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Die subzelluläre Expression der Co-Regulatoren RIP140 und LCoR in uni- und multifokalen Mammakarzinomen

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1. Publikationsliste der Kumulativen Dissertation

Bei der vorliegenden kumulativen Dissertation handelt es sich um eine publikationsbasierte Darstellung der Forschungsergebnisse. Die ausführlichen Ergebnisse wurden bereits in folgenden Fachzeitschriften veröffentlicht:

Katharina Müller, Sophie Sixou, Christina Kuhn, Stéphan Jalaguier, Doris Mayr, Nina Ditsch, Tobias Weissenbacher, Nadia Harbeck, Sven Mahner, Vincent Cavaillès, Udo Jeschke: **Prognostic Relevance of RIP140 and ERβ Expression in Unifocal Versus Multifocal Breast Cancers: A Preliminary Report.** International Journal of Molecular Sciences. January 2019.

Sophie Sixou, **Katharina Müller**, Stéphan Jalaguier, Christina Kuhn, Nadia Harbeck, Doris Mayr, Jutta Engel, Udo Jeschke, Nina Ditsch, Vincent Cavaillès: **Importance of RIP140 and LCoR Sub-Cellular Localization for Their Association With Breast Cancer Aggressiveness and Patient Survival**.Translational Oncology, October 2018.

Inhaltsverzeichnis

2. Abkürzungsverzeichnis

LCoR	Ligand Dependent Corepressor
RIP140	Receptor Interacting Protein of 140 kDa
RKI	Robert-Koch-Institut
BRCA1/2	Breast Cancer 1/2 - Gen
PTEN	Phosphatase (Phosphatase and Tensin homolog)
ATM	Ataxia teleangiectatica
TNM	Tumor Node Metastasis
UICC	Union Internationale Contre Le Cancer
Ki-67	Antigen Ki-67
DCIS	Duktales Carcinoma In Situ
LN	Lobuläre Neoplasien
ADH	Atypische Duktale Hyperplasie
FEA	Flache Epitheliale Atypie
WHO	World Health Organisation
NST	No Special Type
ILC	Ivasives Lobuläres Carcinom
IPLC	Invasives Pleomorphes Lobuläres Carcinom
TC	Tubuläres Carcinom
MC	Muzinöses Carcinom
ER	Estrogen Rezeptor
PR	Progesteron Rezeptor
HER2	Human Epidermal Growth Factor Receptor 2
IRS	Immunoreactive Score
EGFR	Epidermal Growth Factor Receptor
FISH	Fluoreszenz-In-Situ-Hybridisierung
MAP-Kinase-Weg	Mitogen-Activated-Protein
PI3K/AKT	Phosphoinositide 3-Kinase/Proteinkinase B
E2F	E2F Transcription Factor 1
Sp/KLF-Familie	Krüppel-like factor 67
KRAB	Krüppel-associated box
GLUT4	Glucosetransporter Typ 4
EMT	Epitheliale Mesenchymale Transition
CD133	Cluster Of Differentiation 133
CSC	Cancer Stem Cell
HTH-Struktur	Helix-Turn-Helix-Struktur
ROC	Receiver Operating Characteristic Curve

3. Einleitung

3.1 Epidemiologische Daten

Das Mammakarzinom stellt weltweit die häufigste maligne Tumorentität, sowie die zweithäufigste Krebstodesursache der weiblichen Population dar (1). Weltweit gab es nach Schätzungen der *Global Cancer Statistics* im Jahr 2018 knapp über 2 Millionen Neuerkrankungen (2). Im Jahr 2013 wurden in Deutschland 71.640 Neuerkrankungen bei Frauen und 682 bei Männern erfasst und für das Jahr 2020 wird nach Schätzungen eine weitere Zunahme bis auf über 77.000 Erkrankungsfälle erwartet (1). Ca. 5% aller Neuerkrankungen treten bei Frauen unter 40 Jahren auf und mit zunehmendem Alter steigt die Inzidenz deutlich an (3). Das mittlere Erkrankungsalter bei Frauen beträgt 64,3 Jahre; das Lebenszeitrisiko, an Brustkrebs zu erkranken, beträgt für Frauen ca. 12 %, für Männer ca. 0,1%, damit erkrankt etwa jede achte Frau in ihrem Leben am Mammakarzinom, bei Männern etwa einer von 800 (1). Bezogen auf die Erhebungen des Robert-Koch-Institutes (RKI) konnte aufgrund besserer Diagnostik- und Therapieoptionen seit den 1990er Jahren eine stetige Abnahme der Mortalität verzeichnet werden. Außerdem zeigen sich in den letzten Jahren nach Einführung des Mammographie-Screenings ab 2005 eine häufigere Detektion von Karzinom-Vorstufen und frühen Tumorstadien, sowie konsekutiv eine reduzierte Inzidenz an Brustkrebsfällen in fortgeschrittenen Stadien (1). Im Jahr 2013 gab es in Deutschland gemäß den vorliegenden Daten des RKI 17.853 Sterbefälle, die auf eine Brustkrebserkrankung zurückzuführen waren. Weltweit werden die Todesfälle in Folge eines Mammakarzinoms im Jahr 2018 auf über 620.000 geschätzt (2). Die Inzidenzraten zeigen regional deutliche Unterschiede, so findet sich innerhalb Europas und sogar innerhalb Deutschlands bis heute ein deutliches West-Ost-Gefälle (1). Beispielsweise zeigen sich hohe Inzidenzraten in Belgien (145 Fälle/100.000) im Vergleich zu niedrigeren Erkrankungsraten in Polen (66 Fälle/100.000) (4). Darüber hinaus ergeben sich weitere globale Unterschiede hinsichtlich Inzidenz und Mortalität zwischen westlichen Industrienationen und Entwicklungsländern; die höchsten Inzidenzraten werden wiederum in Westeuropa (Belgien, Dänemark, Niederlande) verzeichnet im Gegensatz zu den geringsten Raten in Mongolia oder Lesotho (5). Diese Differenz ist einerseits auf die bessere flächendeckend vorhandene Diagnostik und Früherkennungsmaßnahmen, andererseits auf bestimmte westliche Lebensstilfaktoren (hinsichtlich Reproduktion, Ernährung, Aktivität, Umwelt) zurückzuführen (3), (5). Auch die limitierten therapeutischen Möglichkeiten in Entwicklungsländern spiegeln sich in den erheblich geringeren Überlebensraten wieder (6).

3.2 Risikofaktoren und genetische Ursachen

Ungefähr 10% aller Brustkrebserkrankungen sind erblich bedingt (3). Häufiger zeigt sich eine Assoziation mit Reproduktionsverhalten, hormonellen Faktoren, Umwelt- oder Lebensstilfaktoren. Eine frühe Menarche und späte Menopause gelten traditionell als hormonelle Risikofaktoren, jedoch konnte 2013 von *Li et al.* in ihrer Analyse, in welcher das reproduktive Risiko erstmals an die Mammakarzinom-Subtypen angepasst wurde, dargelegt werden, dass eine frühe Menarche nicht signifikant mit einem erhöhten Risiko an Brustkrebs zu erkranken einhergeht (7), (8). Eine späte Menopause geht mit einem leicht erhöhten relativen Risiko einher (9). Des Weiteren gelten Nulliparität oder wenige Schwangerschaften, eine späte erste Geburt, sowie eine kurze Stillzeit als hormonell vermittelte Risikofaktoren (3). Eine kombinierte Hormonersatztherapie aus Estrogen und Gestagenen zur Behandlung von postmenopausalen Beschwerden ist mit einem deutlich erhöhten Erkrankungsrisiko assoziiert (10). Auch unabhängig vom Hormoneinfluss zählen zu weiteren karzinogenen Einflussfaktoren beeinflussbare Lebensstilfaktoren wie Adipositas, mangelnde physische Aktivität, westliche Ernährungsmuster, sowie ein hoher Alkohol- und Nikotinkonsum (3). Ein wesentliches Risiko besteht in der familiären Belastung mit Brustkrebs- oder anderen gynäkologischen Tumorerkrankungen. Eine familiäre Belastung liegt in weniger als ein Drittel der Fälle vor, hierbei sollte eine genetische Untersuchung angeboten werden, die in ca. 10% zu einem Mutationsnachweis führt (11). Ein hohes Lebenszeitrisiko besteht bei den Mutationen *BRCA1* und *BRCA2*, welche autosomal-dominant vererbt werden. Das kumulative Risiko an Brustkrebs zu erkranken, steigt bei Mutationsträgerinnen schon in frühen Jahren an, das mediane Erkrankungsalter liegt bei 38 bzw. 45 Jahren (3). Das kumulative Lebenszeitrisiko bei Mutationsträgerinnen wird auf etwa 60% bis 80% geschätzt (12). Auch das seltene Li-Fraumeni-Syndrom folgt einem autosomal-dominanten Erbgang und ist zurückzuführen auf eine Mutation im Tumorsuppressorgen *TP53*, was über die Malfunktion des Proteins *p53* zur Dysregulation des Zellzyklus führt und assoziiert ist mit verschiedenen Tumoren (Mammakarzinome, Hirntumore, Leukämien, adrenokortikale Tumore) (13). Die Fanconi-Anämie ist eine seltene autosomal-rezessiv vererbte Erkrankung, die auf der Mutation von bisher 15 identifizierten Genloci beruht und zur chromosomalen Instabilität, sowie zu dysfunktionalen DNA-Reparatur-Mechanismen führt. Die Erkrankung zeichnet sich mitunter durch das Auftreten von aplastischen Anämien, Leukämien, sowie gynäkologischen Tumoren aus (14). Das PTEN- oder Cowden-Syndrom ist ein seltenes autosomal-dominant vererbtes Syndrom mit erhöhtem Risiko für multiple Hamartome, sowie Schilddrüsen-, Mamma- und Endometriumkarzinome (3). Des Weiteren findet sich ein vermehrtes Auftreten von Mammakarzinomen beispielsweise bei Genveränderungen beim Peutz-Jeghers-Syndrom (*STK11*-Mutation), bei der Ataxia teleangiectatica (*ATM*), sowie beim Lynch-Syndrom (*MLH1*, *MSH2*, *MSH6*, *PMS2*, *EPCAM*) (15), (16). Diese und weitere Gene sind Bestandteil verschiedener diagnostischer Genexpressionsanalysen, beispielsweise mittels der Testung durch *Oncotype Dx* (17). Anhand der Gentestung wird ein Punktwert (Risikoscore) ermittelt, welcher zur Abschätzung des Rezidivrisikos dient und beim operablen Mammakarzinom die

Entscheidung zur Durchführung einer adjuvanten Chemotherapie neben klinischen und histopathologischen Kriterien weiter standardisieren soll (18). Seit Juni 2019 soll nach Empfehlung des Gemeinsamen Bundesausschusses (G-BA) die Testung durch Genexpressionsanalysen erstattet werden (19).

3.3 Klassifikationen des uni- und multifokalen Mammakarzinoms

Das Mammakarzinom umfasst eine Vielzahl an Tumorveränderungen, wobei non-invasive Risikoläsionen von invasiven Karzinomen unterschieden werden.

Die Einteilung der Neoplasien erfolgt nach der TNM-Klassifikation gemäß der Union Internationale Contre Le Cancer (UICC) und erfasst die Tumorgröße und Ausbreitung in das angrenzende Gewebe (T), eine Metastasierung in regionäre Lymphknoten (L) oder ferne Metastasierung (M), des Weiteren kann das Tumorresektat pathologisch eingeordnet werden (pTNM) (20), (21). Der Differenzierungsgrad (Grading) invasiver Mammakarzinome wird anhand der histo- und zytologischen Analyse nach *Elston und Ellis* bestimmt: Es werden die Kriterien der tubulären Differenzierung, der nukleären Polymorphie, sowie der Mitoserate semiquantitativ bewertet und zu einem Summenscore zusammengefasst (22), (23), (11). Die Abstufung der Malignitätsgrade reicht von G1 (gut differenziert), G2 (mäßig differenziert) bis G3 (gering differenziert). Bei mäßig differenzierten Tumoren kann die Diagnostik um die immunhistochemische Analyse des Proliferationsfaktors Ki-67 ergänzt werden (24). Das Protein Ki-67 ist ein Mitosemarker und selbst maßgeblich an der Regulation des Zellzyklus, sowie am koordinierten Ablauf der Mitose und der perichromosomalen Stabilität beteiligt (25). Bei einer erhöhten Proliferationsaktivität (Ki-67-Positivität größer oder gleich 25%) kann von einem erhöhten Risiko ausgegangen werden, was unter anderen Faktoren in der Entscheidung zur Durchführung einer adjuvanten Chemotherapie berücksichtigt werden kann (24), (11). Zusätzlich kann anhand der Parameter Tumorgröße, Grading und Lymphknotenstatus die Prognoseabschätzung anhand des Nottingham-Prognose-Index bewertet werden, welcher das 15-Jahres-Überleben prognostisch abbildet (26), (11).

In der histologischen Typisierung der non-invasiven Neoplasien finden sich zu 95% Läsionen, die von den Brustdrüsengängen ausgehen und als Duktales Carcinoma In Situ (DCIS) definiert werden (27). Zu 5% finden sich lobuläre Neoplasien (LN), die von den Brustdrüsenläppchen ausgehen (4). Des Weiteren gehören zu dieser Gruppe die atypische duktale Hyperplasie (ADH), die flache epitheliale Atypie (FEA) und das intraduktale Papillom. Eine Sonderform stellt die seltene mamilläre Paget-Erkrankung dar, eine epidermale Neoplasie, die sich klinisch durch ein erythematöses Ekzem der Mamille präsentiert und in über 90% mit einem intraduktalen Karzinom assoziiert ist (28).

Nach der WHO-Klassifikation soll in der feingeweblichen Untersuchung des invasiven Mammakarzinoms eine Abgrenzung von speziellen und gemischten Tumortypen erfolgen; ein spezieller Tumortyp liegt vor, wenn über 90% ein spezifisches Muster abbilden, wohingegen bei einem gemischten Typ 10-49% kein charakteristisches Muster aufweisen (29). Am häufigsten findet sich demnach das invasive Karzinom, kein spezieller Typ (no special typ, NST) mit 50-80%, hierunter werden unterschiedliche Tumoren ohne vorherrschendes Tumormuster gezählt (11). Zu 5-15% zeigt sich ein invasives lobuläres Karzinom (invasive lobular carcinoma, ILC) mit einem charakteristischen kleinzelligen Bild, sowie infiltrierendem Wachstum; ein seltener Subtyp hierunter besteht in der Entität des invasiven pleomorphen lobulären Karzinoms (invasive pleomorphic-type lobular carcinoma, IPLC) (30). Nur 1-4% aller invasiven Karzinome macht das tubuläre Karzinom (tubular carcinoma, TC) aus, welches sich durch das Auftreten von ovalen oder runden Tubuli auszeichnet und mit einer günstigen Prognose assoziiert ist (31), (11). Das muzinöse Karzinom (mucinous carcinoma, MC) präsentiert sich durch eine deutliche extrazelluläre Schleimanhäufung, liegt in etwa 2% aller Fälle vor und ist mit einer sehr günstigen Prognose assoziiert (32),(29). Des Weiteren umfasst eine Subgruppe in der WHO-Klassifikation die Karzinome mit medullären Eigenschaften und inkludiert das reine medulläre Karzinom, welches in weniger als 1% der Fälle vorliegt; charakteristischerweise zeigen sich eine scharfe Abgrenzbarkeit, wenig differenzierte Zellen, eine synzytiale Architektur und lymphoide Infiltration des Tumors und der Peripherie (33), (29).

Eine Sonderstellung nimmt das inflammatorische Mammakarzinom ein, welches sich klinisch als meist Quadranten übergreifende, entzündliche Läsion mit Rötung und Schwellung zeigt und als fortgeschrittenes Tumorstadium betrachtet werden muss, folglich nach der TNM-Klassifikation in das Stadium T4d eingeordnet wird (11), (34).

Neben der histologischen Einordnung gehört die Bestimmung der Expression der Hormonrezeptoren Estrogen Rezeptor (ER) und Progesteron Rezeptor (PR), sowie des Human Epidermal Growth Factor Receptor 2 (HER2) zu jeder standardisierten Diagnostik. Die Expression der Hormonrezeptoren ER und PR wird immunhistochemisch abgebildet und etwa 70% aller invasiven Mammakarzinome zeigen einen positiven Hormonrezeptorstatus (35). Hierbei wird die Färbeintensität mit der Quantität der positiven Zellen kombiniert und in Prozent angegeben, zusätzlich kann eine Beschreibung mittels des Immunreaktiven Scores (IRS) nach Remmele und Stegner oder des Allred-Scores stattfinden (11), (36). Der HER2 Rezeptor ist eine membranständige Rezeptor-Tyrosinkinase aus der Familie der epidermalen Wachstumsfaktorrezeptoren (EGFR), der über verschiedene intrazelluläre Signalkaskaden eine maßgebliche Rolle in der Zellproliferation und Hemmung der Apoptose einnimmt (35). Eine HER2-Überexpression findet sich in etwa 20-25% aller invasiven Mammakarzinome und ist mit einem aggressiveren Tumorverhalten und einer schlechteren Prognose assoziiert (37). Eine HER2 Überexpression wird über immunhistochemische Analyse anhand einer vierstufigen Skala erhoben, eine genauere Methode stellt die In situ-Hybridisierung, FISH-Testung dar (11). Als Konsequenz kann bei HER2-Positivität eine Therapie mit dem monoklonalen Antikörper

Trastuzumab begonnen werden (35). Der HER2 Status trägt zur weiteren Klassifikation der invasiven Mammakarzinome bei: in der Zusammenschau der verschiedenen immunhistochemischen Parameter werden klinisch relevante molekulare Subtypen zur Risikoeinstufung und Entscheidungsgrundlage für eine adjuvante Chemotherapie gebildet. Hauptsächlich erfolgt eine Unterscheidung in Luminal A, Luminal B, HER2-positiv und „Triple-Negative" oder „Basal-Like"-Typ (37). Die Subtypen Luminal A und B zeigen eine ER- und PR-Positivität und können zusätzlich HER2-positiv sein, der Subtyp „Triple Negativ" oder „Basal-Like"-Typ ist negativ für ER, PR und HER2 (38). Je nach Ausprägung des Subtyps soll nach den St. Gallen- Konsensus-Empfehlungen eine adjuvante Chemotherapie durchgeführt werden (39). Die neu eingeführten Genexpressionsanalysen erörtern eine weitere molekulare und genetische Subkategorisierung (40).

Die Fokalität beim Mammakarzinom unterliegt bisher keiner international einheitlich standardisierten Definition. Häufig werden Multifokalität als zwei oder mehrere Läsionen innerhalb eines Quadranten, Multizentrizität dagegen als zwei oder mehrere Läsionen in mehreren Quadranten beschrieben. Auch die aktuelle S3-Leitlinie zum Mammakarzinom definiert Multifokalität als makroskopisch getrennte Herde innerhalb eines Quadranten oder bei einem Abstand zwischen den Herden von weniger als 4 cm und Multizentrizität dagegen bei mehreren Läsionen innerhalb eines Quadranten oder einem Abstand über 4cm (11), (41), (42). Neuere Daten, wie auch die neueste Version der TNM Klassifikation (21) definieren Multifokalität als mehrere simultane ipsilaterale Tumorläsionen und verzichten auf eine Bezugnahme auf die Quadranteneinteilung, die ohnehin keiner streng anatomischen Einteilung folgt (43). Eine Unterscheidung zwischen Multifokalität und Multizentrizität wird hierbei nicht mehr vorgenommen. Hinsichtlich der Inzidenz von multifokalen Mammakarzinomen gibt es wenige Studien, die sich zum Teil erheblich in ihren Fallzahlen unterscheiden. *Wolters et. al.* detektierten in einer großen (n=8935) retrospektiven multizentrischen Studie (*BRENDA group*) eine Inzidenzrate von 20,8% für multifokale Läsionen (44). Das multifokale Mammakarzinom zeigt eine Assoziation mit regionaler Metastasierung der Lymphknoten, Fernmetastasen, sowie kürzeren Überlebens- und höheren Mortalitätsraten (44), (45), (46), (47), (48). Bei nachgewiesener Multifokalität wird der Hormonstatus (ER, PR und HER2) für die größte Läsion erhoben; eine Analyse aller Läsionen ist Gegenstand aktueller Diskussionen und wird von einigen Autoren empfohlen (43). In einer früheren Arbeit von 2013 von *Weissenbacher et. al.* konnte bereits gezeigt werden, dass der Fokalitätsstatus assoziiert ist mit einer veränderten Tumorbiologie, sowie einer veränderten Expression von E-Cadherin als Marker der epithelialen mesenchymalen Transition bei multifokalen Tumoren (49).

3.4 Interaktion und Einfluss der Transkriptionsfaktoren RIP140, LCoR und ERβ auf die Tumorigenese des Mammakarzinoms

Die Tumorigenese und -progression des Mammakarzinoms werden neben klinischen, histopathologischen Parametern, TNM-Klassifikation, Hormonrezeptor- und HER2-Status durch ein komplexes Netzwerk und Wechselspiel von nukleären Rezeptoren (besonders ERα und ERβ), sowie deren Co-Aktivatoren und Co-Repressoren beeinflusst (50). Die nukleären Rezeptoren ERα und ERβ sind ligandenaktivierte Transkriptionsfaktoren, die nach Bindung von Estrogen (mit verschiedenen Affinitäten: Estron, Estradiol, Estriol, Estetrol oder ähnliche Strukturen) ein Homo- oder Heterodimer bilden und im Zellkern an die entsprechende Promotorregionen binden (51), (50). Die Estrogen-vermittelte Signaltransduktion umfasst genomische (durch Bindung des dimerisierten Rezeptors an spezifische DNA-Sequenzen, sog. hormone response elements) und nicht-genomische Effekte (Interaktion mit Zellmembranproteinen, G-Proteinen, Rezeptor-Tyrosinkinasen, und damit Einfluss auf multiple Signalkaskaden, beispielsweise auf den MAP-Kinase-Weg oder Phosphoinositide 3-Kinase (Pl3K/AKT) Signalweg) (52), (36). Estrogen steigert die Proliferation des Brustdrüsengewebes, ebenso wird das Zellwachstum bei ERα-positiven Tumoren stimuliert (51). Im Tumorgewebe findet sich ein verändertes Expressionsverhältnis von ERα und ERβ mit hohem ERα/ERβ-Verhältnis in Karzinomen aufgrund von verringerter Expression von ERβ (51). Die beiden nukleären Rezeptoren ERα und ERβ haben in ihrer Wirkungsweise zum Teil antagonistische Effekte: ERβ hat inhibierenden Einfluss auf ERα und scheint abhängig durch eine ERα-Induktion zu einer Hemmung des Zellwachstums zu führen (51). Die Estrogen-vermittelte Stimulierung der Zellproliferation ist somit abhängig von der vorherrschenden Expression der ER-Isoformen, sowie des realen ERα/ERβ-Verhältnisses und darüber hinaus von der Expression der Co-Regulatoren, welche ERα oder/und ERβ aktivieren oder inhibieren (51).

In der vorliegenden Arbeit herrscht ein besonderer Fokus auf die Co-Regulatoren RIP140 (Receptor Interacting Protein of 140 kDa) und LCoR (Ligand Dependet Corepressor). RIP140 ist ein einzigartiger dualer Co-Regulator, welcher zusammen mit Steroidhormonen rekrutiert wird und in den Zellkern translokiert, als Transkriptionsfaktor gleichzeitig als Co-Aktivator und Co-Repressor agiert und in mehreren Signalkaskaden maßgeblich an Prozessen der Zellproliferation, Zelldifferenzierung und Apoptose beteiligt ist (53). Das Protein wird in verschiedenen Geweben exprimiert und ist an der Regulation von Brustdrüsenwachstum und -entwicklung, Ovulation und Fertilität beteiligt, sowie an weiteren metabolischen Prozessen beispielsweise der Lipogenese, Gluconeogenese, intestinalen Homöstase, sowie des zirkadianen Rhythmus (53), (54), (55). Im Wechselspiel mit ER konnte eine stärkere Interaktion mit ERβ als mit ERα nachgewiesen werden (51), (56). Der Transkriptionsfaktor interagiert neben nukleären Rezeptoren auch mit verschiedenen Signalkaskaden der Zellproliferation, beispielsweise mit dem Wnt/β-Catenin Signalweg, sowie mit Zellzyklus-regulierenden Genen, beispielsweise der E2F-Familie (57). Es konnte gezeigt werden, dass RIP140 eine wichtige Rolle in

der Karzinogenese verschiedener solider Tumore hat und in die Tumorbiologie des Ovarialkarzinoms, des Kolorektalen Karzinoms, sowie des Hepatozellulären Karzinoms eingreift (57).
LCoR ist ein weiterer Co-Regulator, der hauptsächlich mit Liganden-aktiviertem ERα interagiert und die ER-Aktivität vermittelt über Histondeacetylasen oder unabhängig davon inhibiert (58). Über die physiologische Rolle von LCoR ist bisher wenig bekannt; beschrieben wurde eine wichtige Rolle in der Regulation der hepatischen Homöostase, sowie der Lipogenese (59). Im Mausmodell konnte gezeigt werden, dass LCoR eine Rolle in der Karzinogenese des Prostatakarzinoms spielt (59). LCoR interagiert mit verschiedenen weiteren Transkriptionsfaktoren, beispielsweise der Sp/KLF-Familie (Krüppel-like factor 67 und KRAB-assoziiertem Protein 1), welche eine Rolle in der Erythropoese spielen (59).

3.5 Fragestellung und Studiendesign

In der diagnostischen Aufarbeitung von Mammakarzinomen gelingt zunehmend eine Subkategorisierung der verschiedenen heterogenen Entitäten, welche die bestmögliche klinische und molekulare Tumorcharakterisierung zum Ziel hat. Die Herausforderung hierbei ist es, ein möglichst genaues molekularbiologisches Abbild zu schaffen und die klinisch-therapeutische Herangehensweise anhand einer exakten diagnostischen Einordnung optimal anzupassen. Durch histopathologische Aufarbeitung, sowie der kürzlich eingeführten Genexpressionsanalysen wird eine zunehmende Risikostratifizierung der Patienten vorgenommen, um daraus konkrete klinisch-therapeutische Handlungsweisen abzuleiten (18). Einige Autoren sprechen hinsichtlich einer patientenindividualisierten Verschmelzung von Diagnostik und Therapie zunehmend vom Begriff der „Thera(g)nostik“ (60).

Der Faktor Multifokalität wurde hierbei in der Literatur hinsichtlich einer veränderten Tumorbiologie und –progression bzw. daraus abgeleiteter diagnostischer oder therapeutischer Konsequenz wenig behandelt (61). Die vorliegende Arbeit widmet sich der genaueren Differenzierung zwischen Uni- und Multifokalität und befindet sich an der Schnittstelle zwischen immunhistochemischer Tumorcharakterisierung und Assoziation mit klinischen Parametern. Sie befasste sich einerseits mit der Frage, ob die subzelluläre Lokalisation der Transkriptionsfaktoren RIP140, LCoR und ERβ assoziiert ist mit einer veränderten Tumorprogression (hauptsächlich Bestandteil der Originalarbeit I). Des Weiteren wurde untersucht, ob sich die Tumorbiologie, sowie die Expression verschiedener Transkriptionsfaktoren (hier besonders RIP140, LCoR und ERβ) zwischen uni- und multifokalen

Mammakarzinomen unterscheiden (hauptsächlich in Originalarbeit II erarbeitet). Beide Arbeiten haben zum Ziel, ein besseres Verständnis der Interaktion von nukleären Rezeptoren und deren Coregulatoren zu ermöglichen, sowie deren unterschiedlichen Einfluss auf die Tumorigenese bei uni- vs. multifokalen Tumoren. Darüber hinaus stellt sich die zentrale Frage, ob ein dysreguliertes Expressionsverhalten der untersuchten Coregulatoren bei uni- vs. multifokalen Tumoren mit einem veränderten Patientenüberleben korreliert und damit die immunhistochemische Statuserhebung der genannten Transkriptionsfaktoren vor allem bei multifokalen Tumoren prognostische Relevanz aufzeigt.

Für die vorliegende Arbeit wurden zwei unterschiedliche Studiendesigns gewählt. In einem ersten Schritt wurden zwischen 2000 und 2002 an der Frauenklinik des Klinikums der Universität München ohne Vorselektion alle 320 uni- und multifokale Patientenfälle gesammelt, das Tumorgewebe mittels Paraffineinbettung asserviert und nach den üblichen Kriterien, TNM, Grading, Hormonrezeptor- und HER2-Status, sowie Histopathologie eingeordnet. Die Paraffinschnitte dieser Kohorte wurden anschließend immunhistochemisch auf die subzelluläre Expression der Rezeptoren RIP140, LCoR und ERβ und deren Präsenz in verschiedenen Zellkompartimenten (Nukleus *vs.* Zytoplasma) untersucht und auf ihre Assoziation mit klinischen Parametern, beispielsweise hinsichtlich des Patientenüberlebens geprüft.

In einem zweiten Schritt ließen sich aus dem oben genannten Patientenkollektiv nach bestimmten Matching-Kriterien zwei exakt äquivalente Patientengruppen mit Hilfe eines Matched-Pair-Studiendesigns bilden. Es wurden hierbei 21 unifokale mit 21 multifokalen Patientenfällen verglichen, die Matching-Kriterien umfassten Tumorgröße, Lymphknotenstatus und Grading. Durch das beschriebene Studienmodell kann eine Merkmalsausprägung (hierbei die Expression von Kernrezeptoren) zwischen zwei äquivalenten Gruppen untersucht werden, die sich nur im Fokalitätsstatus unterscheiden. Die Expression von RIP140, LCoR und ERβ wurde anschließend auf die Assoziation mit klinischen Parametern, beispielsweise dem Patientenüberleben, hin untersucht.

Die zugrundeliegende Eigenleistung der vorliegenden Dissertation und bei beiden Publikationen bestand in der Literaturrecherche, sowie in der Auswahl und der Akquise der Patientendaten für die Studie, sowie in der selbstständigen Durchführung der beschriebenen Methoden. Anschließend erfolgte als Eigenanteil die mikroskopische Analyse und Evaluation anhand der beschriebenen Scoring-Systeme. Nach Abschluss des experimentellen Teiles erfolgte die eigenständige statistische Datenauswertung in Korrelation zu den Patientendaten mit Berechnung und Formatierung aller vorliegenden Daten. Die aufgeführten Veröffentlichungen entstanden unter Supervision der genannten Co-Autoren, wurden eigenständig verfasst und im Verlauf von den genannten Co-Autoren Korrektur gelesen.

4. Kumulativer Teil der Dissertation

4.1 Originalarbeit I:

Importance of RIP140 and LCoR Sub-Cellular Localization for Their Association With Breast Cancer Aggressiveness and Patient Survival. Translational Oncology. October 2018.

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Importance of RIP140 and LCoR Sub-Cellular Localization for Their Association With Breast Cancer Aggressiveness and Patient Survival[1]

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Abstract

New markers are needed to improve diagnosis and to personalize treatments for patients with breast cancer (BC). Receptor-interacting protein of 140 kDa (RIP140) and ligand-dependent corepressor (LCoR), two transcriptional co-regulators of estrogen receptors, strongly interact in BC cells. Although their role in cancer progression has been outlined in the last few years, their function in BC has not been elucidated yet. In this study, we investigated RIP140 and LCoR localization (cytoplasm *vs* nucleus) in BC samples from a well-characterized cohort of patients (n = 320). RIP140 and LCoR were expressed in more than 80% of tumors, (predominantly in the cytoplasm), and the two markers were highly correlated. Expression of RIP140 and LCoR in the nucleus was negatively correlated with tumor size. Conversely, RIP140 and LCoR cytoplasmic expression strongly correlated with expression of two tumor aggressiveness markers: N-cadherin and CD133 (epithelial mesenchymal transition and cancer stem cell markers, respectively). Finally, high RIP140 nuclear expression was significantly correlated with longer overall survival, whereas high total or cytoplasmic expression of RIP140 was associated with shorter disease-free survival. Our study strongly suggests that the role of RIP140 and LCoR in BC progression could vary according to their

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prevalent sub-cellular localization, with opposite prognostic values for nuclear and cytoplasmic expression. The involvement in BC progression/invasiveness of cytoplasmic RIP140 could be balanced by the anti-tumor action of nuclear RIP140, thus explaining the previous contradictory findings about its role in BC.

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Introduction

Breast cancer (BC) is the most frequent cancer and the leading cause of mortality in women worldwide [1]. The involvement of nuclear receptors in BC progression and aggressiveness is widely accepted. Human epidermal growth factor receptor 2 (HER2), estrogen receptor (ER) and progesterone receptor (PR) are key prognostic and predictive markers, and their expression is routinely determined in primary BCs [2]. Nuclear expression of ER/PR in tumor tissue is correlated with good outcome and an expected sensitivity to endocrine therapy, such as selective ER modulators (SERMs; *e.g.*, tamoxifen). Conversely, HER2 expression is correlated with poor prognosis in untreated patients with BC and an expected sensitivity to the humanized anti-HER2 antibody trastuzumab [3].

The main nuclear receptor activities are precisely regulated through complex and dynamic interactions of transcriptional co-regulators. Several families of coactivators and corepressors are involved in the development, progression, invasion, and therapy resistance of solid tumors, especially hormone-responsive cancers, such as breast, ovarian and prostate cancers [4,5]. Among the many nuclear receptor co-regulators, Receptor Interacting Protein of 140 kDa (RIP140), also called Nuclear Receptor Interacting Protein 1 (NRIP1), acts predominantly as a corepressor [6–9] through recruitment of histone deacetylase (HDAC) and C-terminal binding proteins (CtBPs) [10,11]. RIP140 plays pivotal roles in normal cell metabolism, especially in lipid metabolism [12,13], and is required for ovulation and mammary gland development [14]. RIP140 could also function as a tumor suppressor in ovarian and colon cancer. Specifically, in ovarian cancer, RIP140 interacts mainly with ERβ and could be involved in the repression of ERα activity by ERβ [15]. In colon cancer, RIP140 inhibits cell proliferation through the Wnt signaling pathway [16]. Similarly, it has been suggested that RIP140 is a favorable prognostic marker in chronic lymphocytic leukemia [17]. In BC, RIP140 acts as a coactivator of ERα-responsive genes, and might regulate tumor progression and response to endocrine therapy [18]. On the other hand, RIP140 is the immediate downstream target of nucleolar protein 14 (NOP14), an RNA binding protein that acts as a tumor suppressor gene in BC through the Wnt/APC/βcatenin pathway [9]. Moreover, RIP140 is overexpressed in BC cell lines and tumors compared with normal breast cell lines and adjacent healthy tissues [7,9]. Importantly, RIP140 expression is higher in the nucleus of epithelial cells in malignant BC, whereas it is stronger in the cytoplasm of stromal cells in benign tumors [7].

We recently demonstrated that RIP140 directly interacts with Ligand-dependent CoRepressor (LCoR) and that the two proteins colocalize in the nucleus of human BC cells. RIP140 positively regulates LCoR expression and is necessary for LCoR-mediated inhibition of gene expression and cell proliferation in BC cells [19]. LCoR is a nuclear protein that interacts with ERα and the repressive activity of which is driven through HDAC and CtBPs recruitment, as described for RIP140 [20–22]. LCoR shows repressive activity in BC cells [19,23], and also inhibits prostate cancer growth in murine models [24]. Moreover, high RIP140 and LCoR mRNA expression were associated with longer survival in a cohort of 183 patients with BC [19]. Very recently, a study confirmed the relevance of LCoR in BC by demonstrating that it inhibits mammary cancer stem cell (CSC) activity [25].

In this retrospective study, we wanted to determine the specific role of nuclear and cytoplasmic RIP140 and LCoR expression in BC. To this aim, we analyzed the tumor sub-cellular expression of these two transcriptional co-regulators in a cohort of 320 patients with BC, and evaluated the correlation with clinicopathological features and the expression of tumor aggressiveness markers.

Materials and Methods

Patient Characteristics

For this study, a well characterized collection of paraffin-embedded breast tumor tissue samples from 320 patients with BC was used. As only eight patients had metastatic BC at the time of diagnosis, the cohort was considered to be composed of patients with primary BC. The study was approved by the Ethics Committee of the Ludwig Maximilians University (LMU) of Munich, Germany (approval number 048–08). BC tissue samples were collected from patients treated for BC at the LMU Department of Obstetrics and Gynecology between 2000 and 2002. All tumors were classified using the tumor-node-metastasis (TNM) classification that includes the tumor size (primary tumor size, or pT, as defined in the TNM classification: pT1a-c, pT2, pT3, pT4a-d), the involvement of regional lymph nodes (N), and presence or absence of metastases (M). The BC histological grade was determined by an experienced pathologist (Dr D. Mayr) of the LMU Department of Pathology, according to the Elston and Ellis modification of the Bloom and Richardson grading system [26]. Patient data, such as age, hormone receptor status (ERα and PR), HER2-amplification, histological grade, metastases, local recurrence, progression and survival, were retrieved from the Munich Cancer Registry. The patients' characteristics are shown in Table 1.

Immunohistochemistry

Expression of ERα, PR and HER-2 was determined in all BC samples of this cohort at the LMU Department of Pathology, Germany, at diagnosis. ERα and PR expression was evaluated by immunohistochemistry, as described previously [26]). Samples showing nuclear staining in more than 10% of tumor cells were considered as hormone receptor-positive, in agreement with the guidelines at the time of the analysis (2000–2002). HER2 expression was analyzed with an automated staining system (Ventana; Roche, Mannheim, Germany), according to the manufacturer's instructions.

Table 1. Patients' Clinicopathological Characteristics

	n	%
Patients	320	100%
ER status *		
Negative	45	14.1%
Positive	201	62.8%
Unknown	74	23.1%
PR status *		
Negative	93	29.1%
Positive	153	47.8%
Unknown	74	23.1%
HER2 status *		
Negative	95	29.7%
Positive	94	29.4%
Unknown	131	40.9%
Triple negative *		
No	169	52.8%
Yes	20	6.3%
Unknown	131	40.9%
Histologic type		
Invasive lobular	42	13.1%
Invasive medullar	12	3.8%
Invasive mucinous	4	1.3%
No Special Type (NST) †	174	54.4%
DCIS (only or with NST)	83	25.9%
Unknown	5	1.6%
Tumor size *		
pT1 a, b, c	205	64.1%
pT2	90	28.1%
pT3	4	1.3%
pT4 a, b, c, d	17	5.3%
Unknown	4	1.3%
Grade *		
I	15	4.7%
II	109	34.1%
III	48	15%
Unknown	148	46.3%
Lymph node metastasis		
No	167	52.2%
Yes	133	41.6%
Unknown	20	6.3%
Local recurrence		
No	263	82.2%
Yes	43	13.4%
Unknown	14	4.4%
Distant metastases ‡		
No	239	74.7%
Yes	67	20.9%
Unknown	14	4.4%

* All data refer to the primary tumor.
† NST include the formerly called "Invasive ductal" and "other" types.
‡ Distant metastases were detected in 8 patients (2.5%) at diagnosis and in 59 patients during the follow-up (18.44%).

Data on N-cadherin and CD133 expression in these BC samples were extracted from a previously published study [27]. For RIP140 and LCoR analysis, samples were processed as previously described [27,28]. Specifically, 3 µm tissue sections, cut from paraffin-embedded BC samples, were dewaxed in xylol (Carl Roth GmbH & Co. KG, Karlsruhe, Germany) at room temperature for 15 min. To block endogenous peroxidases, sections were immersed in a solution of 3% hydrogen peroxide (VWR International S.A.S., Fontenay-sous-Bois, France) in methanol (Sigma-Aldrich, Steinheim, Germany) for 20 min. After rehydrating in decreasing concentrations of ethanol (100–0% in distilled water), sections were boiled in a pressure cooker with sodium citrate buffer (pH 6) for 5 min (for epitope retrieval). Then, sections were washed with distilled water and phosphate buffered saline (PBS), before blocking with Powerblock (Biogenex, San Ramon, CA, USA) in distilled water (1:10) for 5 min. Sections were then incubated with the rabbit polyclonal anti-NRIP1 HPA046571 (1:400 in PBS; Sigma-Aldrich) and the mouse polyclonal anti-LCoR NBP1–83477 antibody (1:50 in PBS; Novus Biologicals, Littleton, CO, USA) at 4 °C for 16 hours. After incubation with the corresponding biotinylated secondary anti-rabbit and anti-mouse IgG antibodies, and with the associated avidin-biotin-peroxidase-complex (both Vectastain Elite ABC Kit; Vector Laboratories, Burlingame, CA, USA), interactions were visualized with the substrate and chromogen 3,3-diamino-benzidine (Dako, Glostrup, Denmark). Sections were counterstained with acidic hematoxylin and dehydrated in increasing concentrations of ethanol (70–100%). They were immediately mounted with Eukitt (Merck, Darmstadt, Germany) before manual analysis with a Diaplan light microscope (Leitz, Wetzlar, Germany) with 2.5x, 10x or 40x magnification. Images were acquired with a digital CCD camera system (JVC, Tokyo, Japan). Negative controls were performed by replacing the primary antibodies with the species-specific isotype control antibodies (Dako, Glostrup, Denmark). Appropriate positive controls (placenta samples) were included in each experiment.

Data Analysis

For RIP140 and LCoR expression, the immunoreactive score (IRS) was determined by evaluating the percentage of positive tumor cells and their staining intensity (IRS = percentage score x intensity score). For the quantification of positive cells (percentage score), BC samples were classified in four groups: no visible staining (score = 0), <10% of stained cells (score = 1), 10–50% of stained cells (score = 2), 51–80% of stained cells (score = 3), and 81–100% of stained cells (score = 4). Staining intensity (intensity score) was evaluated as: absence of staining (score = 0), weak (score = 1), moderate (score = 2), or strong staining (score = 3). Therefore, the maximum IRS value is 12. In doubtful cases, slides were evaluated by two or three independent examiners and the IRS represented the final consent. Staining localization (cytoplasmic and nuclear) was evaluated in parallel, leading to the determination of the cytoplasmic IRS and nuclear IRS separately. When needed, the total IRS was calculated by adding the cytoplasmic and nuclear IRS. For N-cadherin and CD133 expression, the IRS values corresponded to the total expression (*i.e.,* nuclear and cytoplasmic staining) [27].

Statistical Analysis

Statistical analyses were performed using SPSS 23 (IBMSPSS Statistics, IBM Corp., Armonk, NY, USA). The correlations presented in Tables 2 and 3 were obtained by calculating the Pearson or Spearman's rho correlation coefficient (p values of Spearman's rho presented). Data distribution was displayed using box and whisker plots and the Kruskal-Wallis non-parametric one-way analysis of variance was used to detect significant differences. The p value and the number of patients/BC samples analyzed in each subgroup are

Table 2. Correlation Between Total, Nuclear and Cytoplasmic Expression of RIP140 and LCoR

Correlation coefficient n = 299 to 309		RIP140			LCoR		
		Total	Nuclear	Cytoplasmic	Total	Nuclear	Cytoplasmic
RIP140	Total	1.000					
	Nuclear	0.793**	1.000				
	Cytoplasmic	0.874**	0.427**	1.000			
	Total	0.414**	0.248**	0.459**	1.000		
LCoR	Nuclear	0.331**	0.284**	0.327**	0.819**	1.000	
	Cytoplasmic	0.397**	0.173**	0.465**	0.898**	0.536**	1.000

** $P \leq .01$ (Spearman's rho test).

Table 3. Correlation Between RIP140/LCoR with Tumor Size (pT) and EMT/CSC Markers

Correlation coefficient	RIP140 n = 179 to 304			LCoR n = 185 to 309		
	Total	Nuclear	Cytoplasmic	Total	Nuclear	Cytoplasmic
pT	**−0.134***	**−0.181****	−0.074	**−0.134***	**−0.149****	−0.086
NCAD	0.116	0.049	**0.137***	**0.258****	0.111	**0.317****
CD133	**0.222****	**0.155***	**0.201****	**0.189****	0.107	**0.198****

* $P \leq .05$ or
** $P \leq .01$ (Spearman's rho test).

given for each chart. For comparison of survival times, Kaplan–Meier curves were generated. Mantel-Cox (log-rank) tests were performed to compare survival curves (disease-free survival, DFS; or overall survival, OS). For all analyses, p values below 0.05 were considered statistically significant.

Results

RIP140 and LCoR Expression in BC Samples

The tumor samples evaluated for this study were from 320 patients with BC (mean age 59.9 years, range 26–94 years) who were followed for 10–12 years. As patients were treated between 2000 and 2002, hormone receptor and HER2 status were not recorded for all of them at the time of diagnosis (unknown ER and PR status in 19.5% and unknown HER2 status in 37.1% of patients) (Table 1). Most patients (n = 239; 74.7%) had a primary BC without metastases at diagnosis, and 59 (18.44%) developed distant metastases during the follow-up. Distant metastases were detected in 8 patients (2.5%) already at diagnosis, and the metastasis status at diagnosis was unknown in 14 patients (4.38%).

Analysis of RIP140 and LCoR expression in all BC samples showed that 304 and 309 samples were positive for RIP140 and LCoR, respectively. As staining was observed in the nucleus and/or cytoplasm of tumor cells, the IRS was calculated for each subcellular location (Figure 1). Some tumors displayed similar nuclear and cytoplasmic IRS values for the same protein (Figure 1, *A* and *E*, and *B* and *F*), whereas in other BC samples the nuclear and cytoplasmic IRS values were very different (Figure 1, *D* and *H*, and *C* and *G*).

Analysis of the distribution of the nuclear and cytoplasmic IRS values for RIP140 (Supplementary Fig. 1A and C) and LCoR (Supplementary Fig. 1B and D) showed that the highest IRS values was 9 in the nucleus and 8 in the cytoplasm for LCoR, whereas it was 6 in both compartments for RIP140. However, as very few samples had very high LCoR IRS values, the mean IRS were similar for LCoR and RIP140 (1.31 and 2.71 in the nucleus and cytoplasm respectively for LCoR, and 1.71 and 2.12 respectively for RIP140). For both proteins, the mean IRS was higher in the cytoplasm than in the nucleus.

Analysis of the correlations between nuclear, cytoplasmic and total IRS for each protein independently using the Spearman rho (Table 2) showed that for RIP140 (n = 304 samples), the total IRS was strongly correlated with both nuclear and cytoplasmic IRS, and that the nuclear and cytoplasmic IRS were correlated between them ($P < .01$). Similar results were obtained for LCoR (n = 309) ($P < .01$).

The correlations between nuclear and cytoplasmic IRS values for RIP140 and LCoR were confirmed also when BC samples were classified in two groups based on the absence (IRS = 0) and presence (IRS >0) of nuclear expression of RIP140 or LCoR (Supplementary Fig. 2A-B) (box plots and Kruskal-Wallis non-parametric test, $P < .001$).

Correlation Between RIP140 and LCoR Expression

Concerning the total expression of RIP140 and LCoR, both transcription co-regulators were negative (IRS = 0) in 1.7% of BC samples (Figure 2), whereas they were both positive (IRS >0) in 81.3% of tumors. Positivity for only one was detected in 17% of samples. A similar distribution was observed for the nuclear and cytoplasmic IRS values. More than 60% of tumor samples expressed

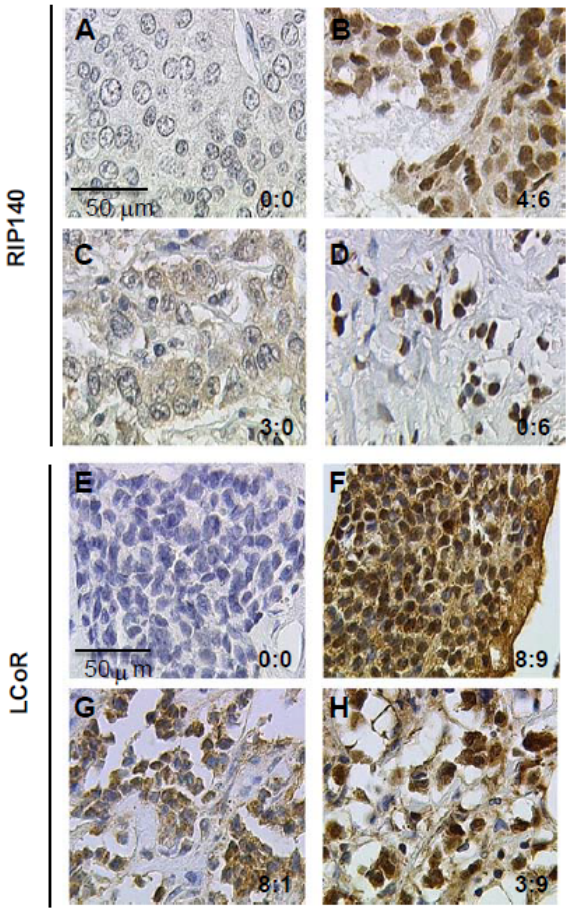

Figure 1. Immunohistochemical analysis of RIP140 and LCoR expression. Evaluation of RIP140 (A to D) and LCoR (E to H) expression in primary BC samples showing no or low nuclear expression (A, C, E, G) and high nuclear expression (B, D, E, F) of the two transcription co-regulators. The cytoplasmic and nuclear IRS values are indicated for each BC sample. Scale bars: 50 μm.

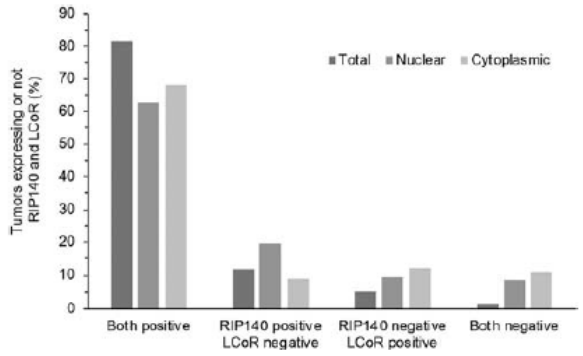

Figure 2. Distribution of RIP140 and LCoR expression in primary BC samples. The graph shows the percentage of tumors expressing both proteins, only RIP140, only LCoR, or none (IRS = 0).

both markers, whereas 8% (nuclear IRS) and 11% (cytoplasmic IRS) of tumors were negative for both RIP140 and LCoR.

In agreement, the total, cytoplasmic and nuclear IRS for RIP140 were positively and significantly correlated with the relevant IRS values for LCoR ($P < .01$, n = 299 samples with both stainings) (Table 2). The correlation between nuclear and cytoplasmic IRS values for RIP140 and LCoR was confirmed when BC samples were classified in two groups based on the absence (IRS = 0) and presence (IRS >0) of nuclear or cytoplasmic expression of LCoR (Supplementary Fig. 2C-D) (box plots and Kruskal-Wallis non-parametric test, $P < .001$). Similar results were obtained for the total IRS values (data not shown).

Correlation of RIP140 and LCoR Expression with Clinicopathological Parameters and Tumor Aggressiveness Markers

Expression of ER and PR (two main prognostic markers for BC) did not correlate with RIP140 or LCoR expression (total, cytoplasmic or nuclear IRS values, data not shown). Conversely, the total ($P < .05$) and nuclear ($P < .01$) IRS values for RIP140 and LCoR were negatively correlated with pT (Table 3). This result was confirmed after separating BC samples in two groups based on the pT: pT1 (tumor size ≤20 mm at its widest area) and pT2–4 (tumor larger than 20 mm) (Supplementary Fig. 3A-B).

No other clinicopathological parameter (age, HER2, histologic type, grade, node status, distant metastases, triple negative status, contralateral BC and local recurrence) was correlated with RIP140 or LCoR expression (data not shown). However, in the specific subgroup of patients with nuclear expression of both RIP140 and LCoR (n = 188), nuclear RIP140 was negatively correlated with ERα and PR (rho = − 0.164 and − 0.181 respectively, $P < .05$), and

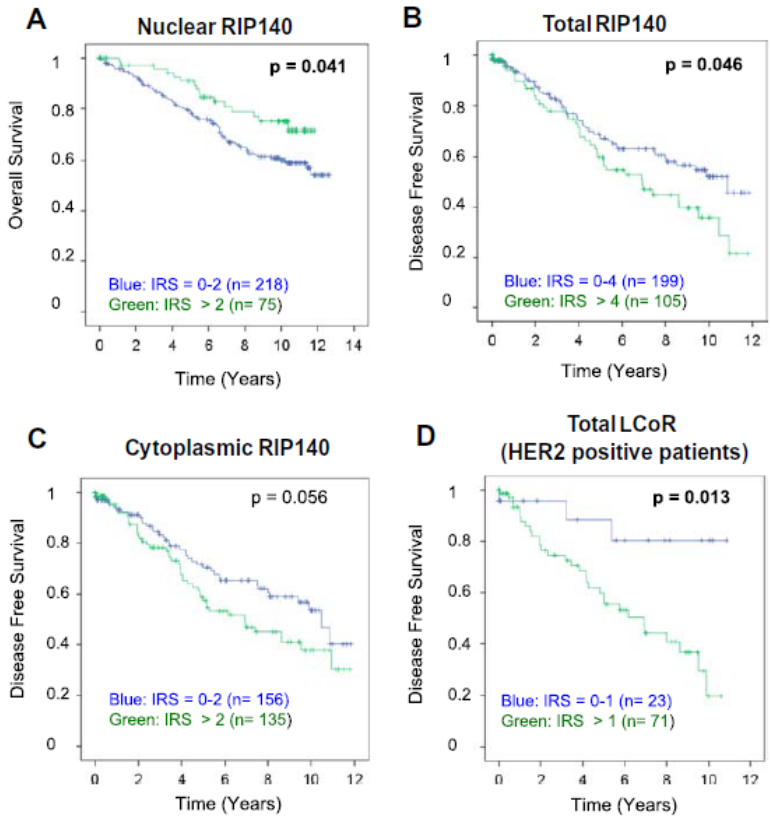

Figure 3. Kaplan–Meier analysis of patient survival according to the RIP140 or LCoR IRS values. For this analysis, optimized IRS cut-off values for low and high RIP140 or LCoR expression were determined by ROC-curve analysis. Overall survival was longer in patients with nuclear RIP140 IRS >2 (A). Disease-free survival (12-year follow-up) according to total (B) or cytoplasmic (C) RIP140 IRS values. Disease-free survival was associated with total LCoR IRS values only in the sub-population of patients with HER2-positive BC (n = 94) (D).

positively correlated with the triple negative status (rho = 0.214, $P < .01$).

Besides these widely used clinicopathological features, BC aggressiveness is known to be driven by other parameters, such as epithelial mesenchymal transition (EMT) and CSCs. N-cadherin (EMT marker) and CD133 (CSC marker) expression correlated with cytoplasmic RIP140 ($P < .05$ for N-cadherin and $P < .01$ for CD133) and cytoplasmic LCoR expression ($P < .01$ for N-cadherin and for CD133), and also with total LCoR ($P < .01$). Moreover, CD133 correlated also with nuclear and total IRS for RIP140 ($P < .01$) (Table 3).

The correlations between cytoplasmic RIP140/LCoR and N-cadherin (Supplementary Fig. 3C-D) and CD133 expression (Supplementary Fig. 3E-F) were confirmed after grouping the BC samples according to the presence (IRS >0) and absence (IRS = 0) of N-cadherin or CD133 expression, respectively. Moreover, in the whole cohort, N-cadherin was correlated with CD133 expression (rho = 0.432, n = 261, $P < .01$).

Correlation with patient survival

Kaplan–Meier analyses identified significant correlations between RIP140 and LCoR expression and DFS and OS (Figure 3). For this analysis, optimized IRS cut-off values for low and high RIP140 or LCoR expression were determined by receiver operating characteristic curve (ROC-curve) analysis, based on the maximal differences of sensitivity and specificity.

Patients with tumors with low nuclear RIP140 expression (IRS ≤2) had a worse OS than those with high IRS values (IRS >2) (mean OS: 9.37 ± 0.30 years *vs* 10.14 ± 0.38 years; $P = .041$). Conversely, DFS was significantly longer in patients with low total RIP140 expression (IRS ≤4) than in those with high expression (IRS >4) (mean DFS: 8.15 ± 0.39 years *vs* 6.89 ± 0.51 years; $P = .046$) (Figure 3, *A* and *B*). A similar trend, although not significant, was observed for cytoplasmic RIP140 expression ($P = .056$, Figure 3*C*).

LCoR expression did not have a significant effect on DFS or OS in the whole population. However, within the subgroup with HER2-positive tumors (n = 94), DFS was significantly longer in patients with low (total IRS ≤1) than in those with higher LCoR expression (total IRS >1) (mean DFS: 9.38 ± 0.77 years *vs* 6.23 ± 0.52 years; ($P = .013$)(Figure 3*D*).

Finally, multivariate analysis using the Cox regression model with RIP140 and LCoR expression (total, nuclear or cytoplasmic), N-Cadherin and CD133 levels and 11 clinicopathological features (ER, PR, HER2, triple negative status, histologic type, age, grading, pT, pN, local recurrence, and distant metastases) showed that besides age, pT, pN, and distant metastasis, no other parameter was an independent prognostic factor for OS in this cohort (data not shown).

Discussion

The purpose of this study was to elucidate the expression localization of the two transcription co-regulators RIP140 and LCoR in BC, and to correlate their expression in different cell compartments with tumor aggressiveness markers, clinicopathological features and patient survival.

Both RIP140 and LCoR were expressed in most of the 320 BC samples analyzed. Overall, they were moderately expressed, and predominantly in the cytoplasm with a strong correlation between cytoplasmic and nuclear expression for each protein. We and others previously described their expression in both nucleus and in cytoplasm [7,19,29]. Aziz et al. [7] reported a preferential increase of RIP140 nuclear localization in epithelial cancer cells. Various post-translational modifications, including lysine acetylation [30] or conjugation to vitamin B6 [31], have been proposed to explain RIP140 nucleo-cytoplasmic shuttling. Fewer data are available concerning LCoR post-translational modifications and it should be interesting to monitor its phosphorylation status, particularly in HER2-positive BC in view of our findings (see data from Figure 3*D*). In the MCF-7 cell line used as a ER/PR-positive BC model, LCoR is evenly distributed in both compartments, whereas RIP140 is expressed predominantly in the nucleus [19]. Therefore, the MCF7 cell line, like the tumors of our patient cohort, is characterized by a low cytoplasmic/nuclear IRS ratio for RIP140 and a ratio close to 1 for LCoR.

By comparing the expression of both RIP140 and LCoR, we found strong correlations between their cytoplasmic, nuclear and total expression. More than 80% of tumors expressed both proteins, whereas only 1.7% was negative for both. For both RIP140 and LCoR, the mean IRS values were higher in the cytoplasm than in the nucleus. These results are fully concordant with our previously published data obtained by mRNA analysis and showing that RIP140 can transactivate the *LCOR* gene promoter in BC cells [19].

We then analyzed the correlations between expression of RIP140/LCoR and of N-cadherin (EMT marker) and CD133 (CSC marker). We previously demonstrated that N-cadherin and CD133 expression correlate positively in 307 primary BC tumors from this cohort, and that N-cadherin positivity is associated with shorter survival time for patients without lymph node metastases [27]. Moreover, N-cadherin expression was significantly higher in metastases than in the related primary tumors. Here, we found that RIP140 and LCoR cytoplasmic expression were positively correlated with N-cadherin and CD133 expression, suggesting that in the cytoplasm, RIP140 and LCoR could specifically interact with these pathways to promote BC progression.

Analysis of the correlations between the patients' clinicopathological and RIP140 and LCoR IRS values highlighted that only tumor size was negatively correlated with nuclear RIP140 and LCoR expression, suggesting that nuclear RIP140 and LCoR may play a role in tumor growth inhibition. Moreover, nuclear RIP140 was negatively correlated with ERα and PR and positively correlated with the triple negative status in the subgroup of patients with nuclear expression of both RIP140 and LCoR.

Altogether, these findings suggest that RIP140 and LCoR may have different roles in tumor development according to their subcellular location. This hypothesis is supported by the results of our survival analyses. Indeed, high total or cytoplasmic expression of RIP140 was associated with shorter DFS, whereas high nuclear expression predicted longer OS. This suggests opposite roles for cytoplasmic and nuclear RIP140 in survival. Similarly, low total LCoR expression was strongly correlated with longer DFS in patients with HER2-positive cancer. A previous study demonstrated that low *RIP140* or *LCOR* mRNA expression is associated with poor OS [19].

Although RIP140 and LCoR expression are well correlated with each other in BC samples, the present study also demonstrates that these two transcription co-regulators may play different roles in breast tumorigenesis, according to their subcellular location. Indeed, nuclear RIP140 correlated with smaller tumor size and longer OS, whereas cytoplasmic LCoR correlated with markers of poor prognosis (N-cadherin, CD133) and poor DFS in HER2-positive tumors. However, the result of the multivariate analysis indicated that only age, pT, pN, and distant metastasis are independent prognostic factors for OS in this cohort. Therefore, further studies are needed to

delineate the specific roles of cytoplasmic and nuclear RIP140 and LCoR in BC progression as well as their relevance as potential new independent prognostic markers in BC. Especially, it would be relevant to investigate the involvement the association of nuclear and cytoplasmic expression of RIP140 and LCoR with the response of BC patients to systemic or targeted therapies.

It is however noteworthy to highlight that, in this first study dealing with the specific analysis of nuclear/cytoplasmic expression of RIP140 and LCoR in breast tumors, data showing correlations of expression with patient survival or other parameter such as tumor size or expression of CD133/N-Cadherin, is helpful to better appreciate the biological roles of these two transcriptional co-regulators in breast tumorigenesis.

Competing Interests

The authors declare that they have no competing interests. We confirm that the authors have full control of all primary data and agree that the journal is allowed to review these data if requested.

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Not applicable.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.tranon.2018.06.006.

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4.2 Originalarbeit II:

Prognostic Relevance of RIP140 and ERβ Expression in Unifocal Versus Multifocal Breast Cancers: A Preliminary Report. International Journal of Molecular Sciences. January 2019.

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Prognostic Relevance of RIP140 and ERβ Expression in Unifocal Versus Multifocal Breast Cancers: A Preliminary Report

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Abstract: The aim of this study was to investigate the expression of two nuclear receptor transcriptional coregulators, namely RIP140 (receptor-interacting protein of 140 kDa) and LCoR (ligand-dependent corepressor) in unifocal versus multifocal breast cancers. The expression of these two proteins was analyzed by immunohistochemistry in a matched-pair cohort of 21 unifocal and 21 multifocal breast tumors. The expression of the two estrogen receptors (ERα and ERβ) was studied in parallel. RIP140 and LCoR levels appeared lower in unifocal tumors compared to multifocal samples (decreased of immune-reactive scores and reduced number of high expressing cells). In both tumor types, RIP140 and LCoR expression was correlated with each other and with expression of ERβ. Very interestingly, the expression of RIP140, LCoR, and ERβ was inversely correlated with overall survival only for the unifocal group. The negative correlation with overall and recurrence free survival was more pronounced in patients whose unifocal tumors expressed high levels of both RIP140 and ERβ. Altogether, this preliminary report indicates that the ERβ/RIP140 signaling is altered in unifocal breast cancers and correlated with patient outcome. Further investigation is needed to decipher the molecular mechanisms and the biological relevance of this deregulation.

Keywords: breast cancer; tumor focality; RIP140; LCoR; estrogen receptors

1. Introduction

The last decade provided clear evidence for the importance of focality regarding breast cancer (BC) aggressiveness [1,2]. Although a standard classification of focality status is still debated, multifocality was initially defined as two or more separate invasive tumors in the same quadrant and multicentricity as two or more separate invasive tumors in more than one quadrant of the same breast

[3]. Nonetheless, since the quadrant definition is not linked to breast anatomy, the difference between multifocality and multicentricity is becoming less and less important. More recently, multifocality has been defined as multiple simultaneous ipsilateral BC lesions, provided they are macroscopically distinct and measurable, irrespective of the localization of the lesions [4]. Multifocality has been associated with lymph node and distant metastases [5,6], with shorter survival [7], and higher mortality rates [8]. Our previous work demonstrated that multicentricity/multifocality was a significant independent predictor for local relapse, distant metastasis, and reduced overall survival (OS) [9]. This previous study emphasized the importance of the combined tumor volume rather than that of the larger tumor diameter. More recently, using a well-balanced matched-pair cohort, we demonstrated a significant lower expression of E-cadherin in the multifocal group [10].

Together with the tumor node metastasis (TNM) classification, the hormone receptor (estrogen receptor ER, progesterone receptor PR) and the HER2 receptor status are standard diagnostic parameters to describe tumor biology and support therapy decisions. For multifocal tumors, assessment of ER, PR, and HER2 status only in the largest lesion (if the lesions do not differ in grade or histological subtype) is still questionable, and an accurate biological characterization of all lesions should be recommended [4]. Regarding estrogen signaling, the expression ratio between ERα and ERβ has a great effect on tumorigenesis in breast and ovarian cancers; ERβ was shown to have inhibiting effects on ERα and thus on cell proliferation. ERβ, especially the isoform 2, is expressed at a higher level than ERα in the normal human mammary gland, but its expression decreased in BC cells, particularly in ERα expressing cells [11,12].

BC signaling and progression is also influenced by the complex interplay of nuclear receptors and their transcriptional coactivators and corepressors [5]. The importance of nuclear receptors and coregulators networks which appear disrupted in BC and have prognostic significance was previously demonstrated [13]. RIP140 (receptor-interacting protein of 140 kDa) is one of the first transcriptional coregulators shown to interact with ERs [14] and to regulate BC cell proliferation and invasion in vitro [15–17]. RIP140 was found to be an important transcriptional cofactor for estrogen signaling in ovarian and BC cells [18,19]. The effect of RIP140 appeared stronger on ERβ than on ERα (better in vitro interaction and greater modulation of estradiol-dependent transactivation) [20,21]. Moreover, RIP140 was shown to be more efficiently upregulated by estradiol in ERβ expressing BC cells [22]. Finally, analysis of knock-out mice revealed that RIP140 is an essential factor for normal mammary gland development through its functions on estrogen signaling [23].

LCoR (ligand-dependent corepressor) is another transcriptional coregulator which interacts with agonist-activated ERα and represses its activity via histone deacetylase-dependent and independent mechanisms [24]. The physiological role of LCoR is poorly understood but biological effects on prostate cancer and liver homeostasis have been reported [25,26]. Previously, we reported that LCoR was engaged in a complex with RIP140 and negatively regulated BC cell proliferation in a RIP140-dependent manner [27]. Moreover, we recently analyzed RIP140 and LCoR expression at the protein level in BC biopsies showing that the two proteins were highly correlated in more than 80% of tumors and that RIP140 expression was significantly correlated with patient survival [28].

The present work analyzes the expression of RIP140 and LCoR in BC samples according to their focality status. We used a previously characterized, small but well-balanced matched-pair cohort of 42 unifocal and multifocal BC samples [10]. This cohort is homogenous for the two types of tumors in terms of tumor size, histology grade, lymph node status, and patient survival. Using immunohistochemistry, RIP140 and LCoR expression was monitored in parallel to that of ERα and ERβ, and data were correlated with OS. The results highlight a highly significant negative prognostic impact of RIP140/ERβ coexpression, only in unifocal tumors.

2. Results

2.1. RIP140 and LCoR Expression in Unifocal vs. Multifocal Tumors

Using immunohistochemistry, we evaluated RIP140 and LCoR expression on 21 matched pairs of unifocal or multifocal tumors described in Table 1.

Table 1. Clinicopathological features of the matched-pair cohort.

Parameters	Unifocal (n = 21)	Multifocal (n = 21)
Age		
Mean (years)	58.5	63.7
Histological type		
Ductal	13 (61.9%)	16 (71.4%)
Lobular	3 (14.3%)	4 (19%)
Ductal-Lobular	1 (4.8%)	2 (9.5%)
Medullary	1 (4.8%)	0 (0%)
Micropapillary	2 (9.5%)	0 (0%)
Unknown	1 (4.8%)	0 (0%)
Tumor size		
pT1 a, b, c	18 (85.7%)	18 (85.7%)
pT2	2 (9.5%)	2 (9.5%)
pT3	0 (0%)	0 (0%)
pT4a, b, c, d	1 (4.8%)	1 (4.8%)
Grade		
I	0 (0%)	0 (0%)
II	17 (81.0%)	17 (81.0%)
III	4 (19.0%)	4 (19.0%)
Lymph node metastasis		
No	16 (76.2%)	16 (76.2%)
Yes	4 (19%)	4 (19%)
Unknown	1 (4.8%)	1 (4.8%)
Local recurrence		
No	13 (61.9%)	17 (81%)
Yes	8 (38.1%)	4 (19%)
Overall survival		
Mean time (months)	130.79	133.41
ERα status		
Negative	5 (23.8%)	5 (23.8%)
Positive	16 (76.2%)	16 (76.2%)
ERβ status		
Negative	10 (47.6%)	5 (23.8%)
Positive	11 (52.4%)	16 (76.2%)
HER2 status		
Negative	18 (85.7%)	16 (76.2%)
Positive	3 (14.3%)	5 (23.8%)

Semi-quantitative immunoreactive scores (IRS) were quantified by assessing the percentage of positively stained cells together with staining intensity. Figure 1 illustrates RIP140 and LCoR staining results with low or high IRS, in unifocal and multifocal samples. The mean IRS of RIP140 and LCoR expression were compared in unifocal vs. multifocal tumors and found to be lower in the unifocal group compared to the multifocal one, with a significant difference only for LCoR.

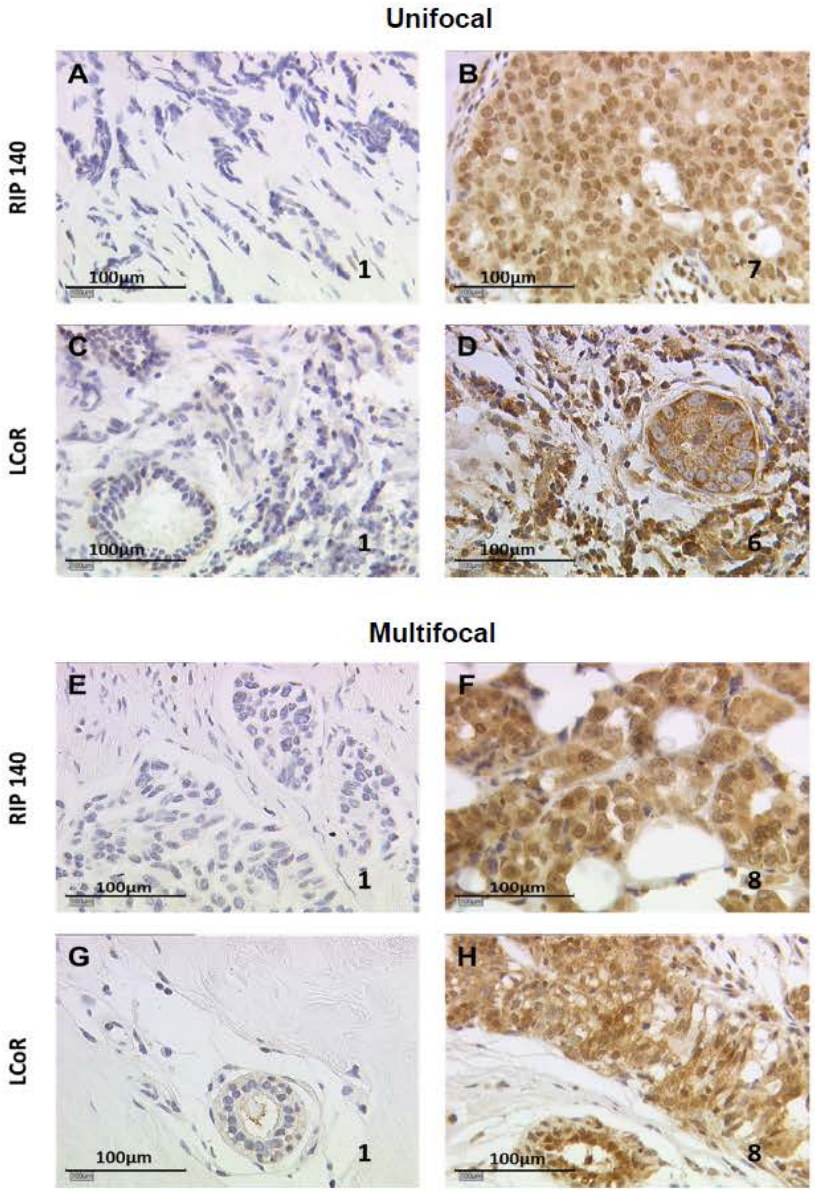

Figure 1. Immunohistological staining of receptor interacting protein of 140 kDa (RIP140) and ligand dependent corepressor (LCoR) in unifocal or multifocal breast cancers. RIP140 (A,B,E,F), and LCoR (C,D,G,H) expression was evaluated by immunohistochemistry in unifocal (A–D) and multifocal (E–H) cases. Positive staining appears in brown color, nuclei appear in blue. Examples of low expression (A,C,E,G) vs. high expression (B,D,F,H) have been selected (immunoreactive score (IRS) values indicated in each photograph).

As shown in Table 2, the mean IRS for RIP140 was 2.61 in the unifocal group vs. 2.98 in the multifocal, and for LCoR expression, 2.38 vs. 3.38, respectively ($p < 0.05$). Considering IRS values ≥3.25 as positive, the percentage of samples expressing low or high levels of each coregulator was analyzed in the two types of tumors, as shown in Table 2. The majority of the 42 tumors expressed low levels of RIP140, and the percentage of RIP140 high expressing tumors decreased in the unifocal group

(23.8% high expressing vs. 42.9% in the multifocal group). Similarly, the percentage of LCoR high expressing tumors decreased in the unifocal group (19% high expressing vs. 57.1% in the multifocal group, $p = 0.011$). Finally, a positive and significant correlation between RIP140 and LCoR expression was observed in the two tumor types ($p < 0.01$) confirming our previous observation [28].

Table 2. Distribution and correlation of RIP140 and LCoR expression in unifocal versus multifocal breast cancers.

Parameters	Unifocal ($n = 21$)	Multifocal ($n = 21$)
RIP140 expression		
Mean IRS ± SE	2.61 ± 1.74	2.98 +/− 2.12
Low expressing tumors n (%)	16 (76.2%)	12 (57.1%)
High expressing tumors n (%)	5 (23.8%)	9 (42.9%)
LCoR expression		
Mean IRS ± SE	2.38 * ± 1.49	3.38 ± 1.93
Low expressing tumors n (%)	17 * (80.1%)	9 (42.9%)
High expressing tumors n (%)	4 * (19%)	12 (57.1%)
Correlation between RIP140 and LCoR		
Spearman's Rho correlation coefficient	0.714 **	0.686 **

The cut-off value between low and high expression is defined for RIP140 and LCoR as an IRS ≥ 3.25. The difference or correlation are statistically significant for $p < 0.05$ (*) and for $p < 0.01$ (**), using mean or percentage bilateral analysis and Spearman's Rho test.

2.2. Correlation with Clinical and Biological Parameters

RIP140 and LCoR expression was then correlated with clinical parameters linked to tumor aggressiveness and prognosis. These parameters were the recurrence status, pT, pN, pM, grade, histology types (classified as ductal, lobular, ductal-lobular, medullary, micro papillary, as described in Table 1), as well as expression of the two ER (ERα and ERβ), and HER2. No correlation was observed between RIP140 or LCoR, and most of the clinical and biological parameters analyzed, except for ERβ, as shown in Table 3. Indeed, we observed positive and significant correlations between ERβ and RIP140 or LCoR, both in unifocal and multifocal tumors. The correlations did not appear statistically different in unifocal cases ($r = 0.741$ and 0.783, for RIP140 and LCoR, respectively) and multifocal tumors ($r = 0.699$ and 0.612, respectively).

Table 3. Correlation analysis of RIP140 and LCoR expression with clinical parameters and estrogen receptor (ER) expression in unifocal versus multifocal breast cancers.

	RIP140		LCoR	
	Unifocal	Multifocal	Unifocal	Multifocal
Recurrence Status	0.368	−0.01	0.353	0.091
pT	−0.157	0.083	0.017	0.091
pN	−0.204	−0.054	0.018	−0.096
pM	−0.257	0.159	−0.34	0.011
Grade	−0.152	−0.01	0.071	0.081
Histology	0.034	0.124	−0.12	0.004
ERα	0.012	0.126	0.181	−0.212
ERβ	0.741 **	0.699 **	0.783 **	0.612 **
HER2	0.397	0.196	0.239	0.309

Spearman's Rho correlation coefficient are presented. Correlation are statistically significant for $p < 0.01$ (**), using a Spearman's Rho test. pT: primary tumor size; pN: lymph node involvement; pM: state of metastasis.

2.3. Correlation with Patient Survival

To analyze whether patient survival was linked to expression of the transcription factors, we then performed Kaplan–Meier analyses using the optimal threshold IRS values determined by

receiver operating characteristic curve (ROC-curve) analysis for each selected parameter (as detailed in the "Survival Analysis" section of "Materials and Methods"). In the matched-pair cohort, the mean survival time of patients with unifocal cancer was not significantly different from that of patients with multifocal cancer, as shown in Table 1. As shown in Figure 2, we observed a significant correlation of either high RIP140 ($p = 0.003$), high LCoR ($p = 0.028$), or high ERβ ($p = 0.001$) expression with poor OS in the unifocal group, whereas OS analysis in the multifocal group showed no significant correlation to transcription factor expression levels. Mean survival times in the unifocal group were 153.9 and 53.4 months for low vs. high RIP140 expression, 146.3 and 56.8 months for low vs. high LCoR expression, and 161.9 and 55.7 months for low vs. high ERβ expression, whereas no correlation in either group was observed for ERα expression.

We then combined the parameters to evaluate whether the positivity of two markers could better predict OS. The combination of ERα expression with either RIP140, LCoR, or ERβ expression did not reveal any change in correlation with OS, neither in the unifocal nor in the multifocal group. As illustrated in Figure 3, high LCoR expression combined with high RIP140 expression (A,B) or ERβ expression (C,D) maintained a significant correlation with a poor OS in the unifocal cases only ($p < 0.01$) but did not improve the prognostic impact compared to the individual markers, as shown in Figure 2. In contrast, combined RIP140 and ERβ expression (E,F) had an even stronger correlation with poor OS ($p = 0.0004$), again only in the unifocal group (mean survival times of 160.9 and 44.2 months for low vs. high combined expression).

Taking into account this strong correlation of the combined RIP140 and ERβ expression with poor OS, we calculated the correlation with recurrence free survival (RFS), as shown in supplementary Figure A1. We observed again a significant correlation of either high RIP140 ($p = 0.004$) or high ERβ ($p = 0.0002$) expression with poor RFS in the unifocal group (A and C, respectively), whereas RFS analysis in the multifocal group showed no significant correlation (B and D, respectively). The combined RIP140 and ERβ expression (E,F) had again an even stronger correlation with poor RFS ($p = 0.00001$) only in the unifocal group.

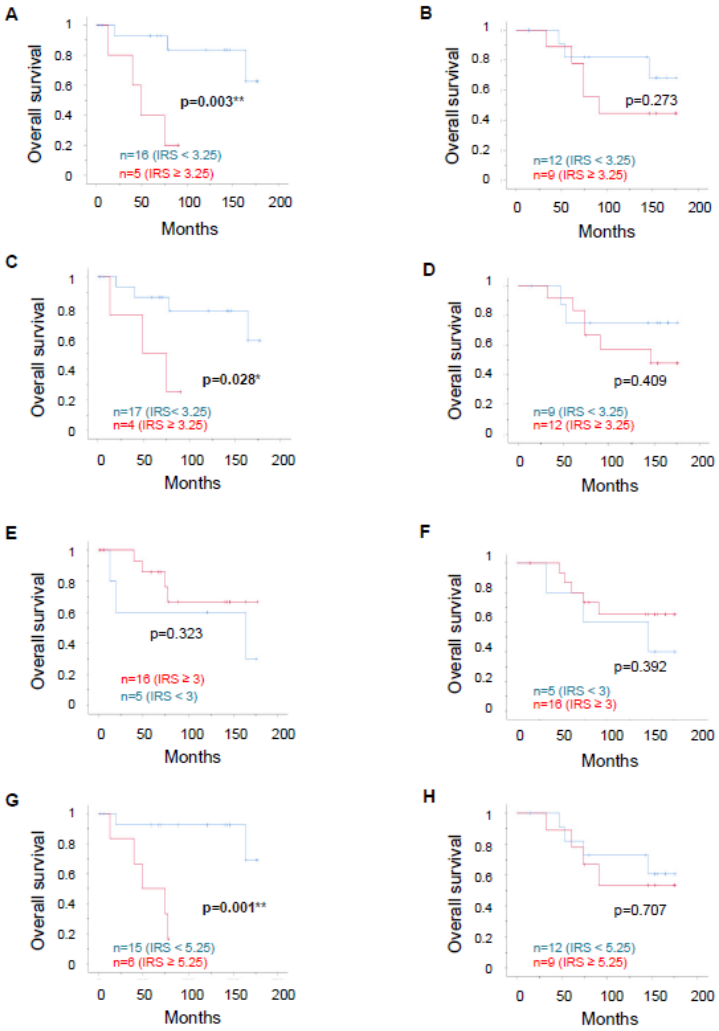

Figure 2. Patient overall survival (OS) according to RIP140, LCoR, ERα, or ERβ expression. Kaplan–Meier analysis of the correlation between RIP140 (**A**,**B**), LCoR (**C**,**D**), ERα (**E**,**F**), or ERβ (**G**,**H**) expression with OS in the matched pairs of unifocal (**A**,**C**,**E**,**G**) or multifocal (**B**,**D**,**F**,**H**) breast cancers. The IRS cut-off values together with the number of cases in each arm are indicated in each panel. Correlations are statistically significant for $p < 0.05$ (*) and for $p < 0.01$ (**).

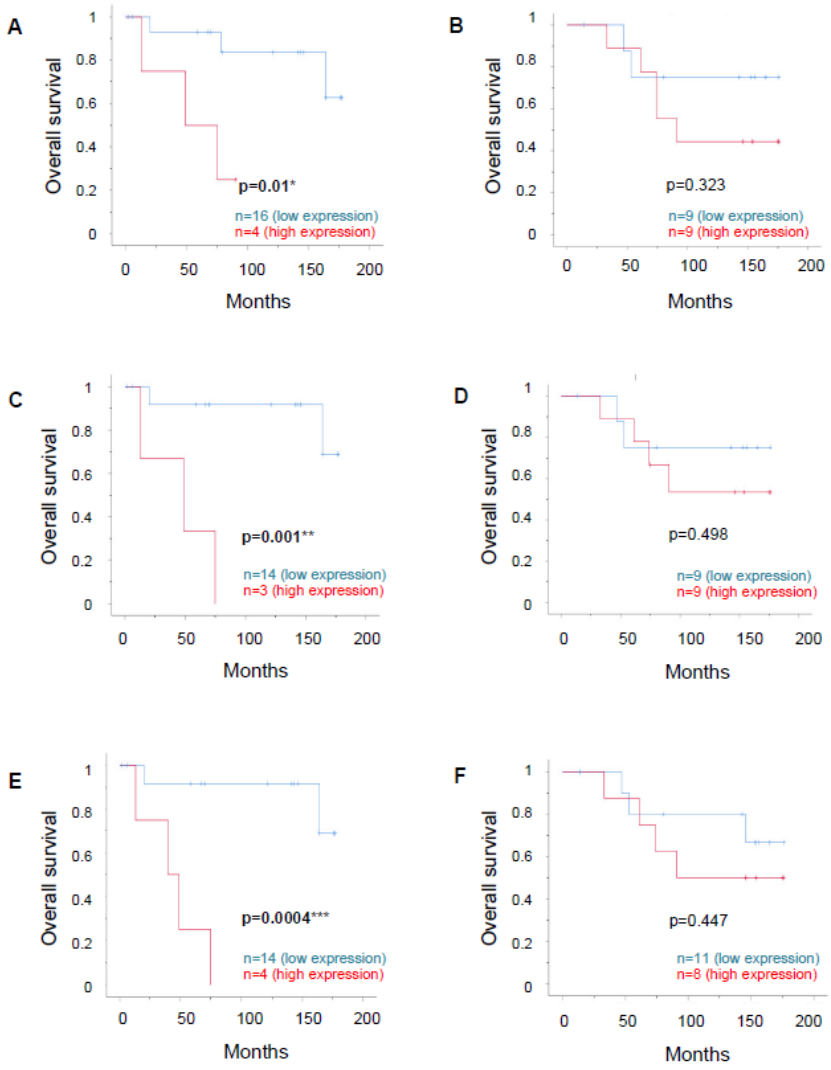

Figure 3. Overall survival according to combined expression of RIP140, LCoR, or ERβ. Kaplan–Meier analysis of the correlation between combined low vs. high expression of two markers with OS in the matched pairs (unifocal for **A**, **C**, **E** or multifocal for **B**, **D**, **F**). The analysis was performed with LCoR expression combined with RIP140 (**A**,**B**) or ERβ (**C**,**D**) expression, or with RIP140 expression combined with ERβ expression (**E**, **F**). Correlations are statistically significant for $p < 0.05$ (*), for $p < 0.01$ (**), and for $p < 0.001$ (***).

3. Discussion

The aim of this study was to compare RIP140 and LCoR expression in unifocal and multifocal BC samples, and to identify correlations with clinical parameters. We did not analyze multifocal and multicentric cancers separately but grouped them in the multifocal cohort and compared them to unifocal tumors.

In our matched-pair cohort of BC samples, LCoR appeared expressed at lower levels in the unifocal tumors (lower average IRS values and lower percentage of high expressing tumors). The same trend was noticed for RIP140 although the difference was not significant. Nonetheless,

expression of the two transcription factors was highly correlated in both groups, as recently described in a large BC patient cohort [28].

RIP140 or LCoR expression did not correlate with clinicopathological parameters, neither in unifocal nor in multifocal samples, except with ERβ levels. Indeed, a strong and significant correlation with ERβ expression was observed for both coregulators in the unifocal and in the multifocal group. In contrast, no significant correlations were observed between RIP140, LCoR, or ERβ expression and cell adhesion-related glycoproteins, namely E-cadherin, MUC1, and β-catenin compared in the unifocal vs. multifocal groups (data not shown). We previously quantified these three proteins in the same matched cohort, demonstrating that cytoplasmic β-catenin was associated significantly with reduced OS in unifocal patients [10].

Survival analysis demonstrated that high LCoR expression, and to a stronger extent high RIP140 or ERβ expression, correlated significantly with poor OS only in the unifocal BC samples. No significant correlation with survival was seen in the multifocal BC cohort, indicating a difference in the expression patterns and prognostic relevance of these transcription factors according to the focality status. It should be mentioned again that other biological parameters, such as tumor size, histology grade, and lymph node status, were similar between the two groups.

Concerning RIP140, these results are in accordance with our data obtained on a cohort of 320 samples showing that high RIP140 protein levels were correlated with short disease-free survival (DFS) [28]. With respect to ERβ, the biological relevance of high expression and its consequences on clinical outcome are still controversially discussed, depending on patient cohorts, analysis method (ERβ mRNA or protein) or ERβ isoforms tested [29–32]. We analyzed ERβ1, the full-length fully functional ERβ isoform [29]. Indeed, ERβ overexpression was found to be correlated either with poor [33,34] or with favorable [35] DFS. A study performed on 139 ER-positive BC samples demonstrated that ERβ protein levels were correlated with small tumor size, while ERβ mRNA levels were associated with poor DFS and were found to be an independent predictor of disease recurrence [33]. However, none of these studies analyzed survival according to tumor focality.

Interestingly, our data demonstrated an enhanced prognostic impact of combined ERβ/RIP140 expression on OS. RIP140 exerts a strong inhibitory effect on estrogen signaling; it was previously shown to interact more efficiently with ERβ than with ERα and to inhibit its activity with greater efficacy [20]. Cistrome and transcriptome analyses combined with clustering algorithms also supported a preferential recruitment of RIP140 by ERβ [22]. Moreover, the induction of RIP140 appeared mainly driven by ERβ in ovarian cancer cells [20]. Being aware of the small number of patients in our study, and the limitations of the immunostaining as a semi-quantitative assessment technique, we looked for confirmation of this significant effect on unifocal BC patients. We could confirm the negative impact of RIP140 or ERβ alone, and of the combined high ERβ/RIP140 expression on DFS also, suggesting that the two proteins may control various aspects of BC progression.

4. Materials and Methods

4.1. Collective

As previously described [10], our total collective was formed of a consecutive patient cohort consisting of 112 patients documented and surgically treated for primary BC between 2000 and 2002 at the Department of Gynecology of the University Hospital in Munich-Innenstadt; 57 unifocal BC patients and 55 patients with multicentric/multifocal disease. Data were entered into the database in an anonymized and coded fashion.

Because of the uneven distribution of prognostic factors in our original patient group, a matched pair analysis was performed. From this collective, two equivalent groups of 23 BC patients with multicentric/multifocal vs. unifocal tumors were selected according to the highest degree of equivalence in the following hierarchical and sequential order: tumor size at the time of primary diagnosis, histology grading, and lymph node status. We deliberately matched patients based on the criteria at the time of primary diagnosis. Kruskal–Wallis one-way analysis of variance was used,

which tests the equality of population medians among groups in a non-parametric way (continuation of the Mann–Whitney U test to analyze ≥3 groups). Hereby BC were equally allocated into the two groups (p = 1.000). The Institutional Review Board of the Ludwig Maximilian University (LMU) Munich, Germany, approved the study (approval number 048–08, 18 03. 2008) and all the patients gave informed consent. For the present study, from these 2 groups of 23 patients, only tumors of 2 groups of 21 patients could be stained for RIP140. Our study was then based upon a total of 42 primary BC, with 21 tumors classified as primary unifocal BC and 21 as multifocal or multicentric BC.

The focality status was evaluated by clinical examination, ultrasound, and mammography. In some cases, further investigation including nuclear magnetic resonance imaging (NMRI), galactography, or pneumocystography was necessary to accurately describe the focality status. Tumors with unconfirmed or questionable focality status (prior to histological examination) were excluded. All patients included in this project had to be free of any other disease at the time of the primary diagnosis and to be treated for resectable BC.

Tumor stage at primary diagnosis was histologically evaluated using the "Union internationale contre le cancer" (UICC) TNM classification which includes tumor size (primary tumor size, or pT, classified as: pT1a-c, pT2, pT3, pT4a-d), involvement of regional lymph nodes (N), and presence or absence of metastases (M). The tumor grade was determined by an experienced pathologist (Dr D. Mayr) of the LMU Department of Pathology and classified according to the WHO (Nottingham grading respectively to Elston and Ellis modification of Bloom–Richardson grading) [36]. For multifocal samples, each tumor was analyzed and classified as ERα or HER2 positive BC if at least one tumor lesion was positive. Additional data, such as age, ERα status, HER2-status, histological grade, metastases, local recurrence, progression, and survival, were retrieved from the Munich Cancer Registry.

All patient data were fully anonymized, and all diagnostic procedures had already been fully completed when samples were collected for the study. Authors were blinded from the clinical information during the experimental analysis. This study was approved by the Ethical Committee of the Medical Faculty, LMU, Munich, Germany and informed consent was obtained from all patients.

4.2. Immunohistochemistry

Expression of ERα and HER2 was determined at diagnosis, in all BC samples of this cohort at the LMU Department of Pathology, Germany. ERα expression was evaluated by immunohistochemistry, as previously described [36]. Samples showing nuclear staining in more than 10% of tumor cells were considered as hormone receptor-positive, in agreement with the guidelines at the time of the analysis (2000–2002). HER2 expression was analyzed with an automated staining system (Ventana; Roche, Mannheim, Germany), according to the manufacturer's instructions. Data on MUC-1, β-catenin, and E-cadherin expression in these BC samples were extracted from a previously published study [10]. For ERβ, RIP140, and LCoR analysis, samples were processed as previously described [28,37,38]. Formalin-fixed and paraffin-embedded sections of 3 µm were deparaffinized using xylol for 20 minutes, rehydrated in a descending ethanol gradient (100%, 96%, and 70%) and subjected to epitope retrieval for 5 min in a pressure cooker using sodium citrate buffer (pH 6.0). After returning to room temperature, sections were washed twice in PBS (phosphate buffered saline). The sections were immersed in 3% H_2O_2 in methanol for 20 min to block endogenous peroxidase activity. To prevent non-specific binding of the primary antibody, the sections were treated by the appropriate blocking solution. Incubation with the primary antibody (RIP140: polyclonal antibody, Sigma Aldrich; LCoR: polyclonal antibody, Novus Biologicals; ERβ1: monoclonal antibody, Dako, Glostrup) was performed for 16 hours at a temperature of 8 °C. After washing with PBS, reactivity was detected by the Vectastain Elite ABC-Kit (Vector Laboratories, Burlingame, CA, USA) according to the producer's protocol. Visualization was reached with DAB substrate and chromogen (3, 3′-diaminobenzidine DAB, Dako, Glostrup, Denmark) for 2 minutes. Then the slides were counterstained with Maier's acidic hematoxylin and dehydrated in an ascending alcohol series (50–98%), then immersed in xylol. The sections were embedded and covered. Placenta

tissue served as positive control staining. Replacement of the primary antibody with mouse or rabbit IgG was used as negative control.

The slides were investigated using a Leitz Diaplan microscope (Wetzlar, Germany) with a 3CCD color camera (JVC, Victor Company of Japan, Yokohama, Japan). To differentiate the intensity and distribution patterns, the semi-quantitative IRS was used. The IRS assesses the percentage of positively stained cells (graded as 0 = none, 1 = weak, 2 = moderate, and 3 = strong) with the cells' intensity of staining (0 = no staining, 1 = <10% of cells, 2 = 11–50% of cells, 3 = 51–80%, and 4 = >81% of cells) by multiplying. The reproducibility of RIP140 and LCoR stainings was checked by triplicate stainings of some sections and for all sections, the stainings were analyzed by two independent observers.

4.3. Statistical Analyses

Statistical analyses were performed on the 21 matched pairs resulting in a total collective of 42 patients. Besides the collective characterization described above, the differences were calculated using mean or percentage bilateral analysis, as shown in Table 2, and the correlations using Spearman's Rho test, as shown in Tables 2 and 3. All differences or correlations are statistically significant for $p < 0.05$ (*), $p < 0.01$ (**) or $p < 0.001$ (***). The IBM Statistical Package for the Social Sciences 24.0 (SPSS Inc., Chicago, IL, USA) was used to test for statistical significance.

4.4. Survival Analysis

To compare the mean immunoreactivity levels described by the IRS, the groups were divided into low vs. high expressing (RIP140, LCoR, ERβ, and ERα) cases. Therefore ROC-curve analyses were performed and the maximum difference between sensitivity and specificity was used for identification of the cut-off level for RIP140, LCoR, ERβ, and ERα. The following thresholds were determined regarding OS: RIP140 ≥ 3.25, LCoR ≥ 3.25, ERβ ≥ 5.25, ERα ≥ 3.0. These thresholds were used to determine the percentages of low or high RIP140 and LCoR expression described in Table 2, besides the survival analysis.

Survival times were compared by Kaplan–Meier graphics and differences in OS, as shown in Figures 2 and 3, and DFS, as shown in Figure S1, were tested for significance using the chi-square statistics of the log rank test. Data were assumed to be statistically significant in case of p-value < 0.05. Kaplan–Meier graphics were then provided for each subgroup and each marker as well as for the combined expression of two markers in order to compare the differences of survival times between unifocal and multifocal tumors and between low and high receptor expression. Only mean survival times are presented, as median survival times were not reached for all sub-groups.

5. Conclusions

In conclusion, this preliminary report shows that the prognostic value of ERβ/RIP140 coexpression differs according to tumor focality and significantly correlates with poor OS and DFS only in patients with unifocal BC. While being small, our cohort presents the great advantage of being a matched-pair cohort with perfect matching criteria. Moreover, despite the relatively limited number of cases, the results obtained in unifocal tumors for RIP140 and ERβ were highly significant. These data strengthen the need to further investigate the relevance of these two genes as independent prognostic markers in extended cohorts and to enlarge the analysis to other nuclear receptors and coregulators. It would also be of interest to understand why ERβ and RIP140 lose their prognostic impact (as single markers or in combination) in multifocal BC. Altogether, our results may lead to a better understanding of key transcription networks involved in multifocal BC and to define the clinical potential of new biological markers.

Supplementary Materials: Supplementary materials can be found at www.mdpi.com/xxx/s1. Figure S1: Recurrence free survival (RFS) according to the expression of RIP140 and ERβ, alone or combined.

Author Contributions: S.S., V.C., and U.J. conceived and supervised the project. D.M., N.D., T.W., and S.M. provided the samples and the related clinical data. K.M. performed most experiments, with the help of C.K. K.M.

wrote the first draft of the paper. S.S., V.C., U.J., S.J., and N.H. contributed to manuscript writing and editing. All authors read and approved the final manuscript.

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Abbreviations

BC	breast cancer
DFS	disease free survival
DCIS	ductal carcinoma in situ
ER	estrogen receptor
HER2	human epidermal growth factor receptor 2
IRS	immunoreactive score
LCoR	ligand dependent corepressor
MUC-1	epithelial mucin-1
NST	non-special type
pN	lymph node involvement
LMU	Ludwig Maximilians University
M	Metastasis
NMRI	nuclear magnetic resonance imaging
NR	nuclear receptor
OS	overall survival
PBS	phosphate buffered saline
pT	primary tumor size
PR	progesterone receptor
RFS	recurrence free survival
ROC-curve	receiver operating characteristic curve
RIP140	receptor interacting protein of 140 kDa
TNM status	tumor node metastasis status

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5. Zusammenfassung/Summary

5.1 Subzelluläre Lokalisation von RIP140 und LCoR

In der ersten vorliegenden Originalarbeit wurde Tumorgewebe von 320 Patientinnen mittels immunhistochemischer Analysen auf ihre totale, nukleäre und zytoplasmatische Expression von RIP140 und LCoR hin untersucht. Zugrundeliegend wurde die Hypothese formuliert, ob die beiden Co-Regulatoren bei Mammakarzinomen in verschiedenen Zellkompartimenten unterschiedlich exprimiert werden und dies wiederum einhergeht mit einer veränderten Tumorbiologie, sowie assoziiert ist mit histopathologischen Merkmalen und einem veränderten Patientenüberleben. Verschiedene Studien belegen die Co-Expression von RIP140 im Zellkern und Zytoplasma; je nach stattgehabter posttranslationaler Modifikation erfolgt eine Verschiebung von RIP140 in den Zellkern bzw. ins Zytoplasma (62), (63). Neben den Liganden-abhängigen genomischen Funktionen konnten neue zytoplasmatische Funktionen in verschiedenen Signalkaskaden im Zytoplasma (beispielsweise im Wnt/β-Catenin Signalweg, sowie innerhalb der Insulin-abhängigen Glukoseaufnahme über GLUT4) beschrieben werden (64), (65), (66).

Über 80% der untersuchten Tumore exprimieren beide Transkriptionsfaktoren, ca. 17% nur einen von beiden (siehe *Abbildung 2*). Konsekutiv zeigte sich eine hohe und signifikante Korrelation der Expression von RIP140 mit LCoR. Für beide Proteine konnte eine höhere Präsenz im Zytoplasma als im Zellkern beobachtet werden. Im Folgenden wurden Korrelationsanalysen mit weiteren histopathologischen und klinischen Parametern durchgeführt: die Tumorgröße zeigte sich hierbei negativ korrelierend mit der Gesamtexpression, sowie der nukleären Expression von RIP140 und LCoR (siehe *Tabelle 3*). Außerdem fand sich eine nukleäre Expression von RIP140 negativ korrelierend mit ERα und PR. Zusätzlich wurden weitere interagierende Signalwege, die eine Rolle bei der Tumorigenese des Mammakarzinoms spielen, untersucht. Das Adhäsionsmolekül N-Cadherin ist ein zentrales Protein der epithelialen mesenchymalen Transition (EMT), welche die Tumorprogression des Mammakarzinoms über Invasion und vaskuläre Ausbreitung beeinflusst (67). Das Protein cluster of differentiation 133 (CD133) spielt eine große Rolle in der Tumorigenese und Zellproliferation und gilt als Tumorstammzellmarker (Cancer Stem Cell, CSC) (68). Es zeigte sich hierbei eine Korrelation zwischen der Expression von N-Cadherin und CD133 mit der zytoplasmatischen Expression von RIP140 und LCoR (siehe *Tabelle 3*). Darüber hinaus wurden mittels Kaplan-Meier-Analysen Assoziationen zwischen einer Expression von RIP140 und LCoR und dem Patientenüberleben untersucht. Hierbei wurde ein signifikant schlechteres Gesamtüberleben bei einer verminderten nukleären Expression von RIP140 beobachtet; das Rezidivfreie Überleben war signifikant länger bei Patienten mit einer niedrigen Gesamtexpression, sowie einer zytoplasmatischen

Expression von RIP140 (siehe *Abbildung 3*). In der Gesamtkohorte zeigte sich keine Assoziation zwischen Überlebensdaten und LCoR-Expression, jedoch fand sich bei den HER2-positiven Tumoren ein längeres Rezidivfreies Überleben bei geringer zytoplasmatischer Expression von LCoR (siehte *Abbildung 3D*).

Subcellular Localization of RIP140 and LCoR

For the first original article tumor tissue from 320 patients was analysed using immunohistochemistry regarding their total, nuclear and cytoplasmic expression of RIP140 and LCoR. The underlying hypothesis questioned if the expression of the coregulatory proteins in breast cancer is varying according to their different cellular compartment and hence is associated with modified tumor biology, histopathologic features and different patient survival. Various studies prove the co-expression of RIP140 in the nucleus and cytoplasma; depending on the different posttranslational modifications a shifting of RIP140 into nuclear or cytoplasmic cellular compartment is carried out (62), (63). Beside ligand-dependent genomic functions new cytoplasmic functions could be found within various cytoplasmic signal transduction cascades (for example within the Wnt/β-catenin signal pathway, as well as the insulin dependent glucose uptake via GLUT4) (64), (65), (66).

More than 80% of the analysed tumor tissue show an expression of both transcriptional factors, approximately 17% show only expression of one of them (see *figure 2*). Consequently there is a high and significant correlation between the expression of RIP140 and LCoR. Both of the proteins show a stronger presence in the cytoplasma than in the nucleus. Following additional correlational analyses were obtained regarding further histopathologic and clinical parameters: tumor size was negatively correlated with the total expression, as well as the nuclear expression of RIP140 and LCoR (see *table 3*). Moreover nuclear expression of RIP140 was negatively correlated with ERα and PR. Furthermore various interacting signal transduction pathways important for the tumorigenesis of breast cancer were investigated. The adhesion molecule N-cadherin is a central protein of the epithelial mesenchymal transition (EMT), which influences tumorprogression in breast cancer by invasion and vascular spreading (67). The protein cluster of differentiation 133 (CD133) plays an important role in tumorigenesis and cell proliferation and is respected as tumor stem cell marker (cancer stem cell, CSC) (68). A high correlation between the expression of N-cadherin and CD133 with the cytoplasmic expression of RIP140 and LCoR could be detected (see *table 3*). In the following research using Kaplan-Meier calculation the association of the expression of RIP140 and LCoR and patient survival was observed. A significantly reduced overall survival correlated with low expression of RIP140, whereas a significantly increased disease-free survival was seen in patients with a lower total expression, as well as in patients with a cytoplasmic expression of RIP140 (see *figure 3*). The overall cohort showed no association between survival data and expression of LCoR, but in HER2-positive cases a favourable disease-free survival was seen in patients with low cytoplasmic expression of LCoR (see *figure 3D*).

5.2 Assoziation zwischen der Expression von RIP140, LCoR und ERβ und Überlebensdaten

In der zweiten vorliegenden Originalarbeit wurde mittels immunhistochemischer Analysen die Expression von RIP140 und LCoR innerhalb einer Matched-Paired-Analyse bei 21 uni- und multifokalen Tumoren untersucht. Die 21 Paare zeichnen sich durch statistische Äquivalenz in den Matching-Kriterien (Tumorgröße, Lymphknotenstatus und Grading) aus (siehe *Tabelle 1*). Anhand der ermittelten IRS-Werte wurde die vorliegende Expressionsausprägung der untersuchten Tumore als „niedrig“ oder „hoch“ beschrieben. Die Co-Regulatoren RIP140 und LCoR sind in den unifokalen Tumoren vermindert, in den multifokalen Fällen dagegen stärker exprimiert, signifikant ist diese Differenz jedoch nur bei unifokalen Tumoren mit hoher Expression von LCoR zu beobachten (siehe *Tabelle 2*). Es zeigte sich eine statistisch signifikante ($p<0,01$) Korrelation zwischen der Expression von RIP140 und LCoR (siehe *Tabelle 2*), konkordant zu unseren Ergebnissen aus der ersten vorliegenden Originalarbeit (69). Es wurden Korrelationsanalysen der Expression von RIP140 und LCoR mit klinischen und weiteren histopathologischen Parametern, sowie mit der Expression von ERα und ERβ durchgeführt. Hierbei zeigte sich eine signifikante Korrelation zwischen der Expression von ERβ und RIP140, sowie LCoR bei uni- und multifokalen Tumoren (siehe *Tabelle 3*). In einem weiteren Schritt wurden die Expressionsanalysen mit klinischen Überlebensdaten verknüpft. Zwischen den gebildeten äquivalenten Paaren der uni- und multifokalen Gruppe zeigte die mittlere Überlebenszeit keinen signifikanten Unterschied. Anhand von ROC-Kurven (Receiver Operating Characteristic Curves) wurden optimale Schwellenwerte der Expressionsausprägung von RIP140, LCoR und ERβ erstellt. Anschließend wurden anhand dieser Expressionsunterschiede (niedrige vs. hohe Expression) Kaplan-Meier-Analysen erstellt. Hierbei wurde beobachtet, dass eine hohe Expression von jeweils RIP140, LCoR oder ERβ signifikant und hoch signifikant mit einem geringeren Gesamtüberleben in der Gruppe der unifokalen Tumore, nicht aber bei multifokalen Fällen assoziiert ist (siehe *Abbildung 2*). Die Analysen mit ERα zeigten keine signifikante Korrelation mit dem Gesamtüberleben. Auch die Co-Expression der Transkriptionsfaktoren und Kernrezeptoren wurde untersucht: Eine hohe Co-Expression von LCoR und RIP140 oder ERβ zeigte eine, ähnlich zu den Einzelexpressionen, signifikante Assoziation mit schlechterem Gesamtüberleben in unifokalen Tumoren (siehe *Abbildung 3*). Eine Kombination aus hoher Expression von RIP140 und ERβ zeigte jedoch eine hoch signifikante Assoziation mit geringem Gesamtüberleben in den untersuchten unifokalen Fällen und lieferte eine hohe prognostische Aussagekraft (siehe *Abbildung 3*). Darüber hinaus detektierten wir in weiteren Kaplan-Meier-Analysen bezugnehmend auf das Rezidivfreie

Überleben eine hoch signifikante Assoziation zwischen hoher Co-Expression von RIP140 und ERβ und kürzerem Rezidivfreien Überleben (siehe *Abbildung A1*).

Association between the Expression of RIP140, LCoR and ERβ and Survival Data

The second original article focussed on the expression of RIP140 and LCoR within a matched-paired study of 21 uni- and multifocal tumor cases using immunohistochemistry. The 21 pairs were characterised by statistical equivalence in the matching criteria (tumor size, lymph node status and grading) (see t*able 1*). On the basis of the identified IRS values the characteristic expression of the investigated tumor tissue was described as 'low' or 'high'. The expression of the coregulatory proteins RIP140 and LCoR is reduced in unifocal tumor tissue, whereas there is a higher expression in multifocal cases; a significantly difference was observed only for unifocal tumors with high expression of LCoR (see *table 2*). A statistically significant ($p<0.01$) correlation was detected between the expression of RIP140 and LCoR (see *table 2*), consistent with the results of the first original article (69). Further correlational analyses were performed regarding the expression of RIP140 and LCoR with clinical and histopathologic parameters, as well as with the expression of ERα und ERβ. A significant correlation could be shown between the expression of ERβ, RIP140 and LCoR in unifocal and multifocal tumor tissue (see *table 3*). The next step linked the expression analyses with clinical survival data. Between the built equivalent pairs of the uni- and multifocal group there was no significant difference in the mean survival time. On the basis of ROC curves (Receiver Operating Characteristic Curves) cut off values were determined for the characteristic expression of RIP140, LCoR and ERβ. Afterwards Kaplan-Meier calculation was performed using the differences in expression (low vs. high) depending on the cut off values. A high expression of each RIP140, LCoR and ERβ was correlated significantly and highly significant with a reduced overall survival in the unifocal group, but not in multifocal cases (see *figure 2*). Analyses with ERα show no correlation with overall survival. Consequently the co-expression of the transcriptional and nuclear receptors was investigated: a high co-expression of LCoR and RIP140 or ERβ showed, similarly to the single expressions, a significant association with reduced overall survival in unifocal tumor cases (see *figure 3*). A combination of high expression of RIP140 and ERβ showed a strong an highly significant association with reduced overall survival in the unifocal cases and provided high prognostic value (see *figure 3*). Furthermore we detected in various Kaplan-Meier calculations regarding the disease-free survival a highly significant association between the co-expression of RIP140 and ERβ and reduced disease-free survival (see *figure A1*).

5.3 Diskussion

Kernthemen der vorliegenden Arbeit sind die Expressionslokalisation der beiden Co-Regulatoren RIP140 und LCoR, sowie deren Expressionsverhalten bei uni- und multifokalen Tumoren. Herausgearbeitet wurde in beiden Originalarbeiten, dass die subzelluläre Lokalisation der beschriebenen Co-Regulatoren, sowie der Fokalitätsstatus relevante Faktoren hinsichtlich Tumoraggressivität und Patientenüberleben darstellen. In der vorliegenden ersten Originalarbeit, sowie in weiteren Studien konnte gezeigt werden, dass RIP140 und LCoR sowohl nukleär, als auch überwiegend zytoplasmatisch exprimiert werden mit einer hohen Korrelation zwischen beiden Proteinen (69), (59). Diese Interaktion wird über Bindung der N- und C-terminalen Strukturen von RIP140 an die C-terminale Region von LCoR, sowie über die HTH-Struktur von LCoR möglich und führt zu einer RIP140 vermittelten Aktivierung von LCoR, sowie zu einer vermehrten Expression von LCoR über Transaktivierung der *LCOR* Promotorgene in Mammakarzinomzellen (59). Die zentrale Rolle der Expressionslokalisation von RIP140 zwischen Nukleus und Zytoplasma wurde unter anderem von *Aziz et. al.* beschrieben; hierbei zeigte sich eine höhere zytoplasmatische Expression von RIP140 bei benignen Tumoren, jedoch eine höhere nukleäre Expression in malignen Epithelzellen (64) . Je nach posttranslationaler Modifikation wird eine Verschiebung der RIP140 Expression in den Zellkern oder ins Zytoplasma diskutiert, beispielsweise über posttranslationale Acetylierung oder Konjugation an Vitamin B6 (62), (63). Eine zytoplasmatische Expression von RIP140 und LCoR war positiv korrelierend mit einer Expression von N-Cadherin und CD133, Markerproteine der EMT und CSC, welche wiederum assoziiert sind mit lymphogener Metastasierung und kürzerem Patientenüberleben (70). Die Tumorgröße, sowie die Expression von ERα und PR waren dagegen negativ korrelierend mit einer nukleären Expression von RIP140 und LCoR, was hinweisend sein kann auf eine Ligandenabhängige Inhibition des Tumorwachstums, sowie der Expression der Hormonrezeptoren durch die untersuchten Co-Regulatoren. Auch beim Patientenüberleben zeigte sich eine hohe zytoplasmatische Expression von RIP140 assoziiert mit einem kürzeren Rezidivfreien Überleben, wohingegen eine vorwiegend nukleäre Expression von RIP140 mit einem besseren Gesamtüberleben korreliert. Eine geringe Gesamtexpression von LCoR war assoziiert mit einem längeren Rezidivfreien Überleben in HER2-positiven Tumoren.

Hinsichtlich des Fokalitätsstatus in der Matched-Paired-Studie zeigte sich wiederum eine hohe Korrelation beider transkriptionaler Co-Regulatoren sowohl bei uni- als auch bei multifokalen Tumoren. Bemerkenswert bleibt eine hohe Korrelation zwischen der Expression von RIP140 und LCoR mit der Expression von ERβ in uni- und multifokalen Tumoren. Hinsichtlich des Patientenüberlebens konnte beobachtet werden, dass eine hohe Expression von LCoR, RIP140 und

ERβ signifikant mit einem geringeren Gesamtüberleben in unifokalen Tumorfällen, nicht aber bei multifokalen Tumoren assoziiert war. Hervorzuheben bleibt, dass eine Co-Expression von LCoR, jedoch stärker von RIP140 und ERβ hoch signifikant mit einem geringeren Gesamtüberleben und kürzerem Rezidivfreien Überleben bei unifokalen Tumoren assoziiert war. Bezüglich RIP140 zeigten sich diese Ergebnisse konkordant mit den Daten der größeren Patientenkohorte der ersten vorliegenden Originalarbeit. In der Literatur wird hinsichtlich eines hoch exprimierten ERβ und den klinischen Auswirkungen kontrovers diskutiert. Zum einen konnte von *Kim et. al.* und *Guo et. al.* belegt werden, dass eine Überexpression von ERβ mit einem geringeren Rezidivfreien Überleben einhergeht (71), (71), wohingegen in Untersuchungen von *Tan et. al.* eine Assoziation mit einem günstigeren Rezidivfreien Überleben besteht (52). Die vorliegende Diskrepanz erklärt sich am ehesten durch verschiedene getestete ERβ-Isoformen, sowie durch unterschiedlich angewandte Methoden (Testung auf mRNA- oder Proteinebene). Der inhibierende Effekt auf Estrogen-vermittelte Signalkaskaden wird durch RIP140 stärker in Interaktion mit ERβ als mit ERα ausgeübt (51). Des Weiteren konnte beim Ovarialkarzinom beobachtet werden, dass eine ERβ-abhängige Induktion von RIP140 stattfindet (51).

Die vorliegenden Ergebnisse zeigen, dass die Expression von RIP140 und LCoR abhängig von ihrer subzellulären Lokalisation entgegengesetzte Effekte auf nukleäre und zytoplasmatische Signalkaskaden und damit auf die Tumorigenese haben. Eine nukleäre Expression von RIP140 korreliert mit geringer Tumorgröße, sowie längerem Gesamtüberleben, wohingegen eine zytoplasmatische Expression von LCoR mit geringem Rezidivfreien Überleben in HER2-positiven Tumoren, sowie mit der Expression von Tumorprogressionsmarkern N-Cadherin und CD133 korreliert. Nach Auswertung der vorliegenden Daten bleibt zu vermuten, dass nicht nur eine Inhibition des Tumorwachstums durch die Co-Regulatoren vornehmlich im Zellkern (ligandenabhängige, genomische Effekte durch Interaktion mit Kernrezeptoren) vermittelt wird, sondern auch eine prokarzinogene Wirkung durch Interaktion mit verschiedenen Signalkaskaden im Zytoplasma erfolgt. Das nukleäre vs. zytoplasmatische Expressionsverhältnis von RIP140 und LCoR und seine oppositionellen Auswirkungen auf die Tumorigenese bleiben damit Gegenstand weiterer Untersuchungen. Darüber hinaus konnte herausgearbeitet werden, dass eine Co-Expression von RIP140/ ERβ in Abhängigkeit vom Fokalitätsstatus prognostische Relevanz aufweist und assoziiert ist mit einem geringeren Gesamt- und einem kürzeren Rezidivfreien Überleben in unifokalen Tumoren. Die untersuchten Co-Regulatoren RIP140 und LCoR nehmen eine dual wirkende Sonderstellung im komplexen Netzwerk pro- und antikarzinogener Interaktionspartner ein und könnten Schlüsselproteine hinsichtlich neuer prognostischer oder therapeutischer Zielproteine darstellen.

Discussion

Central themes of the present work are the expression localisation of the coregulatory proteins RIP140 and LCoR, as well as their expression behaviour in uni- and multifocal tumor tissue. In both original articles it could be pictured that the subcellular localisation of the specified coregulatory proteins, as well as their focality status are relevant factors regarding tumor aggressiveness and patient survival. In the first original article, as well as in further studies, it could be shown that the expression of RIP140 and LCoR is as well nuclear as predominantly cytoplasmic with a high correlation between both proteins (69), (59). This interaction is possible through binding of N- and C-terminal structures of RIP140 onto the C-terminal region of LCoR, as well as across the HTH structure of LCoR and leads to an activation of LCoR and to augmented expression of LCoR through upregulated transcription of the *LCOR* genes in breast cancer cells (59). Among other studies, *Aziz et. al.* described the central role of the expression localisation of RIP140 between nucleus and cytoplasma, observing a higher cytoplasmic expression of RIP140 in benign tumors, however a higher nuclear expression in malign cells (64). It has been discussed a nuclear and cytoplasmic shifting of the expression of RIP140 depending on the posttranslational modification, for example via posttranslational acetylation or linking with vitamin B6 (62), (63). A cytoplasmic expression of RIP140 and LCoR was positively correlating with expression of N-cadherin and CD133, which represent marker proteins of EMT and CSC and are associated with lymphogen metastasis and shorter patient survival (70). However tumor size and expression of ERα und PR were negatively correlated with a nuclear expression of RIP140 and LCoR, which can be pointing to a ligand-dependent inhibition of tumor growth and expression of hormone receptors by the investigated coregulatory receptors. Also regarding patient survival a high cytoplasmic expression of RIP140 was associated with reduced disease-free survival, whereas a predominantly nuclear expression of RIP140 was correlated with better overall survival. A low total expression of LCoR showed association with augmented disease-free survival in HER2-positive tumor cases.

Regarding the focality status in the matched-paired study a high correlation of both transcriptional coregulatory proteins was observed in uni- and multifocal tumor cases. Remarkable is a high correlation between the expression of RIP140 and LCoR with the expression of ERβ in uni- and multifocal cases. Concerning patient survival a high expression of LCoR, RIP140 and ERβ was significantly associated with a reduced overall survival in only unifocal tumor cases, but not in multifocal tumors. Highlighting the co-expression between LCoR, but even stronger between RIP140 and ERβ showed highly significant association with reduced overall and shorter disease-free survival in only unifocal tumor cases. Respecting RIP140 these findings were consistent with the data of the

patient cohort of the first original article. The overexpression of ERβ and its clinical effects remain subject of current discussion. On the one hand *Kim et. al.* and *Guo et. al.* could provide evidence about the association between the overexpression of ERβ and reduced disease-free survival (71), (72), whereas investigations by *Tan et. al*. found opposing results (52). The discrepancy may be explained by differently tested ERβ isoforms or by different methods (testing mRNA or protein levels). The inhibitory effect on estrogen signalling is mediated by RIP140 even stronger if there is an interaction with ERβ rather than with ERα (51). Furthermore observing ovarian cancer cells showed an ERβ-dependent induction of RIP140 (51).

The present results show the opposing effects of the expression of RIP140 and LCoR onto nuclear and cytoplasmic signal transduction pathways in dependency on their subcellular localisation. The nuclear expression of RIP140 is correlating with small tumor size, as well as with increased overall survival, whereas a cytoplasmic expression of LCoR is associated with reduced disease-free survival in HER2-positive tumors and expression of tumor progression markers, N-cadherin and CD133. The present data questions if an inhibition of tumor growth is not only mediated by the coregulatory proteins predominantly in the nucleus (via ligand-dependent genomic effects in interaction with nuclear hormone receptors), but also a procarcinogenic effect can be mediated through the same proteins by interaction with various signal transduction pathways in the cytoplasma. The nuclear vs. cytoplasmic expression ratio of RIP140 and LCoR and its opposing effects on tumorigenesis remain subject of further investigation. Moreover the prognostic relevance of the coexpression of RIP140/ERβ was found to be dependent to the focality status and is associated with reduced overall and disease-free survival in unifocal breast cancer. The coregulatory proteins RIP140 and LCoR take an exceptional position in the complex network of pro- and anticarcinogenic interaction partners and could be key proteins regarding new prognostic or therapeutic target proteins.

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7. Eidesstattliche Versicherung

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Ich erkläre hiermit an Eides statt,
dass ich die vorliegende Dissertation mit dem Thema

Die subzelluläre Expression der Co-Regulatoren RIP140 und LCoR in uni- und multifokalen Mammakarzinomen

selbstständig verfasst, mich außer der angegebenen keiner weiteren Hilfsmittel bedient und alle Erkenntnisse, die aus dem Schrifttum ganz oder annähernd übernommen sind, als solche kenntlich gemacht und nach ihrer Herkunft unter Bezeichnung der Fundstelle einzeln nachgewiesen habe.

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München, den 12.07.2020 Katharina Johanna Müller

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