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Bedeutung von Hormonrezeptoren und potenziellen Co-Faktoren in Gynäkologischen Malignomen sowie deren Vorstufen

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Abkürzungsverzeichnis

Akt	Serine/Threonine-Specific Kinase
BRAF	Proto-Oncogene B-Raf oder v-Raf Murine Sarcoma Viral Oncogene Homolog B1
CIN	Cervical Intraepithelial Lesions
cAMP	Zyklisches Adenosinmonophosphat
Dkk2	Dickkopf2
EGFR	Epidermal Growth Factor Receptor
EOC	Epithelial Ovarian Cancer
EP2/ 3	Prostaglandin 2/3 Rezeptor
ER α / β	Nuclear Estrogen Receptor alpha/ beta
GPOR	G Protein-Coupled Estrogen Receptor
HGSC	High-Grade Serous Carcinoma
HSIL	High Grade Squamous Intraepithelial Lesions
IRS	Immune-Reactivity Score
KRAS	Kirsten Rat Sarcoma Virus Proto-Oncogene
LEF	Lymphoid Enhancer Factor
LGSC	Low-Grade Serous Carcinoma
LHCRG	Luteinizing Hormone/Choriogonadotropin Receptor
LRP5/6	Low-Density-Lipoprotein-Related Protein 5/6
LSIL	Low Grade Squamous Intraepithelial Lesions
NCOR	Nuclear Co-Repressor
OS	Overall Survival
PFS	Progression Free Survival
PI3K/CA	Phosphatidylinositol-4,5-Bisphosphat-3-Kinase/ katalytische Untereinheit alpha
PLD	Pegyliertes liposomales Doxorubicin
SIL	Squamous Intraepithelial Lesion
TCF	T-Cell Factor
TMA	Tissue Microarrays

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Publikationsliste

Publikation I

Patricia Fraungruber, Till Kaltofen, Sabine Heublein, Christina Kuhn, Doris Mayr, Alexander Burges, Sven Mahner, Philipp Rathert, Udo Jeschke, Fabian Trillsch

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Publikation II

Juliane Reichenbach, Patricia Fraungruber, Doris Mayr, Christina Buschmann, Fabian B. T. Kraus, Nicole Elisabeth Topalov, Anca Chelariu-Raicu, Thomas Kolben, Alexander Burges, Sven Mahner, Mirjana Kessler, Udo Jeschke, Bastian Czogalla, Fabian Trillsch

Nuclear receptor co-repressor NCOR2 and its relation to GPER with prognostic impact in ovarian cancer

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Publikation III

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The role of EP-2 receptor expression in cervical intraepithelial neoplasia.

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1. Einleitung

1.1 Übergeordnetes Forschungsziel der Veröffentlichungen

Der verbindende vorrangige Aspekt dieser kumulativen Promotion war das Bestreben, molekulare Mechanismen im Signalweg der Hormonrezeptoren bei gynäkologischen Tumoren zu finden und geeignete Biomarker für den klinischen Verlauf zu identifizieren. Relevante Fragestellungen der Forschung in diesem Bereich sind dabei die mögliche Beteiligung dieser Proteine an der Karzinomentstehung sowie dem Krankheitsverlauf und dem Ansprechen auf onkologische Therapien. Darüber hinaus sollen molekulare Mechanismen der Resistenzentstehung gegenüber Chemotherapeutika und neue zielgerichtete Therapieansätze identifiziert werden.

Im Rahmen des vorliegenden Promotionsprojektes wurden dabei neben Zervixdysplasien ein Kollektiv primärer Ovarialkarzinome immunhistochemisch untersucht und mit verschiedenen Fragestellungen ausgewertet.

Bei den Färbungen von Gewebeschnitten der zervikalen intraepithelialen Neoplasien (CIN) ging es vorwiegend um die Dynamik der Rezeptorexpression in den verschiedenen Stadien der Dysplasie und somit der Karzinomentstehung.

Hierbei könnten geeignete Biomarker einen entscheidenden Einfluss auf die Therapieentscheidung haben. Vor allem die Unterscheidung zwischen niedriggradigen Dysplasien (CIN I, Low Grade Squamous Intraepithelial Lesions (LSIL), PAP IIID1) und höhergradigen Dysplasien (CIN II - CIN III, High Grade Squamous Intraepithelial Lesions (HSIL), PAP IIID2) kann therapieentscheidend und gegebenenfalls folgenschwer sein, besonders für Frauen im gebärfähigen Alter mit Kinderwunsch (1).

Bei Ovarialkarzinomen sind es die hohe Letalität und die fehlenden Methoden zur Früherkennung, welche die Suche nach geeigneten prädiktiven, sowie prognostischen Biomarkern notwendig machen (2). Hier lag der Schwerpunkt der Arbeit auf dem Einfluss der Biomarker auf die klinische Prognose der Patientinnen, gemessen an klinischen Parametern und dem potenziellen therapeutischen Nutzen der Rezeptoren als Zielstrukturen für spezifische Therapieansätze.

1.2 Zervixdysplasien

1.2.1 Epidemiologie

Ab der Implementierung der gynäkologischen Vorsorgeuntersuchung 1971 in Deutschland bis zum Jahr 2000 sanken die Inzidenz- sowie Mortalitätsraten des Zervixkarzinoms stark (3), seither stagnieren die Zahlen (4). Im Jahr 2018 gab es in Deutschland 4320 Neuerkrankungen (5). Weltweit stellt das Zervixkarzinom noch immer die vierthäufigste Krebserkrankung bei Frauen dar (7).

Die Neuerkrankungsrate des Zervixkarzinoms ist zwischen dem 35. und 44. Lebensjahr am höchsten (5). Einer amerikanischen Studie zufolge sind die Inzidenzraten für Zervixdysplasien bei noch jüngeren Frauen besonders hoch. Bei Frauen zwischen 20 bis 24 Jahren belief sich die Inzidenz von CIN I auf 5,1 pro 1.000 Frauen pro Jahr, während die Inzidenz über alle Altersgruppen bei 1, 2 pro 1.000 Frauen liegt. Bei 25 bis 29-jährigen Frauen wurden die höchsten Inzidenzraten für CIN II und CIN III gefunden (8).

Dieser Aspekt des jungen Alters ist von besonderer Bedeutung bei der Abwägung der Behandlungsmöglichkeiten der Karzinomvorstufen sowie des Zervixkarzinoms bei Frauen mit Kinderwunsch. Hinweisende, nicht-invasive Marker für den Verlauf von CINs (Progress/ Regress) fehlen derzeit (9).

CIN I haben generell eine sehr hohe Spontanremissionsrate von 70-90 % innerhalb von zwei Jahren (10-12). Erst wenn die Läsion über zwei Jahren besteht, erfordert das klinische Management einen Wechsel von einer beobachtenden Haltung zu einer chirurgischen Behandlung (13).

Wie bei allen Screeninguntersuchungen gilt es auch bei der Gebärmutterhalskrebsvorsorge abzuwägen zwischen dem potenziellen Schaden und dem Nutzen der Maßnahmen. Die neue Einteilung der Zervixdysplasien nach der WHO in LSIL / HSIL wurde sehr kontrovers diskutiert und die Implementierung von US-amerikanischen Wissenschaftlern forciert. Sie favorisieren ein früheres operatives Vorgehen bei CIN II (HSIL) (14). Der Vergleich der relativen 5-Jahres-Überlebensrate für das Zervixkarzinom zwischen Deutschland (65-69 %) und den USA (72,5 %) kann diesen radikaleren Ansatz möglicherweise unterstützen (15).

Von Bedeutung ist allerdings auch, dass die Infektion mit den Hochrisiko-Typen 16 und 18 des Humanen Papillomavirus (HPV) in kausalem Zusammenhang mit der Entstehung des Zervixkarzinoms sowie dessen Vorstufen steht (1). In Deutschland wurde sich darauf geeinigt, die bisherige Einteilung in CIN I-III beizubehalten (14). Ausschlaggebend dafür waren zum einen die hohen

Ausheilungsraten von HPV-Infektionen im Allgemeinen. 80–90 % heilen innerhalb von zwei Jahren aus (16). Zum anderen weisen CIN I und CIN II häufig eine spontane Remission im Verlauf auf (10-12). Dem gegenüber stehen die möglichen Komplikationen einer Übertherapie (14).

1.2.2 Diagnostische Verfahren

Die Verlässlichkeit der diagnostischen Tests ist vor allem bei höhergradigen Dysplasiegraden entscheidend und vor insbesondere bei jungen Frauen mit potenziellem Kinderwunsch relevant (1).

Die angewandten Verfahren zur Sekundärprävention des Zervixkarzinoms sind der zytologische Abstrich nach Papanicolaou (PAP), die HPV-Testung, die Bestimmung von p16/ Ki67, sowie die Kolposkopie (14).

Beim zytologischen Abstrich ist die Qualität der Präparate entscheidend, um Ergebnisse mit hoher Sensitivität und Spezifität zu gewährleisten. Die Rate an falsch-positiven Ergebnissen liegt bei 2-3 % und führt zu weiteren unnötigen diagnostischen sowie therapeutischen Schritten, die für die Patientinnen potenziell schädigend sein können (17). Aufgrund des langen Beobachtungszeitraums und der wiederholten Kontrolluntersuchungen stellen CIN auf mehreren Ebenen eine Belastung für betroffene Frauen dar (18).

Derzeit bekannte Biomarker (mRNA 5 HPV-Typen, p16/Ki-67, E6-Protein) zeigten sich in Querschnittsstudien im Vergleich zur konventionellen Zytologie überlegen, während sie jedoch verglichen mit Hochrisiko-HPV DNA Testverfahren keine Vorteile bieten (19, 20). Derzeit fehlen longitudinale Studien, weswegen diese Biomarker laut Leitlinien auch nicht als primäres Screening-Instrument verwendet werden sollen (1).

Die immunhistochemische Färbung für p16 alleine oder in Kombination mit Ki67 dient dem Zweck, tatsächlich HPV-induzierte Präkanzerosen von anderweitigen Läsionen, wie (post-) entzündlichen Veränderungen, Meta- oder Hyperplasien abzugrenzen. Sie ist derzeit diesem differentialdiagnostischen Zweck vorbehalten und hat noch keine Bedeutung für die Vorsorgemaßnahmen (1).

1.2.3 Primärprävention

Ein dringend notwendiger Ansatz neben den Vorsorgeuntersuchungen als Sekundärprävention ist die seit 2006 in Europa zugelassene HPV-Impfung als Primärprävention. In Deutschland sind seit 2007 der bivalente Impfstoff Cervarix ® gegen die HR-HPV-Typen 16, 18 und seit 2015 der nonavalente Impfstoff Gardasil ® 9 gegen HPV-Typen 6, 11, 16, 18, 31, 33, 45, 52, 58 zugelassen und

verfügbar. Die Ständige Impfkommission (STIKO) empfiehlt mittlerweile die HPV-Impfung sowohl für Mädchen als auch für Jungen im Alter von 9 bis 14 Jahren. Die Kosten werden von den Krankenkassen getragen. Um die maximale Schutzwirkung zu erreichen, soll die Impfung vor dem ersten Sexualkontakt erfolgen. Eine Nachholimpfung ist möglich, macht aber drei statt zwei Impfdosen erforderlich und zeigt eine reduzierte Wirksamkeit (1, 21, 22). Zwei große Studien aus Schweden (21) und England (23) zeigen, dass die Impfung das Zervixkarzinom sowie dessen Vorstufen verhindern kann. In Schweden verzeichnen 75,4% der Mädchen, die zwischen 2004 und 2005 geboren wurden, eine vollständige HPV-Impfung (24). Im Gegensatz dazu weisen in Deutschland nur 45,3% der Mädchen, die zwischen 2000 und 2003 geboren wurden, eine vollständige Immunisierung gegen HPV auf (25). Die HPV-Impfquote stagniert in Deutschland seit Jahren bei diesen knapp 50 %, was bedeutet, dass das präventive Potenzial der Impfung hierzulande bei weitem nicht ausgeschöpft wird (25). Der Sekundärprävention und Forschung kommt daher in diesem Bereich weiter eine hoch relevante Bedeutung zu.

1.2.4 Therapie und Komplikationen

Bei Persistenz bzw. Fortschreiten höhergradiger Dysplasien ist nach einem bestimmten Beobachtungszeitraum die operative Konisation Therapie der Wahl (1). Dies birgt bei den häufig jungen Patientinnen für mögliche darauffolgende Schwangerschaften ein erhöhtes Risiko für Komplikationen, wie eine Zervixinsuffizienz, oder eine Frühgeburt (26). Davon abgesehen kann eine Konisation weitere Komplikationen, wie primäre oder sekundäre Blutungen, Schmerzen, übelriechender Fluor und postoperative Zervixstenosen nach sich ziehen. Außerdem können Dysplasienareale zurückbleiben, was meist einen weiteren Eingriff notwendig macht (27).

Geeignete Biomarker könnten bei oben genannten Abwägungen bezüglich der Therapie von CINs wegweisend sein und damit die therapiebegleitende Morbidität verringern. Zudem bleibt die Grundlagenforschung zur Sekundärprävention des Zervixkarzinoms weiterhin notwendig, solange die Impfquote hierzulande so niedrig liegt (22, 25).

1.3 Ovarialkarzinome

1.3.1 Epidemiologie

Ovarialkarzinome sind die gynäkologischen Tumoren mit der höchsten Letalitätsrate (28). 2018 starben in Deutschland 5326 Frauen an einem Ovarialkarzinom und die Inzidenz betrug 7300 neue Fälle. Diese Zahlen in Zusammenschau mit einer relativen 5-Jahres Überlebenszeit von 43 % verdeutlichen die Dringlichkeit, geeignete Screeningmethoden, Therapieansätze und prognostische Marker für das klinische Outcome dieser malignen Erkrankung zu finden (29).

1.3.2 Histologische Subtypen

Derzeit werden Ovarialkarzinome (epithelial ovarian cancer, EOC) in fünf histologische Subtypen aufgeteilt, die durch unterschiedliche Mutationsereignisse entstehen und klinisch unterschiedlich verlaufen (30-33). Am Häufigsten sind seröse Adenokarzinome (> 70 %). Diese werden dichotom in die zwei Tumorentitäten, die high-grade serösen Tumoren (HGSC) und die low-grade serösen Tumoren (LGSC) unterteilt. HGSC weisen Mutationen im Tumorsuppressorgen p53 auf und sind durch einen klinisch aggressiven Verlauf charakterisiert. LGSC stehen mit stabilen Kirsten Rat Sarcoma Virus Proto-Oncogene (KRAS) und Proto-Oncogene B-Raf (BRAF) Mutationen in Zusammenhang und werden als langsam proliferierend gewertet (30). Muzinöse Tumoren (4 %) weisen ebenfalls oft KRAS und BRAF Mutationen auf. Durch das langsamere Wachstum werden diese Tumorentitäten häufig früher diagnostiziert. EOCs vom endometrioiden (15 %) und klarzelligen (10 %) Typ weisen Mutationen im AT-rich interactive domain-containing protein 1A und der Phosphatidylinositol-4,5-bisphosphat-3-Kinase (PIK3CA) auf. Sie können beide mit atypischer Endometriose assoziiert sein (29-33).

Obwohl sich sehr deutlich zeigt, dass sich die einzelnen Tumoren auf molekularer Ebene stark unterscheiden, werden diese Eigenschaften bei der Therapieplanung der EOCs noch nicht direkt berücksichtigt. Bisher fehlen aussagekräftige Studien, die ein Abweichen vom Therapiestandard rechtfertigen würden (2).

1.3.3 Screening

Geeignete Methoden zur Früherkennung von EOCs fehlen derzeit noch komplett. Zum einen ist die Inzidenz zu gering und zum anderen fehlen Möglichkeiten, relevant frühere Tumorstadien verlässlich zu detektieren, sodass sich bisher kein

Nutzen von Screeninguntersuchungen nachweisen lässt (34, 35). Weder für die Allgemeinbevölkerung, noch für Risikopopulationen ist ein Screening mit transvaginaler Ultraschalluntersuchung oder der Bestimmung von CA125 empfohlen, denn keines der beiden Verfahren führte in Studien zu einer Reduktion der Mortalität (2).

1.3.4 Therapie

Für die Festlegung des therapeutischen Vorgehens ist vor allem die Tumorausbreitung entscheidend, welche durch die FIGO Klassifikation erfasst wird (Tab.1). Über alle Tumorstadien hinaus kommt der operativen Therapie eine große Bedeutung zu, durch die der Krankheitsverlauf wesentlich beeinflusst werden kann.

Tabelle 1 Stadien der Ovarialkarzinome (FIGO 2014), modifiziert mit Therapieempfehlungen nach vgl. (2) Tabelle 17 S.65-66

FIGO	TNM	Tumorausdehnung	Therapie
I A-B	T1 a-b	Tumor auf die Ovarien oder Tuben beschränkt, Oberfläche tumorfrei, Kapsel/ Serosa intakt, negative Spülzytologie	Komplettes operatives Staging mittels Längsschnittlaparotomie inkl. Adnexektomie bds. und Hysterektomie + G1 Observanz + G3 adjuvant 6 Zyklen Carboplatin
I C 1-3	T1 c 1-3	Ein/ beide Ovarien oder Tuben befallen, zusätzlich C1 iatrogene Kapsel- / Serosaruptur C2 präoperative Kapsel- / Serosaruptur oder Befall der Oberfläche C3 maligne Zellen im Aszites oder in Spülzytologie	
II	T2	Ein/ beide Ovarien oder Tuben befallen mit Ausbreitung ins kleine Becken	Explorativlaparotomie zum Tumordebulking + postoperative Chemotherapie mit 6 Zyklen Carboplatin/Paclitaxel
IIA	T2a	Tumorausbreitung auf Uterus +/- Tuben, Ovarien	
IIB	T2b	Befall weiterer intraperitoneale Strukturen im Bereich des kleinen Beckens (Sigma, Rektum, Peritoneum)	

III A-C	T3 a-c und/ oder N1	Ein/ beide Ovarien oder Tuben befallen mit Ausbreitung außerhalb des kleinen Beckens +/- retroperitoneale LK-Metastasen	Explorativlaparotomie zum Tumordebulking + Erstlinien-Chemotherapie mit 6 Zyklen Carboplatin + Paclitaxel + Bevacizumab +/- PARP-Inhibitor (je nach BRCA/HRD-Status)
IV A-B	T1-3 N0/ 1 M1	Fernmetastasen (Leber, Milz etc.)	

1.3.4.1 Operative Therapie

Beim frühen EOC (FIGO I–IIA) erfolgt das operative Staging mittels Längsschnittlaparotomie. Im Rahmen dieser OP erfolgt neben der Entnahme von Biopsien und einer Peritonealzytologie, eine beidseitige Adnexektomie, eine Hysterektomie, mindestens eine infrakolische Omentektomie und gegebenenfalls eine Appendektomie (2). Zur Festlegung des Tumorstadiums und der weiteren Systemtherapie wird zusätzlich die systematische pelvine und paraaortale Lymphonodektomie durchgeführt (2).

Bei fortgeschrittenen EOCs (FIGO IIB–IV) kann durch eine makroskopische Komplettresektion die mediane Gesamtüberlebenszeit um bis zu 60 Monate verlängert werden. Kein anderer Faktor beeinflusst die Prognose so maßgeblich, wie das Erzielen der makroskopischen Tumorfreiheit (36). Zum Erreichen dieses operativen Ziels wird eine radikale Resektion mit zum Teil multiviszeralen Operationsschritten in Kauf genommen, um den deutlichen Überlebensvorteil zu erzielen und sogar einen kurativen Ansatz verfolgen zu können (37). Limitationen für die OP kann die Infiltration der Mesenterialwurzel oder der Leberpforte sein sowie ein ausgedehnter Befall des Dünndarms. Liegen diese Befunde vor, steht eine Reduktion der Tumorlast im Vordergrund, um z.B. eine Darmobstruktion zu verhindern (2).

1.3.4.2 Systemtherapie

Die Wirksamkeit einer neoadjuvanten Chemotherapie ist bisher nicht verlässlich nachgewiesen und wird daher explizit nicht empfohlen (2).

Bei frühen EOC profitieren alle Patientinnen hinsichtlich Overall Survival (OS) und Progression Free Survival (PFS) von einer adjuvanten platinhaltigen Chemotherapie (6 Zyklen Carboplatin-Monotherapie) mit der Ausnahme von Patientinnen mit einem FIGO IA low-grade Tumor (38).

Bei fortgeschrittenen EOCs (FIGO IIB–IV) soll laut Leitlinie eine Kombinationstherapie aus 6 Zyklen Carboplatin und Paclitaxel 175 mg/m² alle 3

Wochen erfolgen, sowie ab dem FIGO Stadium III eine Erhaltungstherapie mit Bevacizumab und/ oder Poly-ADP-Ribose-Polymerase (PARP)-Inhibitoren (2). Bevacizumab vermag, parallel zum Standardregime verabreicht, das PFS signifikant zu verlängern, ein Effekt auf das Gesamtüberleben konnte jedoch nicht gezeigt werden (39). Die PARP-Inhibitoren Olaparib und Niraparib können seit 2015 bzw. 2017 bei Patientinnen (FIGO-Stadien III und IV) unabhängig vom BRCA1/2-Mutationsstatus mit abgeschlossener platin-basierter Chemotherapie angewendet werden, wenn das Tumorwachstum auf die Chemotherapie angesprochen hatte. Olaparib und Bevacizumab können im Rahmen der Primärtherapie bei vorliegender BRCA1/2-Mutation oder positivem HRD-Status miteinander kombiniert werden (2).

Die Rezidivpopulation wird derzeit noch anhand des Ansprechens des Tumors auf die platinhaltige Chemotherapie eingeteilt. Von Platinresistenz wird gesprochen, wenn innerhalb von 6 Monaten nach Abschluss der platinhaltigen Erstlinien-Therapie ein Rezidiv auftritt. Bei platinsensiblen Tumoren dagegen ist das platinfreie Interfall definitionsgemäß größer als 6 Monate (2).

Bei platinresistenten Rezidivtumoren sind Monochemotherapien mit pegyliertem liposomalem Doxorubicin (PLD), Topotecan, Gemcitabin, oder Paclitaxel wöchentlich mögliche Optionen. Eine Kombinationstherapie bringt keinen zusätzlich Vorteil bei deutlich erhöhter Morbidität (2).

Bei platinsensiblen Rezidiven werden platinhaltige Kombinationstherapien empfohlen, bei denen Carboplatin mit Gemcitabin, PLD oder erneut Paclitaxel kombiniert werden. Falls zuvor keine anti-angiogene Therapie verabreicht wurde, kann die Kombinationstherapie auch um Bevacizumab ergänzt werden oder nach Therapieansprechen ein PARP-Inhibitor verabreicht werden.

1.4 G-Protein-gekoppelte Rezeptoren

1.4.1 Prostaglandinrezeptoren

Der Prostaglandin E2 Rezeptor (EP2) hat sieben Transmembrandomänen und ist an einen heterotrimeren G-Proteinkomplex gebunden. Dieser enthält $G\alpha$ - und $G\beta/\gamma$ -Untereinheiten. Physiologischerweise wird er von Prostaglandin E2 aktiviert (40).

Die Aktivierung der $G\alpha$ Subunit bewirkt einen Anstieg des zyklischen Adenosinmonophosphats (cAMP) und in weiterer Folge die Aktivierung der Proteinkinase A, welche darauffolgende Transkriptionsfaktoren, wie cAMP response element-binding protein beeinflusst (41). Wenn die $G\alpha$ -Untereinheiten

gebunden ist, können freie $G\beta/\gamma$ -Untereinheiten über die Aktivierung des Phosphatidylinositol-4,5-Bisphosphat-3-Kinase (PI3K) / serine/threonine-specific kinase (Akt)-Signalwegs zur Phosphorylierung und somit Inaktivierung der Glykogen-Synthase-Kinase-3 β führen. Dies wiederum bewirkt die Akkumulation von β -catenin welches über T-cell factor (TCF) / lymphoid enhancer factor (LEF) die Transkription von Genen wie c-myc, cyclin d1 und Vascular Endothelial Growth Factor bewirken kann (42).

Zusätzlich zu den G-Protein-gekoppelten Signalwegen, kann EP2 einen Komplex mit β -arrestin bilden und dadurch zahlreiche Signalkaskaden aktivieren (PI3K, Akt, Tyrosinkinase Src, Extracellular Signal-Related Kinase [Erk], c-Jun N-terminal Kinasen, Epidermal Growth Factor Receptor [EGFR]) (43). Insgesamt sind die Signalwege der Prostaglandinrezeptoren allgemein und der G-Protein-gekoppelten Rezeptoren im Speziellen hoch komplex und interagieren mit zahlreichen anderen zellulären Signalwegen (44).

Ein möglicher Einfluss der Signalkaskaden von Prostaglandinrezeptoren auf das Wachstum von Tumorzellen wurde unter anderem für das Kolon-, Mamma- und Zervixkarzinom untersucht (45). Die Signalwege des EP2-Rezeptors tragen einerseits zur Zellproliferation, Apoptose und Angiogenese bei, andererseits können sie eine Immunsuppression bewirken (44). Die Immunsystem supprimierende Komponente scheint in Hinblick auf die Entstehung von CINs und Zervixkarzinomen interessant, deren Hauptrisikofaktor die Persistenz und Ausbreitung von HPV ist (46). Eine HPV-Infektion kann nur persistieren, wenn das Immunsystem das Virus nicht eliminieren kann (47).

1.4.2 GPER

Der G-Protein-gekoppelte Östrogenrezeptor (GPER) wird im menschlichen Körper ubiquitär exprimiert, seine Funktionen sind zahlreich (48). Anders als die nukleären Östrogenrezeptoren $ER\alpha$ und $ER\beta$, die vorwiegend als Transkriptionsfaktoren wirken, wird die Wirkung des GPER durch eine G-Protein-gekoppelte Untereinheit vermittelt (49). Die Lokalisation des GPER ist Gegenstand der Forschung, denn die Rezeptoraktivität lässt sich weder klar auf die Plasmamembran, noch auf intrazelluläre Kompartimente (z.B. Golgi Apparat, Endoplasmatisches Retikulum, Mitochondrien) begrenzen (48, 50, 51). Die Verteilung des Rezeptors scheint in Abhängigkeit von Zelltyp, Geschlecht und Gewebe zu variieren (48).

Die Bindung eines Liganden an den GPER führt primär zur Aktivierung schneller Signalkaskaden, indirekt kann aber auch die Gentranskription beeinflusst werden (52). Die Aktivierung der unmittelbaren Signalkaskaden über den GPER kann

zum intrazellulären Anstieg von cAMP führen (49), die Aktivierung der Extracellular Signal-Related Kinase 1/ 2 (53) sowie von PI3K bewirken und die Freisetzung von Ca^{2+} zur Folge haben (50). Die daraus resultierende Induktion von Matrix-Metalloproteinasen beeinflusst wiederum den EFGR (54). Die beschriebenen Beeinflussungen von Signalwegen stammen von in vitro Experimenten an unterschiedlichen Zell- und Gewebetypen (48). Welche patho- / physiologischen Funktionen der GPER im Menschen in vivo hat, ist aktuell noch Gegenstand der Forschung.

Eine Arbeit unserer Forschungsabteilung gab Hinweise, dass die zytoplasmatische Expression von GPER in frühen Stadien (FIGO I) des Zervixkarzinoms mit einem längeren OS und PFS assoziiert ist. Der genaue Mechanismus blieb allerdings unklar (55). Die gegenseitige Beeinflussung von GPER und den Gonadotropinrezeptoren für LH/ Choriogonadotropin (LHCGR) und FSH in EOCs war Gegenstand zweier Forschungsarbeiten an unterschiedlichen Tumorentitäten. Granulosazelltumoren des Ovars mit starker GPER Expression zeigten ein signifikant vermindertes OS. Dies wurde darauf zurückgeführt, dass der GPER sowohl von Östrogenen als auch Gonadotropinen reguliert wird. Der Östrogenspiegel ist bei Granulosazelltumoren in der Regel hoch und der Gonadotropinspiegel durch den negativen Feedback-Mechanismus niedrig. Die Rezeptorkonstellation könnte somit ein Faktor bei der Entscheidung für oder gegen die Anwendung einer Hormontherapie sein (56). EOC Patientinnen mit LHCGR / FSHR negativen, aber GPER positiven Tumoren wiesen ein verlängertes OS auf. Die medikamentöse Aktivierung des GPER mit G1 verminderte in diesen Fällen die Tumorzellproliferation (57). Neuesten Erkenntnissen zufolge reguliert der GPER die epigenetische Modifikation des Histon H3-Komplex und hemmt über diesen Mechanismus die Zellproliferation in EOCs, was sich positiv auf das OS auswirkt (58). In der Arbeit „G Protein-Coupled Estrogen Receptor Correlates With Dkk2 Expression and Has Prognostic Impact in Ovarian Cancer Patients“ ging es um die Erforschung möglicher Zusammenhänge zwischen den Signalwegen des GPER und des wnt-Modulators Dickkopf2 (Dkk2). Patientinnen, deren EOCs sowohl Dkk2, als auch GPER stark exprimierten, zeigten hier ein signifikant längeres OS (59).

1.5 Dkk2

Dkk2 ist ein Mitglied der Dickkopf-Proteinfamilie, das als Modulator des Wnt-Signalwegs fungiert. Der Wnt-Signalweg ist ein evolutionär konservierter Signaltransduktionsweg, der bei der Regulation von Zellproliferation, Differenzierung, Zellmigration und Zelladhäsion eine entscheidende Rolle spielt.

Dickkopf-Proteine sind spezielle Modulatoren des Wnt-Signalwegs und haben signifikante Auswirkungen auf die zelluläre Differenzierung und Entwicklung (60). Wnt-Glykoproteine entfalten ihre Funktion über die Bindung an Frizzled-Rezeptoren und deren Co-Rezeptoren, wie z.B. Low-density-lipoprotein-related protein 5/6 (LRP 5/6) (61). Die Affinität von Dkk zu LRP5/6 ist höher, als die von wnt (62). Abhängig vom zellulären Kontext und den vorhandenen Ko-Faktoren wie z.B. Kremen2 scheint Dkk2 sowohl als Agonist als auch als Antagonist auf Wnt/LRP6 wirken zu können (63-65). Gemäß Forschungsergebnissen von Zhu et al. beeinflusst Dkk2 die Wnt Signalkaskaden bei Ovarialkarzinomen inhibitorisch (66). Dies ist kohärent zu unserer Arbeit, bei der sich ein signifikant längeres OS der Frauen mit EOCs zeigt, die eine hohe Expression von Dkk2 und GPER aufweisen (59).

1.6 NCOR2

Nuclear receptor corepressor 2 (NCOR2) ist ein Proteinkomplex, welcher die Gentranskription über Histonmodifikationen beeinflussen scheint. Wie der Name „corepressor“ suggeriert, hemmt NCOR2 die Gentranskription überwiegend (67). NCOR2 interagiert dabei mit zahlreichen nukleären Rezeptoren, wie peroxisome proliferator-activated Rezeptoren und Steroidrezeptoren ($ER\alpha/\beta$, Progesteron- und Androgenrezeptoren) (68). Die Wirkweise von NCOR2 ist dabei kontextabhängig. Der NCOR2-Komplexe beeinflusst zahlreiche Gene und Signalwege, was sich in im Falle einer Dysfunktion durch die Begünstigung von Tumorwachstum bemerkbar machen kann (69). So scheint NCOR2 über eine Beeinflussung des $ER\alpha$ das Wachstum von Brustkrebs zu fördern (70). Zudem zeigten Gong et al. welchen Einfluss ein gestörter NCOR2-Komplex auf den Therapieerfolg mit dem $ER\alpha$ -Antagonisten Tamoxifen nehmen kann. So konnte durch die Bestimmung des NCOR2-Levels eine mögliche Resistenz gegen Tamoxifen prognostiziert werden (71). Tamoxifen wirkt unter anderem durch die Stabilisierung des Komplexes aus NCOR2 und $ER\alpha$ (72). In einer Gen-Array-Studien bei Patientinnen mit serösem Ovarialkarzinom korrelierte eine Überexpression von NCOR2 mit einer Platin-Chemotherapieresistenz (73). Im hier vorgelegten Paper zu NCOR2 hatte die Expressionshöhe von NCOR2 isoliert betrachtet keinen Einfluss auf das OS, wohingegen die Kaplan-Meier-Analyse in der Kombination mit einer hohen GPER Expression ein signifikant längeres OS zeigte. Die Ergebnisse weisen auf ein mögliches Zusammenspiel des Östrogenrezeptors GPER mit NCOR2 hin (74).

1.7 Beitrag zur Publikation I

In der Publikation “G Protein-Coupled Estrogen Receptor Correlates With Dkk2 Expression and Has Prognostic Impact in Ovarian Cancer Patients” (59) war die Autorin der Arbeit neben Fabian Trillsch, Udo Jeschke und Phillipp Rathert mitverantwortlich für die Konzipierung und das Design der Studie mit Identifizierung der Marker für die immunhistochemischen Untersuchungen.

Die TMAs der Gewebeproben wurden vom Institut der Pathologie der LMU München (Doris Mayr) zur Verfügung gestellt. Nach einer Einarbeitung im Labor durch Christina Kuhn (medizinisch-technische Assistentin) führte die Autorin die im Material und Methoden-Teil beschriebenen immunhistochemischen Färbungen der Gewebeschnitte eigenverantwortlich durch, wertete die Färbeergebnisse aus und erstellte eine Fotodokumentation.

Diese Arbeitsschritte sowie die statistische Verarbeitung der generierten Daten erfolgte durch Patricia Fraungruber unter Supervision von Udo Jeschke und Fabian Trillsch. Die Autorin war verantwortlich für die Erstellung des Manuskripts inklusive aller Tabellen und Abbildungen. Zudem war sie maßgeblich am Review-Prozess beteiligt.

1.8 Beitrag zur Publikation II

Patricia Fraungruber führte die immunhistochemischen Färbungen für die Publikation „Nuclear receptor co-repressor NCOR2 and its relation to GPER with prognostic impact in ovarian cancer“ durch. Auch die Auswertung der Färbeergebnisse und die Fotodokumentation oblagen der Autorin.

Die Datenanalyse erfolgte von Patricia Fraungruber gemeinsam mit Juliane Reichenbach unter der Supervision von Fabian Trillsch. Die anschließende Interpretation und Manuskripterstellung wurde von Patricia Fraungruber als Co-Autorin maßgeblich unterstützt.

1.9 Beitrag zur Publikation III

Bei der Publikation “The role of EP-2 receptor expression in cervical intraepithelial neoplasia” war die Zweitautorin verantwortlich für die Durchführung der Experimente und deren Auswertung. Darüber hinaus wurden von ihr die Datenanalyse unter Supervision von Udo Jeschke und Helene Hildegard Heidegger durchgeführt.

Die ersten Entwürfe des Manuskripts inklusive Abbildungen und Tabellen stammten von der Autorin. Die Korrektur des Manuskriptes und die Durchführung

des Review-Prozesses wurden durch Elisa Schmoeckel durchgeführt und von Patricia Fraungruber als Co-Autorin unterstützt.

2. Zusammenfassung

Das übergreifende Konzept hinter den drei Publikationen war es, molekulare Mechanismen in Signalwegen der Hormonrezeptoren bei gynäkologischen Tumoren zu finden und einzelne Proteine auf ihre Nützlichkeit als Biomarker zu prüfen. Es wurden jeweils Gewebeschnitte immunhistochemisch untersucht und in Hinblick auf verschiedenen Fragestellungen ausgewertet.

In den beiden Publikationen zu Ovarialkarzinomen „G Protein-Coupled Estrogen Receptor Correlates With Dkk2 Expression and Has Prognostic Impact in Ovarian Cancer Patients“ und „Nuclear receptor co-repressor NCOR2 and its relation to GPER with prognostic impact in ovarian cancer“ wurden die Auswirkungen von Dickkopf 2 (Dkk2) und des nukleären Rezeptor-Co-Repressors (NCOR2) in Verbindung mit dem G-Protein-gekoppelten Östrogenrezeptor (GPER) auf den klinischen Verlauf bei EOC betrachtet. Dkk2 übt als Modulator des Wnt-Signalweges wesentlichen Einfluss auf die zelluläre Differenzierung und NCOR2 hemmt als Proteinkomplex die Gentranskription kontextabhängig. Alle zwei interagieren dabei mit einer Vielzahl anderer Signalwege. Unter anderem auch mit GPER, wie die Ergebnisse unserer Publikationen vermuten lassen. In beiden Publikationen wies eine starke Expression des jeweiligen Markers in Kombination mit einer starken GPER Expressionen auf ein längeres Gesamtüberleben hin. Weitere Forschung ist erforderlich, um ein tiefergehendes Verständnis des Einflusses von Dkk2, NCOR2 und GPER auf die Tumorbiologie zu erlangen und damit deren Nutzbarkeit als prognostische Marker oder therapeutische Zielstrukturen zu klären.

Die Publikation „The role of EP-2 receptor expression in cervical intraepithelial neoplasia“ untersucht die Rolle des Prostaglandinrezeptors EP2 bei zervikalen intraepithelialen Neoplasien (CIN). Es wurde festgestellt, dass die EP2 Expression signifikant mit zunehmender Schwere der Dysplasie ansteigt und höhere EP2 Expressionen mit einem progredienten Verlauf von CIN II korrelieren. Diese Ergebnisse legen nahe, dass EP2 als Biomarker für den Verlauf von CIN geeignet sein könnte.

Zusammengefasst betonen die Publikationen die Bedeutung molekularer Marker wie Dkk2, NCOR2, GPER und EP2 für die Prognose und Therapie von gynäkologischen Krebserkrankungen. Zukünftige Forschungen könnten die Grundlage für maßgeschneiderte Therapieansätze bilden und die Prognose sowie Behandlungsmöglichkeiten der Erkrankungen verbessern.

3. Abstract

The overarching concept behind the three publications was to find molecular mechanisms in signaling pathways of hormone receptors in gynecological tumors and to test individual proteins for their usefulness as molecular markers. Tissue sections were examined immunohistochemically and analyzed with regard to various questions.

In the two publications on epithelial ovarian carcinomas (EOC) "G Protein-Coupled Estrogen Receptor Correlates With Dkk2 Expression and Has Prognostic Impact in Ovarian Cancer Patients" and "Nuclear receptor co repressor NCOR2 and its relation to GPER with prognostic impact in ovarian cancer", the effects of Dickkopf 2 (Dkk2) and the nuclear receptor co-repressor (NCOR2) in conjunction with the G protein-coupled estrogen receptor (GPER) on the clinical course of EOC were examined. As a modulator of the Wnt signaling pathway, Dkk2 exerts a significant influence on cellular differentiation. NCOR2, as a protein complex, inhibits gene transcription in a context-dependent manner. All two interact with a variety of other signaling pathways. Among others also with GPER, as the results of our publications suggest. In both publications, strong expression of the respective marker in combination with strong GPER expression indicated longer overall survival. Further research is needed to gain a deeper understanding of the influence of Dkk2, NCOR2 and GPER on tumor biology and thus to clarify their usefulness as prognostic markers or therapeutic targets.

The publication "The role of EP-2 receptor expression in cervical intraepithelial neoplasia" examines the role of the prostaglandin receptor EP2 in cervical intraepithelial neoplasia (CIN). It was found that EP2 expression increases significantly with increasing severity of dysplasia and that higher EP2 expression correlates with a progressive course of CIN II. These results suggest that EP2 may be suitable as a biomarker for the progression of CIN.

In summary, the publications emphasize the importance of molecular markers such as Dkk2, NCOR2, GPER and EP2 for the prognosis and therapy of gynecological cancers. Future research could form the basis for customized therapeutic approaches and improve the prognosis and treatment options of the diseases.

4. Publikation I

Patricia Fraungruber, Till Kaltofen, Sabine Heublein, Christina Kuhn, Doris Mayr, Alexander Burges, Sven Mahner, Philipp Rathert, Udo Jeschke, Fabian Trillsch

G Protein-Coupled Estrogen Receptor Correlates With Dkk2 Expression and Has Prognostic Impact in Ovarian Cancer Patients

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G Protein-Coupled Estrogen Receptor Correlates With Dkk2 Expression and Has Prognostic Impact in Ovarian Cancer Patients

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Purpose: Wnt pathway modulator Dickkopf 2 (Dkk2) and signaling of the G protein-coupled estrogen receptor (GPER) seem to have essential functions in numerous cancer types. For epithelial ovarian cancer (EOC), it has not been proven if either Dkk2 or the GPER on its own have an independent impact on overall survival (OS). So far, the correlation of both factors and their clinical significance has not systematically been investigated before.

Methods: Expression levels of Dkk2 were immunohistochemically analyzed in 156 patient samples from different histologic subtypes of EOC applying the immune-reactivity score (IRS). Expression analyses were correlated with clinical and pathological parameters to assess for prognostic relevance. Data analysis was performed using Spearman's correlations, Kruskal-Wallis-test and Kaplan-Meier estimates.

Results: Highest Dkk2 expression of all subtypes was observed in clear cell carcinoma. In addition, Dkk2 expression differed significantly ($p < 0.001$) between low and high grade serous ovarian cancer. A significant correlation of Dkk2 with the cytoplasmic GPER expression was noted ($p = 0.001$) but not for the nuclear estrogen receptor alpha (ER α) or beta (ER β). Patients exhibiting both, high expression Dkk2 (IRS > 4) and GPER (IRS > 8), had a significantly better overall survival compared to patients with low expression (61 months vs. 33 months; $p = 0.024$).

Conclusion: Dkk2 and GPER expression correlates in EOC and combined expression of both is associated with improved OS. These findings underline the clinical significance of both pathways and indicate a possible prognostic impact as well as a potential for treatment strategies addressing interactions between estrogen and Wnt signaling in ovarian cancer.

Keywords: Dickkopf 2, G protein-coupled estrogen receptor, Wnt signaling, estrogen, epithelial ovarian cancer

INTRODUCTION

Epithelial ovarian cancer (EOC) causes most deaths of gynecological malignancies (1) with a relative 5-year survival of almost 45% (2). The need to identify suitable screening methods, prognostic markers and efficient therapies is crucial. So far, standard treatment for primary disease consists of debulking surgery and a platinum-based chemotherapy with antiangiogenics and/or Poly-ADP-Ribose-Polymerase (PARP) inhibitors (3). Apart from clinicopathological aspects such as the stage in the system of the International Federation of Gynecology and Obstetrics (FIGO), volume of residual disease after debulking surgery, patients' age, and histological subtype (4–7), there are no reliable prognostic factors to predict the clinical course. With regards to the molecular background and specific gene mutations, EOC is histologically separated into clear cell, endometrioid, mucinous, and serous carcinoma of low or high grade (LGSC/HGSC) (8).

Revealing molecular events that cause ovarian cancer and are responsible for its progression represent a major challenge for translational research. One approach is to understand the importance and complexity of the Wnt signaling pathway and its regulation (9–11). Secreted Wnt glycoproteins translate their function *via* binding to Frizzled receptors and co-receptors such as low-density-lipoprotein-related protein 5/6 (LRP5/6) (11). Subsequently, Wnt proteins exhibit their effects on several cellular processes by activating either the canonical Wnt/ β -catenin or at least two non-canonical β -catenin-independent pathways (12). Alterations in Wnt signaling components, such as APC (adenomatous polyposis coli) protein, AXIN and β -catenin and downregulation of modulatory Wnt antagonists have been described to be involved in the onset of several cancer types (10, 13, 14). As a consequence, modulators of the Wnt pathway like members of the Dickkopf family (Dkk1–4) may play an essential role during development (15, 16) and tumorigenesis (17, 18). Dkks bind to LRP5/6 with higher affinity than Wnt (19). Dkk2 seems able to act as agonist as well as antagonist for Wnt/LRP6 signaling depending on the cellular context and therefore co-factors such as *krm2* (18–20). In EOC Zhu et al. suggest that Dkk2 may function as a Wnt pathway inhibitor (13).

Estrogen (E2, 17 β -estradiol) has numerous cellular functions in the human body including gynecologic cancer biology (21) and interactions between estrogen and Wnt signaling have been described (22–25). In this context an interplay of Dkk2 and estrogen receptors (ER) could link these two mechanisms and classical nuclear ER α or ER β as well as the G protein-coupled estrogen receptor (GPER) could be involved in this process.

GPER is a transmembrane receptor with intracellular domains binding E2 (26), which mediates rapid non-genomic estrogen signaling (27). Its activation *via* agonists like G1 or E2 (28) leads to cAMP production (29), activation of extracellular signal-related kinase 1 and 2 (Erk1/2) (28), mobilization of intracellular Ca²⁺, phosphatidylinositol 3-kinase (PI3K) activation (26) and the induction of metalloproteinases which then transactivates the epidermal growth factor receptor (30). GPER can also indirectly impact gene transcription (31). Since its role in ovarian cancer has been conflicting so far (32–34) this analysis focused on the correlation of Dkk2 with GPER to identify a possible link between Wnt and estrogen and investigating their potential prognostic significance.

METHODS

Patients

In this study 156 formalin-fixed and paraffin-embedded tissue specimens of epithelial ovarian cancer from patients who had been treated in the Department of Obstetrics and Gynecology at Ludwig-Maximilians-University of Munich between 1990 and 2002 were analyzed. Numerous markers were already examined in this collective in preceding studies (35–37). Clinical data was collected from the patient's charts and information about the follow up was acquired from the Munich Cancer Registry.

Only patients with malignant, non-borderline tumors were included in the study. Seventy-three patients (46.8%) were older or age 60 years at the initial diagnosis and 83 patients (53.2%) were younger than 60 years. There were no data available about estrogen replacement therapy in postmenopausal women. Pathologists categorized the histological subtypes of the samples: LGSC (n=24), HGSC (n=80), endometrioid (n=21), clear cell (n=12), mucinous (n=13). According to the updated FIGO classification from 2014, specimens of serous ovarian cancer were re-evaluated and attributed to low-grade (G1) and high-grade (G3) histology. Endometrioid and mucinous ovarian cancer samples were related to G1, G2, and G3. Clear cell cancer was always categorized as G3 (38). Staging was done following the FIGO classification: I (n=35), II (n=10), III (n=103), IV (n=3) (Table 1).

Sampling and Microarray Construction

Three core biopsies for each EOC patient were taken from paraffin-embedded and formalin-fixed tumor blocks in our archive. The biopsies were assembled in tissue microarrays (TMA) paraffin blocks. Those TMA paraffin blocks were cut into serial sections at 2 μ m and fixed on slides. A pathologist verified that representative areas of the tumor were aligned on the slides.

Immunohistochemistry

Immunohistochemical staining of paraffin-embedded and formalin-fixed tissue micro arrays of ovarian cancer specimens for Dkk2 was performed as previously described (39). The TMA slides were dewaxed in Roticlear (Carl Roth Karlsruhe,

Abbreviations: Dkk2, Dickkopf2; EOC, epithelial ovarian cancer; E2, estrogen, 17 β -estradiol; ER α , nuclear estrogen receptor alpha; ER β , nuclear estrogen receptor alpha beta; Erk1, extracellular signal-related kinase 1; Erk2, extracellular signal-related kinase 2; GPER, G protein-coupled estrogen receptor; HGSC, high-grade serous carcinoma; IRS, immune-reactivity score; LGSC, low-grade serous carcinoma; LRP5, low-density-lipoprotein-related protein 5; LRP6, low-density-lipoprotein-related protein 6; OS, overall survival; PI3K, phosphatidylinositol 3-kinase; ROC curve, receiver operating characteristics curve; TCF, T-cell factor; TMA, tissue microarrays.

TABLE 1 | Correlation between Dkk2 expression and clinicopathologic characteristics of ovarian cancer patients.

Characteristics	Total	Dkk2 low expression	Dkk2 high expression	P value
	Number of cases(%)	Number of cases	Number of cases	
Age(y)				
≥60y	73 (46.8)	28	21	<0.001
<60y	83 (53.2)	17	38	
Tumor histology				
LGSC	24 (15.4)	2	17	0.001
HGSC	80 (51.3)	28	27	
Clear cell	12 (7.7)	0	6	
Endometrioid	21 (13.5)	7	4	
Mucinous	13 (8.3)	6	2	
Missing	6 (3.8)			
FIGO				
I	35 (22.4)	8	14	0.615
II	10 (6.4)	2	4	
III	103 (66.0)	33	39	
IV	3 (1.9)	1	0	
Missing	5 (3.2)			
Expression of GPER				
low expression (IRS ≤ 8)	83 (53.2)	33	28	0.005
high expression (IRS>8)	70 (44.9)	11	31	

Dkk2, Dickkopf2; GPER, G protein-coupled estrogen receptor; HGSC, high-grade serous carcinoma; LGSC, low-grade serous carcinoma; FIGO, International Federation of Gynecology and Obstetrics.

Bold numbers represent p-values < 0.05.

Germany) for 20 min. The endogenous peroxidase was suppressed with 3% hydrogen peroxide (Merck, Darmstadt, Germany) in methanol (20 min). The specimens were rehydrated in a descending alcohol series (100%, 70%, 50% ethanol). The epitopes were retrieved by putting the slides in a pressure cooker with sodium citrate buffer (pH 6.0) for 5 min. After cooling to room temperature, the slides were washed in distilled water and phosphate-buffered saline (PBS). To evade unspecific staining reagent 1 of the polymer kit (ZytoChem Plus HRP Polymer System, Berlin, Germany) was administered for 5 min. Next the slides incubated at +4°C for 16 h with the primary anti-body Anti-Dkk2 polyclonal rabbit IgG (ProteinTech, Manchester, UK). As negative controls the primary antibody was replaced by normal rabbit immunoglobulin G(IgG) supersensitive rabbit negative control; BioGenex, Fremont, California). Washing in PBS and the application of reagents 2 (20 min) and 3 (30 min) of the polymer kit anticipated the substrate-staining with chromogen diaminobenzidine (Dako, Hamburg, Germany). Counterstaining in Mayer acidic hematoxylin (Waldeck-Chroma, Münster, Germany) and dehydration in an ascending series of alcohol followed by Rotoclear was performed. Cervical tissue was served as positive control.

Using a microscope (Leitz, Wetzlar, Germany) the immunoreactivity score (IRS) was applied to assess the immunostaining extent semi-quantitatively by two blinded examiners. The IRS is composed of the staining intensity (0=negative, 1=low,

2=moderate, 3=strong) multiplied with the percentage of stained cells (0=no staining, 1%≤10% positive cells, 2 = 11%–50% positive cells, 3 = 51%–80% positive cells, 4%≥81% positive cells). The immunoreactivity score ranges from 0 to 2: negative, 3 to 4: weak, 6 to 8: moderate, and 9 to 12: strong (40). Formerly published staining results of GPER in this panel recorded in the archive of the laboratory were recaptured (36).

Staining Evaluation

In order to define reliable cut-off points for the IRS of the Dkk2 staining the receiver operating characteristics (ROC) curve was used. ROC curve illustrates sensitivity on the y-axis plotted against (1-specificity) on x-axis (41). With Youden Index (42) the optimal cut-off was defined with highest possible values for sensitivity and specificity. For the Dkk2 staining IRS 0-4 was considered as weak and IRS 6-12 as high. Regarding its components, the IRS can never have a value of 5. GPER expression was divided into low (IRS ≤ 8) vs. high (IRS>8) according to the median (36).

Statistical Analysis

Statistical analysis was operated with SPSS 25 (IBM, Chicago, IL, USA). With the Kruskal-Wallis analysis the null hypothesis was tested against its opposite. Further Spearman's correlation analysis and Kruskal-Wallis analysis was applied for testing correlation of Dkk2 and GPER scores. The Kaplan-Meier estimate was used for analyzing times to event variables. Correlations between mean Dkk2 expression and clinicopathologic characteristics were assessed with Chi-Square tests (Table 1, crosstab). For all tests p-values ≤ 0.05 were considered as statistically significant. Figures were designed with SPSS 25 and Microsoft Power Point 2016 (Microsoft, Redmond, WA, USA).

RESULTS

Correlations between Dkk2 expression and clinicopathologic characteristics of EOC patients are displayed in Table 1. A median IRS of 6 for anti-Dkk2 staining was observed in the 131 of 152 cases (86%) with adequate staining. Applying ROC curve analysis, an IRS>4 was selected as cut-off.

Dkk2 expression differed significantly between the histological cancer subtypes (Figures 1A–F) with clear cell carcinomas showing the highest median IRS of 12 compared to the other subtypes (range: 9–12; p<0.001). While endometrioid and mucinous EOCs exhibited both a median IRS of 4, the overall cohort of serous EOCs had moderate staining extent at IRS of 6 which subdivided into high-grade serous histology with an IRS of 4 (range 0–12) and significantly higher for low-grade serous histology with an IRS of 6 (range 4–12; p<0.001).

Performing correlation analysis of Dkk2 expression and clinicopathologic parameters such as distant metastasis, affected lymph nodes, FIGO classification, and grading, no significant results were found. In addition, Dkk2 expression was examined in comparison to other potentially pathological

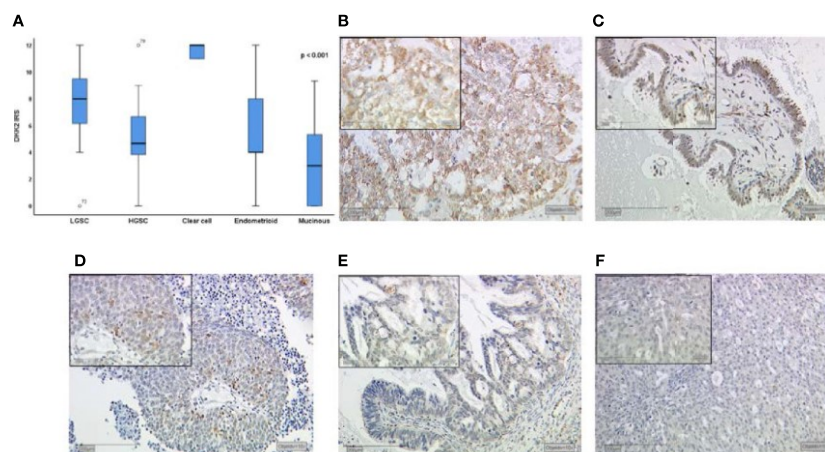


FIGURE 1 | Dkk2 expression patterns in different histological subtypes of EOC after immunohistochemical staining was performed as shown in a Kruskal-Wallis analysis for histological subtypes (A). Clear cell carcinomas (B) presented the strongest staining patterns. Low-grade serous carcinomas (LGSC; C) had shown moderate Dkk2 expression. For endometrioid (D), mucinous (E) and high-grade serous carcinomas (HGSC; F) the median IRS was lower. Scale bars equal 200 μ m.

markers with a possible impact on the prognosis of EOC. Cytoplasmatic Dkk2 was observed to correlate significantly with cytoplasmic GPER expression ($cc=0.304$, $p=0.001$). Further analysis revealed that high Dkk2 expression is correlated to high GPER expression (Figure 2). In contrast,

Dkk2 did not correlate with either ER α or ER β expression (Table 2).

Patients with high Dkk2 expression (IRS>4) exhibited longer OS with a median of 65 months compared to 35 months in Kaplan-Meier analysis, although this difference was not

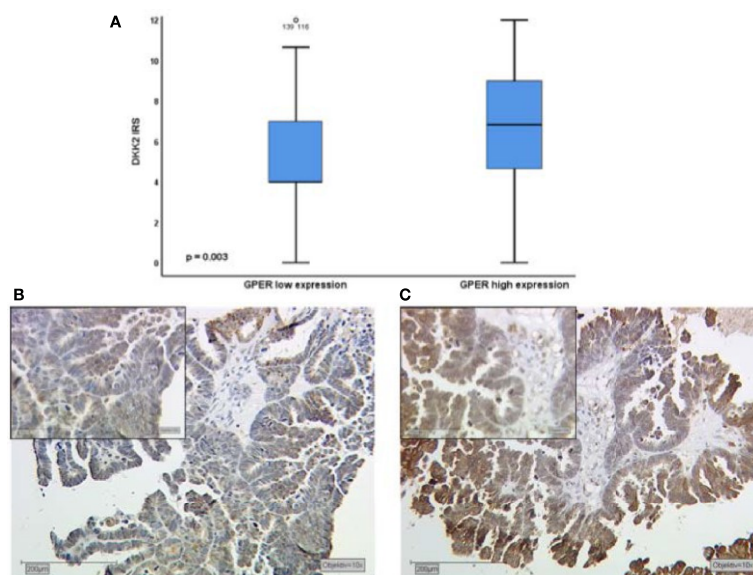


FIGURE 2 | Kruskal-Wallis analysis for correlation of Dkk2 and GPER expression (A). High expression of Dkk2 (B) correlates with high GPER expression (C) in tissue samples of the same patient. Scale bars equal 200 μ m.

TABLE 2 | Results of Spearman's correlation analysis of Dkk2 with the different estrogen receptors (GPER, ERα, ERβ).

Staining	DKK2	GPER	ERα	ERβ
DKK2				
cc	1.000	0.304	0.092	-0.080
p	.	0.001	0.298	0.366
n	125	124	131	128

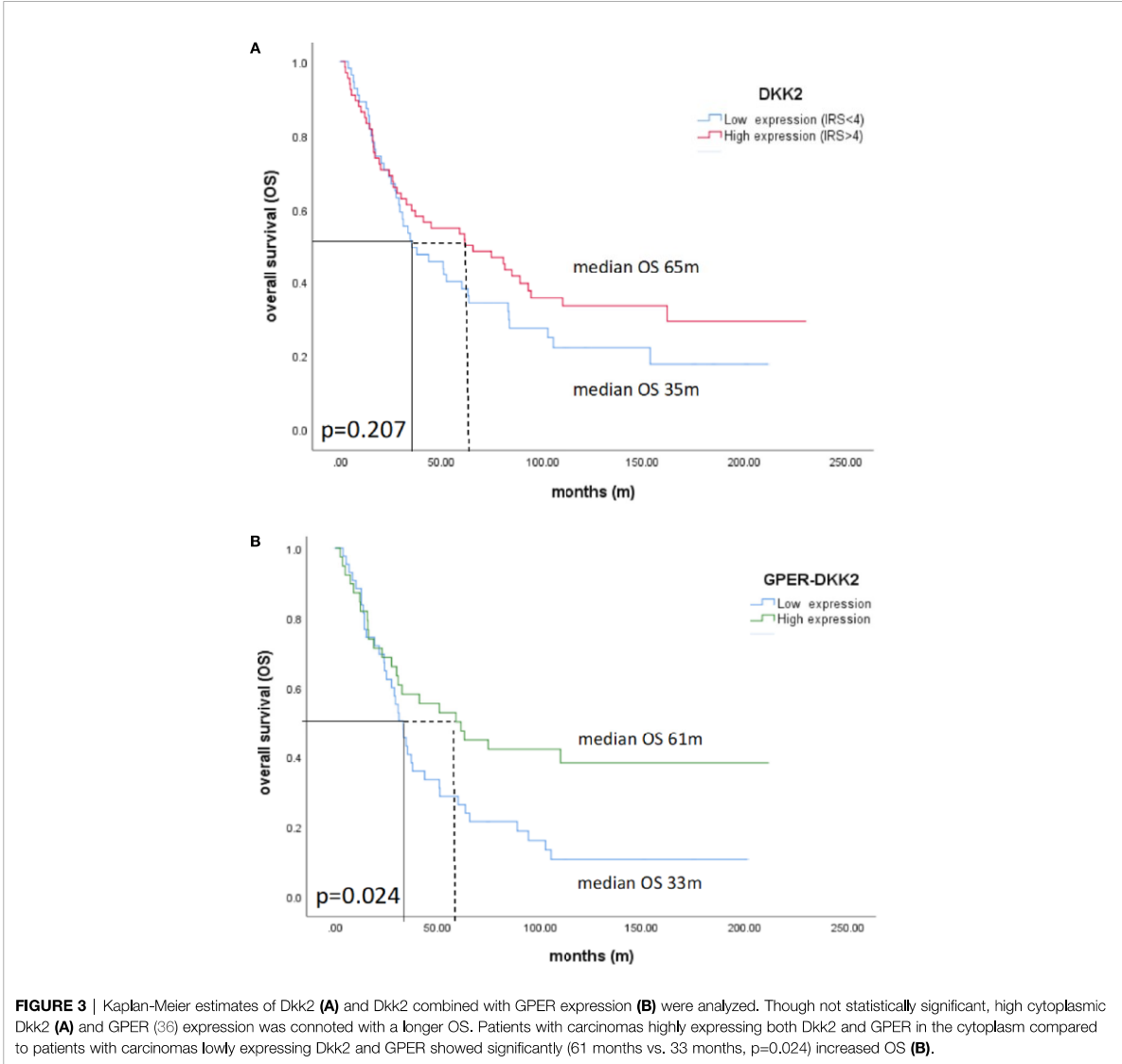
Dkk2, Dickkopf 2; GPER, G protein-coupled estrogen receptor; cc, correlation coefficient; p, two-tailed significance; n, number of patients. Bold numbers represent p-values < 0.05.

statistically significant (p=0.207; **Figure 3A**). The same trend was observed for GPER expression as published before with longer OS for patients with high expression but without statistical

significance (36). When the expression analyses of the two markers were combined, patients with high Dkk2 (IRS>4) as well as high GPER (IRS>8) expression had a significantly longer OS with 61 months compared to 33 months in patients with low expression of both influenced OS (p=0.024; **Figure 3B**).

DISCUSSION

Dkk2 as a Wnt/β-catenin antagonist may play an important role in ovarian cancer (13, 18, 43). In this analysis, we investigated the expression of Dkk2 in the different histological subtypes of epithelial ovarian cancer, its relation to clinicopathological aspects and its impact on OS. Clear cell carcinoma exhibited



the highest Dkk2 expression at all and LGSC showed significantly higher expression compared to the other histologies, which could reflect the different pathogenesis and origins of the histological subtypes (44).

In a previous study from Zhu et al. it has been shown that Dkk2 is frequently methylated and therefore epigenetically silenced in ovarian cancer. Lower Dkk2 expression levels correlated with tumor progression and advanced tumor stages (FIGO III-IV). By treating mice with the DNA methyltransferase inhibitor 5-aza-2'-deoxycytidine (decitabine) in order to re-establish Dkk2 expression in mice tumor growth was impaired (13). This is in accordance with our findings, suggesting an impact of Dkk2 on OS although this was not significant. Seemingly aberrant DNA methylation patterns also play a major role in platinum resistance, therefore the potential of epigenetic modulator decitabine to restore sensitivity towards platinum has been successfully tested in a phase II clinical trial (45). So far, agents for epigenetic therapy may cause severe adverse effects, in particular when they are administered in combination with chemotherapy. This underscores the necessity of more selective epigenetic modulators (46).

The impact of GPER on the OS of ovarian cancer patients has been controversially discussed so far (32–34). The conflicting results in these studies may arise from application of different concentrations for the agonists E2 and G1 and the investigation in different cancer cell lines. Accounting for these and the current results, GPER may not be sufficient to predict OS on its own. However, in combination with other factors like Luteinizing Hormone/Choriogonadotropin Receptor and Follicle Stimulating Hormone Receptor (36) or Dkk2, it could serve as a positive prognostic factor for patients suffering from epithelial ovarian cancer.

As previous studies elucidated a possible connection between estrogen and Wnt signaling (22–25), we investigated the relationship of Dkk2 with estrogen receptors. Subcellular localization of the DKK2 staining pattern was noted which has been previously attributed to the Golgi apparatus (www.proteinatlas.org). Unlike other studies in breast cancer which have shown an association between plasma membrane expression and outcome, plasma membrane expression of GPER was not detected in the ovarian cancer samples evaluated here (47). We could demonstrate a strong correlation of high cytoplasmic Dkk2 and high cytoplasmic GPER expression levels in EOC samples. In contrast, no correlation of Dkk2 with the traditional estrogen receptors ER α or ER β was noted. To the best of our knowledge, a possible connection of GPER and Dkk2 has not yet been investigated. The described association of higher Dkk2 expression in younger patients may be reflected by more patients in premenopausal status and therefore relate to the estrogen levels in these patients.

In our study, a high Dkk2 expression in combination with a high cytoplasmic level of GPER had a significant prognostic impact on OS which might help to find new approaches for possible treatment strategies accounting for the correlation of estrogen and Wnt signaling pathways. As Dkk2 is a modulator of the Wnt pathway, therapeutics addressing this cascade could be

combined with agents modulating GPER. Although promising in early stage development, previous strategies targeting Wnt proteins like tumor associated MUC1 (TA-MUC1) inhibitor gatipotuzumab and others have not led to durable responses and not reached clinical significance so far (48). Very recently, a Wnt modulator of Dkk1 (DKN-01) has shown interesting activity and is currently in a phase 2 basket trial which still supports the rationale for this approach (49).

In renal cancer cells, the selective estrogen receptor modulator genistein reportedly abolished miR-1260b, which is able to suppress Wnt signaling modulators like Dkk2, and therefore preserved levels of these proteins (24). Genistein is not exclusively binding to GPER though, it also inflects ER α and/or ER β (50). In hepatocytes administering the GPER antagonist G15 attenuated β -cat Ser675 phosphorylation and T-cell factor (TCF) expression suggesting an involvement of GPER in β -cat/TCF activities (51). Beside cell culture experiments, analyzing methylation patterns with methylation-specific polymerase chain reaction could help to further investigate the suggested interactions of GPER and Dkk2. Implementing TCF/LEF (lymphoid enhancing factor) reporter assays, could be assessed to evaluate possible effects of GPER agonists or antagonists on the Wnt signaling pathway.

There are some factors limiting our study. First of all, it is retrospective based on a single dataset with a relatively low sample size which may not be sufficient to elucidate all subtype-specific differences in a heterogeneous tumor like ovarian cancer (44). Additional specific information of patient characteristics like an history of hormonal replacement therapy could enrich the investigation how estrogen levels interact with Dkk2 and better account for possible environmental toxicants. In Kaplan-Meier analysis, subtype-specific evaluation did not reveal significant differences regarding OS between patients with high and low Dkk2 expression so that results can be considered as a base for further research in ovarian cancer. Further methods will be necessary capture the extensive complexity of GPER and Wnt signaling pathways with their possible interaction as indicated.

However, aside from these limitations our data is in accordance with previous findings in EOC literature (13, 33, 36, 45, 52) and elucidate that targeting the GPER receptor as well as the Wnt pathway could represent promising therapeutic strategy in ovarian cancer. The study might provide an impetus to further investigate the crosstalk between estrogen and Wnt signaling in regard to the therapeutic potential in EOC.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Ludwig-Maximilians-University, Munich,

Germany. Written informed consent for participation was not required for this study in accordance with the national legislation and the institutional requirements.

AUTHOR CONTRIBUTIONS

PF, UJ, SH, AB, SM, and FT contributed conception and design of the study. DM performed histological examinations on the patient tumour tissue. PF, CK, UJ, and PR did the laboratory work. PF, TK, UJ, and PR performed the statistical analysis. PF

and UJ wrote the first draft of the manuscript. All authors contributed to the article and approved the submitted version.

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The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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5. Publikation II

Juliane Reichenbach, Patricia Fraungruber, Doris Mayr, Christina Buschmann, Fabian B. T. Kraus, Nicole Elisabeth Topalov, Anca Chelariu-Raicu, Thomas Kolben, Alexander Burges, Sven Mahner, Mirjana Kessler, Udo Jeschke, Bastian Czogalla, Fabian Trillsch

Nuclear receptor co-repressor NCOR2 and its relation to GPER with prognostic impact in ovarian cancer

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RESEARCH



Nuclear receptor co-repressor NCOR2 and its relation to GPER with prognostic impact in ovarian cancer

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Abstract

Purpose The significance of the non-classical G-protein-coupled estrogen receptor (GPER) as positive or negative prognostic factor for ovarian cancer patients remains still controversial. Recent results indicate that an imbalance of both co-factors and co-repressors of nuclear receptors regulates ovarian carcinogenesis by altering the transcriptional activity through chromatin remodeling. The present study aims to investigate whether the expression of the nuclear co-repressor NCOR2 plays a role in GPER signaling which thereby could positively impact overall survival rates of ovarian cancer patients.

Methods NCOR2 expression was evaluated by immunohistochemistry in a cohort of 156 epithelial ovarian cancer (EOC) tumor samples and correlated with GPER expression. The correlation and differences in clinical and histopathological variables as well as their effect on prognosis were analyzed by Spearman's correlation, Kruskal–Wallis test and Kaplan–Meier estimates.

Results Histologic subtypes were associated with different NCOR2 expression patterns. More specifically, serous and mucinous EOC demonstrated a higher NCOR2 expression ($P=0.008$). In addition, high nuclear NCOR2 expression correlated significantly with high GPER expression ($cc=0.245$, $P=0.008$). A combined evaluation of both high NCOR2 (IRS > 6) and high GPER (IRS > 8) expression revealed an association of a significantly improved overall survival (median OS 50.9 versus 105.1 months, $P=0.048$).

Conclusion Our results support the hypothesis that nuclear co-repressors such as NCOR2 may influence the transcription of target genes in EOC such as GPER. Understanding the role of nuclear co-repressors on signaling pathways will allow a better understanding of the factors involved in prognosis and clinical outcome of EOC patients.

Keywords Ovarian cancer · Nuclear co-repressor 2 · G-protein-coupled estrogen receptor · Immunohistochemistry · Epigenetic regulation · Chromatin remodeling

Abbreviations

cAMP	Cyclic adenosine monophosphate
cc	Correlation coefficient
CI	Confidence interval
EOC	Epithelial ovarian cancer
ER α	Estrogen receptor α
ER β	Estrogen receptor β
FDA	Food and Drug Administration
FFPE	Formalin fixated and paraffin embedded
FIGO	Fédération Internationale de Gynécologie et d'Obstétrique

GPER	G-protein-coupled estrogen receptor
GPS2	G-protein pathway suppressor 2
HDAC3	Histone deacetylase 3
HGSOC	High-grade serous ovarian cancer
IgG	Immunoglobulin G
IRS	Immunoreactive Score
LGSOC	Low grade serous ovarian cancer
MDa	Megadalton
<i>N</i>	Number of patients
NCOR2	Nuclear co-repressor 2
ns	No significance
OS	Overall survival
<i>P</i>	Two-tailed significance
ROC	Receiver operating characteristic
SD	Standard deviation

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TBL1	Transducin b-like protein 1
TBLR1	TBL1-related protein 1
TMA	Tissue micorarrays
vs.	Versus
WHO	World Health Organization

Introduction

Epithelial ovarian cancer (EOC) remains the most life-threatening gynecological malignancies with an estimated 5-year survival of less than 45% and only 29% for advanced stage disease (Siegel et al. 2022). Initially, around 70% of the patients are primarily diagnosed at an advanced staged disease with dissemination to the entire abdominal cavity after presenting with unspecific symptoms and with the lack of reliable biomarkers (Barnholtz-Sloan et al. 2003). Due to molecular characteristics and differences in histogenesis and oncologic signaling pathways, EOC forms a heterogenous group of cancers consisting of five histological subtypes (Duska and Kohn 2017) which are major factors influencing clinical and biological behavior with impact on therapy and prognosis (Lalwani et al. 2011).

Signaling pathways important for cell growth, metabolism and inflammation are mediated by nuclear receptors, that are regulated through transcriptional co-regulatory proteins including co-activators and co-repressors (Mangelsdorf et al. 1995). The homeostatic balance between repressing and activating co-factors is key to regulate cell development. In contrast, dysregulation of these processes can promote either pro- or even anti-tumorigenic effects (Wong et al. 2014). NCOR2 is a nuclear co-repressor that was firstly known for its transcriptional silencing role of retinoid and thyroid hormone receptors and is referred to as SMRT (silencing mediator for retinoid and thyroid hormone receptors) (Hussein-Fikret and Fuller 2005; Sasaki et al. 2008). Appearing in large protein complexes up to 1.6–2 MDa, NCOR2 is forming the core, supported by protein components such as histone deacetylase 3 (HDAC3), transducin β -like protein 1 (TBL1) or TBL1-related protein 1 (TBLR1) and G-protein pathway suppressor 2 (GPS2) (Li et al. 2000). By chromatin remodeling, the co-repressor complex reduces transcriptional activity and consequently regulates different epigenetic cellular processes (Jepsen and Rosenfeld 2002). In malignant cells, the co-repressor machinery inhibits the normal transcriptional cycle under contribution of histone modifying enzymes and the DNA methylation machinery and leads to reduced transcriptional plasticity and consequently to an altered gene expression (Battaglia et al. 2010). Accordingly, NCOR2 expression was found in more than 70% of ovarian cancers (Havrilesky et al. 2001).

Several estrogen receptor isoforms such as ER α , ER β and the membrane receptor GPER regulate ovarian cell

differentiation and follicle and oocyte development and maturation (Bai et al. 2000). While especially serous and clear cell ovarian carcinomas derive from the fallopian tube and endometrium and not directly from the ovarian surface, the classical nuclear estrogen receptors α (ER α) and β (ER β), demonstrated only minor effect for therapeutic approaches in EOC (Langdon et al. 2020). Non-genomic signaling on the other hand, is induced by binding of the non-classical G-protein-coupled estrogen receptor (GPER) (Filardo and Thomas 2012). G-protein-coupled receptors represent a large family of transmembrane molecules that mediate rapid intracellular responses to their extracellular ligands via cAMP or phosphatidylinositol signal transduction pathways and serve as potential drug targets (Hauser et al. 2018). Promoting estrogen-dependent physiological and pathophysiological processes, GPER expression was found in various cancer cell lines of reproductive tissues such as breast, endometrial, testicular and ovarian cancer (Prossnitz and Barton 2011; Pavlik et al. 2011; Revankar et al. 2005; He et al. 2009; Chevalier et al. 2012; Fujiwara et al. 2012). While the exact intracellular localization of GPER remains unclear, cytoplasmatic and nuclear occurrence were described (Otto et al. 2008; Zhu et al. 2018). Interestingly, foremost nuclear GPER expression served as significant independent negative prognostic factor for overall survival in EOC patients (Zhu et al. 2018).

The conflicting findings for GPER in EOC suggest that the complex regulation of transcriptional activity might be involved in both the pathogenesis and prognosis of EOC. Regulation by nuclear co-factors and co-repressors possibly alternates clinical outcome. The current study aims to explore a putative involvement of the nuclear co-repressor NCOR2 on the regulation of the gene GPER. A comprehensive understanding of the co-repressors' role in EOC and their interaction with transcription factors and the target genes will allow a better understanding of the factors involved in prognosis and clinical outcome.

Methods

Patients and specimens

For the present study, 156 tumor samples with the clinical information, respectively, of ovarian cancer patients who underwent oncological surgery at the Department of Obstetrics and Gynecology, Ludwig-Maximillan's-University Munich from 1990 to 2002 were collected, formalin fixated and paraffin embedded (FFPE). Additionally, the Munich Cancer Registry provided data about follow-up. All tumor samples included in this study originated from patients with malignant, non-borderline tumors and were further classified by a pathologist according to the histological subtypes

serous ($n=110$), endometrioid ($n=21$), clear cell ($n=12$), mucinous ($n=13$). By WHO classification, serous ovarian cancer samples were defined as low- versus high-grade cancer, while endometrioid and mucinous subtypes as low- (G1), intermediate- (G2) and high-grade (G3) cancer. Clear cell ovarian cancer samples were always classified as high-grade cancer (G3) (Duska and Kohn 2017).

Of each EOC patient, three tissue specimen were obtained from the paraffin-embedded and formalin-fixated tumor blocks and compiled in tissue microarrays (TMA) paraffin blocks. By supervision of a pathologist, representative tumor sections of 2 μm were cut and aligned onto slides.

In the same sample set of 156 patients, various biomarkers for risk and prognostic assessment were already assessed in previous studies and the obtained data was hereby utilized to perform further analyses (Czogalla et al. 2019; Heublein et al. 2013).

Immunohistochemistry

For NCOR2 staining of the FFPE microarrays of ovarian cancer samples, immunohistochemical procedures were conducted as previously described by our laboratory (Heidegger et al. 2017). Anti-NCOR2 IgG (Abcam, Cambridge, United Kingdom) served as primary antibody and was detected by polymer method (ZytoChem Plus HRP Polymer System mouse/rabbit, Zytomed Systems Berlin, Germany). The staining of FFPE ovarian cancer tissue samples was previously conducted by our lab incubating the sections with rabbit GPER IgG (BioGenex, Fremont, USA) (Heublein et al. 2012). Visualization was performed using chromogen diaminobenzidine (Dako, Hamburg, Germany). Counterstaining was performed using hematoxylin (Waldeck-Chroma, Münster, Germany). At all steps, system controls were included.

The immunohistochemical staining reaction in the nuclei and cytoplasm of the tumor cells was assessed by a Leitz photomicroscope (Wetzlar, Germany) applying a semi-quantitative immunoreactivity scoring system (IR-score, Remmele's score). Therefore, staining intensity (no staining = 0, weak staining = 1, moderate staining = 2, strong staining = 3) is defined in relation to the percentage of stained cells (less than 10% of positive cells = 1, 11–50% of positive cells = 2, 51–80% of positive cells = 3, more than 81% of positive cells = 4) (Remmele et al. 1986).

Staining evaluation

A receiver operating characteristic curve (ROC) was utilized to generate reliable cut-off values for the IR-Score of the NCOR2 staining. Hereby, the true positive rate (corresponding to the sensitivity) is related in a plot to the false positive rate (calculated as $1 - \text{specificity}$). The most error-free points, which are those with highest sensitivity and

specificity are determined by Youden's J statistic (Lasko et al. 2005). For NCOR2, samples with $\text{IRS} \leq 6$ were considered to show low and samples with $\text{IRS} > 6$ to show high expressed according to a median IRS of 6 as cut-off point.

Staining results for GPER in the same tumor sample group that defined a median IRS of 8 as cut-off point for low ($\text{IRS} \leq 8$) and high ($\text{IRS} > 8$) expression were already published by our laboratory and taken into consideration (Heublein et al. 2013).

Statistical analysis

Statistical analysis was conducted with IBM SPSS Statistics Version 25.0 (PASW Statistic, SPSS Inc., IBM, IL, USA). Spearman analysis was carried out for correlation analysis of NCOR2 and GPER. Overall survival was calculated by Kaplan–Meier estimates (log-rank). P -values < 0.05 were considered as statistically significant.

Results

Clinical and pathological characteristics

Clinical and pathological characteristics of our ovarian cancer patient collective, such as data on histology, lymph node status, FIGO classification, age and information about follow-up, were already described and published previously by our group (Table 1) (Czogalla et al. 2019; Heublein et al. 2013).

NCOR2 expression

For 123 of the evaluable 152 cases (82%), NCOR2 nuclear staining was conducted properly and median immunoreactivity with an IRS of 6.0 ($\text{SD} = 3.1$) was detected. In terms of histologic subtypes, significantly higher median IR-scores were found in serous ($\text{IRS} = 6$) and mucinous carcinomas ($\text{IRS} = 6$, compared to weaker expression in endometrioid EOC ($\text{IRS} = 3$, $P = 0.008$) (Figs. 1A–D, 2A). For serous EOC, different immunoreactivity was observed depending on grading, with weaker NCOR2 expression in high-grade ($\text{IRS} = 6$) compared to low-grade serous histology ($\text{IRS} = 9$, $P = 0.002$) (Fig. 2B). No further correlations with statistical significance were noted for NCOR2 with regard to other clinicopathological data as listed in Table 1.

Correlation analysis for NCOR2 and GPER

Nuclear NCOR2 immunoreactivity was correlated with the previously reported expression of estrogen receptors. Here, a significant correlation of high NCOR2 expression with GPER immunoreactivity was noted ($P = 0.008$;

Table 1 Clinicopathological data

Clinicopathological aspects	N	Percentage (%)
Histology		
Serous	104	70.50
Low grade	24	21.80
High grade	80	72.70
Clear cell	12	7.70
Endometrioid	21	13.50
Mucinous	13	8.30
Lymph node		
pNX	61	39.10
pN0	43	27.60
pN1	52	33.30
FIGO		
I	35	23.10
II	10	6.60
III	103	68.20
IV	3	2.00
Age		
≤ 60 years	83	53.20
> 60 years	73	46.80

Clinicopathological data about the ovarian cancer patient collective (Table modified according to Czogalla et al. 2019)

cc = 0.245), while no significant correlation was observed for ER α and ER β receptors (Table 2).

High NCOR and GPER expression is associated with improved overall survival

In Kaplan–Meier analysis to detect possible influences of the immunophenotypes on overall survival (OS), high NCOR2 expression was not associated with significant impact on prognosis (median OS 52.3 months for NCOR2 IRS > 6 versus 50.9 months for NCOR2 IRS < 6; $P = 0.600$) (Fig. 4A). Patients with a high GPER expression had a median OS improvement of 17.3 months in comparison to patients with low GPER expression (median OS 35.0 months for IRS < 8 versus median OS 52.3 months, for IRS > 8), however not statistically significant ($P = 0.176$) (Fig. 4B).

Consistent with the hypothesized contribution of NCOR2 to GPER guided signaling pathways, the combination of high NCOR2 as well as GPER expression was associated with a significant effect on OS ($P = 0.048$). OS was significantly longer for patients with NCOR2 IRS > 6 and GPER IRS > 8 with a median OS of 105.1 months compared to 50.9 months with low expression of both markers (Figs. 3, 4C).

Multivariate analysis

A multivariate Cox regression analysis was developed to evaluate the influence of the variables age, histological type, FIGO stage and expression of NCOR2 and GPER on OS. In this context, only FIGO stage was identified as a significant independent prognostic factor with a Hazard ratio of 1.733 (CI 1.113–2.698; $P = 0.015$) (Table 3).

Discussion

In the present study, we investigated the expression of the nuclear co-repressor NCOR2 in EOC and its potential role in estrogen-dependent physiological and pathophysiological processes promoted by the G-protein coupled estrogen receptor GPER through non-genomic signaling. Co-expression of both GPER and NCOR2 showed significant changes in clinical outcome in EOC patients, highlighting not only the molecular, but also the prognostic significance of the transcriptional regulation altered by NCOR2. In accordance to differences in the pathogenesis of EOC (Kurman and Shih 2011), the NCOR2 expression varied significantly according to histological subtypes and was independent from other clinical–pathological data. Highest NCOR2 immunoreactivity was observed in serous carcinomas and was dependent on tumor grading. Since subtype-specific impact of NCOR2 expression on overall survival by Kaplan–Meier estimates was not statistically significant, the present study further evaluated general molecular mechanisms of ovarian cancer biology rather than focusing on subtype-specific analyses. Isolated evaluation of high NCOR2 co-repressor as well as high GPER expressing tumors could not reveal significant impact on OS, whereas evaluation of both factors together indicated that the tumors exhibiting the combination of both, high NCOR2 and high GPER expression, seem to have significantly improved OS with a median improvement of 54.2 months. Consequently, these results suggest an association with a possible regulating role of the nuclear co-repressor NCOR2 in GPER-mediated signaling, which merits further evaluation.

As a transcription co-regulator of metabolic processes, NCOR2 is involved in the development of various cancer entities by unbalancing pro- and anti-inflammatory signaling pathways (Mottis et al. 2013). For primary ovarian cancers, its expression was found in up to 70% of tumor tissues (Havrilesky et al. 2001). Structural investigations detected NCOR2 appearing in a large co-regulatory complex that assembles multiple and context-dependent partner proteins (Oberoi et al. 2011). The NCOR2 co-repressor complex disrupts transcription of various target genes through chromatin remodeling, leading to altered cellular processes and to malignancy through induced transcriptional arrest (Battaglia

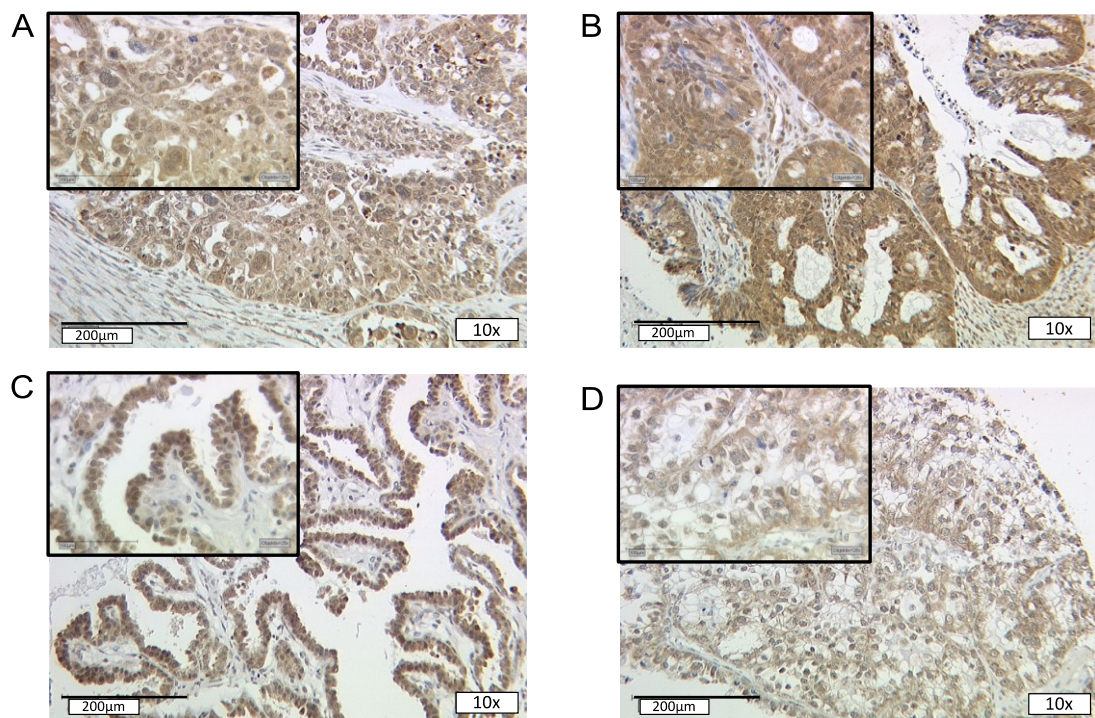


Fig. 1 Detection of NCOR2 with immunohistochemistry. Nuclear NCOR2 staining in the subtypes serous (A), mucinous (B), endometrioid (C) and clear cell (D)

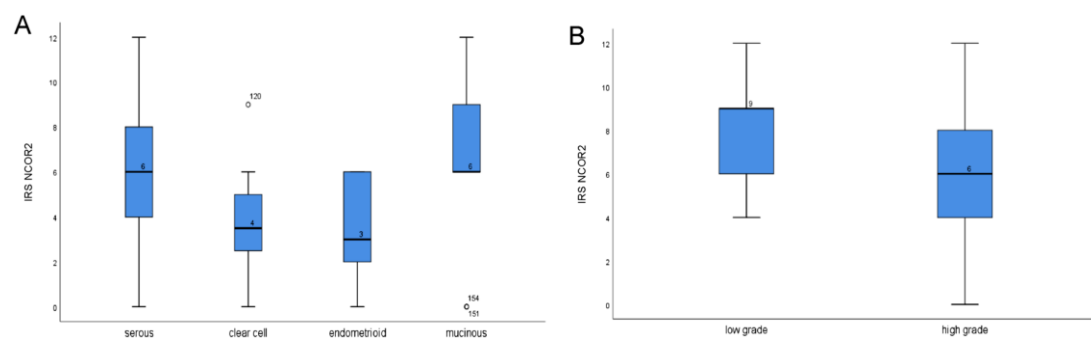


Fig. 2 Boxplot graphs for NCOR2 IR-scores of the respective subtypes. Significantly different median IR-scores were found for the histological subtypes, respectively ($P=0.008$), **A** for serous, clear cell, endometrioid and mucinous EOC and **B** for low-grade and high-grade serous EOC

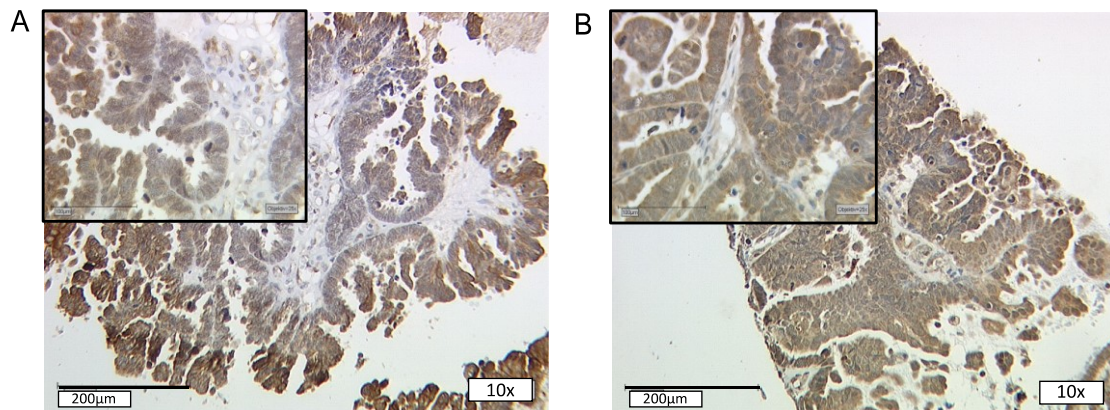
et al. 2010). Some of the complex' proteins represent substantial core components as they account for the repressive function (Oberoi et al. 2011). Each of these components was shown to get involved in carcinogenesis. Transducing β -like related 1 (TBLR1), for instance, is taking an ambiguous role, having either co-repressing or co-activating function

depending on the affected cell type and interacting hormone receptor (Wu et al. 2016). It appeared to be significantly upregulated in ovarian cancer cells and served as predictor for the clinical outcome of EOC patients (Havrilesky et al. 2001; Wu et al. 2016; Ma and Yu 2017). Showing a significantly higher expression in both the nucleus and cytoplasm

Table 2 Correlation analysis of NCOR2 with GPER, ER α and ER β

			NCOR2	GPER	ER α	ER β
Spearman's rho	NCOR2	cc	1.000	0.247	0.071	0.173
		<i>p</i>	–	0.008	ns (0.446)	ns (0.064)
		<i>N</i>	–	115	117	115

Spearman correlation showed positive correlation of NCOR2 immunoreactivity with GPER expression, while no correlation was found with ER α and ER β staining

**Fig. 3** GPER (A) and NCOR2 (B) staining in the same individual. For the same patient with serous EOC high GPER and high NCOR2 staining was detected

of breast and ovarian cancer cells compared to benign tissues, it activates cell proliferation and accelerates migration and invasion through ER-independent and ER-dependent pathways (Wu et al. 2016). However, in EOC, granulosa cell tumors of the ovary and healthy ovarian tissue, no significant correlation of NCOR1 and NCOR2 with ER β was found, consistent with our results of NCOR2 expression and their correlation to ER α /ER β (Hussein-Fikret and Fuller 2005).

Depending on the hormonal signaling context, the NCOR2 complex gets involved into the transcriptional activity of different nuclear receptors, including steroid hormone receptors such as estrogen, androgen and progesterone receptors and is incorporated in different regulation processes (Jepsen and Rosenfeld 2002; Wong et al. 2014). Various studies affirm that an appropriate suppression of NCOR2 expression is a key aspect for proper cell signaling. In contrast, aberrant function can promote cancer and disease progression. In breast cancer cells, NCOR2 was proposed as a candidate to initiate cancer cell growth by modifying the transcriptional activity of ER α or directly influencing the ER α expression (Ciriello et al. 2013; Dobrzycka 2003; Kurebayashi et al. 2000). In prostate cancer, loss of the repressive function of NCOR2 altered the AR response to ligands and contributed to cancer development (Godoy et al. 2012). By interacting with other co-repressing proteins, NCOR2 was identified as a fundamental modulator

of the estrogen receptor in breast cancer patients treated with tamoxifen as adjuvant antihormonal therapy. In these, aberrant NCOR2 appeared to modify the hormone receptor response to tamoxifen while low NCOR2 levels predicted drug resistance against tamoxifen (Gong et al. 2018). For serous ovarian cancer patients, NCOR2 was identified as a possible predictive biomarker of chemotherapy response among seven other genes in a gene array study, correlating with resistance to chemotherapy (Fekete et al. 2020). By epigenetic regulation, co-repressors play a context-dependent role in biological processes and deregulated function might unbalance homeostasis and therefore accelerate malignant degeneration (Mottis et al. 2013).

Estrogen-dependent signaling regulated by GPER was found in several tumor entities (Chevalier et al. 2012; Fujiwara et al. 2012; He et al. 2009; Prossnitz and Barton 2011; Revankar et al. 2005). Thereby, the role of GPER expression in ovarian cancer tumorigenesis either as promoting or as suppressing factor remains still subject to current research: for either ER α -negative or ER α -positive ovarian cancer cells, GPER-mediated signaling pathways were found to promote ovarian cancer cell proliferation (Liu et al. 2014; Albanito et al. 2008). In contrast, GPER expression might inhibit cell growth and therefore serve as tumor suppressor and as a positive prognostic factor for disease-free survival (Ignatov et al. 2013). While comparable nuclear and cytoplasmatic

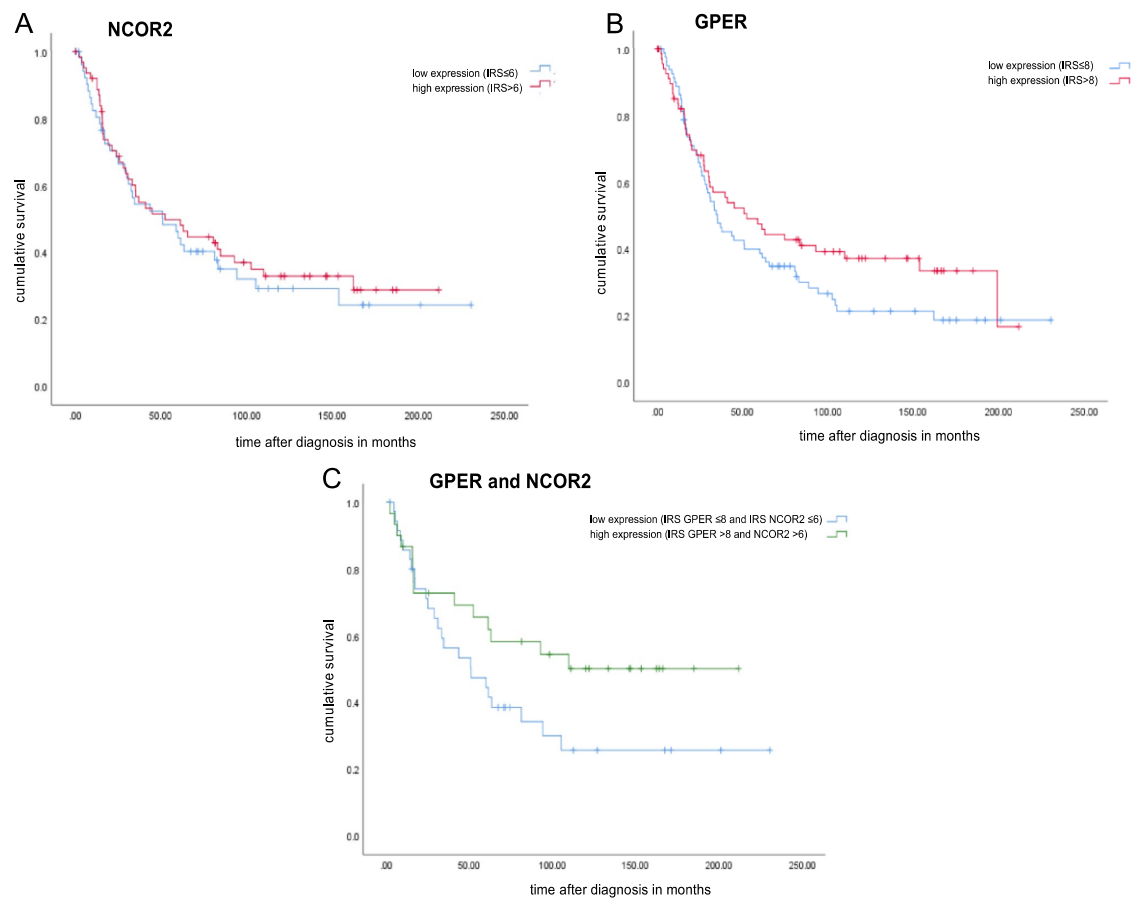


Fig. 4 Kaplan–Meier estimates. Kaplan–Meier estimates of NCOR2 expression (A), GPER expression (B) and combined GPER and NCOR2 expression (C) were analyzed. Both NCOR2 and GPER expression were associated with prolonged overall survival (A, B),

however, without statistical significance. A significant effect on overall survival was detected for the combined high GPER and NCOR2 expression (C)

Table 3 Multivariate analysis

Covariate	Coefficient	Hazard ratio	P value	95% CI
Age (<60y vs. ≥60y)	0.223	1.250	0.395	0.748–2.089
Histology (HGSOC vs. LGSOC vs. clear cell vs. endometrioid vs. mucinous)	−0.128	0.880	0.453	0.630–1.229
FIGO (I vs. II vs. III vs. IV)	0.550	1.733	0.015*	1.113–2.698
NCOR2 (low vs. high expression)	0.044	1.045	0.908	0.491–2.227
GPER and NCOR2 (expression of either GPER or NCOR2 vs. expression of both)	0.045	1.046	0.874	0.600–1.824

In the multivariate Cox regression analysis only the FIGO stage was detected as a significant independent prognostic factor as indicated by asterisks (* $P < 0.05$)

occurrence was described, the specific intracellular localization and signaling pathway has not been identified yet. On a differentiated analysis of a previous study, only nuclear GPER expression was identified as an independent negative prognostic factor (Zhu et al. 2018). The study assumed that nuclear rather than cytoplasmatic occurrence drives carcinogenesis and therefore leads to impaired outcome (Zhu et al. 2018). Further investigation with a discrimination of nuclear and cytoplasmatic expression of GPER in EOC might serve as a future approach to explain these contradictory findings.

However and in accordance with our results, effects on overall survival are not depending on GPER expression alone. The present results indicate a possible regulating role of the NCOR2 nuclear co-repressor complex on GPER-regulated pathways in EOC. High expression of both immunophenotypes was shown to be significantly correlated with a positive impact on OS in ovarian cancer patients possibly indicating a role to serve as a reliable prognostic marker. Approximately 34% of drugs approved by the American Food and Drug Administration (FDA) exert their therapeutic effects by targeting G-protein-coupled receptors (Hauser et al. 2018). Our new insights to the previously relatively unknown role of GPER in EOC carcinogenesis make GPER a potential novel target for treatment strategies. Furthermore and more importantly, drugs with epigenetic targets such as Small inhibiting molecules, Histone deacetylase inhibitors and demethylation agents might be able to modulate the function of the NCOR2 complex and might therefore alter response to chemotherapy and affect the clinical outcome of EOC patients (Graham et al. 2009).

Based on the results of the present study, we hypothesize that NCOR2 mediated modulation of nuclear GPER-mediated signaling pathways is responsible for the improved prognosis. Some limitations of our study should be taken into consideration for the interpretation and evaluation of the present data. Since EOC comprise a heterogeneous group of several histological subtypes that differ in biological, clinical and prognostic behavior, our study is limited by a relatively small sample subset that allows rather general than subtype-specific conclusions regarding the ovarian cancer biology. As a retrospective dataset, additional data on patient characteristics are lacking for a deeper exploration considering factors such as menopausal status, estrogen levels and exposure to hormonal replacement therapy. Moreover, further studies and experimental methods will be necessary to elucidate not only the static receptor expression by immunohistochemistry, but also mechanisms regarding the regulation of gene transcription, modulation of receptor dynamics and ultimately its exact biological function. Based on the presented results, these consecutive experiments are already in preparation by our group and will be prospectively followed. Accordingly, the current study might serve as a potential starting point to further explore the complex

molecular implications of the epigenetic regulator NCOR2 and its components on processes involved in ovarian cancer development, such as GPER-mediated signaling pathways. A comprehensive understanding of the mechanisms of chromatin regulation and transcription activity in ovarian cancer development enables future patient-specific therapeutic approaches.

Author contributions JR, PF, UJ, BC and FT contributed to the concept and design of the study. PF and UJ performed the laboratory work and statistical analysis. JR, PF, BC and FT wrote the manuscript. DM was responsible for the pathological evaluation of the patient tumor tissue. All authors analyzed and interpreted the data, read, revised and approved the final version of the manuscript.

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Data availability The datasets generated during and analyzed during the current study are available from the corresponding author on reasonable request.

Declarations

Competing interests S.M. received research support, advisory board, honoraria and travel expenses from AstraZeneca, Clovis, Medac, MSD, PharmaMar, Roche, Sensor Kinesis, GSK/Tesaro, and Teva. F.T. declares research support, advisory board, honoraria and travel expenses from AstraZeneca, Eisai, Clovis, ImmunoGen, Medac, MSD, PharmaMar, Roche, and GSK/Tesaro. The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Ethical approval Approval by the Ethics Committee of the Ludwig-Maximilian-University, Munich, Germany was obtained (approval number 227-09 and 18-392). The analyzed tumor samples were formerly utilized for pathological diagnostics and then archived by the Department of Obstetrics and Gynecology, University Hospital, LMU Munich, Munich, Germany. The patient's personal data was entirely anonymized and investigators were blinded during the analysis.

Consent to participate The studies involving human participants were reviewed and approved by Ludwig-Maximilian-University, Munich, Germany. Written informed consent for participation was not required for this study in accordance with the national legislation and the institutional requirements.

Consent to publish Not applicable.

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6. Publikation III

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The role of EP-2 receptor expression in cervical intraepithelial neoplasia.

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ORIGINAL PAPER



The role of EP-2 receptor expression in cervical intraepithelial neoplasia

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Abstract

Prostaglandin induced signalling is involved in different cancers. As previously described, the EP3 receptor expression decreases with increasing stage of cervical intraepithelial lesions (CIN). In addition, in cervical cancer EP3 is an independent prognosticator for overall survival and correlates with FIGO stages. Currently the role of Prostaglandin 2 receptor 2 (EP2) in CIN is unknown. The aim of this study was to analyse the expression of EP2 for potential prognostic value for patients with cervical dysplasia. EP2 expression was analysed by immunohistochemistry in 33 patient samples (CIN1–3) using the immune-reactivity scoring system (IRS). Expression levels were correlated with clinical outcome to analyse prognostic relevance in patients with CIN2. Data analysis was performed using non parametric Kruskal–Wallis and Spearman rank sum test. Cytoplasmic expression levels of EP2 correlated significantly ($p < 0.001$) with different grades of cervical dysplasia. Median EP2-IRS in CIN1 was 2 ($n = 8$), 3 in CIN2 ($n = 9$) and 6 in CIN3 ($n = 16$). Comparing regressive ($n = 3$, median IRS = 2) to progressive ($n = 6$, median IRS = 4) CIN2 cases the median IRS differed significantly ($p = 0.017$). Staining intensity ($p = 0.009$) and IRS ($p = 0.005$) of EP2 and EP3 correlate inversely. EP2 expression level significantly increases with higher grade of CIN and could qualify as a potential prognostic marker for the regressive or progressive course in CIN2 lesions. These findings emphasize the significant role of PGE2 signalling in CIN and could help to identify targets for future therapies.

Keywords EP-receptor · EP2 · Prostaglandin E2 · CIN · Cervical intraepithelial neoplasia · Cervical cancer HPV

Introduction

After breast-, colorectal- and lung cancer, cervical cancer represents the fourth most common malignant tumour in women worldwide (Wallis 2014; Watson et al. 2014).

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Approximately 500,000 women worldwide are newly diagnosed with cervical cancer per year. 260,000 women die from the disease each year (Gottlieb 2016; Jiang et al. 2015; Landy et al. 2016). Incidence and mortality of cervical cancer correlates negatively with the Human Development Index and varies extremely in geographic contexts (Wentzensen 2016). Regarding Germany, 4500 women were diagnosed with cervical cancer in 2014 and 1500 of these patients died tumour associated (Zentrum für Krebsregisterdaten 2019). After the implementation of Pap smear screening, which detects precursor lesions of cervical epithelium, incidence dropped considerably (Hester et al. 2019). The persistent infection with specific types of high-risk papillomaviruses is considered the main risk of intraepithelial neoplasia and especially in the development of cervical cancer (Schiffman et al. 2011). The precursor lesions were formerly called cervical intraepithelial neoplasia (CIN) and ranged from CIN1 to CIN3 (Santesso et al. 2016). In 2014 the histological WHO classification has been altered, and cervical intraepithelial neoplasia is referred to as squamous

intraepithelial lesion (SIL) since 2014 (Lu and Chen 2014). The lesions are divided in low grade (LSIL) and high grade squamous intraepithelial lesions (HSIL). CIN2 and CIN3 are now combined in HSIL (Lu and Chen 2014). However, pathologists still specify their diagnosis with CIN2/CIN3 due to the risk of progression to a cervical carcinoma that may differ between CIN2 and CIN3 (Luo et al. 2018; Papoutsis et al. 2017). Consequently, the therapy options also vary from conservative approaches to surgical treatments (Saah-Briffaut et al. 2006). Young women in childbearing age could especially profit from a watchful waiting strategy as conization increases the appearance of pregnancy complications such as cervical insufficiency and preterm labour (Wilkinson et al. 2015). However, apart from the size of the lesion there is no established marker for the prediction of progression or remission of CIN2 lesions (Kühn et al. 2015).

Heidegger et al. previously indicated that the prostaglandin E2-receptor EP3 is an independent negative prognostic factor in cervical cancer patients. The expression levels and the clinical outcome were proven to correlate with tumour stage (Heidegger et al. 2017). In addition, Hester et al. demonstrated that EP3 receptor expression levels correlate inversely with grades of CIN (Hester et al. 2019). Our aim was to further investigate the role of prostaglandin receptors in cervical intraepithelial neoplasia. This study is focussed on the EP2 receptor, as it is unique among all EP receptors. The fact that it is not desensitized by Prostaglandin E2 (PGE2) sets it apart from other EP receptors and highlights its role in the deferred phases of cellular response (Kalinski 2012).

Materials and methods

Tissue samples

The cervical tissue samples used in this study were collected from patients treated between 2007 and 2014 in the Department of Gynaecology and Obstetrics from Ludwig-Maximilians-University of Munich, Germany. This cohort was analysed in previous studies from our group (Hester et al. 2019; Kolben et al. 2016; Vogelsang et al. 2020). Due to multiple sections the CIN lesions got lost on the slides in many cases, which therefor had been excluded from the present study.

In total, 38 tissue samples of cervical dysplasia were immunohistochemically stained with anti-EP2-antibody; the staining was successful in 33 cases. Of these, 8 were classified as CIN1, 9 as CIN2 and 16 as CIN3. On their first visit all patients were tested positive for high risk Human Papillomavirus (Hybrid Capture 2, Quiagen). Histopathological grade of dysplasia and diagnosis were confirmed by a second gynaecological pathologist. Regarding the CIN2

collective, only cases with either a histologically confirmed progress ($n=6$) or regress ($n=3$) were used. The follow-up interval for patients with CIN2 ranged from 5 to 14 months. The cases that were classified as CIN2 at the latest possible date and had been ranked as CIN3 previously, were defined as regress. CIN2, which had progressed from a former CIN1 were also defined as progress.

The tissue samples were eligible for this study after all routine histopathological diagnostic procedures were completed. The data of the patients were completely pseudonymized. All analytic procedures complied with the Helsinki Declaration guidelines (Reference No. 167-14). Informed consent of the patients was guaranteed before study participation. The Ethics Committee of the Ludwig-Maximilians-University (Munich, Germany) accepted the design of the study.

Immunohistochemistry

The immunohistochemistry of the paraffin-embedded cervical tissue samples was conducted as according to our IHC-protocol, which is provided in the supplement. First the samples were dewaxed for 20 min in xylol, then washed in 100% ethanol. In order to suppress the activity of the endogenous peroxidase slides were placed into 3% methanol/ H_2O_2 for 20 min. Rehydration in a descending alcohol series followed. The slides were boiled in an airtight pot for 5 min at +100 °C in a trisodium citrate buffer solution (Merck 244 and Merck 6448) with pH = 6 to unmask the antigen from formalin-fixation-associated protein-agglomeration. Washing in distilled water and PBS-buffer followed. The first diluent of the Polymer kit (ZytoChem Plus HRP Polymer System, Berlin, Germany) was applied for 5 min. The samples were incubated overnight at +4 °C for 16 h with the anti-EP2-primary-antibody (anti-PTGER2 antibody polyclonal rabbit IgG; ABCAM, Cambridge, UK). After washing in PBS-buffer Reagents 2 (Post block) and 3 (horseradish peroxidase -Polymer) of the Polymer kit were administered. Substrate-staining was performed for two and a half minutes with DAB (chromogen substrate kit, Dako, Hamburg, Germany). Counterstain by hemalaun colouring and dehydrogenation in an ascending alcohol series followed, before the slides were mounted with "Eukitt" (Orsatec, Bobingen, Germany) (Heidegger et al. 2017). According to information in the human protein atlas sigma and placenta tissue were chosen as positive control. To get a negative control IHC staining was performed as characterized above, replacing the anti-EP2-primary-antibody by a rabbit negative control serum. The immune-reactivity scoring system (IRS, Remmele score) was applied to rate immunostaining semi-quantitatively using a Leitz (Wetzlar, Germany) microscope type Diaplan. The PL

Fluotar objective lens provides a magnification/N.A 10/0.30. Images were captured with a JVC camera type KY-F55B with 440,000 pixel for PAL (JVCKENWOOD GmbH, Bad Vilbel, Germany) and the DISKUS acquisition software version 4.60.2017—#223 (Technisches Büro Hilgers, Königswinter, Germany). All pictures in this paper have got an image bit depth of 8bit in RGB-colourmodell. Figures 1d and 2c have got 150 dpi in height and width, all others have got 72 dpi. The IRS multiplies the intensity of the staining (0 = no, 1 = weak, 2 = moderate, 3 = strong staining) with the percentage of positive cells (0 = no staining, 1 ≤ 10% positive cells, 2 = 11–50% positive cells, 3 = 51–80% positive cells, 4 ≥ 81% positive cells). At an IRS of 0–1 the staining is negative, from 2–3 it is mildly positive, 4–8 is moderately positive and 9–12 strongly positive (Remmele et al. 1986). In order to obtain more precise numbers also the percentages were multiplied with the intensity. The analysis concerning the quantity of EP2-receptor expression in CIN2 was blinded for regress vs. progress of the dysplasia.

Immunohistochemistry regarding the EP3 were derived from a previous study performed by our group (Hester et al. 2019).

Statistical analysis

For statistical analysis SPSS 25 (PASW Statistic, SPSS Inc., IBM, IL, USA) was used. To compare the expression of EP2 in varying levels of the cervical dysplasia the non-parametric Kruskal–Wallis rank-sum test was applied. The correlation between levels of EP3 and EP2 was tested with the non-parametric Spearman rank correlation test. *p* values ≤ 0.05 were considered as statistically significant. Figures were configured with SPSS 25 and Microsoft Power Point 2016 (Microsoft, Redmond, WA, USA).

Results

EP2 expression increases with progressing grade of cervical dysplasia

We compared the EP-2-IR-scores between the groups of CIN1-3 to analyse differences in EP2 expression levels. The expression of EP2-receptors in the cytoplasm increased significantly in correlation with increasing grade of cervical dysplasia as shown in Fig. 1a. This difference was statistically significant when each grade of dysplasia was compared to the next higher one. In CIN1 the median EP2-IRS in the

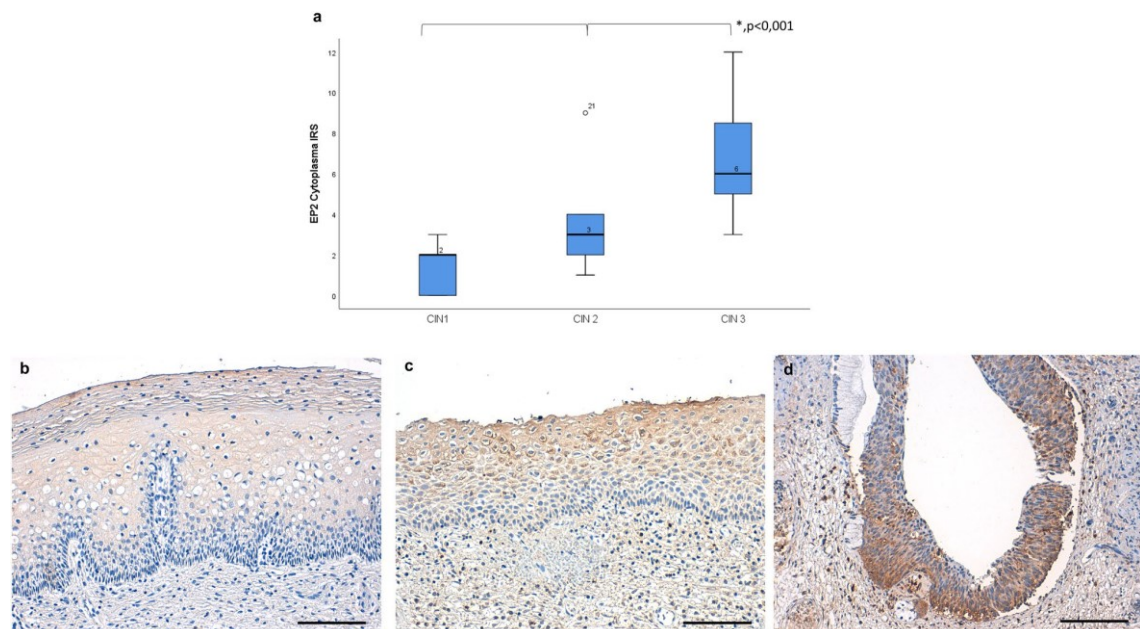


Fig. 1 The expression of EP2-receptors in the cytoplasm increased significantly with increasing grade of cervical dysplasia, displayed by boxplots. The median value is stated above the median-line within the boxes (a). The images show representative microphotographs of

EP2 staining in CIN1 (b IRS 2), CIN2 (c IRS 6) and CIN3 (d IRS 9). 200× magnification was used for picture b, c and d. Scale bars refer to 100 μm. Asterisk represents statistically significant differences in the staining results of CIN1-3

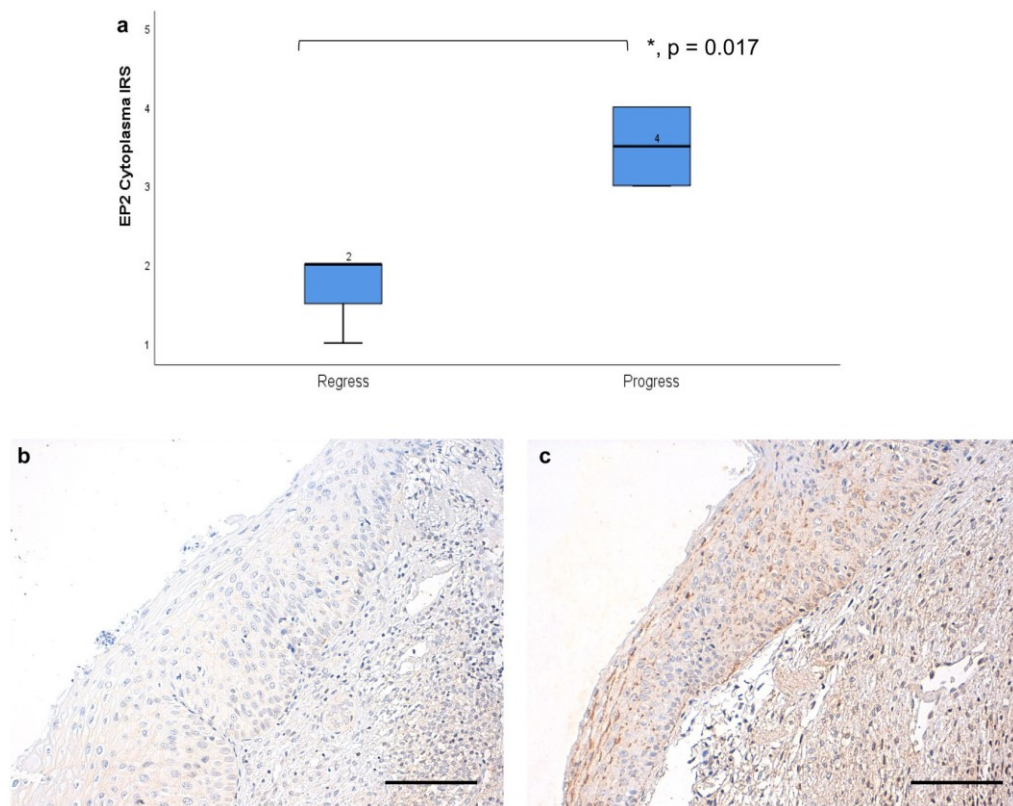


Fig. 2 The median IR-score in regressive cases and in progressive cases differs significantly as shown by the boxplots (a). The different staining results with the anti-EP2-antibody in regressive (b IRS 1) versus progressive (c IRS 6) CIN2 samples ($p=0.017$). 200× magnifi-

cation was used for picture b and c. Scale bars refer to 100 μ m. Asterisk represents statistically significant differences comparing regressive and progressive CIN2 cases

cytoplasm was 2, in CIN2 incidents the value was 3 and in CIN3 cases the median EP2-IRS was 6 ($p < 0.001$).

Exemplary staining for all grades of CIN are shown in Fig. 1b–d.

Cytoplasmic IRS of EP2 positive cells is higher in CIN 2 lesions with a progressive course of the dysplasia

To determine if EP2-receptor expression might serve as a prognostic factor in regard to a progressive or regressive course in cervical dysplasia, we compared EP2 expression between CIN2 cases with histologically confirmed regress or progress. Although the number of cases was little ($n=3$ for regress, $n=6$ for progress) the study revealed statistically significant differences between the cytoplasmatic IRS of EP2-receptor expressions. In regressive cases the median IR-score was 2, while it was 4 in progressive cases ($p=0.017$) as shown in Fig. 2a. Figure 2b and c display the different staining in regressive (Fig. 2b) versus progressive (Fig. 2c) CIN2 samples ($p=0.017$).

Intensity and IR-Score of EP2 correlates negatively with EP3 IRS and intensity

Former research indicated that EP3-receptor expression decreases with increasing grade of cervical dysplasia (Hester et al. 2019). The non-parametric Spearman correlation test revealed that both staining intensity and IRS of EP3 and EP2 correlate negatively on a significant level as presented in Table 1 and Fig. 3a.

Figure 3b and c show the comparison of the staining results in the same tissue sample of CIN1 for a staining with EP2 (IRS 0) and EP3 (IRS 12), representing the inverse correlation of the receptor types.

Discussion

Herein we analysed the expression of the EP2 receptor in CIN samples for potential prognostic information for patients with cervical dysplasia. First, the level of EP2

Table 1 Correlation between EP3 and EP2 staining results

		EP3 intensity	EP3 IRS
Spearman-Rho	EP2 intensity		
	Correlation coefficient	−0.470**	−0.486**
	Sig. (2-tailed)	0.009	0.006
	N	30	30
	EP2 IRS		
	Correlation coefficient	−0.465**	−0.501**
	Sig. (2-tailed)	0.010	0.005
	N	30	30

**represent statistically significant differences at ($p < 0.001$)

Sig. significance, N number of cases, IRS immunoreactive score

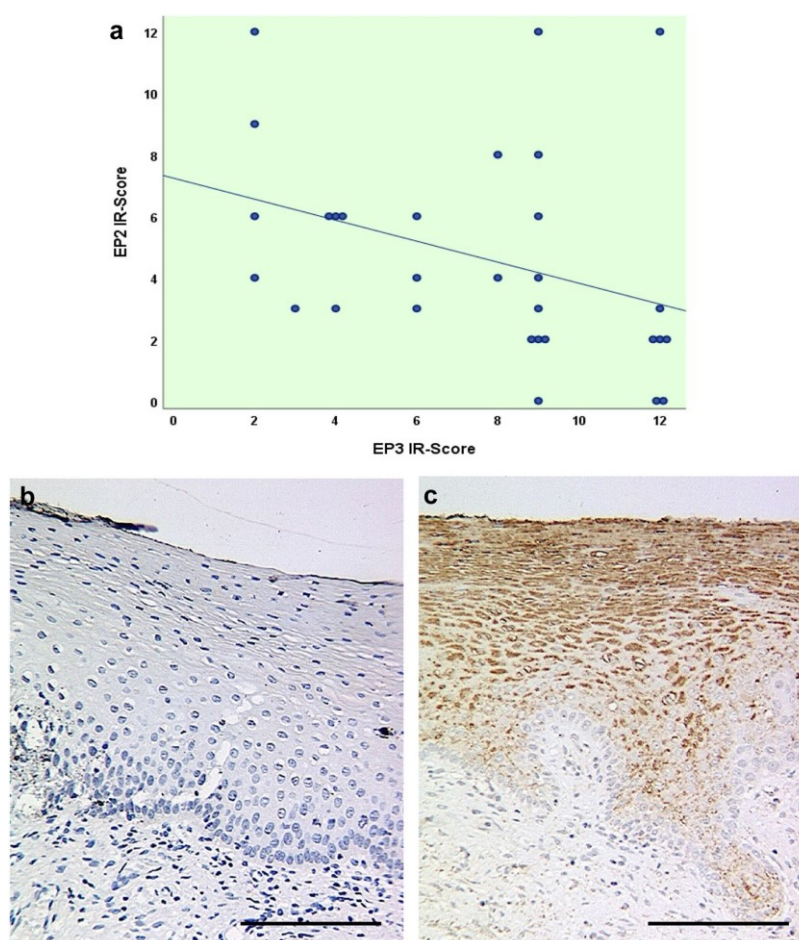
receptor expression was compared to the grade of the dysplasia. In addition, we correlated the receptor expression to the clinical course of CIN2 samples. The analysis revealed that the median IR score of EP2 increases significantly

with increasing grade of dysplasia (CIN1 = 2, CIN2 = 3, CIN3 = 6). CIN2 patients with a regressive clinical course had significantly lower EP2 levels compared to those with a progressive course. Therefore, increasing EP2 expression might indicate a progression of CIN towards cervical cancer.

The small number of CIN samples analysed ($n = 33$) is a critical limitation of our study. The study group proved to be adequate powered and well-reviewed by previous studies of our work group (Hester et al. 2019; Kolben et al. 2016; Vogelsang et al. 2020). However, due to several sections of the cervical biopsies, cases with missing CIN on the slide had to be excluded in the present study. The possibility of a colposcopy sampling error in the follow up check might represent an additional potential problem. In general, larger patient cohorts and further studies are needed to validate our findings.

The EP2 receptor is a G-protein coupled receptor with seven transmembrane domains bound to a heterotrimeric G-protein comprising the stimulatory $G_{\alpha s}$ and $G_{\beta\gamma}$ subunits

Fig. 3 Correlation diagram for IR-score of EP2 and EP3 representing the inverse correlation of the two prostaglandin receptors in CIN tissues (a). The comparison of the staining results in a tissue sample of the same patient with CIN1 for a staining with the anti-EP2-antibody (b) and the anti-EP3-antibody (c) represents this inverse correlation. EP2 was not detected in the staining (IRS 0) whereas EP3 was seemingly highly expressed (IRS 12). 200× magnification was used for picture b and c. Scale bars refer to 100 μ m



(Gilman 1987). It is physiologically activated by PGE₂, a proinflammatory factor with immunosuppressive function (Phipps et al. 1991). PGE₂ derives from arachidonic acids, which is firstly converted to prostaglandin H₂ by cyclooxygenase 1/2 (COX 1/2) enzymes and further processed by PGE₂ synthases (Lambeau and Lazdunski 1999). PGE₂ is known to operate in many processes such as apoptosis, angiogenesis, chronic inflammation, tumour immunity, proliferation, migration and invasion (Kalinski 2012). Compared to other EP receptors, EP₂ is interestingly not desensitized by PGE₂ and therefore may contribute to deferred phases of cellular response (Nishigaki et al. 1996).

The G α activation of the EP₂ receptor can result in increased cAMP levels and activation of protein kinase A which regulates downstream transcription factors such as cAMP response element-binding protein (Fujino et al. 2005). Direct binding of G α to regulator of G protein signalling promotes the release of glycogen synthase kinase-3 β (GSK-3 β) resulting in the activation of β -catenin pathway, which triggers the transcription of genes such as c-myc, cyclin d1 and vascular endothelial growth factor (Vaid et al. 2015). However, activation of serine/threonine-specific kinase (Akt) via G $\beta\gamma$ and phosphoinositide-3-kinase (PI3K) results in the inactivation of GSK-3 β (Castellone et al. 2005). As a consequence, accumulated β -catenin can migrate to the nucleus to stimulate gene transcription via TCF/LEF family of transcription factors (Prasad and Katiyar 2014). When EP₂ forms a complex with β -arrestin it can also function in a G protein-independent manner (Chun et al. 2009). With β -arrestin as a regulator EP₂ can inaugurate pathways of PI3K, Akt, proto-oncogene tyrosine-protein kinase Src, extracellular signal-regulated kinases, c-Jun N-terminal kinases and epidermal growth factor receptors (Sun and Li 2018).

To this point, very little knowledge has been identified of the prostaglandin receptors in cervical intraepithelial neoplasia. Hester et al. showed that EP₃ expression significantly decreases with higher grades of cervical intraepithelial neoplasia (Hester et al. 2019) and the expression levels of EP₃ correlate with tumour stage as well as clinical outcome as Heidegger et al. could confirm (Heidegger et al. 2017). However, currently comparable studies analysing the expression of EP₂ in cervical dysplasia are missing.

The role of EP₂ has been studied in many malignancies as most of the induced pathways play a major role in cell proliferation, migration and angiogenesis (Bonanno et al. 2016; Sobhani et al. 2018). For instance, aberrant expression of EP₂ has been found to be associated with chronic inflammation, deregulation of the immune system, angiogenesis, metastasis as well as multidrug resistance and has been observed in cancer of the colon, liver, breast and cervix (Austing et al. 2017; Cui et al. 2017; Gong et al. 2017; Huynh 2017). Besides the impact of EP₂ activation on cell

proliferation in cervical squamous intraepithelial lesions the immunosuppressive effect of EP₂ seems of interest, as only HPV infections which are not cleared by the immune system can cause SILs and cervical cancer (Westrich et al. 2017).

HPV infections have to evade the host immune defence to persist (Schiffman et al. 2011). Incidence of HPV infections and HPV associated cancer is increased in patients with natural killer cell (NK) deficiencies (Orange 2013). Moreover, a strong cytotoxic T cell (CTL) response correlates with the regression of SILs (Woo et al. 2008). PGE₂ contributes to an acute local inflammation. However, its prolonged immune response can shift cytotoxic T helper cell 1 (Th1), CTL and NK cell mediated type 1 immunity towards a Th2, Th17 and a regulatory T cell mediated immunity (Walker and Rotondo 2004). Thereby PGE₂ prevents damage of lung or reproductive tissue (Huang et al. 2010; Vancheri et al. 2004). Although the limitation of type 1 immunity is pivotal for host self-preservation, it contributes to the establishment of infections with intracellular organisms and cancer, as they both depend on immunosuppression (Kalinski 2012).

den Boon et al. analysed the changes in gene expression patterns from HPV infected cervical tissue to cervical cancer. The study displayed that in early lesions, mostly genes functioning in DNA replication and cell division were upregulated. In transition from CIN3 to cancer the expression of genes serving the mitochondrial electron chain is reduced (den Boon et al. 2015). This suggests a switch from oxidative phosphorylation toward anaerobic glycolysis, and is known as the “Warburg effect” (Hsu and Sabatini 2008). As other DNA viruses, HPV sustain hypoxia inducible factor 1 alpha (HIF1 α), possibly also endorsing the Warburg effect (Mazon et al. 2013; Stover 2009). PGE₂ also takes part in the induction of HIF1 α (Jung et al. 2003).

Grabosch et al. revealed in a systemic review that non-steroidal anti-inflammatory drugs (NSAIDs) and selective COX2 inhibitors (celecoxib, rofecoxib) are not effective in the treatment of CIN (Grabosch et al. 2018). Other structures within the COX downstream signalling pathway like EP receptors might serve as alternative drug targets (Ganesh et al. 2018). Apart from that, levels of EP receptors such as EP₂ and EP₃ (Hester et al. 2019) might serve as potential prognostic biomarkers for patients with CIN2 lesions. In particular women in child bearing age, who might suffer from pregnancy complications after conization could benefit from additional prognostic information (Kühn et al. 2015).

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Data availability The datasets generated and analysed during the current study are available from the corresponding author on reasonable request.

Compliance with ethical standards

Conflict of interest AH has received research Grants from the “Walter Schulz Stiftung” and a speaker and advisory board honorarium from Roche, Germany. TMK is employed at Roche at the time of manuscript submission.

Ethical approval All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards. The current study was approved by the Ethics Committee of the Ludwig-Maximilians-University, Munich, Germany (167-14).

Informed consent Informed consent was obtained from all individual participants included in the study.

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