumor related death as endpoint for ARID1A.

Variable	Hazard Ratio	95% Confidence Interval	P-Value	n
Age (years)	0.990	0.959–1.021	0.514	97
Histological subtype (endometrioid *vs.* clear cell carcinoma)	2.110	0.942–4.728	0.070	97
Grading (G1+G2 *vs.* G3 tumors)	1.064	0.524–2.162	0.864	97
FIGO stage (I+II *vs.* III + IV tumors)	1.079	0.533–2.186	0.833	97
ARID1A (+ vs. -)	3.359	1.152–9.792	0.026	97

https://doi.org/10.1371/journal.pone.0192881.t005

Table 6. Multivariate Cox regression analysis with tumor related death as endpoint for p53.

Variable	Hazard Ratio	95% Confidence Interval	P-Value	n
Age (years)	0.987	0.956–1.019	0.426	97
Histological subtype (endometrioid *vs.* clear cell carcinoma)	1.605	0.716–3.599	0.251	97
Grading (G1+G2 *vs.* G3 tumors)	1.957	0.903–4.239	0.194	97
FIGO stage (I+II *vs.* III + IV tumors)	1.589	0.790–3.196	0.194	97
p53 (+ vs.—o+++)	3.408	1.567–7.415	0.002	97

https://doi.org/10.1371/journal.pone.0192881.t006

Discussion

Endometrial cancer can be subdivided into two histological subtypes, the estrogen-associated type I which includes endometrioid carcinomas and the estrogen-independent type II which comprises mostly high-grade serous and clear cell carcinoma [1]. The type II carcinoma is known to metastasize more often and to have a worse survival. A less accepted paradigm for ovarian cancer also differs between type I tumors which include endometrioid, clear cell and low-grade serous carcinoma and type II tumors containing p53-mutated high-grade serous carcinoma [2].

Many biomarkers, including different tumor suppressors such as ARID1A, p53, p21, p16 and ß-Catenin, were discovered to be changed in expression in endometrioid and clear cell subtypes in recent years [26–29]. The aim of this study was to evaluate their prognostic value, particularly in the synopsis of all the above mentioned factors, by using immunhistochemical methods.

Ovarian clear cell carcinoma usually showed p53 overexpression or no expression (81.8%), while ovarian endometrioid carcinoma mostly was observed to have positive p53 expression (71.4%). Both subtypes consisted in equal parts of p16 overexpression/no expression or positive expression and all stained negatively for p21. These findings confirm the results of various studies [30–32], which indicate that most of the ovarian clear cell tumors show p53 mutations, while most of endometrioid tumors do not. There are several studies showing that p16 is overexpressed in high-grade serous ovarian carcinoma compared with other morphologic types of ovarian cancer [33], whereas inactivation of the gene was observed in 3 out of 9 endometrioid and 1 out of 4 clear cell ovarian carcinomas [34]. Another study explored the expression of p21 in endometrioid and clear cell ovarian carcinoma and found p21 negativity in 59.5% (endometrioid) and 31.2% (clear cell) [35].

For endometrial carcinoma we can report that clear cell subtypes consisted in equal parts of p53 overexpression/no expression or positive expression and all showed p16 overexpression/

Table 7. Multivariate Cox regression analysis with tumor related death as endpoint for ß-Catenin.

Variable	Hazard Ratio	95% Confidence Interval	P-Value	n
Age (years)	0.993	0.961–1.026	0.674	97
Histological subtype (endometrioid *vs.* clear cell carcinoma)	1.743	0.774–3.928	0.180	97
Grading (G1+G2 *vs.* G3 tumors)	1.430	0.691–2.957	0.335	97
FIGO stage (I+II *vs.* III + IV tumors)	1.249	0.633–2.511	0.532	97
ß-Catenin (n-m+ vs. n-m-/n+m+)	2.251	1.096–4.625	0.027	97

https://doi.org/10.1371/journal.pone.0192881.t007

Table 8. Multivariate Cox regression analysis with tumor related death as endpoint for ARID1A/ß-Catenin status.

Variable	Hazard Ratio	95% Confidence Interval	P-Value	n
Age (years)	0.992	0.960–1.024	0.614	97
Histological subtype (endometrioid *vs.* clear cell carcinoma)	1.678	0.736–3.822	0.218	97
Grading (G1+G2 *vs.* G3 tumors)	1.459	0.696–3.058	0.317	97
FIGO stage (I+II *vs.* III + IV tumors)	1.198	0.593–2.421	0.614	97
Others vs. ARID1A-/ß-Catenin n-m-/ß-Catenin n+m+	2.209	1.031–4.734	0.041	97

https://doi.org/10.1371/journal.pone.0192881.t008

no expression while endometrioid subtypes mostly were found to have positive p53 (69.5%) and p16 (69.4%) staining. Both subtypes were stained mostly p21 negative. In several studies, the p53 mutation prevalence among clear cell uterine carcinoma ranges from 28.5% to 76.9% [36,37].

For endometrioid uterine carcinoma however, most of the tumors appear to have no p53 mutation [38], which is in agreement with our results. In endometrioid uterine carcinoma the expression pattern of p16 is typically described as weakly positive unlike the strong expression generally seen in endocervial adenocarcinomas of the usual type [39]. However, another study found up to 33% overexpression and 65% no expression in clear cell endometrial carcinoma and different p16 expression in endometrioid endometrial carcinoma according to FIGO grade [40]. In FIGO grade 1 and 2 endometrioid subtypes were found to be mostly stained p16 negative while in FIGO grade 3 about 25% turned out to be p16 positive. For p21 expression in endometrial cancer some studies showed about 54% p21 negativity, increasing with older age and current smoking [41]. In contrast to these results other studies figured out that p21 seems to be mostly positively expressed in endometrial cancer [26]. The contradictory findings related to p16 and p21 may be due to different staining protocols, cut-off values for p16 and p21 expression, and characteristics of the study populations examined.

In this analysis the ß-Catenin expression of ovarian and uterine tumors was determined. ß-Catenin mutations are particularly common in endometrioid ovarian and uterine cancer [22]. However, their prevalence ranges widely from 16–54% across the several studies [42]. It could be confirmed that ß-Catenin n+m+ staining was only seen in endometrioid tumors, mostly uterine.

ARID1A has been recently classified as a novel tumor suppressor, which regulates p53-controlled genes [43]. Reduced ARID1A expression is mostly induced by nonsense mutations as well as insertions and deletions in the gene-coding region, which lead to mRNA decay or sequence truncation [44]. It has been published that promoter hypermethylation may also be responsible for the loss of ARID1A expression [45]. ARID1A mutations are frequently in various tumors including gastric cancer, colorectal cancer, breast cancer, lung cancer and gynaecological cancer [46]. High rates of ARID1A mutations were observed in 46–57% of ovarian clear cell carcinomas, 30% of ovarian endometrioid carcinomas and 40% of uterine endometrioid carcinomas [15,47,48]. In this study, we also demonstrated that negative expression of ARID1A was common in clear cell (63.6%) and endometrioid (19%) ovarian tumors as well as clear cell (100%) and endometrioid (89.8%) uterine cancer. Moreover, we determined a significant relationship between ARID1A loss and higher FIGO stages which is similar to previous studies [49,50].

No study could be found that examined the combined ARD1A/ß-Catenin status of ovarian and uterine tumors yet. However, few studies indicate that there is an association between

the two tumor suppressor genes. ARID1A silencing seems to be inducing a subcellular redistribution of β-catenin from the plasma membrane to the cytoplasm and nucleus in gastric cancer, which was found to be significantly associated with worse clinical prognosis [51]. Another study demonstrated that the chromatin-remodeling factor ARID1B, forming the BAF complex together with ARID1A, represses the Wnt/ß-Catenin signaling pathway indicating this might contribute to cancer through deregulation of developmental and oncogenic pathways [52]. It also suggests ARID1A might operate in a similar fashion as ARID1B in Wnt/β-Catenin pathway. In this study a significant correlation between ARID1A and ß-Catenin could be shown. Concomitantly ARID1A negative staining and ß-Catenin n+m+ staining respectively ß-Catenin n-m- staining was usually seen in endometrioid uterine cancer with poor grading (G2 and G3) and poor FIGO staging (FIGO III and FIGO IV). In a multivariate Cox Regression analysis this staining combination of ARID1A and ß-Catenin meant a 2.209 fold increased risk for tumor-related death. This indicates that the ARID1A/ß-Catenin pathway connection might play a role in tumor progression in endometrioid endometrial carcinoma. It could already be demonstrated that ARID1A loss and ß-Catenin mutation, each seen individually, are important in progression of different types of human cancer [16,53,54]. Additional studies will have to be conducted to evaluate the association between ß-Catenin and the chromatin remodelling gene ARID1A in uterine endometrioid carcinoma further.

In the present study it was possible to identify three prognostic factors that showed differences in independent survival analysis. For the tumor suppressor gene p53 we were able to show that patients with overexpression or no expression of p53 had a worse overall survival compared to those with normal p53 expression. This is consistent with a number of studies determining the prognostic value of p53 in ovarian and uterine carcinoma [27,35] and in contrast to other studies [54,55].

It could be observed that nuclear and membrane/cytoplasm positive staining as well as nuclear and membrane negative staining of ß-Catenin were markers for worse overall survival which agrees with some studies [56,57] that show reduced ß-Catenin cell surface expression was a predictor for poor survival but not with other studies [58,59] that associate strong membranous β-Catenin expression with shorter progression free survival. Discordance between these results could be due to different patterns of ß-Catenin and p53 expression in ovarian and uterine carcinoma, especially in different staging and grading, and differences in the methods of immunohistochemical interpretation.

Similar to previous studies it could be determined that ARID1A loss in ovarian and uterine tumors is a predictor for poor survival [49,60–62]. ARID1A expression was also reported to be a prognostic marker for several other cancers such as gastric cancer, clear cell renal cell carcinoma and cervical cancer [63–65].

At this point some limitations of this study may be noted which can be addressed in future studies. They include mostly the limited numbers of cases within histological subgroups, especially clear cell carcinoma with a total number of only 17 cases. To prevent the effect, that ovarian and uterine carcinoma can be very heterogeneous in immunohistochemical interpretation the tissue microarray construction was done by using three core biopsies from each specimen. Nevertheless some cases might not have been adequately represented due to loss of cancer tissue material.

In summary, a significant association between AIRD1A and ß-Catenin expression was discovered in endometrioid uterine tumors. This suggests that concomitantly ARID1A negative staining and ß-Catenin nuclear and membrane/cytoplasm positive (n+m+) staining respectively ß-Catenin nuclear and membrane negative staining (n-m-) might play a role in tumor progression in type I endometrial cancer. Furthermore ARID1A, p53 and ß-Catenin turned out to be three promising prognostic factors showing significant differences in independent

survival analysis, indicating that ARID1A, p53 and ß-Catenin could be used along traditional clinical and morphologic factors to predict the prognosis of patients with clear cell and endometrioid ovarian and uterine cancer.

Supporting information

S1 Table. Status of p53 according to tumor grade (N = 97).
(DOCX)

S1 File. Data set.
(XLSX)

S1 Fig. Status of ARID1A according to FIGO stage (N = 97).
(TIF)

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Visualization: Marlene Heckl, Elisa Schmoeckel.

Writing – original draft: Marlene Heckl.

Writing – review & editing: Elisa Schmoeckel, Udo Jeschke, Doris Mayr.

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Overall Survival of Ovarian Cancer Patients Is Determined by Expression of Galectins-8 and -9

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Abstract: The evaluation of new prognostic factors that can be targeted in ovarian cancer diagnosis and therapy is of the utmost importance. Galectins are a family of carbohydrate binding proteins with various implications in cancer biology. In this study, the presence of galectin (Gal)-8 and -9 was investigated in 156 ovarian cancer samples using immunohistochemistry (IHC). Staining was evaluated using semi-quantitative immunoreactivity (IR) scores and correlated to clinical and pathological data. Different types of galectin expression were compared with respect to disease-free survival (DFS) and overall survival (OS). Gal-8 served as a new positive prognostic factor for the OS and DFS of ovarian cancer patients. Gal-9 expression determined the DFS and OS of ovarian cancer patients in two opposing ways—moderate Gal-9 expression was correlated with a reduced outcome as compared to Gal-9 negative cases, while patients with high Gal-9 expression showed the best outcome.

Keywords: galectin-8; galectin-9; immunochemistry; ovarian cancer; prognostic factor; disease-free survival; overall survival

1. Introduction

Ovarian cancer is the fifth leading cause of cancer death among women of all ages [1]. Due to its frequent diagnosis in advanced stages, characterized by a wide cancer dissemination into the peritoneum and the acquisition of chemo resistance after treatment [2], ovarian cancer displays 5-year relative survival rates of less than 50% [3]. Ovarian cancer management lacks effective screening methods and specific treatment options. As prognosticators in ovarian cancer, the histological subtype, disease stage at diagnosis, extent of residual disease after surgery, and volume of ascites can be used [4]. However, except for breast cancer gene (*BRCA*) status, no biological prognostic factor is commonly considered [4]. Various studies have attempted to introduce new prognostic factors in ovarian cancer, and for several proteins a prognostic value independent of clinical parameters has been detected. However, so far none of them can be applied in ovarian cancer therapy or diagnosis. Hence, there is a tremendous need for the evaluation of new prognostic factors that can be targeted in ovarian cancer.

In 1994 the galectin (Gal) family was described as group of proteins sharing a binding affinity for β-galactosides, with significant similarity in the carbohydrate- recognition domain (CRD) [5,6]. Since then, the galectin family has grown in members. In total, 10 different galectins (Gal-1–4, Gal-7–10, Gal-12, and Gal-13) are known to be present in humans [7]. According to the arrangement

of CRDs, galectins can be subdivided into three groups. Prototype galectins contain a single CRD, often forming homodimers, while tandem-repeat galectins contain two CRDs connected by a linker chain, and chimeric galectins (a group containing only member Gal-3) have a second N-terminal domain connected to a single CRD [8]. In this study, we will focus on two tandem-repeat galectins, Gal-8 and -9. By binding β-galactosides on certain glyoproteins with their CRDs, galectins are known to modulate cell–cell and cell–matrix interactions as well as intracellular pathways [6]. Galectins have been discovered to play an important role in several diseases including cancer [9]. Several mechanisms of tumor biology, also referred to as "hallmarks of cancer", are known to be influenced by galectins: enhanced proliferation, resistance to cell death, and induction of angiogenesis, as well as tumor invasion and metastasis [7,10,11]. Therefore, galectin expression in cancer tissues of several malignancies has been found to affect patients' disease-free survival (DFS) or overall survival (OS). For this reason, several studies assessed different galectins as prognostic survival markers, but thus far, most efforts have been spent on Gal-1 and -3. However, Gal-8 and -9 have been evaluated as prognostic markers in few cancer types. In triple-negative breast cancer, for instance, patients displaying Gal-8 expression in nuclei had significantly better DFS and OS [12]. Higher Gal-9 expression, on the other hand, was associated with prolonged OS of gastric cancer patients [13].

In ovarian cancer, however, most previous studies focused on galectin-1, -3 and -7 as prognostic factors [14–19]. Our group recently published an article in the international journal of molecular sciences, presenting high tumor and stroma Gal-1 expression, as well as higher Gal-7 expression as negative prognostic markers for OS of ovarian cancer patients, while nuclear Gal-3 expression was correlated with a better OS [20]. In fact, to our knowledge there is only one very recent study on Gal-8 and Gal-9 in ovarian cancer [21]. In this study, high epithelial Gal-8 expression was associated with the acquisition of chemo resistance. However, no correlation with DFS or OS was observed. In the same study, the Gal-9 expression that was observed in "cytosolic or perinuclear puncta", was correlated with poor OS. However, this special Gal-9 expression was not associated with altered DFS. Cytoplasmic Gal-9 expression, however, showed no association to either DFS or OS. In general, with a 5-year follow-up time, all of the observations were limited to a rather short period of observation and the analysis was performed in a collective of only high-grade serous ovarian cancer samples, with their prognostic role in other than subtypes remaining elusive. Summing up, there are a limited number of studies on Gal-8 and -9 in ovarian cancer and several aspects of their prognostic features still remain to be elucidated.

Therefore, in this study, we evaluated the prognostic influence of Gal-8 and -9 in patients with epithelial ovarian cancer using immunohistochemistry and analyzed correlations to each other and to clinical and pathological parameters. We hypothesize that Gal-8 and -9 are prognostic for overall survival in ovarian cancer patients. Since it is known that galectin function and their effect on patients' survival can be determined by expression in the nucleus or cytoplasm of cancer cells as well as the peritumoral stroma, we paid attention to the specific location of galectin expression in our analysis.

2. Results

2.1. In Silico Analysis of Gal-8 and -9 Expression in Normal Ovarian Tissues and Ovarian Cancer

The human protein atlas (available at www.proteinatlas.org) was used to analyze Gal-8 and -9 expression in normal ovarian tissues as well as ovarian cancer tissues [22]. In ovarian stroma cells, Gal-8 (human Gal-8 gene, *LGALS8*) was not detected via antibody staining. However out of 12 ovarian cancer tissues, 8 showed medium Gal expression. For Gal-9 (human Gal-9 gene, LGALS9), out of 12 ovarian cancer patients, 3 showed medium Gal-9 expression and 5 patients showed low Gal-9 expression. In normal ovarian tissues, Gal-9 was found to have low expression in ovarian stromal cells. According to this, both Gal-8 and Gal-9 seem to be altered in ovarian cancer compared to normal ovarian tissues. This further motivated us to specify Gal-8 and -9 expression in ovarian cancer tissues using immunochemistry.

2.2. Gal-8 is a Positive Prognostic Factor for OS and DFS in Ovarian Cancer Patients

Galectin-8 staining could be evaluated in 143 ovarian cancer samples. Gal-8 expression occurred predominantly in the cytoplasm and nuclei of ovarian cancer cells but not in the peritumoral stroma (Figure 1). In total, 96 cases (67.1%) showed a high Gal-8 expression in the cytoplasm (immunoreactivity score, IRS > 1), while in 47 specimens (32.9%), only low Gal-8 expression was observed (IRS $\leq$ 1). The median IRS of Gal-8 staining in the cytoplasm was 3. According to chi-squared statistics, low Gal-8 expression in the cytoplasm correlated with lymph node metastasis as well as a higher International Federation of Gynecology and Obstetrics (FIGO) stage ($p = 0.019$, $p = 0.033$, respectively) and (Table 1). In 70 of the samples (51.4%) Gal-8 positive nuclei were observed, while 74 specimens (48.6%) did not present with nuclear Gal-8 staining. Positive nuclear Gal-8 staining was more often observed in lower FIGO stages ($p = 0.011$) and (Table 1). Besides, no other correlation of nuclear Gal-8 expression and clinical or pathological data was observed.

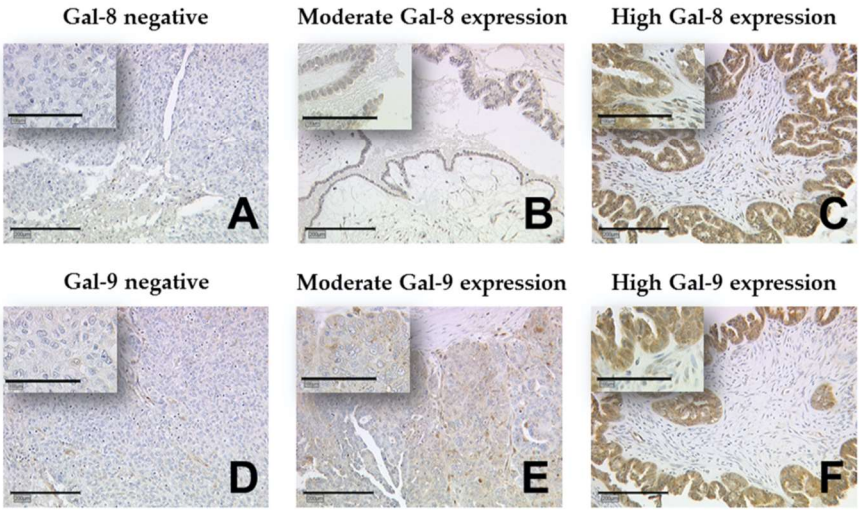

Figure 1. Detection of galectin-8 and -9 using immunohistochemistry (IHC). Gal-8 staining (**A**–**C**) and Gal-9 staining (**D**–**F**) was predominantly present in the cytoplasm of ovarian cancer cells but not in the peritumoral stroma. Representative photomicrographs are shown. There is a 10× magnification for the outer pictures and 25× for the inserts. Scale bar in (**A**) equals 200 µm (outer pictures) and 100 µm (inserts). Gal: galectin.

Table 1. Gal-8 and -9 staining correlated with clinical and pathological data.

	Gal-8 Expression (Cytoplasm)		*p*-Value	Gal-8 Expression (Nucleus)		*p*-Value	Gal-9 Expression (Cytoplasm)			*p*-Value
	Low	High		Negative	Positive		Negative	Moderate	High	
Histology										
Serous	40	62	NS	54	48	NS	24	71	9	0.024
Clear cell	2	9		3	8		1	10	0	
Endometrioid	2	17		8	11		6	10	5	
Mucinous	3	8		5	7		1	6	4	
Tumor Stage										
pT1	10	26	NS	14	23	NS	8	20	9	0.018
pT2+	37	69		55	51		24	77	8	
Lymph node										
pN0/ pNX	25	70	0.019	43	53	NS	26	61	12	NS
pN1	22	26		27	21		6	36	6	
Distant Metastasis										
pM0/pMX	45	94	NS	68	72	NS	32	92	17	NS
pM1	2	2		2	2		0	5	1	
Grading										
G1	7	26	NS	13	21	NS	7	19	8	0.006
G2+	37	62		53	46		24	72	5	
FIGO										
I/ II	8	33	0.033	14	28	0.011	6	25	11	0.002
III/ IV	37	60		55	42		25	69	6	
Age										
≤60 years	24	51	NS	32	43	NS	10	52	17	<0.001
>60 years	23	45		38	31		22	45	1	

TNM staging was accomplished according to the Union for International Cancer Control (UICC); pT1 = tumor stage 1; pT2+ = tumor stage 2 or higher; pN0 = lymph node stage 0; pNX = lymph node stage not evaluated; pN1 = lymph node stage 1; pM0 = distant metastasis stage 0; pMX = distant metastasis not evaluated; pM1 = distant metastasis stage 1; G1 = grade 1; G2+ = grade 2 or higher; NS = Not significant ($p > 0.05$).

Different groups of Gal-8 expression were compared using Kaplan-Meier analysis (Figure 2). Patients presenting with high Gal-8 expression showed a significantly better overall survival and disease-free survival ($p = 0.024$, $p = 0.018$, respectively). Nuclear Gal-8 expression had no significant influence on overall or disease-free survival. In multivariate analysis, Gal-8 staining served as a prognostic factor independent of clinical and pathological variables (Table 2).

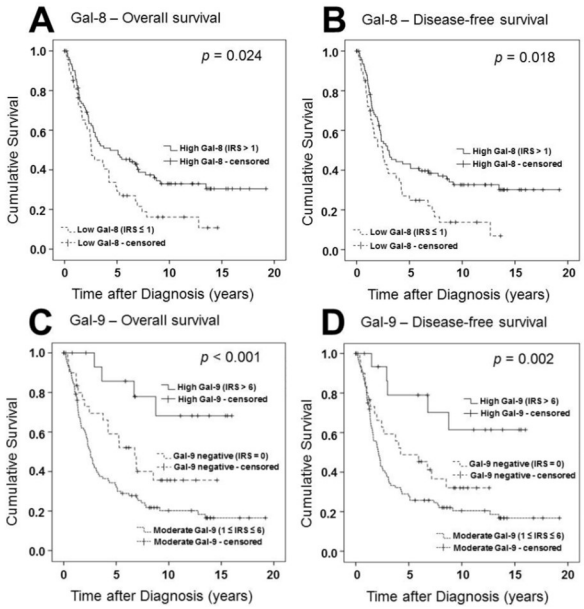

Figure 2. Survival times of patient groups with different galectin-8 and -9 expression levels were compared. Patients with high Gal-8 expression in the cytoplasm showed better progression-free (**A**) and overall survival (**B**) compared to patients without or with low Gal-8 expression. Cases with a moderate Gal-9 expression in the cytoplasm displayed a reduced progression-free (**C**) and overall survival (**D**) compared to Gal-9 negative cases. However, patients with high Gal-9 expression showed the best progression-free (**C**) and overall survival (**D**). Galectin-8 and -9 expression was determined in the cytoplasm of cancer cells using IHC and immunoreactivity (IR) scores. Survival times were plotted as Kaplan–Meier graphs. Graph shows the percentage of living patients (vertical axis) in dependence of time (horizontal axis). Patients without reported death who exited the study before the observation period ended were censored by the software. Censoring events have been marked in the graphs.

Table 2. Multivariate analysis.

Covariate	Coefficient (b_i)	HR Exp (b_i)	95% CI		*p*-Value
			Lower	Upper	
Histology	−0.005	0.995	0.989	1.002	0.135
Grading	0.614	1.848	1.342	2.544	<0.001
FIGO	0.763	2.144	1.503	3.058	<0.001
Patients' age (≤60 vs. >60 years)	0.737	2.089	1.265	3.447	0.004
Gal-8 staining (low vs. high)	−0.487	0.615	0.388	0.973	0.038
Gal-9 staining (neg. vs. low vs. high)	0.687	1.988	1.257	3.145	0.003

HR = hazard ratio; CI = confidence interval.

2.3. Gal-9 Expression Determines DFS and OS of Ovarian Cancer Patients in Two Different Ways

Staining for galectin-9 was assessed in 147 ovarian cancer samples using IR scores. Gal-9 staining was mostly present in the cytoplasm of ovarian cancer cells, but not the nuclei or the peritumoral stroma. Throughout the panel a median IRS of 3 was observed. In total, 32 cases (20.5%) were Gal-9 negative (IRS = 0), 79 cases (50.6%), however, presented with moderate Gal-9 staining ($1 \geq$ IRS ≥ 6) and in 36 cases (24.5%) high Gal-9 expression (IRS > 6) was observed. Gal-9 staining showed different distribution in different histological subtypes (Table 1). Cases with high Gal-9 expression presented more often with low tumor stage, lower grading, early FIGO stage, and younger age. The majority of Gal-9 negative cases, however, showed a high tumor stage, higher grading, advanced FIGO stage, and older age (Table 1).

Using Kaplan-Meier analysis, different groups of Gal-9 expression showed significant differences in overall and disease-free survival. Cases with a moderate Gal-9 expression ($1 \geq$ IRS ≥ 6) displayed a reduced progression-free and overall survival compared to Gal-9 negative cases (IRS = 0). However, the small group of patients presenting with a high Gal-9 expression (IRS > 6) showed the best progression-free (C) and overall survival (D). In multivariate analysis, this correlation proved to be independent of clinical and pathological variables, together with grading, FIGO, patients' age and Gal-8 expression.

2.4. Correlation Analysis

A correlation analysis between IR scores of Gal-8 and Gal-9 staining in the cytoplasm was performed. Results are shown in Table 3. We observed a rather weak, but highly significant correlation between cytoplasmic Gal-8 and Gal-9 staining ($p < 0.001$).

Table 3. Correlation analysis.

	Gal-9 Cytoplasm
Gal-8 cytoplasm	
cc	0.464
p	<0.001
n	142

IR scores for Gal-8 and -9 staining were correlated using Spearman's correlation analysis. cc = correlation coefficient, *p* = two-tailed significance, *n* = number of patients.

3. Discussion

According to our data, high Gal-8 expression in the cytoplasm of cancer cells is a novel positive prognostic factor for DFS and OS in ovarian cancer patients. Cytoplasmic Gal-9 expression, however, determines the DFS and OS of ovarian cancer patients in two opposing ways: On one hand, moderate Gal-9 expression correlates with a reduced overall and disease-free survival, compared to Gal-9-negative cancers, while high Gal-9 expression correlated with the best outcome. Stromal Gal-8 or -9 was not observed in ovarian cancer samples and nuclear expression does not seem to play an important role for survival of ovarian cancer patients.

In 1995, Gal-8 was cloned for the first time from a rat liver cDNA expression library [23]. Later, a homolog gene was detected in the human prostate adenocarcinoma cell line LNCaP, that was identified as prostate carcinoma tumor antigen-1 (*PCTA-1*). Also, altered Gal-8 expressed was found in prostate carcinomas compared to normal prostate and benign prostatic hypertrophy [24]. Several alternative splicing variants have been reported in Gal-8 mRNA processing [25]. In total, seven different isoforms of Gal-8 are encoded by the human Gal-8 gene (*LGALS8*). Three of them belong to the tandem-repeat galectin group and four to the prototype group with only one CRD. However, prototype isoforms of Gal-8 were not found at the protein level [26]. Nevertheless, rather than as a single protein, galectin-8 should be regarded as a discrete subfamily among all galectins. To our knowledge, there are no

antibodies available to target specific isoforms of galectin-8. Hence, immunohistochemistry is limited to the observation of the total expression level of all galectin-8 isoforms. Whether different anti-Gal-8 antibodies have a higher affinity for certain Gal-8 isoforms remains elusive as well. However, this could be a reason for different results evaluating Gal-8 as a prognostic factor using different antibodies in immunohistochemistry [21]. This problem should be addressed in further experiments, e.g., using Western blot analysis to determine the individual Gal-8 subtype expressed in ovarian cancer tissues.

Gal-8 has been found to contribute to several mechanism of tumor biology. Endothelial cell migration and tube formation in vitro as well as angiogenesis in vivo has been demonstrated to be induced by Gal-8 [27]. Cell adhesion in human non-small cell lung carcinoma cells (H1299) and rat hepatoma cells (Fao) as well as Chinese hamster ovary (CHO-P) cells was affected by the presence of Gal-8, either positively or negatively dependent on its concentration [28]. In glioblastoma cell line U87, Gal-8 was shown to promote cell migration and proliferation and has been observed to prevent tumor cell apoptosis [29]. However, none of these effects have been examined in ovarian cancer, and further studies are required to explain the role of Gal-8 in ovarian cancer biology.

One of the first descriptions of Galectin-9 was in 1997, after which it was cloned and identified as a tumor antigen in Hodgkin's lymphoma [30]. Since then, many implications of Gal-9 in cancer have been reported [31]. In melanoma cells, galectin-9 was able to induce cell aggregation and apoptosis, and down-regulation of Gal-9 was associated with distant metastasis [32]. Similarly, in breast cancer, Gal-9 negative tumors were more likely to show distant metastasis and therefore correlated with an unfavorable prognosis [33]. In both, melanoma and breast cancer, tumor cell adhesion has been found to be influenced by Gal-9 expression [32,34]. Furthermore, changing Gal-9 expression was discovered during endothelial cell activation, implying a function in angiogenesis [35]. However, best studied role of Gal-9 is in immunity and inflammation. Most prominent mechanism here is the binding of Gal-9 to TIM3, a T cell-specific surface molecule, leading to intracellular calcium flux, aggregation, and apoptosis of T-helper type 1 cells [36]. Similarly, in CD8+ cytotoxic T-cells, Gal-9 was able to induce apoptosis in vitro and vivo, inhibiting the immune response to alloantigen of a skin graft [37]. The same mechanism can be implicated in the acquaintance of tumor immunity. Furthermore, Gal-9 induced the differentiation of naive T cells to T regulatory (T reg) cells, decreasing the number of CD4(+) TIM3(+) T cells and increasing the number of T reg cells in the peripheral blood of a mouse model [38]. T reg cells, however, are known to suppress the antitumor immune response and therefore enable the tumor immune escape [39]. In line with this, in ovarian cancer, a higher number of T reg cells in lymphoid aggregates surrounding the tumor were associated with significantly reduced patient survival [40]. Summing up, the role of Gal-9 in cancer immunity implicates a reduced survival of patients with Gal-9 expressing cancers, while its functions in apoptosis, cancer cell adhesion, and metastasis could explain a better outcome in Gal-9 expressing ovarian cancers. Both taken together could serve as an explanation for the two opposing ways in which Gal-9 determined the survival of ovarian cancer patients in this study.

Similar to Gal-8, several splice variants have been reported for Gal-9 [31]. Again, varying antibody affinity to different Gal-9 isoforms could explain contradictory results in different studies [21]. Furthermore, heterogeneity in Gal-9 splice variants could explain the complex effects of Gal-9 expression on patients' survival, which is described in literature, but was also observed in this study. Assuming different Gal-9 isoforms can realize different functions in cancer biology, patient survival could be affected by different Gal-9 isoforms in opposing ways. However, since these considerations are rather speculative, further studies are required to address this problem.

4. Materials and Methods

4.1. Patients

Tissue micro arrays (TMAs) were constructed from a collective of formalin-fixed, paraffin-embedded (FFPE) ovarian cancer samples obtained from a collective of 156 female patients who underwent

surgery at the Department of Obstetrics and Gynecology, University Hospital, LMU Munich, Germany between 1990 and 2002. No patient had received chemotherapy before surgery. Four histological subtypes were included into the panel (serous (n = 110), endometrioid (n = 21), clear cell (n = 12), and mucinous (n = 13)). Experienced gynecological pathologists (E.S., D.M.) performed tumor grading (G1 (n = 38), G2 (n = 53), G3 (n = 53)) according to WHO. TNM classification (T = tumor, N = lymph nodes, M = metastasis) was performed according to the Union for International Cancer Control (UICC). Extent of the primary tumor (T1 (n = 40), T2 (n = 18), T3 (n = 93), T4 (n = 4)), lymph node involvement (N0 (n = 43), N1 (n = 52) and distant metastasis (M0 (n = 3), M1 (n = 6) was evaluated. FIGO stage was determined (I (n = 35), II (n = 10), III (n = 103), IV (n = 3)) according to the criteria of the International Federation of Gynecology and Obstetrics (FIGO). Patient follow up data was received from the Munich Cancer Registry. Median patients' age was 62 $\pm$ 12 years with a range between 31 and 88 years. During the study 104 deaths have been observed with a mean overall survival of 3.2 $\pm$ 3.0 years.

4.2. Immunohistochemistry

TMA slides were stained using immunohistochemistry as previously described [16]. All sections were dewaxed in xylol for 20 min, before endogenous peroxidase was quenched with 3% hydrogen peroxide (Merck, Darmstadt, Germany). Next, slides were rehydrated in a descending series of alcohol (100%, 75%, and 50%) and heat-induced antigen retrieval was performed by cooking in sodium citrate buffer (0.1 mol/L citric acid/0.1 mol/L sodium citrate, pH 6.0) in a pressure cooker for 5 min. Tissues were blocked with Blocking Solution (Reagent 1; ZytoChem Plus HRP Polymer System (Mouse/Rabbit); Zytomed Systems GmbH, Berlin, Germany) for 5 min at room temperature (RT). Then, specimens were incubated with Anti-Gal-8 (rabbit, monoclonal, Abcam, Cambridge, UK) at a final concentration of 10 µg/mL (1:100 dilution) in phosphate buffered saline (PBS) for 1 h at RT, and Anti-Gal-9 (rabbit, polyclonal, Abcam, Cambridge, UK) at a final concentration of 3.34 µg/mL (1:300 dilution) in PBS overnight (16 h) at 4 °C. Afterwards, slides were incubated with post-block reagent (Reagent 2) (Zytomed Systems GmbH, Berlin, Germany) for 20 min at RT and HRP-Polymer (Reagent 3) (Zytomed Systems GmbH, Berlin, Germany) for 30 min at RT. After each incubation, slides were washed in PBS twice for 4 min. Visualization reaction was performed with 3,3′-diaminobenzidine chromagen (DAB; Dako, Glostrup, Denmark) and stopped after 2 min in tap water. Counterstaining was performed with Mayer acidic hematoxylin. Specimens were dehydrated in an ascending series of alcohol (50%, 75%, and 100%) followed by xylol. Tissue sections, incubated with PBS instead of a primary antibody, were used as a negative control. Tissue samples of colon mucosa served as a positive control. Staining results were received using a semi-quantitative method analog to the immunoreactivity score. Staining for Gal-8 and -9 was evaluated in the cytoplasm of ovarian cancer cells. The predominant staining intensity (0 = negative, 1 = low, 2 = moderate, and 3 = strong) and the percentage of stained cells (0 = 0%, 1 = 1–10%, 2 = 11–50%, 3 = 51–80%, and 4 = 81–100% stained cells) were assessed and multiplied resulting in values of the IRS. For survival analysis, Gal-8 was grouped into low (IRS $\leq$ 1) and high expression cases (IRS > 1). Gal-9 was divided into negative (IRS = 0), moderate (1 $\geq$ IRS $\geq$ 6) and high (IRS > 6) expression.

4.3. Statistical Analysis

Statistical data was processed using SPSS 23.0 (v23, IBM, Armonk, New York, NY, USA) statistic software. Chi-squared statistics were used to test for correlation to clinical and pathological variables. Correlations between staining results were calculated using spearman's correlation analysis. Kaplan–Meier curves and the log-rank test (Mantel–Cox) were used for survival analysis. Data are presented with the mean $\pm$ standard deviation. Significance was assumed for $p < 0.05$.

4.4. Ethics Statement

The current study was approved by the Ethics Committee of the Ludwig Maximilians University, Munich, Germany (approval number 227-09) on 30 September 2009. All tissue samples used for this study were obtained from left-over material from the archives of LMU Munich, Department

Gynecology and Obstetrics, Ludwig-Maximilians University, Munich, Germany, initially used for pathological diagnostics. The diagnostic procedures were completed before the current study was performed. During the analysis, the observers were fully blinded for patients' data. The study was approved by the Ethics Committee of LMU Munich. All experiments were performed according to the standards of the Declaration of Helsinki (1975).

5. Conclusions

We were able to show that Gal-8 expression is a positive prognostic factor for overall and disease-free survival of ovarian cancer patients, while Gal-9 expression determines overall and disease-free survival in two different ways: Moderate Gal-9 expression correlates with a reduced survival, compared to Gal-9 negative cases, while patients with high Gal-9 expression showed the best outcome.

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Author Contributions: Heiko Schulz, Christina Kuhn and Simone Hofmann performed the experiments; and Heiko Schulz analyzed the data and wrote the paper. Elisa Schmoeckel, Doris Mayr and Sven Mahner revised the manuscript for important intellectual content. Udo Jeschke initiated and supervised the study and designed the experiments. All authors read and approved the final version of the manuscript.

Conflicts of Interest: The authors declare no conflict of interest.

Abbreviations

Gal	Galectin
IHC	Immunohistochemistry
IRS	Immunoreactivity score
DFS	Disease-free survival
OS	Overall survival
BRCA	Breast cancer gene
CRD	Carbohydrate-recognition domain
LGALS8	Human Gal-8 gene
LGALS9	Human Gal-9 gene
FIGO	Fédération Internationale de Gynécologie et d'Obstétrique
UICC	Union for International Cancer Control
PCTA-1	Prostate carcinoma tumor antigen-1
CD8	cluster of differentiation 8
CD4	cluster of differentiation 4
TIM3	T-cell immunoglobulin and mucin-domain containing-3
T reg	T-regulatory
TMA	Tissue micro array
FFPE	Formalin-fixed paraffin-embedded
WHO	World Health Organization
TNM	T = tumor, N = lymph nodes, M = metastasis
PBS	phosphate buffered saline

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ORIGINAL ARTICLE – CANCER RESEARCH

Cytoplasmic versus nuclear THR alpha expression determines survival of ovarian cancer patients

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Abstract

Purpose Thyroid hormone receptors (THR) have manifold functions and are involved in the carcinogenesis of several tumor types. Within this study, we aimed to investigate the expression pattern (nuclear versus cytoplasmic) of the THR alpha and its impact on patients survival in ovarian cancer (OvCa).

Methods The presence of the thyroid hormone receptors THRα, THRα1 and − 2 was investigated in 156 ovarian cancer samples using immunohistochemistry (IHC) using semi-quantitative immunoreactivity (IR) scores and correlated with clinical, pathological data, subtype of ovarian cancer, clinical data, staining of 20 already described OvCa marker proteins and overall survival (OS).

Results Among all subtypes of OvCa, clear cell carcinomas showed the highest THRα expression. Furthermore, nuclear THRα was associated with a reduced survival in this subtype. However, nuclear expressed THRα1 turned out to be a positive prognosticator for all subtypes of OvCa patients. Nuclear THRα2 is a positive prognosticator for OvCa patients of the serous subtype. In contrast, cytoplasmic expression THRα2 was associated with a reduced OS in all subtypes of OvCa patients; while, cytoplasmic expression of THRα1 is associated with reduced OS in mucinous OvCa patients only. In addition, THRα expression correlates with gonadotropin receptors, steroid hormone receptors, TA-MUC1 and glycodelin.

Conclusion Depending on nuclear or cytoplasmic expression, our study shows that THRα and its isoforms 1 and 2 provide different prognostic information for ovarian cancer patients. Further investigations should analyze if THRs may represent new endocrine targets for the treatment of ovarian cancer.

Keywords Thyroid hormone receptor · Ovarian cancer · Overall survival · Nuclear versus cytoplasmic

Background

Thyroid-stimulating hormone (TSH) regulates thyroid function by binding to its receptor (thyroid hormone receptor—THR) expressed at the surface of thyroid cells. Recently, it has been demonstrated that THR is abundantly expressed in several tissues apart from the thyroid, among them the normal ovarian surface epithelium. The hormone dependency of the ovaries and the functional similarity of THRs and estrogen- (ER) and progesterone receptors (PR; both act in the nucleus as transcription factors) lead to the hypothesis that THRs may be a prognostic marker in ovarian cancer patients as demonstrated recently for breast cancer patients (Li et al. 2002; Rasmusson et al. 1987; Turken et al. 2003; Ditsch et al. 2013).

The nuclear receptors of thyroid hormones regulate the expression of specific cellular genes by interacting with distinct DNA sequences. They are ligand-activated transcription factors, which regulate the transcription of target genes. THRs are encoded by two genes—THR alpha and beta—located on human chromosomes 17 and 3 (Silva et al. 2002). They have three major isoforms: THRα1, THRα2 and THRβ1 (Ling et al. 2010) with high homology in amino acid composition. The most diversified region between THRα and THRβ is located in the N-terminal area, related to their

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trans-activation activity (Lazar 1993). Recent studies discovered by oligonucleotide microarray transcriptional profiling that THRα and THRβ mRNAs are among the most strongly expressed nuclear hormone receptor genes in cultured human ovarian surface epithelial (OSE) cells (Rae et al. 2004). The presence of THRα1, THRα2, and THRβ1 transcripts in cultured OSE cells is confirmed and the presence of THRα and THRβ proteins in the OSE cell layer has been demonstrated. Although, THRα and β isoforms are encoded by separate genes, differential promoter usage gives rise to two different THRα receptors, THRα1 and THRα2 (Zhang and Lazar 2000). Unlike THRα1 and THRβ1, which are conventional ligand-activated receptors, THRα2 is a ligand-independent negative regulator of active THRs. Thus, the presence of different THR isoforms, in conjunction with the potential for pre-receptor metabolism of thyroid hormones through expression of activating and inactivating deiodinase enzymes, strengthens the likelihood that the OSE is a physiologically important thyroid hormone target tissue (Rae et al. 2004).

Ovulation is a recurrent inflammatory reaction causing regular and frequent local injury to the ovarian surface during follicular rupture (Espey 1994; Rae and Hillier 2005). Ovarian cancer develops when a mutation or genetic change—spossibly caused by repeat episodes of inflammation-associated DNA damage (Murdoch 1998; Murdoch et al. 1999; Beachy et al. 2004)—occurs in the cells on the surface of the ovaries or in the fallopian tubes and leads to uncontrolled cell growth that may often metastasize (Rasool et al. 2014). Suppression of ovulation by e.g. pregnancy, breast feeding, or oral contraception reduces the risk of ovarian cancer, whereas diseases such as endometriosis, ovarian cysts, and hyperthyroidism are associated with increased risk (Ness and Cottreau 1999; Ness et al. 2000).

Ovarian cancer consists of four histopathological subtypes, represents the fourth most frequent type of cancer among females, and is the leading cause of death from gynecological cancer in the western world. Besides the histopathological subtype, grading, clinical staging and the amount of residual tumor, a number of several putative prognostic markers had been suggested for monitoring this disease (Ditsch et al. 2013). As ovarian cancer is also a thyroid hormone-dependent neoplasm (Shinderman-Maman et al. 2016), T3 has been shown to directly exert inflammatory effects on ovarian surface epithelial cell function in vitro and activate expression of genes associated with inflammation (Cohen et al. 2014; Rae et al. 2007). Studies also indicate that T3 increases the expression of ERα, which strongly associates with the development of epithelial ovarian cancer, which may explain the epidemiological linkage between hyperthyroidism and ovarian cancer (Rae et al. 2007).

The current study examines possible alterations of THR expression in ovarian carcinomas and its implication in ovarian cancer survival. Little is known about the context of thyroid function in ovarian carcinogenesis and the role of THR expression outside the thyroid is not completely understood. From our knowledge of therapy modalities, anti-hormonal therapy like tamoxifen, which unfold its effect via steroid hormone receptors, can be affective in ER-positive ovarian cancers. First in this field, our examinations focuses on the prognostic impact of thyroid hormone receptors of the alpha subtype (general alpha, alpha-1 and alpha-2, respectively) on pathological different ovarian cancer tissues.

Methods

Tissue samples

Tissue samples were obtained from 156 patients undergoing gynecological surgery for epithelial ovarian cancer (EOC) at the Department of Obstetrics and Gynaecology of the Ludwig-Maximilians-University Munich. The clinico-pathological parameters are shown in Table 1. Experienced gynaecologic pathologists performed histopathological staining and evaluation according to the criteria of the International Federation of Gynaecologists and Obstetricians (FIGO) and the World Health Organization (WHO). Full slides were used for immuohistochemical stainings. EOC specimens were available in different histological subtypes: serous ($n=110$) thereof 84 high-grade and 26 low-grade cases, clear cell ($n=12$), endometrioid ($n=21$), mucinous ($n=13$). Patients with ovarian low malignant potential tumors (e.g., Borderline tumors) were excluded from the study and no patients had neo-adjuvant chemotherapy. Patient's clinical data were available from patient charts, aftercare files and tumor registry database information. The main outcomes assessed were disease recurrence and patient survival. For

Table 1 Clinico-pathological parameters of the study group ($n=156$)

Histological subtype		FIGO stage		Nodal status		Age (years)	
High-grade serous	84 (54%)	I	35 (23%)	N0	56 (36%)	mean	62
Low-grade serous	26 (17%)	II	10 (6%)	N1	54 (35%)	min	33
Endometrioid	21 (13%)	III	107 (69%)	NX	46 (29%)	max	88
Mucinous	13 (8%)	IV	4 (2%)				
Clear cell	12 (8%)						

survival analysis, survival time was defined as the time between the date of primary ovarian cancer diagnosis and the date of death.

Immunohistochemistry

Our group has extensively described immunohistochemistry of THRα, THRα1 and THRα2 on FFPE sections (Ditsch et al. 2012a, 2013). In brief, rabbit polyclonal antibodies detecting THRα (Abcam, Cambridge, UK); Zytomed, Berlin, Germany), THRα1 (Zytomed) and THRα2 (Zytomed)) were stained by employing commercially available kits (Vectastain Elite rabbit-IgG-Kit (VectorLabs, Burlingame, CA); ZytoChem Plus HRP Polymer System (Zytomed). Reference sources for the used antibodies are listed in the Supplementary Table. Appropriate positive (struma, colon and placental tissue) and negative controls were included in each experiment (Supplementary Figure). Tissue sections treated with pre-immune IgGs (supersensitive rabbit negative control, BioGenex, Fremont, CA) instead of the primary antibody served as negative controls. Immunoreactivity was quantified by applying a well-established semi-quantitative scoring system (IR-score; also known as Remmele's score) by two independent observers (gynecologic pathologists (E.S. and D.M.)) by consensus. This scoring method has already been used in numerous studies (Ditsch et al. 2012b, c; Lenhard et al. 2011, 2012b) of our group. The IRS quantifies immunoreactivity 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). According to the previously published data, tissue samples that had been assigned an IRS higher than 1 were scored as positive (Lenhard et al. 2012a).

Statistical analysis methods

The IBM statistic package SPSS (version 25) was used to test data for statistical significance. Differences in THR expression among three or more groups were tested using the non-parametric Kruskal–Wallis rank-sum test and for pairwise comparisons using the nonparametric Mann–Whitney U rank-sum test. Correlation analysis was performed using the Spearman correlation coefficient. Overall survival (years) was compared by Kaplan–Meier graphics and differences in patient overall survival times were tested for significance using the chi-square statistics of the log rank test. For multivariate analyses, the cox regression model for overall survival was used. Data were assumed to be statistically different in case of $p < 0.05$.

Results

THRα expression according to EOC subtypes

THRα expression showed significant differences within the histological subtype, accounting nuclear as well as cytoplasmic staining. Serous carcinomas showed only faint expression of THRα in the nucleus (median IRS = 2) as well as in the cytoplasm (median IRS = 0; Fig. 1a = 10 × lens, Fig. 1f = 25 × lens). A more intense staining was observed in the clear cell cases in the nucleus (median IRS = 2) as well

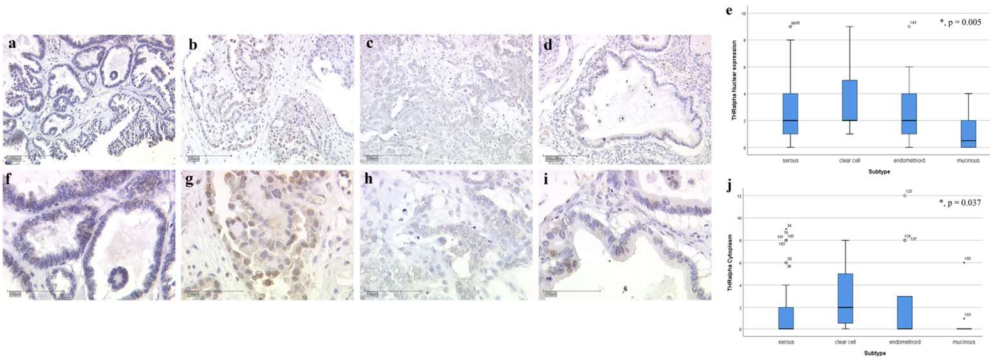

Fig. 1 **a** THRα expression in serous carcinoma (10 × lens). **b** THRα expression in clear cell carcinoma (10 × lens). **c** THRα expression in endometrioid carcinoma (10 × lens). **d** THRα expression in mucinous carcinoma (10 × lens). **e** Summary of THRα expression in different carcinoma subtypes (nuclear expression). **f** THRα expression in serous carcinoma (25 × lens). **g** THRα expression in clear cell carcinoma (25 × lens). **h** THRα expression in endometrioid carcinoma (25 × lens). **i** THRα expression in mucinous carcinoma (25 × lens). **j** Summary of THRα expression in different carcinoma subtypes (cytoplasmic expression)

as in the cytoplasm (median IRS = 2; Fig. 1b = 10 × lens, Fig. 1g = 25 × lens). The endometrioid subtype showed similar expression schemas as the serous subtype in the nucleus (median IRS = 2) as well as in the cytoplasm (median IRS = 0; Fig. 1c = 10 × lens, Fig. 1h = 25 × lens). The lowest expression of THRα was found in the mucinous subtype in the nucleus (median IRS = 1) as well as in the cytoplasm (median IRS = 0; Fig. 1d = 10 × lens, Fig. 1i = 25 × lens). A summary of the staining results is shown in Fig. 1e for the nuclear staining ($p = 0.005$) and Fig. 1j for the cytoplasmic staining ($p = 0.037$).

THRα1 as well as THRα2 showed no significant different expression according to the histological subtype. The median expression of THRα1 in the nucleus was 2 and the median expression in the cytoplasm was 0. The median expression of THRα2 in the nucleus was 6 and, therefore, much more intense compared to THRα and − α1, respectively. The median expression of THRα2 in the cytoplasm was 0. There was no significant different expression of the three THRα subtypes according to grading, FIGO staging or age at surgery.

Correlation analyses

Using recently published data by our institute, we were able to correlate the expression of all THRα subtypes stained with former investigation results. There are significant correlations with the gonadotropin receptors (Lenhard et al. 2011) and the luteinizing hormone (LH)-receptor ligand hCG (Lenhard et al. 2012b); specifically, THRα staining in the nucleus showed a positive correlation to the follicle stimulating hormone receptor (FSHR) (correlation coefficient (cc) = 0.181; $p = 0.027$) and a negative correlation to hCG (cc = − 0.247, $p = 0.003$). In opposite, THRα in the cytoplasm showed a positive correlation with the luteinizing hormone/choriogonadotropin receptor (LH/hCGR) (cc = 0.199, $p = 0.014$) and a positive correlation to hCG (cc = 0.187, $p = 0.027$). The THRα1 expression in the cytoplasm is positively correlated with hCG (cc = 0.278, $p = 0.001$). THRα2 in the nucleus showed a positive correlation to FSHR (cc = 0.185, $p = 0.024$). In addition, there are also positive correlations with the classical steroid hormone receptors, which were analyzed by our research group too (Lenhard et al. 2012a). THRα staining in the nucleus showed a positive correlation with the ERβ (cc = 0.213, $p = 0.009$) and with the PRα (cc = 0.172, $p = 0.035$). The THRα1 expression in the cytoplasm is positively correlated with ERβ (cc = 0.219, $p = 0.006$). THRα2 in the nucleus showed positive correlation with ERα (cc = 0.247, $p = 0.002$) and with PRα (cc = 0.219, $p = 0.007$). In addition to the classical estrogen receptors, also the GPER (Heublein et al. 2014; Heublein et al. 2013a, b) showed positive correlation with THRα staining in the nucleus (cc = 0.219, $p = 0.007$) and with THRα2 in the nucleus (cc = 0.252, $p = 0.002$).

Another positive correlation was found within the tumor-associated mucin 1 epitop (TA-MUC1) detected with the Gatipotuzumab antibody formerly known as PankoMab (Dian et al. 2013; Jeschke et al. 2012) and THRα staining in the nucleus (cc = 0.279, $p = 0.001$). In contrast, THRα1 expression in the cytoplasm is negatively correlated with TA-MUC1 (cc = -0.195, $p = 0.019$). TA-MUC1 as membrane bound protein can also be translocated to the cytoplasm of tumor cells (Heublein et al. 2015). In that case, it is negatively correlated with the expression of THRα1 in the nucleus (cc = − 0.166, $p = 0.048$) and THRα2 in the nucleus (cc = − 0.268, $p = 0.001$). An immunosuppressive glycoprotein that is connected to TA-MUC1 is glycodelin and its specific glycoform glycodelin A (Lenhard et al. 2013; Scholz et al. 2012). Glycodelin A showed a positive correlation with THRα2 in the cytoplasm (cc = 0.170, $p = 0.037$). Glycodelin showed positive correlation with THRα in the nucleus (cc = 0.241, $p = 0.003$) as well as in the cytoplasm (cc = 0.231, $p = 0.004$). THRα2 expression in the nucleus is positively correlated with glycodelin (cc = 0.265, $p = 0.001$).

Survival analyses

The expression of the general THRα is connected to significantly reduced overall survival in the subgroup of clear cell carcinomas. The median survival for THRα-negative patients is 5.24 years in contrast to only 0.29 years for patients showing THRα expression in the nucleus (Fig. 2a, $p = 0.006$).

The THRα isoforms − α1 and − α2 are in general positive prognosticators if expressed in the nucleus and negative prognosticator if expressed in the cytoplasm, respectively. In detail, THRα1 is a general positive prognosticator if expressed in the nucleus with a median survival of 4.22 years for patients positive for THRα1 and 2.08 years for patients that do not express THRα1 in the nucleus (Fig. 2b, $p = 0.024$). Subgroup analyses of mucinous carcinomas showed that THRα1 is a negative prognosticator if expressed in the cytoplasm. The median survival time is 16.59 years for mucinous carcinoma patients that do not express THRα1 in the cytoplasm and 2.87 years for mucinous carcinoma patients with cytoplasmic THRα1 expression (Fig. 2c, $p = 0.037$).

The THRα2 receptor in general is a negative prognosticator if expressed in the cytoplasm. The median survival time is 3.75 years for patients and 1.37 years for patients with THRα2 in the cytoplasm (Fig. 2d, $p = 0.001$). Nuclear expression of THRα2 is not a general positive prognosticator. This can be found in the subgroup of serous high-grade carcinomas. The mean survival time for high-grade serous carcinoma patients with nuclear THRα2 expression

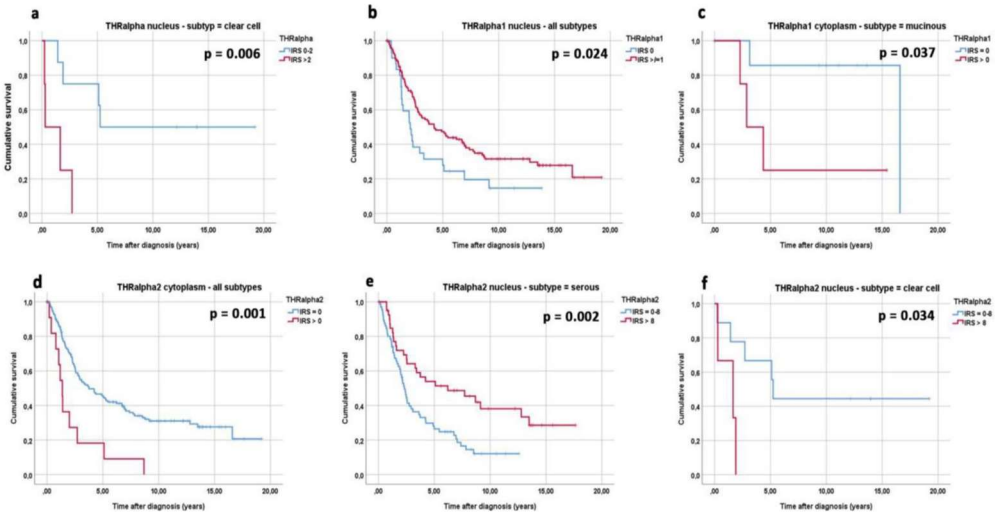

Fig. 2 Kaplan–Meier estimates of THRα expression, THRα1 expression and THRα2 expression were analyzed. In the clear cell subtype, patients with a high nuclear expression of THRα showed a significantly reduced overall survival compared with patients with a low nuclear expression (**a**). In addition, high nuclear THRα1 expression was associated with significantly better overall survival in all ovarian cancer subtypes compared to patients with a low nuclear THRα1 expression (**b**). Patients with high THRα1 expression in the cytoplasm and mucinous subtype had a significantly decreased overall survival compared with those mucinous carcinoma patients with low cytoplasmic expression (**c**). High cytoplasmic THRα2 expression was associated with a significantly reduced overall survival in all ovarian cancer subtypes compared to patients with a low cytoplasmic THRα2 expression (**d**). In the serous subtype, patients with a high nuclear expression of THRα2 showed a significantly better overall survival compared with patients with a low nuclear expression (**e**). Finally, in the clear cell subtype, patients with a high nuclear expression of THRα showed a significantly reduced very low overall survival (all patients deceased within two years) compared to patients with a low nuclear expression (**f**)

is 6.21 years in contrast to 2.32 years for patients with no nuclear THRα2 expression (Fig. 2e, $p=0.002$). It is remarkable that patients with clear cell carcinomas show opposite results. The median survival time for clear cell carcinoma patients with nuclear THRα2 expression is only 1.65 years in contrast to 5.24 years for patients with no nuclear THRα2 expression (Fig. 2f, $p=0.034$).

The results of the survival analyses in correlation with the histological subtype and staining localization of THRα, THRα1 and THRα2 are summarized in Table 2.

Table 2 Results of the survival analyses in correlation to the histological subtype and staining localization of THRα, THRα1 and THRα2

Histological subtype	THRα IRS > 0	THRα1 IRS > 0		THRα2 IRS > 0	
	Nucleus	Nucleus	Cytoplasm	Nucleus	Cytoplasm
Total ($n=156$)	n.s	pos. pro. $p=0.024$	n.s	n.s	neg. pro $p=0.001$
High-grade serous ($n=84$)	n.s	n.s	n.s	pos. pro $p=0.002$	n.s
Low-grade serous ($n=26$)	n.s	n.s	n.s	n.s	n.s
Endometrioid ($n=21$)	n.s	n.s	n.s	n.s	n.s
Mucinous ($n=13$)	n.s	n.s	neg. pro $p=0.037$	n.s	n.s
Clear cell ($n=12$)	neg. pro $p=0.006$	n.s	n.s	neg. pro $p=0.034$	n.s

n.s. not significant; *pos. pro.* positive prognosticator; *neg. pro.* negative prognosticator

Comparison of THRα, – α1 and – 2 expression in low-grade and high-grade serous ovarian cancer

As shown in Fig. 3, the expression of all three α-subunits is higher in the nucleus of low-grade serous ovarian cancer cases with a trend to significance in the general THRα ($p = 0.078$), no significance for THRα1 and a significantly higher THRα2 expression in low-grade serous cancer cases compared to high-grade subtype (the receiver operating characteristic curve (ROC) analyses were performed to calculate the optimal cut-off values between low and high expression of the different THRs).

Cox regression analyses of survival

Cox regression was performed to identify independent predictors for OS. Pattern of age at surgery failed to remain significant within multivariate testing; while grading, FIGO staging, THRα1 in the nucleus (Table 3A, $p = 0.043$) and THRα2 in the cytoplasm (Table 3B, $p = 0.002$) were still predictive in multivariate testing sets regarding all subtypes of the study group. Due to missing clinical data in single cases, cox regression analyses were available in 146 out of 156 cases.

Discussion

Within this study, we analysed the prognostic value of the thyroid hormone receptor alpha forms 1 and 2. The general THRα has prognostic value only in clear cell carcinomas, where it is expressed at the highest immune scores. The differential analyses of nuclear versus cytoplasmic expression of THRα1 and THRα2 revealed striking differences concerning the overall survival of ovarian cancer patients. The thyroid hormone receptor alpha (THRα) exhibits a dual role as an activator or repressor of gene transcription. Former studies showed that THRα, formerly thought to reside solely in the nucleus and tightly bound to the DNA, shuttles rapidly between the nucleus and the cytoplasm (Bunn et al. 2001; Maruvada et al. 2003).

The role of thyroid hormones and its receptors was not very well understood in ovarian cancer biology for a longer time, only very recent publication showed their tremendous roles for this deadly disease.

Early investigations with ovarian cancer cell lines and T3, T4 and reversed T3 stimulation did not result in sufficient stimulation or inhibition outcomes (Martinez et al. 2000). Later, it was found that messenger RNA transcripts for THRα1, THRα2, T3 activating deiodinase 2 and inactivating deiodinase 3 are present in primary ovarian surface epithelial cell cultures (Rae et al. 2007). A more recent study described that for ovarian cancer patients, conflicting results were observed for T3 and T4 levels in the serum. Insignificant differences were found for T3 ($p = 0.209$) and T4 ($p = 0.050$) as compared to controls (Rasool et al. 2014).

An actual study described that αvβ3 integrin, a plasma membrane receptor that binds the thyroid hormones T3 and T4, is overexpressed in ovarian cancer (Shinderman-Maman et al. 2016). Both hormones induced cell proliferation and significantly reduced the expression of genes that inhibit cell cycle particularly in ovarian cancer cells (OVCAR-3) with high integrin expression (Shinderman-Maman et al. 2016). The same group studied the expression of fifteen genes involved in DNA repair, cell cycle, apoptosis, and tumor suppression in OVCAR-3 and A2780 cell lines, using real-time PCR following short incubation with T3 or T4 (Shinderman-Maman et al. 2018). The thyroid hormones downregulated the expression of the majority of genes examined, showing that these hormones influence the expression of cancer-relevant genes in ovarian cancer (Shinderman-Maman et al. 2018). The same group hypothesized that natural thyroid hormone derivatives may antagonize these actions. The three antagonists, tetraiodoacetic acid (tetrac), triiodothyroacetic

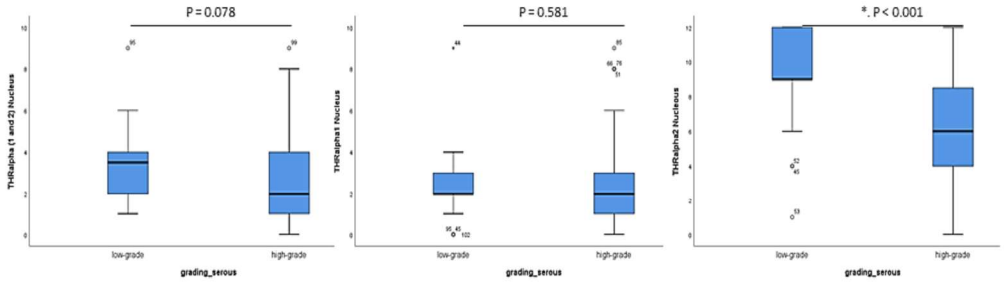

Fig. 3 Comparison of immunohistochemical staining results of the different THR (median values) in the nucleus of the high- and low-grade serous ovarian cancer subtypes. (*IRS* Immunoreactive Score, *THR*: Thyroid Receptor). The expression of THRα2 in the nucleus is significantly different in low-grade compared to high-grade serous carcinomas (marked by an asterisk)

Table 3 Multivariate survival analyses with the overall survival time for (A) THRα1 expression in the nucleus, (B) THRα2 expression in the cytoplasm, regarding patients age, histological subtype, grading, and staging ($n = 146$)

Variables	p value	Hazard ratio	95.0% Confidence Interval	
			Lower	Upper
(A) THRα1 (nucleus)				
IRS > 0 versus IRS 0	0.049	0.618	0.383	0.997
FIGO				
I/II versus III/IV	0.001	2.761	1.510	
Grading				
G1/low grade versus G2/3/high grade	0.002	2.753	1.457	5.199
Histological subtype				
all subtypes versus high-grade serous	0.964	0.994	0.783	1.263
Age				
< 60 versus ≥ 60 years	0.116	0.717	0.473	1.085
(B) THRα2 (cytoplasm)				
IRS > 0 versus IRS 0	0.002	2.790	1.466	5.310
Age				
< 60 versus ≥ 60 years	0.212	0.769	0.509	1.161
Histological subtype				
all other subtypes versus high-grade serous	0.673	0.950	0.747	1.207
Grading				
G1/low-grade versus G2/3/high-grade	0.001	0.325	0.171	0.618
FIGO				
I/II versus III/IV	0.001	0.365	0.201	0.662

acid (triac) and 3-iodothyronamine (T1AM) inhibited cell proliferation and induced cell death and DNA damage in the two ovarian cancer cell lines (OVCAR3 and A2780). Therefore, they concluded that the cytotoxic potential of thyroid hormone derivatives, tetrac, triac and T1AM, in ovarian cancer might provide a much-needed novel therapeutic approach (Shinderman-Maman et al. 2017).

Based on the results of the former study, another group described that thyroid hormone causes elevated phosphorylation and nuclear enrichment of ERα (Hsieh et al. 2017). In addition, confocal microscopy indicated that both T4 and estradiol caused nuclear translocation of integrin αv and phosphorylation of ERα (Hsieh et al. 2017). Within our study, we found a positive correlation between the THRα2 in the nucleus and ERα. We also found positive correlation of THRα in the nucleus and ERβ, assuming that thyroid hormones not only elevate the nuclear enrichment of ERα but also might influence ERβ. However, our correlations referred to the whole study cohort and did not focus on the histological subtypes. Another study showed that THRα1 inhibits the ERα transactivation from the consensus estrogen response element (ERE). In contrast, the ligand bound THRβ1 facilitates ERβ-mediated transactivation (Vasudevan et al. 2001). We also found a positive correlation between the GPER and THRα. Sheng et al. showed that the GPER together with integrin αvβ3 participate in the induction of male germ cell proliferation and thyroid transcription disruption after low-dose Bisphenol A treatment (Sheng et al. 2019). Another correlation of our study was found between THRα in the nucleus and the FSH receptor; whereas, the THRα expression in the cytoplasm showed a positive correlation to the LH/hCG receptor. It has been known for a longer time that LH, FSH, and TSH show low-level cross-reactivity between their respective receptors (Tonacchera et al. 2006). Vissenberg et al. explained that T3 in combination with FSH enhances granulosa cell proliferation and inhibits granulosa cell apoptosis by the PI3K/Akt pathway (Vissenberg et al. 2015). They also described that T3 is considered a biological amplifier of the stimulatory action of gonadotrophins on granulosa cell function (Vissenberg et al. 2015). Because the exclusive expression of the FSHR has already been described by our group as a negative prognosticator in ovarian cancer cases, our finding about enhanced expression of both FSHR and THRα in the nucleus might lead to new treatment strategies for this type of cancer (Lenhard et al. 2011). This assumption might also apply for the antibody Gatipotuzumab and its TA-MUC1 epitope (Heublein et al. 2019), which showed an inverse correlation to THRα1 and -2 expression either in the nucleus or in the cytoplasm, respectively.

In addition, T4 has been shown to promote ovarian cancer cell proliferation via integrin αvβ3. T4 also induced the activation of ERK1/2 and expression of programmed death-ligand 1 (PD-L1) in ovarian cancer cells (Chin et al. 2018). In contrast, resveratrol binds to integrin αvβ3 at a discrete site and induces p53-dependent anti-proliferation in malignant neoplastic cells. T4 impairs resveratrol-induced anti-proliferation in human ovarian cancer cells and T4 inhibited resveratrol-induced nuclear accumulation of COX-2 (Chin et al. 2018). Furthermore, T4 increased expression and cytoplasmic accumulation of PD-L1, which in turn acted to retain inducible COX-2 in the cytoplasm (Chin et al. 2018). Thus, T4 inhibits COX-2-dependent apoptosis in ovarian cancer cells by retaining inducible COX-2 with PD-L1 in the cytoplasm (Chin et al. 2018).

Recently, the interplay between epithelial–mesenchymal transition (EMT) and the thyroid hormones-αvβ3 axis in ovarian cancer was investigated (Weingarten et al. 2018). It was shown that the transcription of mesenchymal markers, β-catenin, zeb-1, slug/snail, vimentin, and n-cadherin was hardly affected by T3 and T4, while that of the epithelial markers, e-cadherin and zo-1, and was inhibited after treatment with thyroid hormones. These results suggest a novel role for the thyroid hormone-αvβ3 axis in EMT, with possible implications for ovarian cancer metastasis (Weingarten et al. 2018).

Finally, a group investigated the role of the thyroid hormone receptor Interactor 13 (TRIP13) in epithelial ovarian cancer (EOC) (Zhou and Shu, 2019). Bioinformatics analysis showed that TRIP13 was one of the most significantly upregulated proteins in EOC. Results of the described study showed that TRIP13 acted as an oncopromotive regulator in EOC development by modulating the Notch signaling pathway (Zhou and Shu, 2019).

A large demographic study, the "Ovarian Cancer Association Consortium", showed that hyperthyroidism within the 5 years before ovarian cancer diagnosis was associated with an increased risk of death (Minlikeeva et al. 2017). These very recent results were accompanied by the fact that a more modest association was observed with the history of hypothyroidism ($n = 624$ cases) and mortality (Minlikeeva et al. 2017).

In sum, the results of the experimental and demographic studies about the roles of thyroid hormones, its receptors and interacting proteins. There is growing body of evidence that they play a major role in ovarian cancer biology and survival of ovarian cancer patients. Only recent studies were able to bring new light into this area of research.

Conclusions

With our study, we could show that there is a direct link between nuclear expression of THRα1 or − 2 and better survival in EOC, except for the subgroup of clear cell carcinomas. The latter group seems to have different properties concerning THRα expression. Shifting the expression of THRα1 or − 2 to the cytoplasm seems to be connected with reduced overall survival in EOC cases. Therefore, the search for THRα interacting factors that prevent this shift to the cytoplasm seems to be a useful new approach for the search of future treatment strategies against the threatening disease of Epithelial Ovarian Cancer.

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Author contributions ND, SH, UJ, DM conceived and designed the experiments. CS, CK, AH, ES performed the experiments. BS, FT, SM, JE analyzed the data. ND, UJ wrote the paper. All authors have read and approved the manuscript.

Compliance with ethical standards

Conflict of interest Sabine Heublein reports grants from FERRING, personal fees from Roche, other from Astra Zeneca, grants from Novartis Oncology, grants and non-financial support from Apceth GmbH, non-financial support from Addex. Fabian Trillsch declares Research support, advisory board, honoraria and travel expenses from AstraZeneca, Clovis, Medac, PharmaMar, Roche, Tesaro. Sven Mahner reports grants and personal fees from AstraZeneca, personal fees from Clovis, grants and personal fees from Medac, grants and personal fees from MSD. He also reports personal fees from Novartis, grants and personal fees from PharmaMar, grants and personal fees from Roche, personal fees from Sensor Kinesis, grants and personal fees from Tesaro, grants and personal fees from Teva, outside the submitted work. The remaining authors declare no conflict of interest.

Ethics approval and consent to participate The Ethics Committee of the Ludwig-Maximilians-University, Munich, Germany (approval number 227–09) on 30 September 2009, approved the study. All tissue samples used for this study were obtained from leftover material from the archives of LMU Munich, Department Gynaecology and Obstetrics, Ludwig-Maximilians-University, Munich, Germany, initially used for pathological diagnostics. When this retrospective study was initiated, diagnostic procedures had already been fully completed; the tissue samples were classified as leftover material and underwent irreversible anonymization. Under these circumstances, no individual written informed consent was needed as per declaration of the Ethics Committee of the Ludwig-Maximilians-University. All experiments were performed according to the standards of the Declaration of Helsinki (1975).

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Extracapsular Lymph Node Involvement in Ovarian Carcinoma

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Abstract: Ovarian cancer (OC) spread to retro-peritoneal lymph nodes is detected in about one out of two patients at primary diagnosis. Whether the histologic pattern of lymph node involvement i.e., intra-(ICG) or extracapsular (ECG) cancer growth may affect patients' prognosis remains unknown. The aim of the current study was to analyze the prevalence of ECG and ICG in lymph node positive ovarian cancer. We further investigated whether ECG may be related to patients' prognosis and whether biomarkers expressed in the primary tumor may predict the pattern of lymph node involvement. Lymph node samples stemming from 143 OC patients were examined for presence of ECG. Capsular extravasation was tested for statistical association with clinico-pathological variables. We further tested 27 biomarkers that had been determined in primary tumor tissue for their potential to predict ECG in metastatic lymph nodes. ECG was detected in 35 (24.5%) of 143 lymph node positive patients. High grade ($p = 0.043$), histologic subtype ($p = 0.006$) and high lymph node ratio (LNR) ($p < 0.001$) were positively correlated with presence of ECG. Both ECG ($p = 0.024$) and high LNR ($p = 0.008$) were predictive for shortened overall survival. A four-protein signature determined from the primary tumor tissue was associated with presence of concomitant extracapsular spread in lymph nodes of the respective patient. This work found extracapsular spread of lymph node metastasis to be a common feature of lymph node positive ovarian cancer. Since ECG was positively associated with grade, LNR and shortened overall survival, we hypothesize that the presence of ECG may be interpreted as an indicator of tumor aggressiveness.

Keywords: ovarian cancer; lymph node metastasis; extra-capsular growth; prognosis

1. Introduction

Metastatic spread to lymph nodes is common in solid tumors and is counted as one of the most potent prognostic determinants. Apart from lymph node involvement per se, the histological growth pattern of neoplastic cells within the lymph node may add further prognostic information [1,2]. Mainly, the phenomenon of extracapsular growth (ECG), i.e., extension of cancer cell invasion beyond the connective tissue capsule of the lymph node, has been linked to tumor aggressiveness in many cancer entities [1–6]. Meanwhile, ECG has become an established prognostic factor for gastrointestinal, breast,

and cervical cancer [1,4,5]. It has reproducibly been linked to advanced cancer stage, earlier disease relapse, treatment resistance, and shortened patient survival [1,2,6]. Regarding squamous cell cancer of the vulva and cancers of the head/neck, ECG has even been incorporated into the official cancer staging systems, respectively [7].

Though an extensive literature search was performed, we did not find published data on ECG in ovarian cancer (OC). So far, the prevalence of ECG in OC remains elusive. Moreover, it is still unknown whether the presence of ECG might be linked to clinicopathological parameters or prognosis in OC patients.

Therefore, the aim of the current study was to investigate the pattern of lymph node involvement (ECG vs. ICG) in lymph node positive ovarian cancer. We further analyzed whether ECG may be related to FIGO (The International Federation of Gynecology and Obstetrics) stage, histological subtype, tumor grade, residual disease or patients' prognosis in OC. Finally, we investigated whether a biomarker signature determined from the primary tumor might be associated with ECG in associated lymph nodes.

2. Results

2.1. Study Cohort

Most patients presented with an extended primary tumor, rated as pT3 (n = 125; 87.4%), fewer patients with pT2 (n = 12; 8.4%) and pT1 (n = 6; 4.2%). According to FIGO 8ed, 127 patients were staged as FIGO III (IIIA: n = 18 (12.6%), IIIB: n = 17 (11.9%), IIIC: n = 92 (64.3%)) while the remaining 16 (11.2%) patients were assigned stage IV disease. Histological characterization revealed serous histologic differentiation in 129 cases (90.2%). Other subtypes were distributed as follows: endometroid (n = 5; 3.5%), clear cell (n = 4; 2.8%), mucinous (n = 1; 0.7%), and undifferentiated (n = 4; 2.8%). A percentage of 85.8% (109 out of 129) of serous cases were graded as high grade. Grading of remaining subtypes was G1 in one, G2 in three and G3 in ten patients.

Data on residual disease after primary debulking surgery were available from 124 out of 143 cases. Complete cytoreduction (i.e., no residual disease) was achieved in 83 (66.9%) out of 124 cases. Numbers of resected lymph nodes ranged from 1 to 94, numbers of positive lymph nodes from 1 to 67. Median count of resected lymph nodes was 37.0 and median count of positive nodes was 5.00 nodes per patient, respectively. Median LNR was 0.17 with a minimum of 0.01 and a maximum of 1.00. Patient's age at primary diagnosis ranged between 33.4 and 87.6 years with a median age of 59.5 years. Median overall survival of the cohort was 45.8 months (95% CI: 32.4–59.3) and median follow up time was 87.6 months (95% CI: 48.5–125.7). There were 80 observed deaths.

Out of 143 patients analyzed we observed ECG (Figure 1) in 35 (24.5%) and ICG in 108 cases (75.5%). While there was no significant correlation to patient age, tumor size, residual disease, and FIGO stage, ECG was detected more often in cases diagnosed with a high grade than with low grade ovarian primary (p = 0.043) (Table 1). Presence of ECG was not related to the number of lymph nodes resected. However, the ratio of positive lymph nodes (LNR) significantly differed depending on the presence of ECG ($p < 0.001$). The proportion of ECG positive cases in patients with a lymph node ratio higher than 0.3 was 22/33 (66.7%) while the presence of ECG in those with lower lymph node ratios was observed in 22 out of 79 patients (27.8%). ECG was more common in those patients whose primary tumor was of non-serous histology (p = 0.006). Finally, patient age was not different between ECG and ICG cases.

To rule out potential confounding by different subtypes, analyses were repeated in the serous subtype only. Again, ECG was not correlated with patient age, tumor size, number of total nodes or FIGO stage. While correlation to grading was lost, a lymph node ratio higher than 0.3 was still closely associated with presence of ECG ($p < 0.001$).

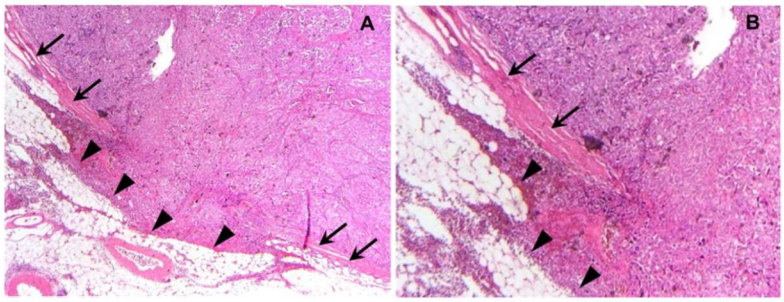

Figure 1. Extracapsular growth of ovarian cancer in a retroperitoneal lymph node: A representative photomicrograph (10× lens (**A**), 20× lens (**B**), HE staining) of extracapsular growth in ovarian cancer is presented. Arrows indicate the intact capsule lining the lymph node that has been invaded by cancer cells. The region of invasive neoplastic cells breaking through the capsule and invading fatty tissue adjacent to the lymph node is indicated by arrowheads (**A**,**B**).

Table 1. Patients' characteristics: Patients' characteristics in cases diagnosed for intra-(ICG) and extracapsular (ECG) are displayed. Grading was binarized for statistical analysis. Low/high grade refers to the serous subtype only while G1/2/G3 represents grading of non-serous cases. *p*-values derive from Fisher's exact test (in the case of categorical variables) and Student's *t*-Test (in the case of continuous variables) are shown. ns: not significant.

Characteristic		ICG	ECG	*p*
		***n* or Median (Range)**	***n* or Median (Range)**	
pT	pT1/2 pT3	12 96	6 29	ns
FIGO	III IV	95 13	32 3	ns
Grade	low or G1 high or G2/3	18 88	1 34	0.043
Histology	non-serous serous	6 102	8 27	0.006
Age	(years)	59.62 (33.4–81.6)	59.46 (36.9–87.6)	ns
Residual disease	none any	66 27	17 14	ns
Total nodes resected		38 (1–94)	37 (6–74)	ns
Fraction positive nodes/total nodes resected	≤0.3 >0.3	79 22	11 22	<0.001

2.2. Histological Pattern of Lymph Node Involvement Is Associated with the Biomarker Profile of the Primary Tumor

Immunostaining data for 27 protein markers (Glycodelin and its glyco-modification Glycodelin A, steroid hormone receptors (ERα, ERβ, PRA, PRB, and GPER), gonadotropins-/receptors (hCG, LHCGR, and FSHR), Galectins (Gal-1, Gal-3, Gal-7, Gal-8, and Gal-9), p53, Mucin-1 (as detected by anti-peptide antibodies: VU3C6, VU4H5, HMFG1) and glyco-modifications of Mucin-1 (TA-MUC1 as detected by Gatipotuzumab, TF (CD176)) were available in 32 ovarian cancer cases. Galectins were determined in cancer cell nuclei, cancer cell cytoplasm and/or tumor stroma.

Nine out of 32 cases were diagnosed with ECG, while ICG was detected in the remaining 23 cases. Neither Glycodelin nor its immunosupressive glyco-modification Glycodelin A were correlated with the presence of ECG. Out of the steroid hormone receptor set only the G-Protein coupled estrogen

receptor (GPER) was associated with the mode of lymph node involvement ($p = 0.038$, Figures 2 and 3A). Median GPER immunopositivity was significantly lower in those primary tumors that presented extracapsular spread in cancer affected lymph nodes (IRS (ICG) = 9.0 vs. IRS (ECG) = 4.0). Though GPER shares some structural similarity with FSHR and LHCGR, neither those two nor HCG, which acts as a ligand on LHCGR, appeared to correlate to the pattern of lymph node involvement. Since there is a strong link between Mucin-1 and metastasis formation, we screened several antibodies detecting either the protein backbone or glyco-modifications of Mucin-1. Only the anti Muc-1 antibody VU4H5 seemed to correlate ($p = 0.028$, Figures 2 and 3B) with ECG in a way that cases that were diagnosed with ECG appeared to overexpress Mucin-1 (IRS (ICG) = 4 vs. IRS (ECG) = 8). Loss of nuclear Galectin-3 (Gal-3) was also predictive ($p = 0.021$) for ECG (Figures 2 and 3C). A similar correlation was found in the case of nuclear Gal-8, though statistically this association was of borderline significance ($p = 0.053$) only (Figures 2 and 3D). Representative photomicrographs of GPER, MUC-1 (VU4H5), Gal-3^{nuc}, and Gal-8^{nuc} as detected using immunohistochemistry in primary tumor tissue are shown in Figure 4. A logistic regression was run to ascertain the effects of GPER, MUC-1 (VU4H5), Gal-3^{nuc}, and Gal-8^{nuc} on the likelihood of extracapsular spread. The logistic regression model was statistically significant, $\chi^2 = 13.29$, $p = 0.01$. The model correctly classified 79.3% of cases. To evaluate the fit of the logistic regression model a receiver operating characteristic (ROC) curve was calculated. The combined signature of GPER, MUC-1 (VU4H5), Gal-3^{nuc} and Gal-8^{nuc} proved to be a characteristic of primary tumors presenting with ECG in associated nodes (AUC = 0.892, $p = 0.001$; Figure 3E). The Youden index of our biomarker combination was 0.75. At this cut point sensitivity was 100% and specificity was 75%.

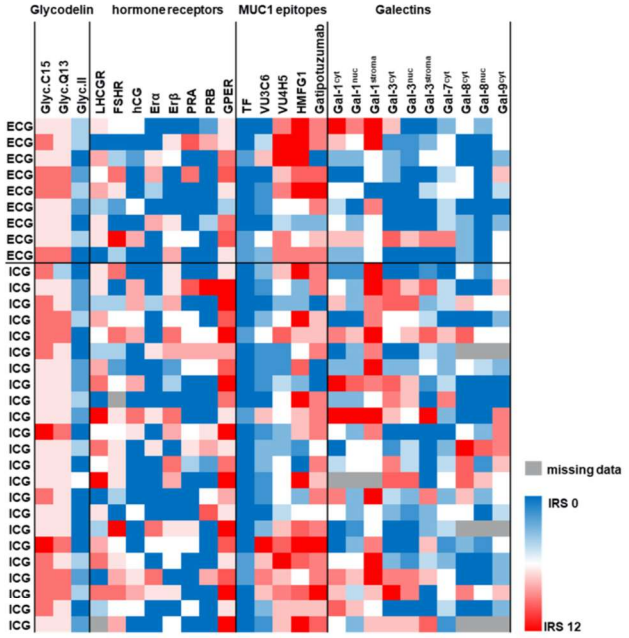

Figure 2. Protein profile of the primary tumor is associated with ECG in associated lymph nodes: Immunostaining data of 27 protein/carbohydrate biomarkers were available from 32 primary tumor tissue cases. IHC data of 26 biomarkers are illustrated as heat map. The protein profile (red—high expression, blue—low expression) of the primary tumor was tested for association with ICG/ECG. IHC was also used to approximate p53 mutational status. Data on p53 in relation to ICG/ECG are presented in the text.

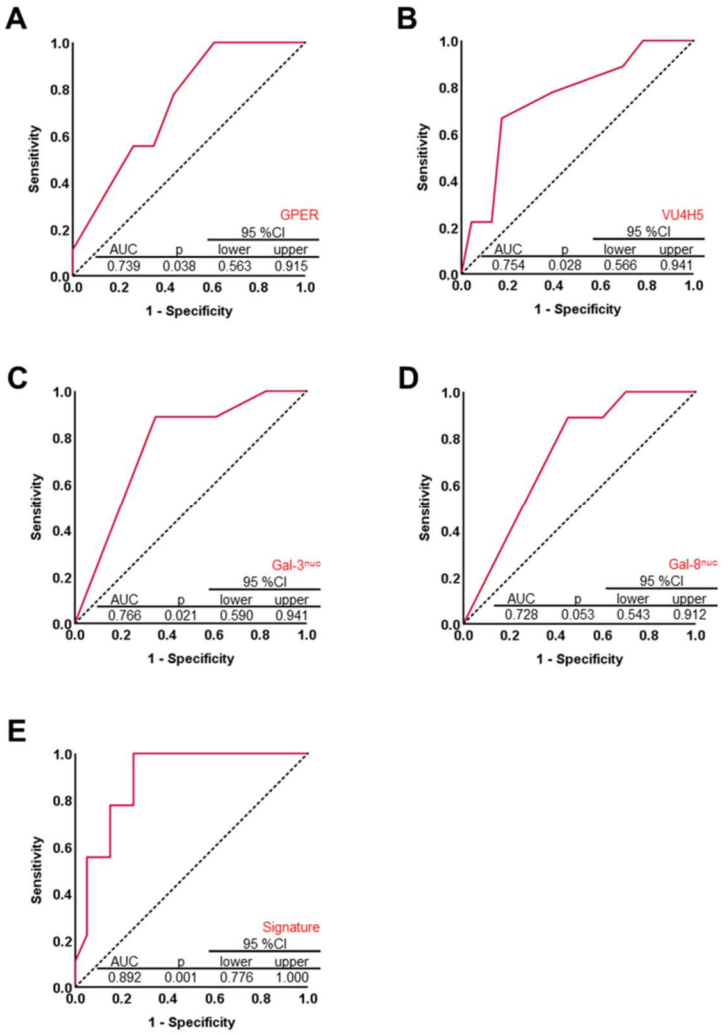

Figure 3. A four-marker signature built of G-Protein coupled estrogen receptor (GPER), MUC-1 and Gal-3/8 is associated with ECG in associated lymph nodes: Four out of 26 markers * turned out to be predictive for presence of ECG in associated retroperitoneal lymph node metastasis (**A**–**D**) as determined by receiver operating characteristic (ROC) analysis using SPSS v22 (**A**–**E**). Diagonal segments are produced by ties. Likelihood of the state event (ECG) was associated with decrease in test variable in case of GPER, Gal-3^{nuc}, and Gal-8^{nuc} (**A**,**C**,**D**). Regarding VU4H5, likelihood of the state event (ECG) was associated with increase in test variable (VU4H5, **B**). Primary tumors expressing GPER, Gal-3^{nuc}, and Gal-8^{nuc} at low and MUC-1 (as detected by VU4H5) at high levels were more likely to present extracapsular metastatic spread in respective, case-matched lymph node specimens (**A**–**D**). A signature combining GPER, VU4H5, Gal-3, and Gal-8 was calculated and tested for its diagnostic strength to discriminate ECG from ICG cases (**E**). * Data on p53 mutational status (i.e., 27th biomarker) in relation to ICG/ECG are presented in the text. There was no difference regarding the prevalence of ECG and ICG with respect to p53 mutation of the primary tumor.

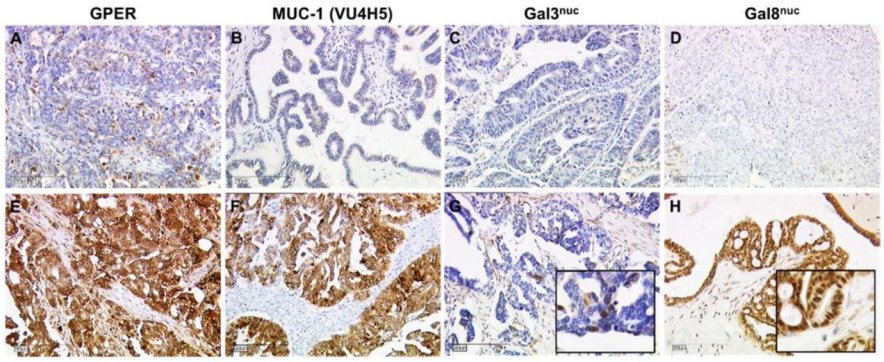

Figure 4. Immunohistochemistry of GPER, MUC-1, and Gal-3n^{uc}/8^{nuc} in primary tumor tissue: GPER (**A**,**E**), MUC-1 (**B**,**F**), Gal-3^{nuc} (**C**,**G**), and Gal-8^{nuc} (**D**,**H**) were determined by IHC in primary OC tumor tissue. Representative images of their staining patterns scored as negative (**A**–**D**) and positive (**E**–**H**) are shown. Scale bars represent 200 µm. Inserts in G and H are magnified twice from the original image thus to illustrate nuclear staining of Gal-3 and Gal-8.

About three-quarters of patients (78.1%, 25/32) were diagnosed with a p53 mutation in their primary tumor—as estimated using the established immunohistochemistry scoring system [8,9]. However, there was no difference regarding the prevalence of ECG and ICG with respect to p53 mutation of the primary tumor.

2.3. Evaluation of ECG as a Potential Prognostic Factor for Overall Survival

The number of deaths observed was 25/35 (71.4%) in the ECG group and 55/103 (53.4%) in the ICG group. Median OS in patients diagnosed with ECG was reduced to 32.6 months (95% CI: 25.1–40.0) as compared to 61.2 months in the ICG cohort (95% CI: 39.2–83.2). OS of ECG vs. ICG cases was significantly different and ECG was prognostic for shortened OS ($p = 0.024$, Table 2, Figure 5E). The total number of resected lymph nodes did not influence overall survival (OS), and neither did FIGO stage, tumor size, histologic subtype or patient age (Table 2). Poor differentiation of the primary tumor (i.e., G2/3 in non-serous or high grade in serous cancers), FIGO stage IV and the presence of residual disease were highly prognostic for shortened OS (Grading: $p = 0.002$; FIGO: $p = 0.012$; residual disease: $p < 0.001$; Figure 5A–C, Table 2). An LNR higher than 0.3 ($p = 0.008$) as well as the combination of LNR and ECG ($p = 0.003$) were also associated with shortened OS (Figure 5D,F, Table 2). To exclude potential biasing by clinico-pathological covariates, subgroup analysis was performed. Presence of ECG remained to be prognostic for shortened OS in cases classified as either pT1/2 ($p = 0.031$), FIGO III ($p = 0.029$), or FIGO IV ($p = 0.030$).

Table 2. Univariate survival analysis: Clinicopathological parameters, lymph node ratio (LNR) and ECG/ICG and the combination of the latter two were tested for their prognostic significance. Significant results ($p < 0.05$) are indicated by bold font and *p*-values derived from relevant log rank tests are shown.

Characteristic	Univariate Analysis	
	Log Rank χ^2 Test	*p*
FIGO (III vs. IV)	6.345	**0.012**
pT (pT1/2 vs. pT3)	0.743	0.389
Grade (G1 vs. G2/3)	9.862	**0.002**
Histology (other vs. serous)	0.425	0.515
Patient age (<60 years vs. ≥60 years)	2.960	0.085
Residual disease (none vs. any)	16.65	**<0.001**

Table 2. *Cont.*

Characteristic	Univariate Analysis	
	Log Rank χ^2 Test	*p*
Total nodes resected (<37 vs. ≥37)	0.223	0.637
LNR (≤0.3 vs. >0.3)	7.059	**0.008**
ECG vs. ICG	5.101	**0.024**
LNR > 0.3 and ECG vs. remaining	8.581	**0.003**

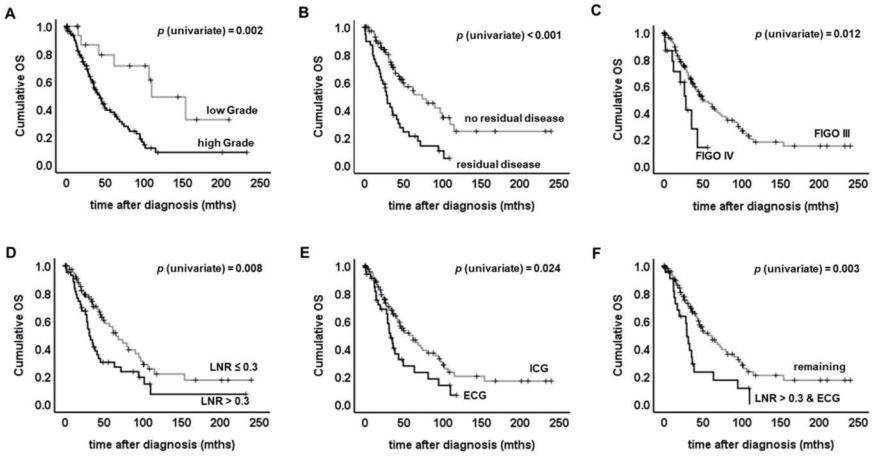

Figure 5. Univariate survival analysis: Log rank tests were performed to determine if there were differences in the survival distribution for the different types of lymph node involvement (ECG vs. ICG), the different fractions of affected lymph nodes (LNR > 0.3 vs. LNR ≤ 0.3) or for clinicopathological parameters. Kaplan–Meier survival curves are shown. High grade (**A**), residual disease after primary debulking surgery (**B**), advanced FIGO stage (**C**) or high LNR (**D**) were found to be associated with shortened overall survival (OS). OS was significantly different when ECG vs. ICG cases were compared (**E**). Long-term surviving patients were only found in within the ICG group (**E**). Those cases presenting both high LNR and ECG at the same time were found to have exceptional reduced OS (**F**).

In non-serous cancers the pattern of lymph node involvement (ECG vs. ICG) remained prognostic for shortened OS ($p = 0.048$). Regarding serous cancers, association of ECG and reduced OS did not reach statistical significance ($p = 0.281$), but survival curves were clearly separated three years after surgery, though the difference was not significant ($p = 0.125$). Within all histologic sub-groups long term survivors (i.e., longer than 10 years after surgery) were only found in those cases diagnosed with ICG.

Finally, univariate survival analysis was restricted to those patients with information on residual disease and survival available ($n = 122$) and ECG continued to be associated with shorter OS ($p = 0.043$) in this smaller cohort, too.

Cox regression (Table S1) was performed to identify independent predictors for OS. Pattern of lymph node involvement (ECG vs. ICG), lymph node ratio and tumor grading failed to remain significant within multivariate testing, while residual disease ($p = 0.01$ and $p = 0.02$) was still predictive in both multivariate testing sets. The second multivariate analysis was run, to test whether the combination of ECG and lymph node ratio may be an independent prognosticator for OS. This combined parameter showed a trend of potentially acting as an independent negative prognosticator ($p = 0.08$) (Table S1).

3. Discussion

We herein report that extracapsular spread is a common phenomenon in ovarian cancer. The current study detected extracapsular spread in 24.5% of advanced staged ovarian cancer cases. A literature search revealed a similar prevalence of ECG in different types of cancer e.g., 19.1% in vulvar [10] and 30.1% in cervical [5] cancer patients. We found ECG to be more common in non-serous OC as compared to the serous histological subtype. So far, this observation cannot be explained satisfactorily. However, this finding may further underline tumor biologic differences among different OC subtypes [11]

In general, ECG was closely correlated with tumor grade and a high lymph node ratio, while being independent of resected lymph nodes or tumor size. These observations support the hypothesis that extracapsular spread may be a surrogate marker for tumor biologic features that drive extravasation and thereby cause tumor aggressiveness. We hence questioned whether the protein profile of the ovarian primary may be related to the presence of ECG in associated lymph nodes.

Metastatic spread is majorly determined by tumor biologic features of the primary tumor. In order to conclude on a biomarker profile that may predict extracapsular spread, a set of 27 protein markers (including p53), that had been determined in the ovarian primary tumor, were correlated with presence of ECG. We found that expression of Mucin-1 (as determined by the antibody VU4H5), Gal-3^{nuc}, Gal-8^{nuc}, and GPER were statistically associated with capsular extravasation. Interestingly, VU4H5—which was positively correlated with ECG in the current study—had been demonstrated to predict reduced overall survival in this ovarian cancer sample [12]. On the contrary, GPER and Gal-3/8 were inversely correlated with presence of ECG and had been found to be positive prognosticators in ovarian cancer [13–15]. We introduced a four-marker signature built by VU4H5, GPER, and Gal-3/8 which was predictive for ECG in associated lymph nodes with relatively good sensitivity and specificity. However, in order to validate the robustness of this signature, an independent validation set as well as testing for interactions would be required. It would also be of interest, if this four-marker signature would predict lymph node involvement as such. If so, such a marker set could help to identify a subgroup of patients with high risk of extracapsular nodal involvement, high extent of nodal involvement and hence a considerably poor prognosis. Whether this subgroup of patients may potentially benefit from systematic lymphadenectomy, would have to be addressed in a prospective trial.

It is well known that complete cytoreduction, FIGO stage and histopathologic subtype are the strongest prognostic factors in epithelial ovarian cancer [16,17]. However, the role of systematic lymphadenectomy within optimal debulking surgery in ovarian cancer has been controversial for years. Recently, results from a large multi-center, prospectively randomized trial of lymphadenectomy in clinically node negative advanced ovarian cancer patients were presented (LION Trial, AGO-OVAR OP.3) [18]. Harter et al. highlighted that omitting lymph node dissection in clinically node negative advanced ovarian cancer patients does not affect prognosis [18]. This has changed the standard of care in many institutions around the world. On the other hand, an earlier, however retrospective exploratory analysis of three prospectively designed randomized chemotherapy trials found that lymphadenectomy was associated with extended overall survival in patients diagnosed with advanced disease that had been optimally debulked [19]. In addition, systematic lymphadenectomy was superior as compared to the removal of bulky nodes only in terms of progression free survival, though it did not affect OS in patients that had undergone optimal tumor debulking [20].

These data seem to be partly contradictory at first glance. To some extent this may be due to the retrospective character of earlier studies, different trial designs and various primary endpoints of the studies cited above. Another interpretation may be the existence of patient subgroups that benefit from lymph node dissection to different extents. Such subgroups—if they exist—have not been identified so far. Biomarkers or clinical predictors that aid identifying those patients that benefit most from systematic lymph node removal remain to be defined. Whether the biomarker signature introduced above may help to select OC cases with ECG and presumably highly aggressive disease can just be speculated by now.

Nowadays, such patients diagnosed as lymph node positive or Stage IIIB or higher may be eligible for a Bevacizumab therapy based on the GOG 218 and ICON 7 trials [21,22]. As a consequence, though potentially missing direct benefit from surgical resection per se, systematic lymph node dissection may aid to perform more accurate staging thereby avoiding adjuvant over- or undertreatment. Whether pattern/extent of lymph node involvement (ECG vs. ICG) may add additional prognostic information in ovarian cancer macroscopically confined to the pelvis may be worth addressing in the future.

Finally, there are several limitations to our study that need to be critically discussed. In general, further studies and larger patient cohorts are needed in order to validate our findings. Future study setups should also rule out potential confounders like longer operation times, higher complication rates or different surgical intents. Tissue samples were collected over a time span of 25 years. Importantly, quality criteria and surgical approaches of ovarian cancer surgery have significantly changed during this period. Although we included 'residual disease status' in our multivariate analysis to correct for this major criterion of ovarian cancer surgery, we were not able to properly control for the aforementioned parameters. Further, due to the retrospective and primarily histopathological character of our study, we were not able to correct for individual patient factors like lifestyle, fitness or comorbidities.

Though sample size certainly is a limitation of our study, the patient cohort studied herein has been extremely well characterized (first and second histopathological review by a specialized gynecological pathologist, broad range of clinical data including follow up available, IHC data available (n = 32)). Although the sample size of n = 143 might appear to be rather small, it is within the range of what several other pre-clinical, translational studies in ovarian cancer do report on [23–25]. We further analyzed serial sections of up to 94 lymph nodes per case. In total, we analyzed a number of 5042 lymph nodes in multiple serial sections. Since the primary aim of this study was to describe the phenomenon of ECG in ovarian cancer and its association with clinicopathological parameters, we think that n (ovarian cancer samples) = 143, n (lymph nodes analyzed) = 5042 is at least a sample size that might serve as a basis for further studies on this topic. Since IHC data were only available for 32 cases regression analysis might be statistically underpowered and needs to be interpreted with care. However, as GPER and Gal-3/8 (= positive prognosticators for overall survival) were positively associated with ICG and VU4H5 (= negative prognosticator for overall survival) was positively associated with ECG [12–15], regression analysis fits with biological rational of ICG/ECG and prognosis.

Finally, whether the univariate prognostic significance of extracapsular spread may one day become of clinical relevance in ovarian cancer management and whether this may influence the decision on whether to perform lymphadenectomy or not, cannot be said until prospective, properly powered trials have been performed on this topic.

4. Materials and Methods

4.1. Patients

Tissue specimens obtained from 143 patients that had been diagnosed and treated with lymph node positive ovarian cancer were analyzed retrospectively. All patients had undergone radical ovarian cancer surgery at the Department of Obstetrics and Gynecology, Ludwig-Maximilians-University of Munich between 1990 and 2015. Surgery was performed by specialized gynecologic oncologists according to the national guidelines for ovarian cancer [8]. Patients received chemotherapy in either an adjuvant (n = 140) or a neo-adjuvant (n = 3) setting.

Histological characterization as well as histological tumor grading according to the WHO criteria were performed by a gynecological pathologist. Grading was binarized for statistical analysis. As long as not stated otherwise the term 'low grade' refers to low grade serous as well as to non-serous cancers graded as G1, and 'high grade' refers to high grade serous as well as non-serous cancers graded as G2 or G3. Staging was performed according to the FIGO criteria (8ed). Lymph node ratio (LNR) was calculated by the absolute number of resected lymph nodes divided by the number of metastatic lymph nodes. A lymph node ratio (LNR) of >0.3 was considered as high, ≤0.3 as low.

Clinical data were retrieved from patients' charts and from the Munich Cancer Registry. Data were analyzed retrospectively. The outcome assessed was patients' overall survival.

4.2. Ethical Approval

This study used tumor tissue that had initially been collected for histo-pathological diagnostics. At the time the tissue was examined for the current study all diagnostic procedures had already been fully completed and the tissue used was thus classified as left-over material. All patient data were fully anonymized, the Ethics Committee of the Ludwig-Maximilians-University (Munich, Germany) approved the study (227-09 and 18-392) and the study was performed according to the standards set in the declaration of Helsinki 1975. As per declaration of our ethics committee no written informed consent of the participants or permission to publish is needed given the circumstances described above. Researchers were blinded from patient data during experimental and statistical analysis.

4.3. Assay Methods

4.3.1. Determination of ECG and ICG

Tissue samples underwent routine histopathology processing. Briefly, specimens were fixed in neutral buffered formalin directly after resection and were submitted to fully automated paraffin embedding. FFPE tissue slides (thickness 2–5 µm) underwent semi-automated H&E staining. HE-stained tissue sections of the resected lymph nodes were evaluated on extracapsular lymph node involvement by two independent observers, including an experienced gynecological pathologist. ECG was defined as growth of tumor cells through or directly beyond the lymph node capsule invading into perinodal fat or into perinodal stroma [4]. Cases diagnosed for ECG in one or more than one lymph node were grouped into the ECG cohort.

4.3.2. Immunohistochemistry

Data regarding the protein-profile of the primary tumor were available in 32 cases and were derived from previous studies performed by our group [12–15,26–29]. Within the currents study staining data were re-analyzed with respect to the presence of either ECG or ICG of the corresponding lymph nodes. Mutation of TP53 was estimated by immunohistochemistry by using a score published earlier by our group and others [8,9].

4.3.3. Statistical Analysis Methods

This study has been carried out according to the REMARK (Reporting Recommendations for Tumor Marker Prognostic Studies) criteria [30]. The IBM statistic package SPSS (version 24) was used to test data for statistical significance. Fisher's exact test was performed to test categorical data for statistical independence. Student's *t*-Tests was applied in the case of continuous variables. A logistic regression was run to ascertain the effects of four biomarkers on the likelihood of extracapsular spread. The Youden index ($J = \max_c \{\text{Sensitivity (c)} + \text{Specificity (c)} - 1\}$) was calculated to illustrate the maximum potential effectiveness of this biomarker combination [31]. Survival analysis was done by applying the Log-Rank test and data are presented as Kaplan–Meier survival curves. Cox regression was performed on ECG and o-variables to test which parameter remains to be prognostic within multi-variate testing. Data were assumed to be statistically different in the case of $p < 0.05$.

5. Conclusions

Our data demonstrate that extracapsular growth (ECG) of lymph node metastasis is a common feature of lymph node positive ovarian cancer. Since ECG was correlated with tumor grade, high lymph node ratio, and shortened overall survival, ECG may be interpreted as an indicator of tumor aggressiveness. Whether the pattern of lymph node involvement (extra- vs. intracapsular growth)

may reproducibly add additional prognostic information in OC and whether this might aid to further individualize adjuvant therapy should be the subject of future studies.

Supplementary Materials: The following are available online at http://www.mdpi.com/2072-6694/11/7/924/s1, Table S1: Multivariate survival analysis.

Author Contributions: Conceptualization, S.H., M.A., S.M., D.M., U.J. and E.S.; Formal analysis, S.H., U.J. and E.S.; Funding acquisition, S.H. and U.J.; Investigation, S.H., H.S., F.M., M.A., B.C., A.B., D.M., U.J. and E.S.; Methodology, S.H., M.A., D.M., U.J. and E.S.; Project administration, S.H. and U.J.; Supervision, S.M., D.M. and U.J.; Validation, F.M., S.M., D.M. and U.J.; Visualization, S.H.; Writing—original draft, S.H. and H.S.; Writing—review and editing, S.H., H.S., F.M., M.A., B.C., A.B., S.M., D.M., U.J. and E.S.

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ORIGINAL ARTICLE

Comprehensive analysis of PD-L1 expression, HER2 amplification, ALK/EML4 fusion, and mismatch repair deficiency as putative predictive and prognostic factors in ovarian carcinoma

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Abstract
Most ovarian carcinomas (OC) are characterized by poor prognosis, particularly the most frequent type high-grade serous carcinoma. Besides PARP inhibitors, target-based therapeutic strategies are not well established. We asked the question which other therapeutic targets could be of potential value and, therefore, analyzed a large cohort of OC for several predictive factors. Two hundred eighty-eight (288) cases of OC including the major histological types were analyzed by immunohistochemistry for PD-L1HER2, ALK, and the mismatch repair (MMR) proteins MLH1, PMS2, MSH2, and MSH6. HER2 amplification and ALK/EML4 fusion were assessed by fluorescence in situ hybridization. The most frequent finding was PD-L1 expression ≥ 1% in 19.5% of the cases, which correlated with a significantly better overall survival in multivariate analysis ($p < 0.001$). HER2 amplification was detected in 11 cases (4%), all high-grade serous carcinomas. Amplification of HER2 did not correlate with patients' survival. ALK/EML4 fusion was found in two cases (0.74%): one high-grade serous and one endometrioid carcinoma. MMR deficiency was only present in one case of stage IV high-grade serous carcinoma. Subsets of high-grade serous carcinomas show PD-L1 expression and HER2 amplification, respectively, and, therefore, could qualify for immune checkpoint inhibitor therapy or anti HER2 therapy. PD-L1 is also of prognostic impact. ALK/EML4 fusion is very rare in OC and not a putative therapeutic target.

Keywords Ovarian carcinoma · HER2 · ALK/EML4 · PD-L1 · Mismatch repair deficiency · Microsatellite instability

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Introduction

Ovarian carcinoma (OC) includes different histological subtypes, which are heterogeneous with respect to underlying pathogenesis, molecular features, and prognosis. This biological diversity of OC is not considered for adjuvant treatment which encompasses relatively uniform chemotherapeutic schemes. In contrast to many other solid tumors, the evaluation of predictive biomarkers and prognostic factors is not an integrated part of the diagnostic procedure of OC, so far. Although a subset of high-grade serous carcinoma (HGSC) can be effectively targeted by PARP inhibitors in addition to chemotherapy, further therapeutic options are of great interest [21, 33]. Currently, the group of "non-HGSC" receives a combination of platin- and taxane-based, adjuvant chemotherapy

depending on stage and grade but regardless of molecular features or histological type [8, 25].

Recently, for several categories of solid cancer, the entity-based therapy has been replaced by a target-based therapy, which addresses to specific tumorigenic pathways. The putative responsiveness of these therapeutic targets, which is usually related to molecular alterations such as mutations, gene fusions, or gene amplifications, may be tested before treatment by molecular pathological analysis. Among the most frequent therapeutic targets, which have not yet been introduced into standard OC therapy, are the immune checkpoint inhibitor molecule programmed cell death 1 ligand 1 (PD-L1), amplified human epidermal growth factor receptor 2 (HER2), fused anaplastic lymphoma kinase/echinoderm microtubule-associated protein-like 4 (ALK/EML4), and mismatch repair deficiency (MMRd).

Anti-HER2-therapy is widely used in breast cancer and in addition in gastric and colorectal cancer [26]. HER2 amplification was studied in OC but by now anti-HER2-therapy was applied only in single cases of OC [28, 29]. Treatment of non-small cell lung cancer by anti-tyrosine kinase inhibitors effectively targets the ALK/EML4 translocation [18, 34], but for OC data are controversial. A few studies suggested a therapeutic application [16, 35] although the TCGA data did not report evidence for ALK/EML4 translocation in OC [4]. Therapeutic inhibition of the immune checkpoint proteins PD-L1 has become a standard procedure for non-small cell lung cancer [37]. Several trials have revealed an improved outcome also for OC [22, 30]. In addition, the responsiveness for immune checkpoint inhibitors is related to microsatellite instability (MSI) due to the stimulation of immunogenicity by the frequent mutations in mismatch repair (MMR)-deficient neoplasms [13, 20, 42]. This has been demonstrated particularly for colorectal carcinoma [2, 41].

We analyzed a cohort of clinically well-documented OC from a single cancer center for alterations in ALK, HER2, PD-L1, and MMRd by immunohistochemistry and molecular analysis. The findings were also correlated to patients' overall survival.

Materials and methods

Study group and clinical data

The study cohort included 288 cases of OC of which 233 were HGSC and the remaining 55 carcinomas consisted of 19 low-grade serous (LGSC), 23 endometrioid (EC), and 13 mucinous (MC) carcinomas. All cases were primarily diagnosed between 2003 and 2007 at the Institute of Pathology, Ludwig-Maximilians-University, Munich, Germany, and underwent a careful histopathological review and reclassification according to the 2014 WHO classification under assistance of immunohistochemistry using PAX8, WT1, p53, Ki67, ER, and PR. Borderline/atypical proliferative tumors were excluded from this study. Complete follow-up data were available for all cases with a mean follow-up time of 44.6 months. One hundred sixty-eight (168) of the 288 patients (58.3%) died of tumor-related disease. Data regarding somatic or hereditary BRCA mutations or Lynch syndrome were not available. The clinicopathological parameters are listed in Table 1. Due to the limited number of cases per group except for HGSC, a detailed subgroup analysis focused on HGSC.

Immunohistochemistry

Immunohistochemical stains were performed using formalin-fixed paraffin-embedded (FFPE) tissues on a tissue microarray (TMA). The TMA was constructed by using two cores of 2.0 mm punched from each donor block and transferred to an empty paraffin block under the guidance of a precision tool.

Serial sections were cut at 4 μm from each paraffin block and mounted on SuperFrost Plus microscope slides (Menzel Gläser, Braunschweig, Germany), deparaffinized, and stained with hematoxylin and eosin (HE). Immunohistochemistry was subsequently performed for ALK, ER, HER2, MLH1, MSH2, MSH6, p53, PAX8, PD-L1, PMS2, ER, PR, and WT1 (Supplementary Table 1). Immunohistochemistry for ALK, ER, HER2, MLH1, MSH2, MSH6, p53, PD-L1, PMS2, and PR was subjected to heat-induced epitope unmasking by heating with a pressure cooker and performed on a Ventana Benchmark XT autostainer (Ventana Medical Systems, Oro Valley, AZ) with the XT UltraView diaminobenzidine kit (Vector Laboratories, Burlingame, CA) and hematoxylin

Table 1 Clinicopathological parameter

Study group ($n = 288$)	
Age (years)	
Mean	62
Minimum	23
Maximum	93
FIGO	*N* (%)
I	28 (9.7)
II	22 (7.6)
III	197 (67.4)
IV	44(15.3)
Histological types	*N* (%)
High-grade serous	233 (80.9)
Low-grade serous	19 (6.6)
Endometrioid	23 (8.0)
Mucinous	13 (4.5)

counterstaning (Vector Laboratories, Burlingame, CA). Positive controls were included.

Evaluation of immunohistochemistry

The interpretation of the immunohistochemical stains for HER2 was based on the three-tiered scoring system according to the guidelines of breast cancer published by the American Society of Clinical Oncology (ASCO) [39]. Scores 0 and 1+ were interpreted as HER2 negative. Score 2+ was interpreted as equivocal for HER2. Score 3+ was interpreted as HER2 positive.

Immunohistochemical staining for ALK was interpreted as positive if tumor cells showed a strong multifocal or diffuse, granular cytoplasmic expression. Weak cytoplasmic immunoreactivity was scored as negative.

PD-L1 staining was graded by using the PD-L1 "Cologne score" [32], referring to the following: score $0 = < 1\%$, score $1 = \geq 1$ and $< 5\%$, score $2 = \geq 5$ and $< 10\%$, score $3 = \geq 10$ and $< 25\%$, score $4 = \geq 25$ and $< 50\%$, and score $5 = \geq 50\%$. A tumor cell was considered "PD-L1 positive" if the cell membrane was partially or completely stained, irrespective the staining intensity. Cytoplasmic PD-L1 staining was disregarded.

For the MMR proteins, complete absence of nuclear staining was considered "loss of expression" and negativity, respectively. Adjacent normal stromal cells served as positive control. Immunohistochemistry was repeated on the original tumor block in all cases of uncertain immunoreactivity.

In order to confirm MSI, the case with immunohistochemical MMRd was further analyzed using a PCR-based technique on a Sanger sequencer according to a standardized protocol.

Fluorescence in situ hybridization

FISH for HER2 and ALK/EML4 was performed according to a standardized protocol. Sections were cut at 3 μm from each TMA block and mounted on SuperFrost Plus microscope slides (Menzel Gläser, Braunschweig, Germany). For HER2-FISH, the dual color fluorescence HER2-specific probe (ZytoLight SPEC ERBB2/CEN 17 Dual Color Probe, Spectrum Green and CEP 17 Spectrum Orange, ZytoVision, Bremerhaven, Germany) was used. The interpretation of the results for HER2-FISH followed the ASCO/CAP guidelines for breast cancer [39]. In all cases of HER2 equivocal status by FISH or technical problems on the TMA, the FISH analysis was repeated on the original large tumor block.

For ALK/EML4 FISH, a triple color break apart single fusion probe (ZytoLight_SPEC ALK/EML4 TriCheck, ZytoVision, Bremerhaven, Germany) was used. The TriCheck probe encompasses two probes (orange and green) flanking the breakpoint cluster region of ALK and third probe (blue) covering the complete echinoderm microtubule-associated protein-like 4 (EML4) gene. ALK/EML4 fusion was defined as the presence of split signals and/or single red signals in ≥ 15% of tumor cells according to the guidelines for non-small lung cancer [36]. The ALK/EML4 FISH was repeated on the original large tumor block in all positive cases and in all cases that could technically not be evaluated on the TMA. For each case, a minimum of 30 cells were analyzed but in most cases 60 cells were evaluated.

The FISH analysis was performed on a ZEISS Axioskop microscope (Carl Zeiss AG, Oberkochen, Germany), equipped with a 100-V OSRAM lamp (OSRAM AG, Munich, Germany) and objectives by ZEISS (Carl Zeiss AG, Oberkochen, Germany). Three independent readers analyzed all samples.

Statistical analysis

For statistical analysis, the SPSS Statistics version 23 (SPSS Inc., Chicago, IL, USA) was used. For testing proportional differences in univariate analysis, the Pearson's Chi-square test or the Fisher's exact test for qualitative variables and unpaired t test for quantitative normally distributed variables were used. The survival curve was generated using the Kaplan-Meier technique and differences between these curves were tested by the log-rank test. All tests were two-sided and the level of statistical significance was accepted at $p \leq 0.05$. For multivariate analyses, the Cox regression model for overall survival was used.

Ethical approval

All patients' data were fully anonymized, and the study was performed according to the standards set in the Declaration of Helsinki 1975. All tumor tissue used was leftover material that had initially been collected for histopathological diagnostics. All diagnostic procedures had already been fully completed when samples were retrieved for the study. The current study was approved in writing by the Ethics Committee of the Ludwig-Maximilians-University, Munich, Germany (approval number 18-130). Authors were blinded for clinical information during experimental analysis.

Results

The results of immunohistochemistry and FISH analyses are summarized in Table 2 and correlated to the histological subtypes.

Table 2 Distribution of HER2 amplification (HER2), ALK/EML4 fusion (ALK), mismatch repair deficiency (MMRd), and PD-L1 status (positivity of ≥ 1% of the tumor cells) according to the histological type of ovarian carcinoma

	High-grade serous ($n = 233$)	Low-grade serous ($n = 19$)	Endometrioid ($n = 23$)	Mucinous ($n = 13$)
HER2 ($n = 11$)	11 (4.7%)	0	0	0
ALK ($n = 2$)	1 (0.4%)	0	1 (4.3%)	0
MMRd ($n = 1$)	1 (0.4%)	0	0	0
PD-L1 ($n = 57$)	55 (23.6%)	0	2 (8.7%)	0

PD-L1 immunohistochemistry

Fifty-seven of the 288 cases (19.5%) were PD-L1 positive (≥ 1%), of which 55 were HGSC (23.6%) (Fig. 1). The detailed results are listed in Table 3. Generally, the PD-L1 status based on the "Cologne score" did not correlate with the histological type ($p = 0.271$); however, positivity ≥ 25% was only present in HGSC. In addition, the number of cases in the non-HGSC categories was limited. In univariate analysis, overall survival was significantly better in cases with PD-L1 immunoreactivity, referring to the "Cologne score" ($p = 0.012$; Fig. 2). This was confirmed by multivariate Cox regression analysis showing PD-L1 expression as a significant and independent prognostic factor with hazard ratio of 2.302 if dichotomized for < 1 and ≥ 1% (Table 4).

HER2 status by immunohistochemistry and FISH

By immunohistochemistry, 3 out of 288 cases (1.0%) were scored as 3+, 42 cases (14.6%) as 2+, and 243 cases (84.4%) as 0 or 1+. HER2-FISH could be analyzed in 284 cases (98.6%), 4 cases were technically not adequate for evaluation. Amplification was found in 11/284

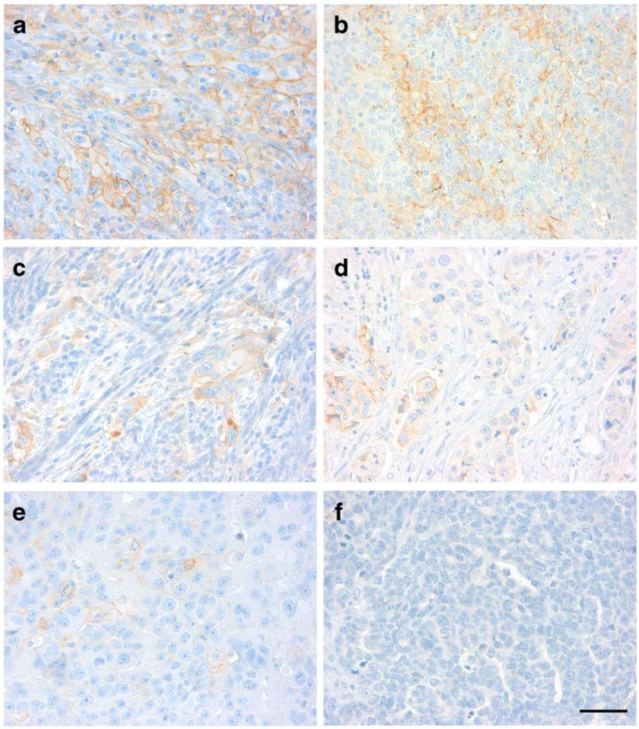

Fig. 1 Immunoreactivity of PD-L1. PD-L1 expression in ≥ 50% (**a**), ≥ 25 and < 50% (**b**), ≥ 10 and < 25% (**c**), ≥ 5 and < 10% (**d**), ≥ 1 and < 5% (**e**), and < 1% (**f**) of tumor cells. For all figures ×400 magnification was used (scale bar refers to 50 μm)

Table 3 PD-L1 score ("Cologne score") related to the histological type of ovarian carcinoma

Histological subtypes		High-grade serous ($n = 233$)	Low-grade serous ($n = 19$)	Endometrioid ($n = 23$)	Mucinous ($n = 13$)	Total (288)
PD-L1	< 1%	178 (76.4%)	19 (100%)	21 (91.2%)	13 (100%)	232 (80.5%)
	≥ 1%	13 (5.6%)	0	1 (4.4%)	0	14 (4.8%)
	≥ 5%	13 (5.6%)	0	0	0	13 (4.5%)
	≥ 10%	21 (9.0%)	0	1 (4.4%)	0	22 (7.6%)
	≥ 25%	7 (3.0%)	0	0	0	7 (2.4%)
	≥ 50%	1 (0.4%)	0	0	0	1 (0.3%)

(3.9%) cases (Fig. 3a, b), and in another 11 cases the HER2 status was originally equivocal by FISH. Based on the guidelines of Wolff et al. 2018, the 11 cases of HER2 equivocal status would be classified as HER2 negative by FISH [40]. The HER2 status was concordant between immunohistochemistry and FISH: The three cases with score 3+ were all amplified. The 42 score 2+ cases consisted of 8 amplified cases and 28 cases without amplification. In five cases with score 2+, the HER2 status remained equivocal by FISH and one case could not be evaluated. The results are detailed in Supplementary Table 2. HER2 amplification was only present in HGSC (4.7% of HGSC), taken into account the small number of cases in the non-HGSC categories. Although 7 of 11 patients with HER2-amplified carcinomas (63.6%) died of disease, HER2 amplification did not correlate with survival ($p = 0.616$).

ALK/EML4 fusion by immunohistochemistry and FISH

ALK/EML4 FISH could be evaluated in 271 cases (94.1%). Seventeen samples were technically not adequate for evaluation. Translocation of ALK/EML4 was detected in 2/271 cases (0.74%), of which one was a HGSC and the other a grade 2 EC (Fig. 3c–f). Both cases presented at FIGO stage III, were HER2- and PD-L1-negative, and showed no MMRd. The patients died of tumor (survival time: HGSC 31.1 months, EC 12.9 months). By immunohistochemistry both cases showed a strong cytoplasmic reactivity, EC additionally nuclear staining (Fig. 3e).

FISH analysis showed for EC ALK split signals in 23.3% and ALK split signals with combined ALK/EML4 inversion in 11.7% of tumor cells; in 75% of tumor cells, ALK signals were normal without breakpoints. The HGSC revealed ALK split signals in 63.3% and ALK split signals with combined ALK/EML4 inversion in 43.3% of the tumor cells; in 16.7% of the tumor cells, ALK signals were normal without breakpoints. ALK immunoreactivity without underlying ALK/EML4 fusion was not found.

Immunohistochemistry for mismatch repair proteins

Loss of immunoreactivity for MSH2 and MSH6 was detected in only one case of HGSC (pT3cpN1pM1; FIGO stage IV)

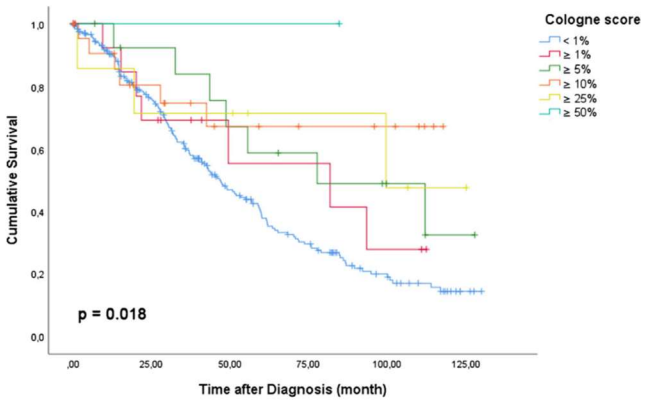

Fig. 2 Kaplan-Meier survival analysis for PD-L1. Censoring events have been marked in the graph

Table 4 Multivariate Cox regression analysis with overall survival for PD-L1 ($n = 288$ for all variables)

Variable	Hazard ratio	95% Confidence interval	p value
Age (per year)	1.209	1.065–1.373	0.003
Grading G1 versus G2/3	1.738	0.831–3.636	0.142
Histological type High-grade serous versus all other histological types versus HGSC	0.989	0.611–1.576	0.707
FIGO (I/II versus II/III)	2.394	1.466–3.535	< 0.001
PD-L1 (≥ 1 versus < 1%)	2.302	1.499–3.535	< 0.001

(Fig. 4) and for this case MSI was confirmed by molecular analysis. Furthermore, this case was negative for HER2, ALK, and PD-L1. The patient was diagnosed at the age of 44 years and died of tumor-dependent death (survival 8.3 months).

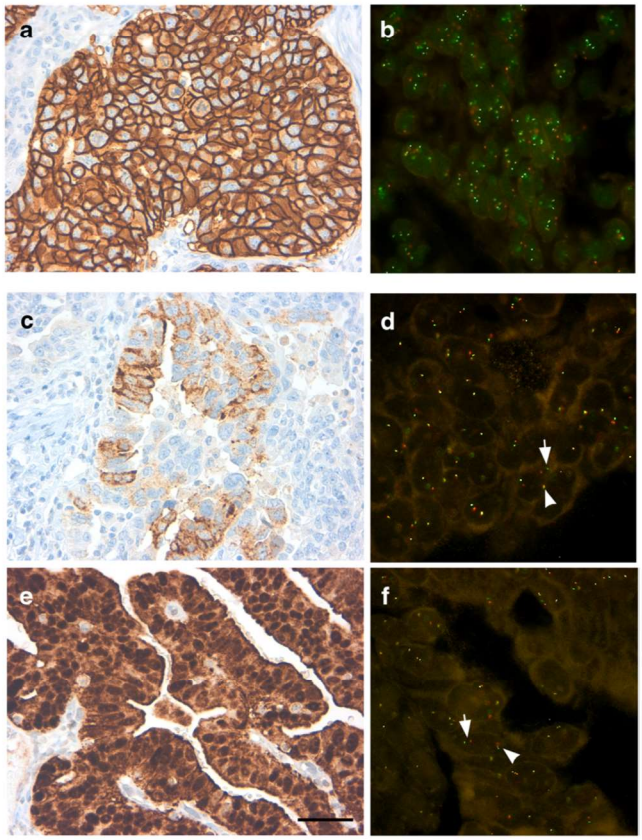

Fig. 3 HER2 amplification and ALK/EML4 translocation: HGSC with strong cytoplasmic and membranous (score 3+) immunoreactivity (**a**) and amplification (**b**) of HER2. HGSC with strong granular cytoplasmic immunoreactivity of ALK (**c**) and ALK/EML4 translocation (**d**). Endometrioid carcinoma with unusual nuclear and cytoplasmic ALK immunoreactivity (**e**) and ALK/EML4 translocation (**f**). Arrows point to ALK split signals and arrow-heads point to ALK fusion signals (**d**, **f**). ×400 magnification was used for all immunohistochemical figures (**a**, **c**, **e**), scale bar refers to 50 μm. ×1000 magnification was used for FISH photomicrographs (**b**, **d**, **f**)

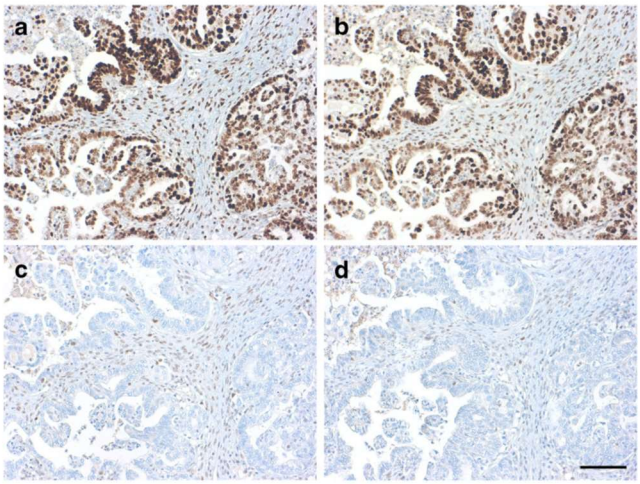

Fig. 4 HGSC with MMRd showing loss of immunoreactivity for MSH2 (**c**) and MSH6 (**d**), while expression of MLH1 (**a**) and PMS2 (**b**) was preserved. ×200 magnification was used for all pictures (scale bar refers to 100 μm)

Discussion

In this study we analyzed various predictive biomarkers for putative targeted therapy in a clinicopathologically well-characterized collective of 288 OC of which 80% were HGSC. We found PD-L1 positivity ≥ 1% in about one quarter of HGSC and in less than 5% of the heterogeneous non-HGSC group. Stronger PD-L1 immunoreactivity (≥ 25%) was limited to HGSC. MMRd was very rare (in < 1% of the cases) and, interestingly, present HGSC. HER2 amplification was found in about 5% of HGSC whereas ALK/EML4 fusion was very uncommon. Therefore, HGSC might be considered for immune checkpoint inhibitor therapy due to frequent PD-L1 expression and less likely for anti-HER2 or tyrosine kinase inhibitor therapy. We would like to stress the strengths of this study represented by the well-characterized patients' collective with long-term follow-up. In addition, this collective underwent systematic histological review and immunohistochemical typing. We are aware that the number of cases in the non-HGSC categories is limited and, therefore, we handled the results of subgroup analysis with caution. Another point of critique could be the use of TMA which contain less amounts of the tumor compared to whole section. Previous studies have shown for several biomarkers, in particular, for PD-L1 that TMA is usually representative for whole sections [10, 17, 23].

Currently available data suggest an increasing importance of immune checkpoint inhibitors in the treatment of OC [3, 14]. So far, it is not clear whether in OC PD-L1 expression of the tumor cells or of the immune cells is predictive for therapeutic response and whether the histological type plays a role. In this study, we analyzed the positivity for PD-L1 in the tumor cells and found positivity in 1% or more in about 20% of all cases and in about 24% of all HGSC, respectively. In addition, PD-L1 expression ≥ 1% turned out to be an independent positive prognosticator. This has been found by others and may be caused by the high numbers of tumor-infiltrating lymphocytes in PD-L1 positive cases [6]. However, the prognostic impact of the PD-L1 status in OC is controversial since PD-L1 expression may correlate with poor prognosis in OC [38, 43].

MMRd and MSI, respectively, have been demonstrated as predictor of an effective immunomodulatory therapy [9, 15]. However, based on our and others' findings, MMRd seems to be very rare in OC. We studied MMRd in all cases and found loss of MSH2 and MSH6 associated with MSI in only one case of HGSC (< 1%). The other OC types showed retained MMR immunoreactivity which is in contrast to the literature [9, 27]. However, these results need to be handled with caution since our EC subgroup was small with 20 cases and clear cell carcinomas were excluded. For our MMRd case, genetic counseling and germ-line testing were not performed. It has been speculated whether this has a favorable prognosis in OC like in colorectal cancer [11, 12], but due to the infrequent occurrence this cannot be assessed [7, 27]. Our single patient was diagnosed at stage IV and survived only 8.3 months.

According to previous studies, HER2 amplification is mainly found in the mucinous type of OC [1, 28]. We found HER2 amplification only in HGSC, whereas all 13 MC were HER2 negative but in this respect the limited number of MC needs to be taken into account. Using the guidance for breast cancer, we were able to show

concurrence between HER2 immunohistochemistry and HER2 FISH and, therefore, immunohistochemistry could be used as primary investigative tool in OC, too. The prognostic impact of HER2 amplification in OC is unclear, which may be related to its rare occurrence. In this and a previous study, there was no correlation between the HER2 status and patients' survival although in a meta-analysis poor outcome was found [24, 29]. Clinical data for anti-HER2 therapy in OC is very limited, although responsiveness was reported for single cases of mucinous type [31].

ALK/EML4 fusion was present in only two cases (0.74%) but it is also an infrequent finding in non-small cell lung cancer by about 4–5% [5]. In non-small cell lung cancer, ALK/EML4 fusion can be very efficiently and reliably detected by immunohistochemistry [19]. Taken a strong, granular cytoplasmic staining as positive, immunohistochemistry for ALK was also in our collective concordant with ALK/EML4 FISH. Interestingly, the case of EC revealed in addition to a cytoplasmic also a strong nuclear immunoreactivity but FISH analysis did not show an uncommon translocation. The prognostic impact of ALK/EML4 fusion is unclear even in non-small cell lung cancer where it is more frequent in younger patients. Both of our OC cases with ALK/EML4 fusion died of tumor. The consideration of ALK rearrangement as a diagnostic and putative therapeutic target needs to be seen with caution. Although the identification of ALK rearrangement is used for therapy in non-small cell lung carcinoma, there is limited evidence for the identification of ALK alterations in OC, only described in few reports and not by the TCGA [4, 16, 35].

In summary, our results show that, particularly, PD-L1 positivity and to a lesser extent HER2 amplification might provide an approach to therapeutic targets in the treatment of OC, which need to be validated in clinical trials. ALK/EML rearrangement is very rare and might, therefore, only be a putative target for selective cases. In addition, the PD-L1 status seems to be an independent positive prognosticator for OC. We are aware that the effort to find a therapeutic target for ovarian cancer needs to be based on solid findings and a proven and demonstrable rationale.

Author's contributions Doris Mayr designed the study. Elisa Schmoeckel designed the study and wrote the first draft of the manuscript. Elisa Schmoeckel, Sophie Hofmann, Daniel Fromberger, and Beate Luthardt carried out the research work and analyzed the data. Miriam Rottmann supported the statistical analyses. Doris Mayr, Alexander Burges, Udo Jeschke, Miriam Rottmann, Thomas Kirchner, and Sigurd F. Lax reviewed and edited the manuscript. All authors gave final approval for publication.

Elisa Schmoeckel takes full responsibility for the work as a whole, including the study design, access to data, and the decision to submit and publish the manuscript.

Compliance with ethical standards All patients' data were fully anonymized, and the study was performed according to the standards set in the Declaration of Helsinki 1975. The current study was approved in writing by the Ethics Committee of the Ludwig-Maximilians-University, Munich, Germany (approval number 18-130).

Conflict of interest There are no business relationships that might lead to a conflict of interest.

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Original article

SATB2 is a supportive marker for the differentiation of a primary mucinous tumor of the ovary and an ovarian metastasis of a low-grade appendiceal mucinous neoplasm (LAMN): A series of seven cases

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ABSTRACT

The differentiation between a primary mucinous ovarian neoplasm and an extra-ovarian metastasis in the ovary is often challenging in the histopathologic practice. Among various ovarian metastases from the gastro-intestinal tract the low grade appendiceal mucinous neoplasm (LAMN) is an important differential diagnosis to consider particularly in case of pseudomyxoma peritonei. A newly recognized marker in the routine diagnostic of a mucinous neoplasm in the ovary is SATB2 (Special AT-rich sequence-binding protein 2). The expression of SATB2 is, within cells of epithelial lineages, mainly restricted to the lower gastro-intestinal tract, indicating colorectal or appendiceal cancer origin. We report seven cases of LAMN, which clinically became apparent due to ovarian metastases in context of pseudomyxoma peritonei or at least small foci of peritoneal tumor spread. An immunohistochemical marker-panel including SATB2, CDX2, CK20, CK7, PAX8, ER and PR revealed a strong expression of SATB2 in all seven cases. On the contrary SATB2-negativity could be demonstrated in the 40 cases of mucinous borderline tumors and primary mucinous carcinomas of the ovary. The histopathologic tentative diagnosis of an ovarian metastasis of LAMN could be confirmed in the findings of the Appendix in six of seven cases. This report supports SATB2 as an additional diagnostic marker for the diagnosis of an ovarian manifestation of LAMN.

1. Introduction

The most important differential diagnosis of a mucinous borderline tumor or a primary mucinous carcinoma of the ovary is a metastatic extra-ovarian mucinous carcinoma. The whole amount of secondary ovarian malignancies of metastatic origin is ranging between 3 and 15% in western countries [4,5,15]. Among various ovarian metastases of the gastro-intestinal tract metastases from the Appendix are rare and represent only 1–2% [12]. Nevertheless in case of pseudomyxoma peritonei the low-grade appendiceal mucinous neoplasm (LAMN) is an important differential diagnosis to consider.

Frequently ovarian metastases of a low-grade mucinous carcinoma present clinically as a cystic lesion mimicking a mucinous borderline tumor or an ovarian mucinous carcinoma. Regarding the histopathologic practice the classification of a mucinous ovarian tumor can be very challenging too [9,13,18]. Immunohistochemistry can help to distinguish primary from secondary ovarian tumors, however immunostains are less useful in mucinous neoplasm. Due to an overlap in expression patterns immunohistochemical marker panels encompassing CDX2, CK20 and CK7 are not able exclude an ovarian metastasis of gastro-intestinal tumor origin by certain [13].

A newly recognized sensitive marker for colorectal adenocarcinomas is SATB2 (Special AT-rich sequence-binding protein 2). SATB2 is a DNA-binding protein and takes part in chromatin remodeling and gene regulation, functioning as a nuclear transcription factor. Among others the physiological role of SATB2 involves growth and skeletal development. In particular SATB2 is important in the differentiation of cortical neurons and osteoblasts [2,6]. Therefore SATB2-deficiency is implicated in some syndromic and non-syndromic disorders of skeletal development, especially of orofacial clefts [1,8]. At present the role for SATB2 in the normal development of the colon or Appendix remains to be identified. However, immunohistochemical analyses revealed SATB2-positivity not only in osteoblasts and cortical neurons [3,21], but also in cells of epithelial lineage. Interestingly within cells of epithelial lineages SATB2-immunoreactivity is mainly restricted to the lower gastrointestinal [7,11]. Furthermore, recently several studies demonstrated a strong expression of SATB2 in the majority of colorectal and appendiceal carcinomas [10,14,17,25]. Therefore SATB2 functions as a useful marker in the routine diagnostic for the differentiation of an intestinal carcinoma of the lower gastro-intestinal tract,

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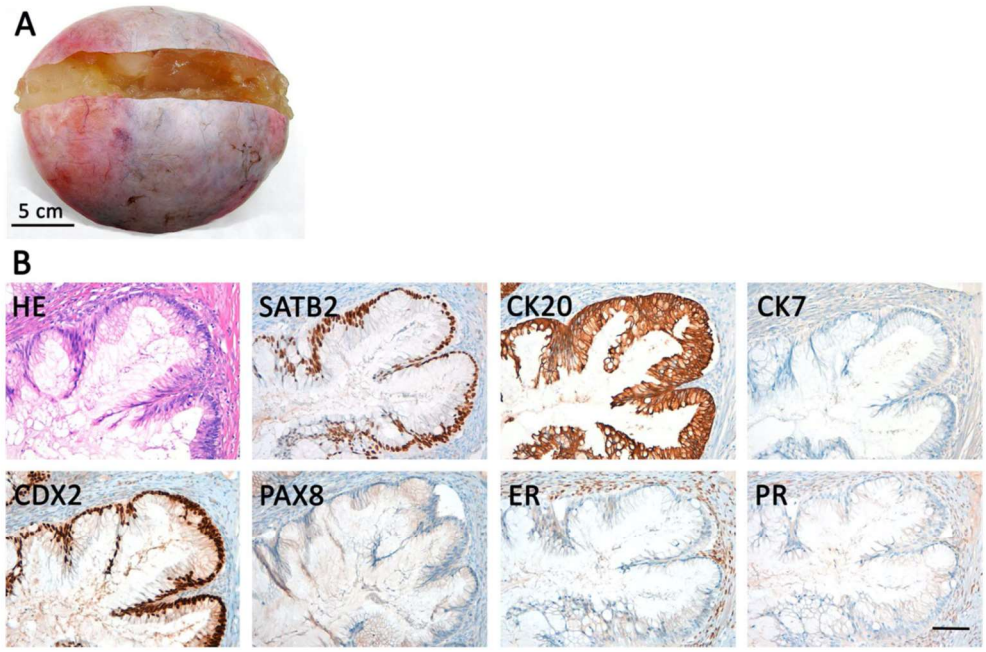

Fig. 1. An example if a cystic ovarian metastasis of a LAMN (case 1). Macroscopy of an unilocular, cystic and mucous-rich ovarian metastasis of a LAMN (A). Immunohistochemistry, showing SATB2-positivity and a typical expression profile of CDX2, CK20, CK7, PAX8, ER and PR; 200× magnification for all pictures, scale bar refers to 100 µm (B).

principally of the colon and the appendix, from other types of carcinomas [7,11]. SATB2 is not expressed by normal ovarian epithelium [17]. However occasionally ovarian tumors may express SATB2 [14,17], although SATB2-positivity is very rare in mucinous ovarian tumors [20].

Here we present seven cases of LAMN, which clinically became apparent due to ovarian metastases. In all cases the diagnosis of an ovarian manifestation of a LAMN was strongly supported by the positivity for SATB2.

2. Materials and methods

2.1. Study groups

The study group encompassed seven cases of LAMN, diagnosed between January and August 2017, with dominant ovarian metastases in context of pseudomyxoma peritonei or at least small foci of peritoneal tumor spread. Patient's age at diagnosis ranged from 50 to 75 years, with an average of 60 years. Four cases of mucinous neoplasms in the ovary (case 2, 3, 4 and 7) were sent to our department for a second consultation from external pathologists. The other three cases (1, 5 and 6) referred to our institute. In one case (case 4) the Appendix was not available for histopathologic diagnostic in our department. However, after consulting the external pathologists we were informed that the diagnosis of LAMN could be confirmed in the appendectomy-specimen of this patient. In summary the diagnosis of LAMN was verified in the findings of Appendix in six cases. In one case (case 7) the patient's follow-up was lost after the ovarectomy, because the after-treatment was not continued in the initial clinic.

40 cases of mucinous borderline tumors and mucinous ovarian carcinomas served as controls.

2.2. Immunohistochemical analysis

Analyzed was an immunohistochemical staining panel including SATB2 (Cell Marque, clone EP281, dilution 1:500), CDX2 (Medac, clone EPR2764y, dilution 1:50), CK20 (Medac, clone KS20.8, dilution 1:500), CK7 (Medac, clone OV-TL12/30, dilution 1:500), ER (Ventana, clone SP1, dilution ready to use), PR (Ventana, clone 1E2, dilution, ready to use) and PAX8 (Cell Marque, clone MRQ-50, dilution ready to use). All stains were prepared by using a Ventana Benchmark XT autostainer (Ventana Medical Systems, Oro Valley, AZ, USA). The immunohistochemical stainings were performed on the ovarian tumor in all cases and additionally on the appendiceal tumor in case 1, 2, 4, 6 and 7. The group of mucinous borderline tumors and mucinous ovarian carcinomas was analyzed for the whole marker panel.

3. Results

3.1. Clinical data histopathologic characteristics

We investigated seven cases of LAMN with dominant ovarian metastases. Ovarectomy was done in all cases. Simultaneous appendectomy was done in four cases (case 1, 2, 3 and 5), and in one case (case 4) in a secondary operation. Case 6 presented a recurrent setting of LAMN with dominant ovarian metastases, which was diagnosed in 2015 (at that time: pT3, G1, pN0 (0/9), R0). The ovarian metastases presented in all cases as a cystic tumor ranging from 2.0 to 21.5 cm in diameter (case example in Fig. 1A). In two cases (case 1 and 6) both ovaries were affected and in another two cases (case 2 and 4) only the left ovary. Macroscopically five cases (case 1, 2, 4, 5 and 6) presented a cystic swelling of the Appendix in terms of a mucocele (case example in Fig. 2A). In case 3 the Appendix was adherent to ovary and therefore not evident on primary surgery. However detailed histopathologic

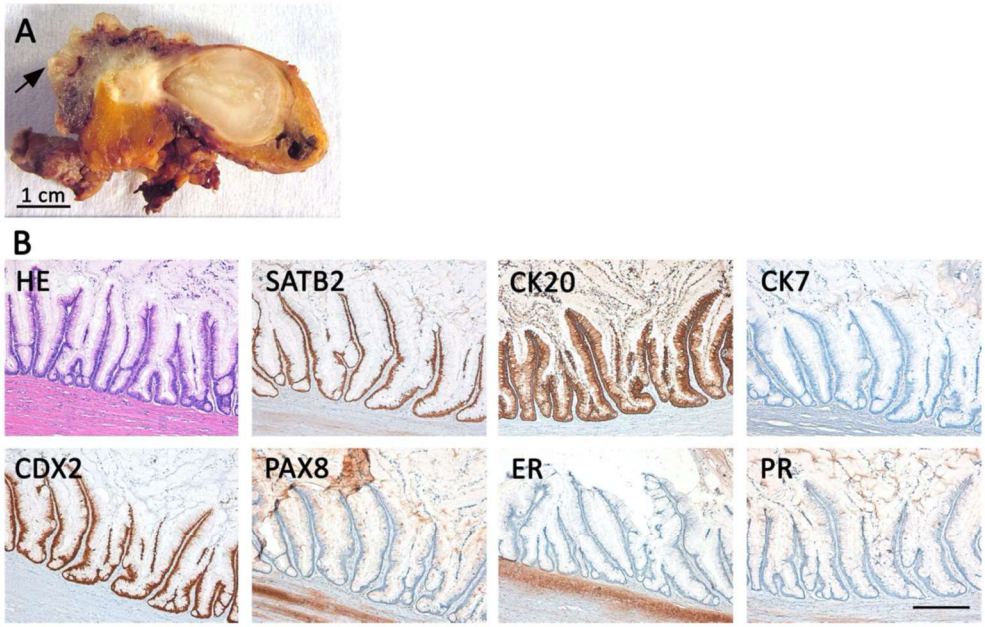

Fig. 2. An example of a LAMN in the appendix (case 5). Macroscopy of a mucocelen-like swollen Appendix and associated perforation (arrow) with mucous on the peritoneal surface (A). Immunohistochemistry, showing SATB2-positivity and a typical expression profile of CDX2, CK20, CK7, PAX8, ER and PR; 100× magnification for all pictures, scale bar refers to 300 µm (B).

Table 1
Clinical and histopathologic characteristics of each case and data of the referring TNM-classification.

Case	Age	Appendix	Ovary		Pseudomyxoma peritonei	TNM-classification
			Side (right/left)	max. diameter		
1	50	Mucocele, perforation	Right and left	3,4 cm (right), 21,5 cm (left)	Yes	pT4a, pNX, pM1b (PER), IVA
2	58	Mucocele, perforation	Left	7 cm	Yes	pT4a, pNX, pM1b (PER), IVC
3	63	Adhesion to the right ovary	Right	13 cm	Yes	pT4b, pNX, pM1b (PER), IVA
4	70	Mucocele, perforation	Left	20 cm	No	pT4a, pNX, pM1b (PER), IVA
5	52	Mucocele, perforation	Right	2 cm	Yes	pT4a, pNX, pM1b (PER), IVA
6	52	Initial Mucocele without perforation (pT3)	Right and left	14,5 cm (right), 19 cm (left)	Yes	Recurrence: rpT0, rpN0 (0/4), rpM1b (PER), IVA
7	75	No data	Right	6.5 cm	No data	(pTX, pNX, pM1b (PER), IVA)

Table 2
Immunohistochemical findings (+ = positive staining, − = negative staining).

Case	SATB2	CDX2	CK20	CK7	PAX8	ER and PR
1	+	+	+	−	−	−
2	+	+	+	−	−	−
3	+	+	+	−	−	+ (focally, weak)
4	+	+	+	−	−	−
5	+	+	+	−	−	−
6	+	+	+	+ (focally)	−	−
7	+	+	+	−	−	−

examinations could discover small remnants of the Appendix in the surgical specimen of this case, which confirmed the presence of LAMN. In six cases pseudomyxoma peritonei was visible on surgery and confirmed by peritoneal biopsies. In one case (case 2) acellular mucous could be found only histologically on the peritoneal surface of the Appendix and the ovary (Table 1).

3.2. Immunohistochemical findings

In all cases of LAMN a strong nuclear expression of SATB2 could be demonstrated. CDX2 and CK7 were consistently expressed in all cases. CK7 was completely negative in six cases (case 1–5 and 7), whereas one case (case 6) showed focally an expression of CK7. All cases were PAX8 negative. Estrogen- and progesterone-receptor were focally positive in one case (case 3) with a very weak intensity and completely absent in other cases. The immunohistochemical findings of the appendiceal and the ovarian tumor in case 1 − 3, 5 and 6 were identical. The immunohistochemical findings are summarized in Table 2. Figs. 1 and 2B show exemplarily the immunohistochemistry in an ovarian and an appendiceal manifestation of LAMN.

All cases of the control group encompassing mucinous borderline tumors and mucinous ovarian carcinomas were negative for SATB2.

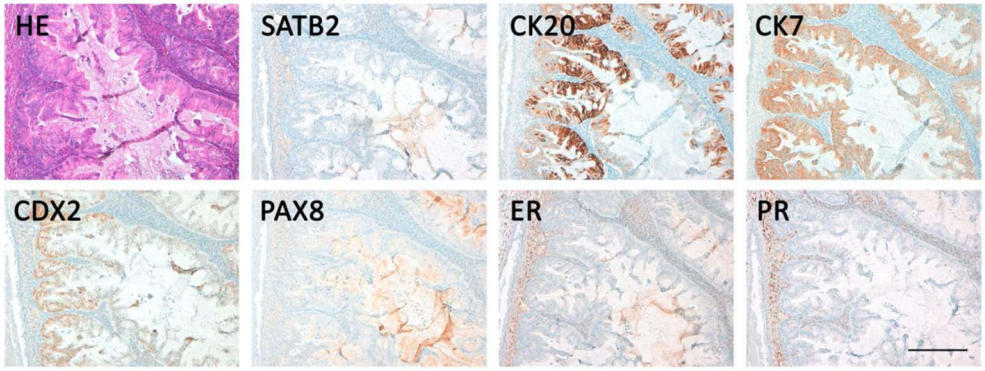

Fig. 3. An example of a primary mucinous ovarian carcinoma. Immunohistochemistry, showing SATB2-negativity and a typical expression profile of CDX2, CK20, CK7, PAX8, ER and PR; 100× magnification for all pictures, scale bar refers to 300 µm.

There was a positive immunostaining for PAX8 in 47%, for CDX2 in 92%, for CK7 in 100%, for CK20 in 85%, for ER in 5% and PR in 5%. Fig. 3 shows exemplarily the immunohistochemical findings in a case of mucinous ovarian carcinoma.

4. Discussion

This case series shows that a dominant ovarian metastasis in progressed LAMN can mimic a primary tumor of the ovary. Therefore the clinical presentation often results in a gynecologic surgery at first [19]. On the other hand pathologists are regularly confronted with missing clinical information about earlier surgical interventions or previous appendectomy that often complicates the correct diagnosis. In addition the primary appendiceal tumor cannot be found at surgery in some cases of advanced tumor stages or might be overlooked. However, concerning the different therapy-strategy and the patienťs prognosis of a mucinous borderline tumor or carcinoma of the ovary and an ovarian manifestation of LAMN is of high importance. The current therapy-management for LAMN is a radical surgery, often followed by a hypertherm intraperitoneal chemoperfusion (HIPEC).

Not only the clinical presentation but also the histopathological diagnosis of an ovarian metastasis of a LAMN is challenging in the majority of cases. Frequently the LAMN appears as a cystic tumor in the ovary mimicking a mucinous borderline tumor or mucinous carcinoma of the ovary. Histopathological characteristics like bilaterality and a mucus-rich, poorly cellular, low-grade adenocarcinoma as well as the $CK20^{+}$ $CK7^{-}$ $CDX2^{+}$ immuno-phenotype favors an ovarian manifestation of LAMN [16]. Nevertheless macroscopic and histopathologic features are not distinct differentiating factors and can occur in both entities. As it is known pseudomyxoma peritonei can arise in settings of an ovarian or an appendiceal mucinous carcinoma.

Searching for the correct diagnosis of a mucinous neoplasm in the ovary immunohistochemical analyses are performed regularly to exclude or to locate an extra-ovarian primary tumor-origin. However due to an overlap expression of CDX2, CK20 and CK7 immunohistochemical stainings cannot exclude a metastasis of a primary gastro-intestinal tumor-origin by certain. Mucinous borderline tumors and mucinous carcinomas of the ovary are typically positive for CK7 and show a variable expression of CDX2, CK20 and PAX8 [23,24]. ER and PR are almost always negative in primary mucinous neoplasm of the ovary [22]. PAX8-positivity indicates ovarian tumor-origin. However PAX8 is not expressed in about 50–60% of primary ovarian mucinous tumors, thus PAX8-negativity does not exclude a primary mucinous tumor of the ovary [23,24]. In particular CDX2 is a helpful marker to confirm an intestinal tumor-differentiation. However CDX2 is not specific and primary ovarian mucinous tumors regularly express CDX2 [24]. Appendiceal adenocarcinomas express regularly CDX2 and CK20, but might be positive for CK7 too [16]. All cases analyzed in this case-series were positive for CDX2 and CK20 and only one out of the seven cases presented a focal CK7-expression.

The recently described SATB2 was shown to be expressed in the epithelium of the lower gastro-intestinal-tract, principally in the colon and the appendix [11]. In contrast to CDX2 SATB2 is not only an indicator for an intestinal differentiation but is also highly specific for a tumor-origin of the lower gastro-intestinal-tract. Consistent with previous studies a strong expression of SATB2 was evident throughout this case series [14,17]. By now there is no study demonstrating a SATB2-negative cases of LAMN.

All cases of our control group of mucinous borderline tumors and primary mucinous carcinomas of the ovary were SATB2-negative. Previous studies demonstrated that mucinous-type ovarian tumors may rarely express SATB2, although SATB2-positive primary ovarian mucinous are largely restricted to an associated teratoma [14,17,20]. On the contrary SATB2-negativity is not synonymous for a primary ovarian neoplasm. In the setting of a SATB2-negative mucinous tumor in the ovary metastasis of the upper gastro-intestinal-tract, the pancreatico-biliar-system and of an endocervical or uterine tumor origin should be considered. Furthermore, concerning the situation of a metastasized non-ovarian intestinal mucinous neoplasm SATB2 can help to differentiate carcinomas of the lower gastro-intestinal tract from other intestinal origins even when metastasized to the ovaries.

In conclusion, this case series strongly supports SATB2 as an additional diagnostic marker that can help to indicate an ovarian manifestation of a LAMN in case of an unknown mucinous, low-grade neoplasm in the ovary.

Conflict of interests

None.

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