Aus der Klinik und Poliklinik für Frauenheilkunde und Geburtshilfe

Klinikum der Ludwig-Maximilians-Universität München

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The roles of the differential subcellular localization of two nuclear receptors in breast cancer--- PPARγ and THRβ1

Dissertation
zum Erwerb des Doktorgrades der Humanbiologie
an der Medizinischen Fakultät
der Ludwig-Maximilians-Universität zu München

vorgelegt von
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aus
Jilin, China
2020

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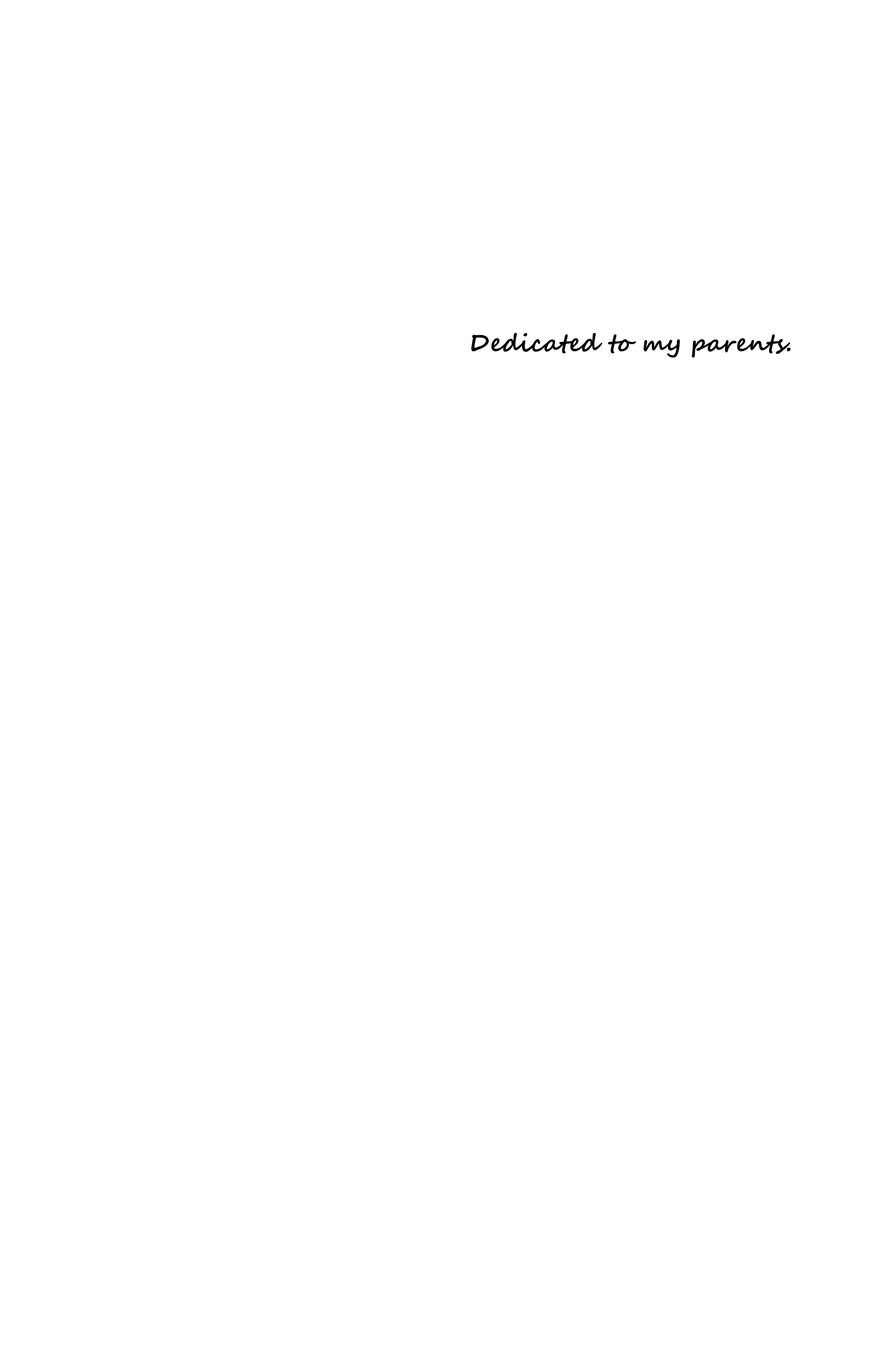
Dedicated to my parents.

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1. Abbreviations

AI	aromatase inhibitor
AA	arachidonic acid
ALND	axillary lymph node dissection
BC	breast cancer
BRL	rosiglitazone
CDK	cyclin-dependent kinase
COX	cyclooxygenase
DBD	DNA-binding domain
DFS	disease-free survival
EGFR/ErbB	epidermal growth factor receptor
ER	estrogen receptor
HER	human epidermal growth factor receptor
HR	hormone receptor
HRE	receptor tyrosine kinases
HSP	heat shock protein
LBD	ligand-binding domain
NLS	localization signal
NR	nuclear receptor
OS	overall survival
pCR	pathologic complete response
PFS	progression-free survival
PG	prostaglandin
PGE2	prostaglandin E2
PPAR	peroxisome proliferator activated receptor
PR	progesterone receptor
RFS	relapse-free survival
RXR	retinoid X receptor
SERD	selective estrogen receptor down-regulator
SERM	selective estrogen receptor modulator
SHR	steroid hormone receptor
SLNB	sentinel lymph node biopsy
TAM	tamoxifen
TGZ	troglitazone
THR	thyroid hormone receptor
TNBC	triple-negative breast cancer
TRE	thyroid hormone response element
TZD	thiazolidinedione

2. Publication list

2.1 Cytoplasmic PPARγ is a marker of poor prognosis in patients with Cox-1 negative primary breast cancers

Wanting Shao, Christina Kuhn, Doris Mayr, Nina Ditsch, Magdalena Kailuweit, Verena Wolf, Nadia Harbeck, Sven Mahner, Udo Jeschke, Vincent Cavaillès, Sophie Sixou

J Transl Med. 2020 Feb 21;18(1):94. doi: 10.1186/s12967-020-02271-6.

2.2 Cytoplasmic and nuclear forms of thyroid hormone receptor β1 are inversely associated with survival in primary breast cancer

Wanting Shao, Christina Kuhn, Doris Mayr, Nina Ditsch, Magdalena Kailuweit, Verena Wolf, Nadia Harbeck, Sven Mahner, Udo Jeschke, Vincent Cavaillès, Sophie Sixou

Int J Mol Sci. 2020 Jan 3;21(1). pii: E330. doi: 10.3390/ijms21010330.

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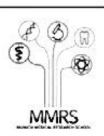

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Name of doctoral candidate

Cytoplasmic PPARγ is a marker of poor prognosis in patients with Cox-1 negative primary breast cancers

Title of publication

Title of article	Cytoplasmic PPARγ is a marker of poor prognosis in patients with Cox-1 negative primary breast cancers
Title of journal	Journal of translational medicine
Year	2020
Volume	18
Pages	94

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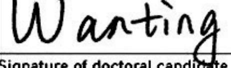

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- their agreement to the submission of the publications, and
- the article in question is not the subject of another (current or completed) thesis.

	Name of co-author	Extent of contribution (content-related and volume)	Signature of co-author
1.	Christina Kuhn	Assisted with IHC staining and techniques	
2.	Doris Mayr	Provided the samples and the clinical data	
3.	Nina Ditsch	Provided the samples and the clinical data	
4.	Magdalena Kailuweit	Performed IHC staining	
5.	Verena Wolf	Performed IHC staining	
6.	Nadia Harbeck	Contributed to manuscript writing and editing	
7.	Sven Mahner	Provided the samples and the clinical data	
8.	Udo Jeschke	Conceived and supervised the project and contributed to manuscript writing and editing; Supervised the research	
9.	Vincent Cavaillès	Conceived and supervised the project and contributed to manuscript writing and editing	
10.	Sophie Sixou	Conceived and supervised the project and contributed to manuscript writing and editing	

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Cytoplasmic and nuclear forms of thyroid hormone receptor β1 are inversely associated with survival in primary breast cancer

Title of publication

Title of article	Cytoplasmic and nuclear forms of thyroid hormone receptor β1 are inversely associated with survival in primary breast cancer
Title of journal	International journal of molecular sciences
Year	2020
Volume	21
Pages	330

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9. Vincent Cavaillès	Conceived and supervised the project and contributed to manuscript writing and editing	
10. Sophie Sixou	Conceived and supervised the project and contributed to manuscript writing and editing	

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4. Introduction

4.1 Breast cancer

4.1.1 Epidemiology

Breast cancer (BC) is the most common female malignancy and the main cause of cancer-related death worldwide [1]. In 2018, 2.1 million newly diagnosed cases and 0.6 million related deaths have been recorded, respectively accounting for 24.2% of total new cancer cases and 15.0% of all deaths in women [2]. It is noteworthy that although the mortality rates are decreasing in developed countries due to the advances in cancer screening and adjuvant therapy, the incidence rates of BC are increasing in most countries whereas death rates are much higher in less developed regions [3, 4]. Therefore, addressing the global BC issue is a huge challenge and it is necessary to develop early detections and novel treatments for BC.

4.1.2 Local management: surgery and radiotherapy

The complexity and heterogeneity of BC require a comprehensive and multidisciplinary approach adapted to each patient. The primary local and regional BC treatment remains surgical intervention, with a constant evolution from the Halsted radical mastectomy [5] from the 19th century to the Fisher modified radical mastectomy currently [6, 7]. Breast-conserving surgery followed by radiotherapy is established for most early BC cases because of developments in surgical techniques and neoadjuvant systematic therapies [8]. Some researchers demonstrated that the overall survival (OS), disease-free survival (DFS) and relapse-free survival (RFS) are equivalent to those of mastectomy [7, 9]. Reconstruction is a selection for women electing mastectomy with a relatively small breast in setting of huge tumor, extensive calcifications, or multicentric disease [10].

In addition to tumor size, axillary lymph node status acted as a prognostic factor in early BC and provides guidance for personalized treatment. Sentinel lymph node biopsy (SLNB) replaced the traditional axillary lymph node dissection (ALND) in node-negative BC patients, preventing them from lymphedema, shoulder dysfunction and other complications [8, 10, 11]. ACOSOG Z0011 trial proved that no survival difference was found between ALND and SLNB [12]. After 10 year follow-

up, early-stage BC patients with 1 or 2 SLN metastases treated by SLNB alone had noninferior outcome in OS compared with those treated with ALND [13].

Radiotherapy was recommended as a critical adjuvant treatment for women after breast-conserving surgery or mastectomy with high-risk clinical or pathologic factors (e.g. positive lymph nodes, large tumor size or lymphovascular invasion), beneficial for reducing local recurrence [14, 15]. In addition, the main complications of radiotherapy comprise cutaneous, pulmonary and cardiac toxicity and radiation techniques development (e.g. intensity modulated radiation therapy) and facilities implementation (e.g. deep inspiration breath hold technique) would contribute to lower rates of adverse events [16].

4.1.3 ER, PR and endocrine therapy

Excessive exposure to estrogen, acting through estrogen receptors, plays an important role in the development of BC by stimulating cell proliferation and initiating mutations during DNA replication [17]. The majority of BC (approximately 70%) express ERα (mostly named ER), progesterone receptor (PR) or both [18, 19] and assessment of ER and PR (together termed as hormone receptor – HR) status has become the standard of care for BC patients. Patients with HR positive BC exhibit lower recurrence and better outcome compared with the HR negative group and HR was identified as an independent predictor in BC [20, 21]. Besides, the expression of PR is primarily regulated by ERα at the transcriptional level [22]. Loss of PR expression is correlated to a worse outcome in luminal cancers [23].

ER and PR belong to the steroid hormone receptor (SHR), a subfamily of nuclear receptor superfamily [22, 24, 25]. Guideline recommendations of immunohistochemical testing suggested 1% or more nuclear ER or PR staining as positive [1] and endocrine sensitivity was determined by the intensity of ER and PR positivity [8]. Gene expression profiling identified a molecular subtype in BC, “luminal-like”, divided to A and B. Luminal B cancers were characterized as higher expression of proliferation genes (Ki-67) compared with luminal A [23, 26].

Endocrine therapy represents an important strategy in the management of early and advanced hormone positive BC [27], including commonly ovarian suppression, selective estrogen receptor modulators (SERMs) and down-regulators (SERDs) and aromatase inhibitors (AIs), which was

given consecutively after surgery or chemotherapy [8, 28]. For premenopausal ER-positive BC patients with sufficient risk factors for recurrence, ovarian suppression was recommended to combine with adjuvant endocrine therapy [29]. Tamoxifen (TAM), a SERM, acts as a competitive inhibition of estrogen binding to ER and consequently suppresses estrogen-dependent gene transcription, cell proliferation and tumor growth [30]. Whereas, fulvestrant, a SERD, binds to ER and makes it accelerated degradation, leading to reduction of cellular ERα levels[31]. AIs (e.g. anastrozole, exemestane and letrozole), usually applied in postmenopausal women by reducing the production of estrogen by blocking the aromatase enzyme activity (also known as CYP19A1 [32]), decrease the recurrence rates and mortality rates compared with TAM [33, 34]. ATLAS trial demonstrated prolongation of TAM treatment for ER-positive BC from 5 years to 10 years produces a further reduction in recurrence and mortality [35].

4.1.4 HER and anti-HER therapy

13-15% of BCs overexpress the HER2 tyrosine kinase receptor, divided to two subgroups: luminal B-like and non-luminal, which have a highest death rate compared with other subgroups [1]. Human EGFR (also called ErbB or HER) family comprises four transmembrane receptor tyrosine kinases: HER1 or EGFR, HER2, HER3 and HER4. When active, formation of homo- and heterodimers could activate downstream pathways: PI3K/AKT, Ras/Raf/MEK/ERK and PLCγ pathway [36]. Among them, HER2, overexpressed in 25%-30% of BC, correlates with poor prognosis and an important therapeutic target [37]. Trastuzumab, a humanized monoclonal antibody targeting HER2, became a successfully clinical biological drug, together or sequential with chemotherapy, as adjuvant or neoadjuvant treatment, which significantly increased OS and DFS in women with HER2-positive breast cancer [38]. Although no ligand is known for HER2, it appears to cooperate with other ErbB receptors (HER3/HER4) in neoplastic progression. Moreover, HER3 serves as an indispensable partner of HER2 dimerization and an essential function of proliferation on HER2-positive BC. Thus, drugs targeting HER3 may enhance the efficacy of dual HER2-targeted approaches [39]. The function of HER4 in BC is controversial, resulting in good or bad outcomes. It works not only in cell cycle arrest, differentiation, apoptosis but also in cell proliferation [40]. Besides, upregulation of nuclear HER4 led to worse trastuzumab response and poorer survival in HER2-positive BC, whereas cytoplasmic HER4 seems related to longer OS [40, 41]. Overexpression of EGFR is

frequently observed in triple-negative BC (TNBC) and inflammatory BC (an aggressive subtype), causing worse prognosis [42, 43]. However, EGFR-targeted therapies, monoclonal antibodies and tyrosine kinase inhibitors, had no significant results in clinical trials of BC [44]. Nevertheless, anti-HER therapy or combined with other targeted drugs may be a promising strategy against BC.

4.1.5 TNBC and other potential targets

TNBC represents approximately 15%-20% of all BC, characterized as lack of ER, PR and HER2 expression. This term is more aggressive with higher relapse rates and poorer overall outcome than other types of BC, distinctly related to large size, high grade and lymph node involvement [1, 45, 46]. Six subtypes were identified by gene expression profile analysis, including basal like 1 and 2, immunomodulatory, mesenchymal, mesenchymal stem–like, and luminal androgen receptor [1, 47, 48]. TNBC patients usually have a better pathologic complete response rates (pCR) after neoadjuvant chemotherapy and those who achieve pCR have a long-term survival [49]. Current treatments for TNBC are limited to cytotoxic chemotherapy, due to the lack of effective targets.

BRCA1/2 mutations are more likely to cause hereditary breast and ovarian cancers and account for around 20% of patients with TNBC, which pattern is susceptible to DNA-damaging agents, platinum compound and poly (ADP ribose) polymerase inhibitors [50]. p53 is another mutant gene considerably associated with TNBC and agents (e.g. PRIMA-1 and APR-246) restoring its wild-type properties maybe new treatments for BC [51]. Dysfunction of PI3K-AKT-mTOR signaling pathway, such as PIK3Ca mutation and loss of PTEN, gives rise to progress in breast tumorigenesis [52]. In addition, PIK3Ca mutation is frequently observed in luminal androgen receptor subtype cell lines and make it sensitive to PI3K/mTOR inhibition [53]. Thus, combination of anti-androgen and other target therapies may optimize current strategies in TNBC. More and more attentions are diverted to individual and personalized therapy from standardized system based on TNM stages. Precision treatment of BC is defined by analysis of immunohistochemical markers and gene expression, guiding treatment plans and response assessments.

4.1.6 Chemotherapy and resistances

Apart from endocrine therapy, anti-HER2 and more recent targeted therapy, chemotherapy was regarded as a conventional and effective adjuvant systemic regime, which indications depend on tumor grade, lymph node involvement or cell proliferation status (widely estimated by Ki-67 index [54]). Moreover, multiparameter gene expression assays were presented for risk assessment and prediction of chemotherapy benefit in patients with luminal-like disease, such as Oncotype DX and MammaPrint [8, 55]. The routine agents of current cytotoxic therapy are anthracylines and/or taxanes given in combination or in sequence, for both early and advanced stage BC [55]. Of note, dose-dense chemotherapy leads to a better prognosis [56]. Besides, the purpose of chemotherapy in metastatic BC is to maintain quality of life, relieve symptoms and prolong life [8].

Drug resistance of BC limiting the chemotherapy efficacy, brings a great challenge to survival of patients, which mechanisms underlying chemoresistance were defined. Higher expressions of twist gene and multidrug resistance 1 gene suggested as a prediction for response to chemotherapy in BC [57, 58]. ATP-binding cassette transporters remove chemotherapeutic drugs from cells and result in chemoresistance [59]. Regulation of the behavior of tumor cells by cytokines and survival of cancer stem cells promoted chemoresistance [60]. In addition, other mechanisms include DNA damage repair [61], tumor microenvironment [62] and microRNAs [63].

Mutations of *ER* gene and lack of ER and PR expression are identified as causes of endocrine resistance in BC [64]. Cyclin-dependent kinases (CDKs) play crucial roles in regulation of cell cycle by synergizing with cyclin. CDK4/6 inhibitors contribute to overcome endocrine resistance BCs combined with anti-estrogen or anti-HER2 therapy [65]. The PI3K/AKT pathway and ER signaling crosstalk is correlated with effectiveness of anti-estrogen drugs [66, 67]. Otherwise, epidermal growth factor receptor (EGFR, ErbB) family, STAT family and NF-κB family are potential targets for combination with endocrine therapeutic strategies in ER-positive BC [68-70]. Furthermore, inhibitors of CDK4/6, PI3K and mTOR have been applied in clinical trials with benefits for advanced HR-positive, HER2-negative BC. Ribociclib (CDK4/6 inhibitor) plus endocrine therapy improved progression-free survival (PFS) and palbociclib, combined with fulvestrant, could increase OS but the difference was not significant [71-73]. PI3K inhibitors, buparlisib and alpelisib,

combined with fulvestrant, resulted in a longer PFS in endocrine-resistant and PI3CA-mutated patients, respectively [74, 75]. Everolimus (mTOR inhibitor) plus an AI, improved PFS in patients with nonsteroidal AIs [76].

Therefore, understanding resistance mechanisms and exploring novel approaches are beneficial to overcoming chemoresistance, and resistance to all targeted therapies.

4.2 Nuclear receptor

4.2.1 An overview

The human nuclear receptor (NR) superfamily contains 48 members, some of which are DNA-binding transcription factors activated by endogenous and exogenous ligands and some of which are so-called "orphan receptors", because the ligands have not been identified [77]. NRs play a crucial role in a range of physiological process, such as metabolism, homeostasis and immune response. Dysfunction of NR signaling pathway lead to numerous diseases including obesity, diabetes and cancer [78, 79]. All NR proteins have a common modular, highly conserved structure with four major domains (Figure 1) [25]. The C-terminal ligand-binding domain (LBD), containing ligand-induced activation function (termed AF-2), involves in transcriptional activity by regulation of ligand binding and coregulator recruitment. The most conserved DNA-binding domain (DBD) located in the central C region of NR protein with two zinc finger motifs. LBD and DBD could mediate the dimerization of NRs in some cases. LBD and DBD are linked by a short hinge region responsible for nuclear localization signal (NLS). In contrast to AF-2, AF-1 is positioned in the poorly identified N-terminal A/B region, interacting with coregulators through a ligand-independent way [80, 81]. Thus, NRs could activate or repress target gene transcription functions by ligand dependent and independent regulations.

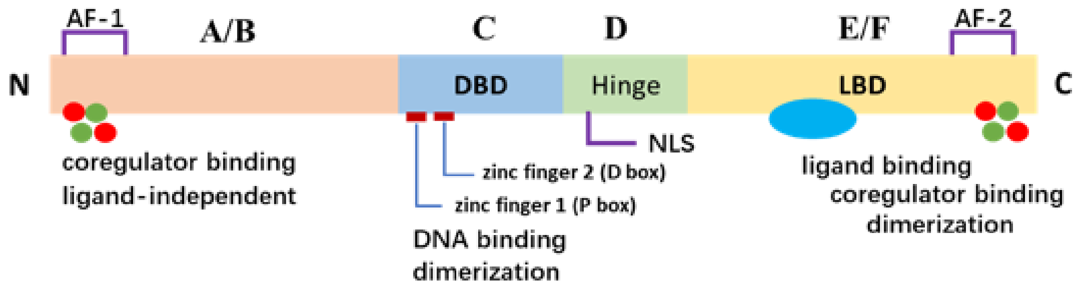

Figure 1: The structure of nuclear receptor.

NRs have been classified as into four subtypes due to the classical genomic mechanisms [79, 82]. Type I NRs include Steroid Hormone Receptors (SHRs), such as ER, PR, androgen receptor, glucocorticoid receptor and mineralocorticoid receptor. They disassociate from heat shock proteins (HSPs) and form homodimers after ligand activation in the cytoplasm. Then dimers translocate to the nucleus and bind to specific sequences of DNA known as hormone response elements (HREs), which subsequently regulate the transcription of target genes by recruiting coactivators [83, 84]. Type II NRs, such as thyroid hormone receptors (THR) and peroxisome proliferator activated receptors (PPAR), are retained in the nucleus binding as heterodimers with retinoid X receptors (RXR) to specific DNA response elements regardless of ligand activation by changes in dissociation of corepressors and recruitment of coactivators [85]. Type III NRs, such as vitamin D receptor, function similarly to type I NRs but bind to direct repeat instead of inverted repeat HREs. Type IV NRs instead bind as a monomer to half-site HREs. Alternate mechanism of NR cross-talk has been recognized as “nongenomic” actions independently of transcriptional regulation [86, 87]. The genomic process generally requires a prolonged series of actions (at least 30 to 60 minutes to modulate the transcription processes), whereas nongenomic type elicits rapid cellular effects within seconds or minutes and is not repressed by inhibitors of transcription or translation [88-91]. The rapid nongenomic actions of NRs initiate by binding to membrane receptors or interacting with molecules, such as G proteins, ion channels, protein kinases, Src tyrosine kinase, PI3K and MAPK. One example is the presence of SHRs or THRs at the mitochondrial or plasma membranes, leading to the rapid nongenomic signaling processes [92-94]. Thus, subcellular localization of NRs may play different roles in genomic and nongenomic actions, which should be considered in the development of NR-related diseases.

In BC, ER and PR, two members of NR superfamily, are of particular importance in tumorigenesis and prognosis, which give rise to more precise routine diagnosis for molecular subtype in all patients. Drugs targeting ER, such as TAM [30], fulvestrant [95], and more recently developed AIs [33] achieve a great success in current BC treatment strategies. However, it is still a tremendous challenge to make relevant therapies for advanced or metastatic cases and TNBC disease. More study of NR-related signaling pathway may provide novel therapeutic targets for BC.

4.2.2 PPARγ

PPARs are ligand-dependent transcription factors, which consist of three major subtypes, commonly designated as PPARα, PPARβ/δ and PPARγ encoded by separate genes [96, 97]. PPARs play critical roles in lipid homeostasis, glucose metabolism, inflammatory response and cancer development [98, 99]. The human PPARγ gene is located in chromosome 3p25 [100]. PPARγ is the most extensively described isoform of PPARs, which influences inflammation, cell proliferation, differentiation, apoptosis and tumor angiogenesis [101]. Positive immunoreactivity of PPARγ was strong in the nucleus of normal and benign breast tissues, however, a decreased or no staining was shown in malignant tissues [102-104]. High levels of PPARγ predominantly in either nucleus or cytoplasm were correlated with a longer survival and favorable clinical characteristics, such as smaller size, lower grade, earlier stage and ER positivity [102-106]. Besides, in a study previously published in our laboratory, cytoplasmic PPARγ showed stronger expression in BRCA1-mutant BC than sporadic cases with no relation to prognosis [107]. In a clinical study with a PPARγ ligand, HER2-positive BC patients with diabetics had a long-term survival after metformin and thiazolidinedione (TZD) therapy [108], indicating activation of PPARγ may play a positive role in repression of BC. However, patients with metastatic BC had no benefits from treatments with troglitazone (TGZ) [109] or rosiglitazone (BRL) [110].

The function of PPARγ in tumorigenesis seems contradictory. The oncogenic role of PPARγ has been reported in several studies, including BC [111-114]. Enhanced PPARγ signaling induced tumor incidence and mortality in transgenic mice with a ligand-independent PPARγ mutant [113]. Besides, T0070907, a selective PPARγ antagonist, and the dominant-negative PPARγ mutant, Δ462, significantly reduces cellular proliferation, migration and invasion in breast cancer cell lines [114].

On the other hand, PPARγ acts primarily as a tumor suppressor in most cancers, especially BCs. BRL suppressed proliferation in MCF-7 cells line with a PPARγ-dependent manner by downregulating PI3K/AKT pathway, which was reversed by ERα antagonist, indicating that ERα negatively mediated PPARγ signaling through binding to PPRE. PPARγ activation also induces overexpression of PTEN tumor suppressor gene [115]. ERα and PPARγ could compete for BRL, mediating each other's transactivation [116]. In mouse tumor model, PPARγ activation inhibited BC progression by upregulating protein tyrosine phosphatase receptor F, a downstream target of PPARγ [117]. Moreover, BRL promoted apoptosis by activating Fas/FasL pathways in human BC cell lines [118] and induce cell differentiation [119]. The biotinylated form of 15d-PGJ2 (b-15d-PGJ2) had obvious anti-proliferative and pro-apoptotic effects on MCF-7 and MDA-MB-231 cell lines compared with 15d-PGJ2, which was attenuated by PPARγ silencing with a decrease of apoptotic markers, PARP-1 and caspase-7 [120]. HER2 overexpression in BC cells was accompanied with a high level of PPARγ protein, inhibiting PPARγ transcription activation and PPARγ ligand-induced cell growth [121]. In addition, PPARγ downregulated CXCR4 expression, which played a pivotal role in mediating the development of BC invasion and metastasis. The mechanism seemed to be reversed by GW9662, a PPARγ antagonist, and decreased levels of phosphorylated FAK, AKT and ERK1/2 in CXCR4 downstream signaling [122]. TGZ inhibited TPA induced NF-κB and AP-1 activation and MMP-9 expression, the critical enzyme for invasion and metastasis, through a PPARγ-dependent mechanism [123].

Besides the genomic effects of the NR, many other nongenomic effects have been described, not only for ER [124], with membrane or cytoplasmic expression. Nuclear export of PPARγ is initiated *via* MAPK/ERK/MEK1/2 signaling, which restrains PPARγ transactivate nuclear target genes and thereby inhibits its genomic function [125, 126]. uPA mediated PON 1 expression in hepatocytes by regulating subcellular compartmentalization of PPARγ and induced PPARγ nuclear export in a MEK-dependent manner [127]. Fatty acids, acting as PPARγ agonists, had antineoplastic effects in BC cells with inhibition of ERK1/2 phosphorylation and nuclear translocation of PPARγ [128-130]. In another study, nuclear immunoactivity of PPARγ was observed in MCF-7 cell line or ER-positive tissues, whereas MDA-MB-231 cells, or ER-negative tissues, showed a cytoplasmic localization strongly related with S-phase kinase protein (Skp2) expression, which is related to

malignancy in certain tumors. Down-regulated Skp2 could reverse tetradecanoyl phorbol acetate-induced nuclear export of PPARγ in MEK1-dependent pathway [131]. These findings suggest that nuclear translocation of PPARγ may play an important role in antitumor effects and suggest that the study of the intracellular distribution of PPARγ may give new insights to identify novel therapy for BC.

4.2.3 THR

As many other NR, TH modulate numerous physiological activities, including development, differentiation, growth and metabolism, again by two distinct pathways, genomic and nongenomic. The classical genomic mechanisms are mediated mostly by T3-THR complex binding to TH response elements (TREs). Two isoforms, THRα and THRβ, are encoded by *THRA* and *THRB* genes which located on chromosome 17 and 3 [132-134]. The nongenomic actions of TH are related to plasma membrane, mitochondria or cytoplasm locations with receptors homologous or nonhomologous to THRs, such as integrin αvβ3 [132, 135]. The TH status and thyroid disorders have a strong correlation with the development of BC. High levels of T3 was observed in BC patients compared to benign breast tumor, positively related to aggressive BC characters, such as larger tumor, lymph node metastases and negative ER and PR expression [136, 137]. In addition, BC patients were inclined to thyroid enlargement and a meta-analysis study showed that BC or thyroid cancer predisposed an individual to developing the other [138, 139]. These findings indicated a significant association between TH signaling and BC.

Several previous studies reported that either THRα or THRβ expression decreased in BC compared with normal breast tissues, indicating downregulation of THR during breast carcinogenesis [140-143]. Loss of nuclear THRα expression was correlated with larger and higher grade tumor [143] and nuclear THRα2 was an independent prognostic factor in improved OS [144, 145]. Other studies figured THRβ functioned as a tumor suppressor in BCs. Low THRβ levels predicted poor outcomes and enhanced resistance to chemotherapy by cAMP-PKA signaling pathway [146]. In BRCA1-mutated BC, THRβ were overexpressed compared with sporadic cases but had a positive prognostic result whereas THRα reduced survival [147]. THRβ inhibited tumor growth by activating apoptosis and decreasing proliferation *via* JAK-STAT-cyclin D pathways in

the xenograft mouse model [148]. The suppression of oncogenic RUNX2 activity was dependent on THRβ, not THRα, in triple negative MDA-MB-231 cell line [149]. Moreover, mutation of THRβ promoted the development of BC *via* aberrant activation of STAT5 [150], which was consistent with the result of another study regarding *THRβ1* gene mutation in tumorigenesis of Chinese BC population [151]. In addition to other preclinical researches, THRβ1 could inhibit cell proliferation, invasiveness and metastasis formation in BC cell lines [152, 153].

Studies of the protein expression and subcellular localization about THRβ were limited. Shuttling of THR between the nucleus and cytoplasm was induced by TH, indicating that THR mislocalization may contribute to the development of some types of cancer [133, 154, 155]. One study reported that THRβ1 expression was predominantly in cytoplasm in BC, and positively associated with ER-positive tumors, small tumors, lymph node negative status and longer survival [156]. In another previous study, THRβ was described as expressed in nuclei of benign and carcinoma *in situ* tissues, and in the cytoplasm of normal breast and infiltrative BC cells [157]. Besides, overlapping genomic and nongenomic actions of TH are observed between integrins and THR [93]. TH binding to αvβ3 induced nuclear translocation of THRβ1 through MAPK/MEK/ERK pathway [158]. In addition, this complex also regulates expression of the *THRβ1*, *ERα*, and *cyclooxygenase-2 (COX-2)* genes and modulates post-translational modifications of THRβ1 [159, 160]. Therefore, exploring nongenomic action of THRs and its subcellular localization is essential in BC development. The cross-talk between genomic and nongenomic actions of THR may provide new targets for BC treatment.

4.3 Cyclooxygenase

Targeting prostaglandins (PGs) pathway potentially plays a positive role in prevention and treatment of cancers. Biosynthesis of PGs, some belonging to PPARγ ligands, from arachidonic acid (AA) is catalyzed by a key enzyme, Cox, which has two isoforms, Cox-1 and Cox-2 [161, 162]. Cox-1 is constitutively expressed in many normal cells, whereas Cox-2 is generally considered induced by inflammatory cytokines and growth factors, resulting in carcinogenesis of many tissues [163, 164]. A meta-analysis study revealed that increased expression of Cox-2 in BC ranged from 27.9% to 81.4%, significantly correlated with poor OS and adverse features, such as large tumor size and

lymph node invasion [165]. Prostaglandin E2 (PGE2), production *via* Cox-2, induced CYP19 expression and aromatase activity, leading to the development of ER-positive BCs [166, 167]. In addition, Cox-2 inhibitors decreased incidence and progression of BC through improving apoptosis and repressing proliferation and angiogenesis [168]. Combination of specific Cox-2 inhibitor and PPARγ agonist resulted in growth inhibition in a mouse model of mammary adenocarcinoma [169]. Compared with Cox-2, less attention was taken to Cox-1 in tumors, although both selective and nonselective Cox inhibitors prevent mammary tumors [170]. Fewer studies demonstrated the tumor suppression of selective Cox-1 inhibitors in BC, such as SC-560, catechin and FR122047. More interestingly, combination of Cox-1 and Cox-2 inhibitors had an addictive effect on tumor repression in BC cell lines [171-173]. Besides, Corticotropin-releasing factor, a hypothalamic neuropeptide, promoted cell motility and invasiveness through production of PGs *via* Cox-1 not Cox-2 in BC cell line [174]. Another study elucidated that the antitumor property of nonsteroidal anti-inflammatory drugs by cell differentiation was not dependent on Cox-2 pathway, indicating that potential role of Cox-1 in the activation of PPARγ [175]. In summary, the literature strongly suggests that both Cox-1 and Cox-2 participate in PGs and PPARγ signaling pathways involved in breast tumorigenesis.

4.4 Aims of the studies

4.4.1 Subcellular expression of PPARγ and correlation with Cox-1 in primary BC tumors

The role of PPARγ, the most extensively described isoform of PPARs, was controversially described as a tumor promoter or suppressor in different cancers. PGs, as PPARγ ligands, are produced from the conversion of AA by Cox-1 and Cox-2. The aim of this study was to analyze the relevance of combined expression of PPARγ and Cox (especially Cox-1) in BC and correlation of the data with several clinicobiological parameters including patient survival. In the Publication I of this thesis, we analyzed by immunohistochemistry the subcellular expression of PPARγ and of the two Cox proteins in a well characterized 308 primary BC specimens in relation to survival, to determine if either one could, independently or in relation to the others, be linked to BC progression.

4.4.2 Expression and subcellular localization of THRβ1 in primary BC tumors

THRβ1, also belonging to NR superfamily, appears to act as a tumor suppressor in many malignant neoplasms. While THRβ1 clearly appears to be a key player in BC carcinogenesis, the importance of its subcellular localization remains to be elucidated. The purpose of this study was designed to explore the different roles of nuclear-cytoplasmic compartmentalization of THRβ1 in BC tissues. Therefore, we investigated the nuclear and cytoplasmic expression of THRβ1 by immunohistochemistry in the same cohort with 274 primary BC tumors and analyzed the correlation of the results with clinicopathological parameters and clinical outcome. All data were published in Publication II of this thesis.

5. Publication I

J Transl Med. 2020 Feb 21;18(1):94. doi: 10.1186/s12967-020-02271-6.

Cytoplasmic PPARγ is a marker of poor prognosis in patients with Cox-1 negative primary breast cancers

Wanting Shao, Christina Kuhn, Doris Mayr, Nina Ditsch, Magdalena Kailuweit, Verena Wolf, Nadia Harbeck, Sven Mahner, Udo Jeschke, Vincent Cavaillès, Sophie Sixou

Shao *et al. J Transl Med* (2020) 18:94
https://doi.org/10.1186/s12967-020-02271-6

Journal of Translational Medicine

RESEARCH **Open Access**

Cytoplasmic PPARγ is a marker of poor prognosis in patients with Cox-1 negative primary breast cancers

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Abstract

Background: The aim of this study was to investigate the expression of the nuclear receptor PPARγ, together with that of the cyclooxygenases Cox-1 and Cox-2, in breast cancer (BC) tissues and to correlate the data with several clinicobiological parameters including patient survival.

Methods: In a well characterized cohort of 308 primary BC, PPARγ, Cox-1 and Cox-2 cytoplasmic and nuclear expression were evaluated by immunohistochemistry. Correlations with clinicopathological and aggressiveness features were analyzed, as well as survival using Kaplan–Meier analysis.

Results: PPARγ was expressed in almost 58% of the samples with a predominant cytoplasmic location. Cox-1 and Cox-2 were exclusively cytoplasmic. Cytoplasmic PPARγ was inversely correlated with nuclear PPARγ and ER expression, but positively with Cox-1, Cox-2, and other high-risk markers of BC, e.g. HER2, CD133, and N-cadherin. Overall survival analysis demonstrated that cytoplasmic PPARγ had a strong correlation with poor survival in the whole cohort, and even stronger in the subgroup of patients with no Cox-1 expression where cytoplasmic PPARγ expression appeared as an independent marker of poor prognosis. In support of this cross-talk between PPARγ and Cox-1, we found that Cox-1 became a marker of good prognosis only when cytoplasmic PPARγ was expressed at high levels.

Conclusion: Altogether, these data suggest that the relative expression of cytoplasmic PPARγ and Cox-1 may play an important role in oncogenesis and could be defined as a potential prognosis marker to identify specific high risk BC subgroups.

Keywords: PPARγ, Cytoplasmic, Cox-1, Cox-2, Overall survival, Breast cancer

Background

Breast cancer (BC), the most commonly diagnosed malignant tumor in women, is also the most frequent cause of cancer death worldwide [1] and a significant global public health problem. BC is highly heterogeneous in its pathological characteristics, which raised a tremendous challenge for treatment selection [2]. So far, few biomarkers have been well recognized in invasive breast carcinomas, including estrogen receptor (ER) and progesterone receptor (PR), which are associated with a better outcome and are predictive of endocrine sensitivity. Overexpression of human epidermal growth factor receptor 2 (HER2) is related with decreased relapse-free survival (RFS) and overall survival (OS) [3, 4]. Agents targeting ER and HER2, such as tamoxifen and trastuzumab, have been very successful as BC therapeutics. However, multifaceted mechanisms emerged in tumors, causing resistance to endocrine treatment in single or combination therapies [5]. Thus,

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comprehensive identification of more biomarkers and molecular targets is essential for optimal and personalized clinical BC management.

Peroxisome proliferator-activated receptors (PPARs) belong to the nuclear receptor (NR) superfamily [6] and function as ligand-activated transcription factors [7]. Following activation by ligands (e.g. 15d-PGJ_2 or the synthetic ligand thiazolidinedione), PPARs heterodimerize with retinoid X receptor (RXR) and interact with proliferator-activated receptor response elements (PPREs) present in target gene promoters [8]. Although the NR superfamily was defined due to genomic actions of the receptors which require nuclear localization, it has been suggested that PPARs localize first in the cytoplasm with specific associated functions [9].

Among the three PPAR isoforms (α, β/δ and γ), PPARγ plays a crucial role in adipogenesis and lipid metabolism [10] and is also found expressed in many human cancers, including BC [11]. PPARγ influences inflammatory processes, cell proliferation, differentiation, apoptosis and tumor angiogenesis [10, 12]. A tumor promoting effect of PPARγ has been reported in some tumors, such as liver [13], cancer [14] or colon cancer [15]. In addition, most of previous studies have revealed that PPARγ acts as a tumor suppressor in BC, inhibiting cell proliferation and inducing apoptosis in different in vivo and in vitro models [16–18]. Besides, PPARγ has been suggested as being involved in chemotherapy resistance of TNBC [19].

Interestingly, some of the PPARγ ligands, prostaglandins (PGs) are produced from the conversion of arachidonic acid by the cyclooxygenases Cox-1 and Cox-2. Cox-1 is constitutively expressed in many normal cells, whereas Cox-2 is generally considered being induced by inflammatory cytokines and growth factors, performing a significant role in carcinogenesis [20, 21]. Studies of Cox importance in tumor progression and invasion were mainly focused on the influence of Cox-2 [22]. However, it was demonstrated that Cox-1 is highly expressed and plays a pivotal role in some carcinomas, such as ovarian [23] and breast cancers [24]. More recently, Cox-1 mRNA and protein levels have been shown to be higher in malignant breast tumors than in normal tissues, whereas Cox-2 mRNA level was lower in malignant tumors. Nonetheless, stromal and glandular Cox-2 immunostaining showed higher levels in malignant breast tumors [25].

It appears therefore obvious that more attention is needed to analyze the relevance of combined expression of PPARγ and Cox (especially Cox-1) in BC. In the present study, we have analyzed expression of PPARγ and of the two Cox proteins in 308 primary BC specimens in relation to survival, to determine if either one could, independently or in relation to the others, be linked to BC progression.

Methods

Patient cohort

A total of 308 formalin-fixed paraffin-embedded primary BC tissues from 303 patients (5 of them are bilateral BC) who received surgeries between 2000 and 2002 at the Department of Obstetrics and Gynecology of the Ludwig-Maximilians-University Munich, Germany were collected. Local and systemic therapy treatment was given according to the guidelines at the time of diagnosis. This study was approved by the Ethical Committee of the Medical Faculty, Ludwig-Maximilian-University, Munich, Germany (approval number 048-08) and informed consent for nuclear factor analysis was obtained from all patients who were alive at the time of follow-up. Data, such as age, histological grade, metastases, local recurrence, progression, and survival were retrieved from the Munich Cancer Registry and anonymized and encoded during statistical analysis and experiments. All tumors were assessed according to UICC TNM classification, containing tumor size and extent of tumors (primary tumor size, or pT, classified as: pT1a-c, pT2, pT3, pT4a-d), lymph node status (N), and presence or absence of metastasis (M). Tumor grade was determined by an experienced pathologist (Dr. D. Mayr) of the Department of Pathology of the LMU, according to a modification of Elston and Ellis grading proposed by Bloom and Richardson [26]. Sixty (19.48%) of the 303 primary BC patients, became metastatic during the follow-up. ER, PR, HER2, Ki-67 and histological status were all determined by an experienced pathologist of the LMU Department of Pathology, as described below. HER2 2+ scores were further evaluated through fluorescence in situ hybridization (FISH) testing.

Immunohistochemistry (IHC)

Expression of ERα, PR, and HER2 was determined at diagnosis in all BC samples of this cohort at the LMU Department of Pathology, Germany. ERα and PR expression were 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 using an automated staining system (Ventana; Roche, Mannheim, Germany), according to the manufacturer's instructions. Ki-67 was stained using an anti-Ki67 monoclonal antibody (Dako, Hamburg, Germany) at a dilution of 1:150 on a VENTANA®-Benchmark Unit (Roche, Mannheim, Germany) as previously described [27]. The Ki-67 cut-off used to differentiate luminal A from luminal B tumors (all HER2 negative) was 14% as this was commonly used at the time of the analysis, although 20% is now preferred

[28]. Data on N-cadherin and CD133 expression in these BC samples were extracted from a previously published study [29]. For PPARγ, Cox-1 and Cox-2 analysis by IHC, samples were processed as previously described [30, 31]. Briefly, sections were first cut and prepared from paraffin-embedded BC samples using standard protocols. Phosphate buffered saline (PBS) was used for all washes and sections were incubated in blocking solution (ZytoChem Plus HRP Polymer System Kit, ZYTOMED Systems GmbH, Berlin, Germany) before incubation with primary antibodies. All primary antibodies were rabbit IgG polyclonal used at a 1:100 dilution for 16 h at 4 °C: anti-PPARγ (ab59256, Abcam, Cambridge, UK) or anti-Cox-1 (HPA002834) and anti-Cox-2 (SAB4502491, both Sigma-Aldrich, Saint Louis, MO, USA). After incubation with a biotinylated secondary anti-rabbit IgG antibody, and with the associated avidin–biotin–peroxidase-complex (both Vectastain Elite ABC Kit; Vector Laboratories, Burlingame, CA, USA), visualization was performed with substrate and chromogen 3,3-diamino-benzidine (DAB; Dako, Glostrup, Denmark). Negative and positive controls were used to assess the specificity of the immunoreactions. Negative controls (colored in blue) were performed in BC tissue by replacement of the primary antibodies by species-specific (rabbit) isotype control antibodies (Dako, Glostrup, Denmark). Appropriate positive controls (placenta samples) were included in each experiment. Sections were counterstained with acidic hematoxylin, dehydrated and immediately mounted with Eukitt (Merck, Darmstadt, Germany) before manual analysis with a Diaplan light microscope (Leitz, Wetzlar, Germany) with 25× magnification. Pictures were obtained with a digital CCD camera system (JVC, Tokyo, Japan). All slides were analyzed by two or three independent examiners.

Immunoreactive score (IRS)

The expression of PPARγ, Cox-1 and Cox-2 was assessed according to the immunoreactive score (IRS), determined by evaluating the proportion of positive tumor cells, scored as 0 (no staining), 1 (≤ 10% of stained cells), 2 (11–50% of stained cells), 3 (51–80% of stained cells) and 4 (≥ 80% of stained cells), and the intensity of their staining, graded as 0 (negative), 1 (weak), 2 (moderate) and 3 (strong) (IRS = percentage score × intensity score). Thus, the range of IRS value is from 0 to 12. As previously described for LCoR and RIP140 [31] and for AhR [32], PPARγ cytoplasmic and nuclear staining were evaluated in parallel, with a separate determination of cytoplasmic IRS and nuclear IRS. Total IRS was calculated by addition of cytoplasmic and nuclear IRS. For all other markers, staining and IRS were determined in the whole cells, without differentiation of nuclear and cytoplasmic staining.

Survival and statistical analysis

Receiver operating characteristic curve (ROC) analyses were performed to calculate the optimal cut-off values between low and high PPARγ, Cox-1 and Cox-2 expressions, based upon the maximal differences of sensitivity and specificity. The threshold determined regarding OS were an IRS ≥ 3.5 for either total or cytoplasmic PPARγ, ≥ 0.5 for nuclear PPARγ and for Cox-1, and finally ≥ 1.5 for Cox-2. These thresholds were used to determine the percentages of tumors expressing low or high PPARγ, Cox-1 and Cox-2 levels described in Table 2, besides the OS analysis detailed below. To present the mean immunoreactivity levels described by the IRS in Table 2, the groups were divided into low- vs. high-expressing cases for total and cytoplasmic PPARγ, Cox-2, or into not expressing vs. expressing cases for nuclear PPARγ, Cox-1 (cut-off values of 0.5).

Differences in nuclear PPARγ expression among three or more groups (Fig. 1, panel k) were tested using the non-parametric Kruskal–Wallis rank-sum test. Correlation analyses presented in Tables 3 and 4 were performed by calculating the Spearman's-Rho correlation coefficient (p values of Spearman's-Rho test presented). Survival times were compared by Kaplan–Meier graphics and differences in OS (or RFS) were tested for significance by using the Chi-square statistics of the log rank test. Data were assumed to be statistically significant in the case of p-value < 0.05. Kaplan–Meier curves and estimates were then provided for each subgroup and each marker. The p value and the number of patients analyzed in each subgroup are given for each chart.

Multivariable analysis for outcome (OS) presented in Table 5 was performed using the Cox regression model, and included cytoplasmic PPARγ expression and relevant clinicopathological characteristics as independent variables. Variables were selected based on theoretical considerations and forced into the model. p values and hazard ratios were indicated, knowing that the hazard ratios of covariates are interpretable as multiplicative effects on the hazard, and holding the other covariates constant.

Statistical analyses were performed using SPSS 24 (IBMSPSS Statistics, IBM Corp., Armonk, NY, USA). For all analyses, p values below 0.05 (*), 0.01 (**), or 0.001 (***) were considered statistically significant.

Results

PPARγ and Cox expression in breast cancers

The total cohort consisted of 308 samples from 303 primary BC (Table 1). Median age of initial diagnosis was 57.98 years (range 26.66–94.62 years) and median

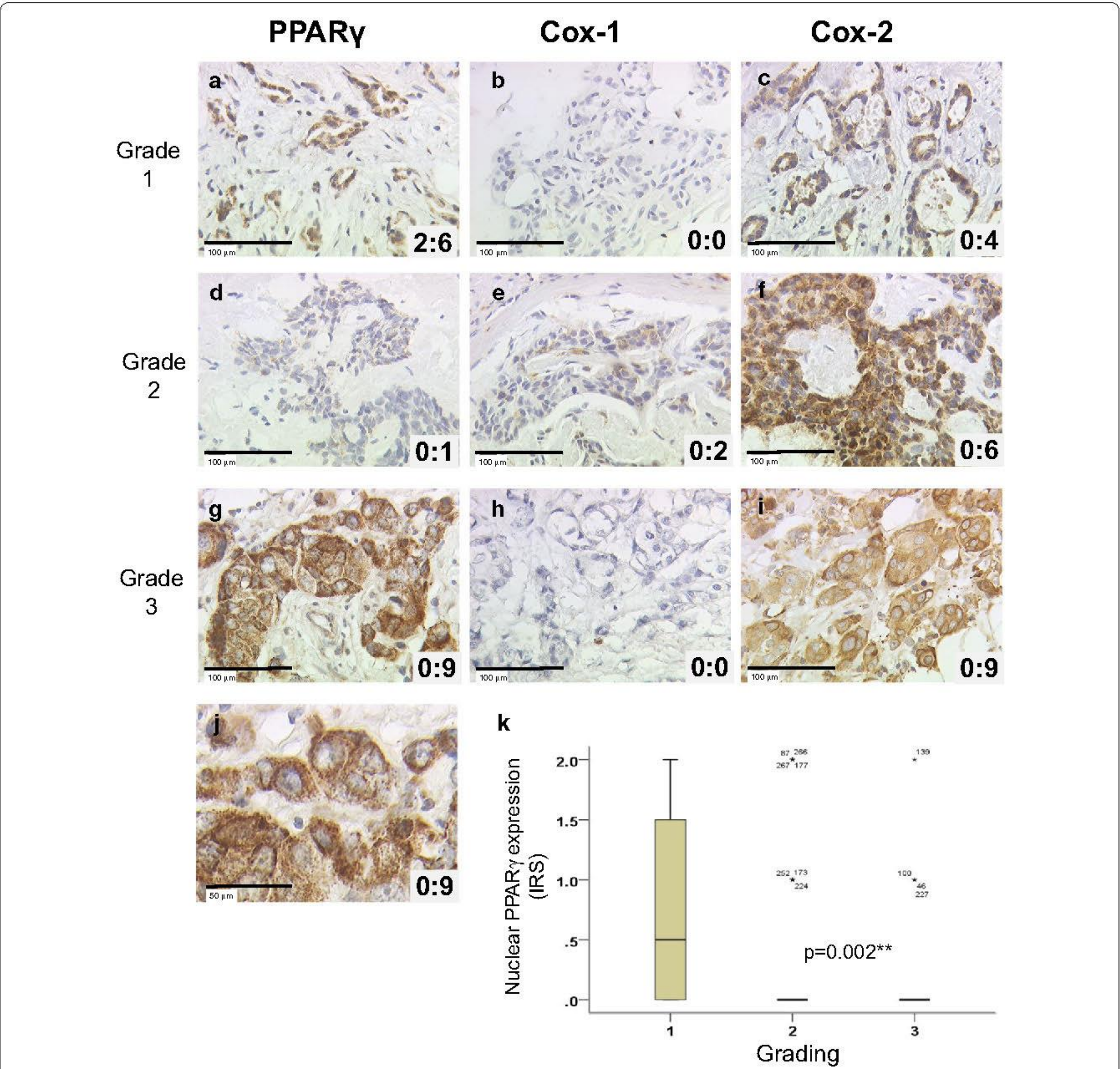

Fig. 1 Immunohistochemical stainings of PPARγ, Cox-1 and Cox-2 expression in breast carcinoma of 3 patients and a box-plot of nuclear PPARγ and Grading. PPARγ (**a**, **d**, **g**), Cox-1 (**b**, **e**, **h**) and Cox-2 (**c**, **f**, **i**) stainings are illustrated for patients with different grading (Grade 1 in **a**–**c**, 2 in **d**–**f** and 3 in **g**–**i**), with examples of null, average or high expressions. Nucleo:cytoplasmic IRS ratios are indicated in each photomicrograph (×25 magnification) and scale bar equals 100 µm. An enlargement of **g** (high cytoplasmic and null nuclear PPARγ staining) is presented (**j**) and scale bar equals 50 µm. Correlation between nuclear PPARγ and grading was presented as box plot (**k**). The boxes represent the range between the 25th and 75th percentiles with a horizontal line at the median. The bars on top and below depict the 5th and 95th percentiles. Values more than 1.5 box lengths from the 75th percentile are indicated by circles (none) and values more than 3.0 box lengths from the 75th percentile are indicated by asterisks. The numbers on asterisks represent the case number. Statistical significance is shown as p-value from Kruskal–Wallis test (**p < 0.01)

follow-up time was 125 months (range 0–153 months). During this period, 41 (13.3%) and 60 (19.5%) cases experienced local recurrence and distant metastasis respectively, and 90 (29.2%) women died.

The expression of PPARγ, Cox-1 and Cox-2 was analyzed by IHC staining, as illustrated in Fig. 1 for 3 patients with Grade 1 (A, B, C), 2 (D, E, F) and 3 (G, H, I) tumors. PPARγ expression (A, D, G) was present both

Table 1 Clinical and pathological characteristics of all patients

Clinical and pathological characteristics[a]	N = 308[b]	%
Age, median (years)	57.98	
Follow up, average (months)	109.89	
Median	125	
Histology[c]		
Invasive lobular	41	13.31
Invasive medullar	10	3.25
Invasive mucinous	3	0.97
No special type (NST)	161	52.27
DCIS with NST	78	25.33
Unknown	15	4.87
ER status		
Positive	248	80.52
Negative	58	18.83
Unknown	2	0.65
PR status		
Positive	178	57.79
Negative	128	41.56
Unknown	2	0.65
HER2 status		
Positive	35	11.36
Negative	271	87.99
Unknown	2	0.65
Molecular subtype		
Luminal A (Ki-67 ≤ 14%)	170	55.19
Luminal B (Ki-67 > 14%)	63	20.45
HER2 positive luminal	27	8.77
HER2 positive non luminal	8	2.60
Triple negative	38	12.34
Unknown	2	0.65
Grade		
I	15	4.87
II	102	33.12
III	45	14.61
Unknown	146	47.40
Tumor size		
pT1	191	62.01
pT2	87	28.25
pT3	4	1.30
pT4	12	3.90
Unknown	14	4.55
Lymph node metastasis		
Yes	126	40.91
No	163	52.92
Unknown	19	6.17
Local recurrence[d]		
Yes	41	13.31
No	253	82.14
Unknown	14	4.55
Distant metastases[e]		
Yes	60	19.48
No	234	75.97
Unknown	14	4.55

[a] All information given refer to the primary tumor

[b] 5 of 303 patients are bilateral primary BC, so we deal with the tumor as individual one (n = 308)

[c] NST include the formerly called "Invasive ductal" and "other" types

[d] Local recurrence has been detected during the follow-up of 40 patients (1 of them are bilateral BC, so n = 41)

[e] Distant metastasis has been detected during the follow-up of 58 patients (2 of them are bilateral BC, so n = 60)

in the nucleus and in the cytoplasm, while Cox-1 and Cox-2 (B, E, H, and C, F, I respectively) were exclusively cytoplasmic. The nucleo:cytoplasmic IRS ratios are presented in each panel (panel J shows the enlargement of PPARγ staining shown in panel G). From now, all Cox-1 and Cox-2 expression refers to their unique cytoplasmic expression, with Cox-1 staining being much fainter than Cox-2 staining, as described in Table 2. As demonstrated in the panel K, nuclear PPARγ exhibited a statistically different expression according to grading, with an inverted correlation ($p = 0.002$). This correlation is illustrated by focusing on the nuclear PPARγ expression observed in panels A, D and G of Fig. 1 (IRS of 2, 0 and 0 respectively) for patients with respectively grade 1, 2 and 3 tumors.

As presented in Table 2, the mean IRS of total and cytoplasmic PPARγ expression were 4.37 and 4.09 respectively, while it was 0.27 for nuclear PPARγ. It clearly appears that, in our cohort, PPARγ expression is dramatically higher (15-fold) in the cytoplasm than in the nucleus, with maximal IRS values of 12 and 4 respectively. This is exemplified in Fig. 1 with cytoplasmic PPARγ IRS values of 1, 6 and 9, and nuclear PPARγ IRS values of 0 and 2 (panels A, D and J). IRS cut-offs were defined by performing a ROC-curve analysis for OS. Of note, the IRS cut-off of 0.5 generated for nuclear PPARγ staining is related to the low expression level of this marker in our cohort, and create sub-groups with negative vs. positive expression, instead of low vs. high expression for other cut-off values. Considering cytoplasmic or total expression of PPARγ being high for IRS value > 3.5, the high expression group is predominant in both cases (52.7 and 57.6% respectively). Only 20 patients out of 262 (7.6%) had no cytoplasmic PPARγ expression (IRS = 0), demonstrating the predominant cytoplasmic expression of PPARγ (92.4% of the tumors).

Table 2 Distribution of expression of PPARγ, Cox-1 and Cox-2

	PPARγ			Cox-1	Cox-2
	Total	Nuclear	Cytoplasmic		
n	262	262	262	297	285
Mean IRS ± SE	4.37 ± 0.17	0.27 ± 0.04	4.09 ± 0.17	0.34 ± 0.04	5.19 ± 0.19
IRS range	12	4	12	4	12
IRS cut-off	3.5	0.5	3.5	0.5	1.5
Number of samples with negative/low expression	111 (42.4%)	213 (81.3%)	124 (47.3%)	224 (75.4%)	36 (12.6%)
Number of samples with positive/high expression	151 (57.6%)	49 (18.7%)	138 (52.7%)	73 (24.6%)	249 (87.4%)

IRS cut-offs were defined by performing a ROC-curve analysis for DFS. The cut-off of 0.5 for nuclear PPARγ and for Cox-1 stainings, related to the low expression level of both markers in our cohort, define negative and positive expressions, instead of low and high expressions sub-groups respectively

Table 3 Correlation between PPARγ, Cox-1 and Cox-2 expression

n = 254 to 297	PPARγ			Cox-1	Cox-2
	Total	Nuclear	Cytoplasmic		
PPARγ					
Total	1.000				
Nuclear	0.037	1.000			
Cytoplasmic	0.959**	−0.215**	1.000		
Cox-1	0.179**	−0.117	0.201**	1.000	
Cox-2	0.261**	−0.124*	0.293**	0.054	1.000

Correlations are statistically significant for $p < 0.05$ (*) or $p < 0.01$ (**), using Spearman-Rho-Test

Besides, the mean IRS of cytoplasmic expression were 0.34 and 5.19 for Cox-1 and Cox-2 respectively. This is again exemplified in Fig. 1 with Cox-1 IRS values of 0 and 2 (panels B, E and H) and Cox-2 IRS values of 4, 6 and 9 (panels C, F and I), for the same 3 selected patients. Similarly to nuclear PPARγ, Cox-1 mean IRS being very low, a cut-off of 0.5 was generated, with sub-groups of negative vs. positive expression, instead of low vs. high expression for Cox-2. In our cohort, 75.4% of the samples were then Cox-1 negative, whereas the samples with a high expression of Cox-2 represented 87.37% of the cases (cut-off of 1.5). Regarding nuclear PPARγ, only 49 samples were positive (18.7%) while for Cox-1, only 73 samples (24.6%) were positive (with maximum IRS of 4 for both markers).

Correlation between PPARγ and Cox expression

The correlations between the expression levels of PPARγ (total, nuclear and cytoplasmic), Cox-1 and Cox-2 were analyzed (Table 3). Cytoplasmic PPARγ expression exhibited a strong and significant positive correlation with total PPARγ, and a negative one with nuclear PPARγ. By

Table 4 Correlation between PPARγ, Cox-1 and Cox-2 expression and clinicopathological or aggressiveness related parameters

	PPARγ n = 143 to 262			Cox-1	Cox-2
	Total	Nuclear	Cytoplasmic	n = 159 to 297	n = 153 to 285
Age	0.004	−0.050	0.002	0.041	−0.015
pT	0.118	−0.037	0.113	−0.049	−0.066
pN	0.069	0.023	0.065	−0.125*	−0.043
Grade	0.007	−0.205*	0.054	−0.007	−0.062
ER	−0.119	0.117	−0.142*	0.009	0.039
PR	−0.048	0.038	−0.049	−0.018	0.012
HER2	0.157**	−0.127*	0.173**	0.137*	0.090
Triple negative	0.076	−0.062	0.085	−0.043	−0.052
Ki-67	0.116	−0.039	0.119	0.084	0.155*
Focality	0.043	0.074	0.016	−0.048	−0.028
CD133	0.221**	−0.007	0.230**	0.132*	0.378**
NCAD	0.412**	−0.196**	0.447**	0.241**	0.461**

Correlations are statistically significant for $p < 0.05$ (*) or $p < 0.01$ (**), using Spearman-Rho-Test

contrast, nuclear and total expression of PPARγ were not correlated together. Regarding Cox expression, Cox-1 and Cox-2 levels were not correlated. Nonetheless, both Cox-1 and Cox-2 expression were significantly correlated with cytoplasmic and total PPARγ expressions. Besides, nuclear PPARγ was significantly negatively correlated with Cox-2 (and not with Cox-1).

Correlation between PPARγ, Cox expression and clinicopathological parameters or aggressiveness markers

We then analyzed the correlations between PPARγ or Cox expression and known clinicopathological characteristics (Table 4). We also quantified the expression of two aggressiveness markers, CD133, a widely used marker for isolating cancer stem cell (CSC) [33, 34], and N-cadherin, a well-known marker for epithelial-to-mesenchymal transition (EMT) [35]. Considering first nuclear PPARγ, significant negative correlations were observed with grade (as already illustrated in Fig. 1k, and by the 3 selected patients in Fig. 1), HER2 and N-cadherin, as well as Cox-2 (as already shown in Table 3). On the contrary, total and cytoplasmic PPARγ were strongly positively correlated with HER2, CD133 and N-cadherin. Only cytoplasmic PPARγ was negatively correlated to ER. Besides, Cox-1 was positively correlated with HER2, CD133, and N-cadherin, while Cox-2 was positively correlated with Ki-67, CD133, and N-cadherin. Only Cox-1 was statistically negatively correlated with lymph node status (LNM), and only Cox-2 was positively correlated with the proliferation marker Ki-67.

Correlation between PPARγ, Cox expression, and patient survival

In order to analyze the correlation between PPARγ and survival, we performed Kaplan–Meier analyses. We used the cut-off IRS values determined by ROC-curve analysis, allowing the maximal difference of sensitivity and specificity (as described in Table 2). In Fig. 2, considering the OS of the whole cohort, the cytoplasmic PPARγ expression was able to discriminate high expressing tumors with a significantly worse survival than patients with low expressing tumors (mean OS: 10.55 years *vs* 9.44 years, $p=0.027$; Fig. 2a). On the contrary, neither nuclear PPARγ (Fig. 2b) nor total PPARγ (Additional file 1: Figure S1A) had any significant correlation with OS.

RFS analysis were performed in parallel for total, cytoplasmic and nuclear PPARγ expression (Additional file 1: Figure S1B–D respectively). Both total and cytoplasmic PPARγ significantly discriminated patients with worse RFS (when PPARγ was highly expressed) from those having better survival when PPARγ expression was low (mean RFS: 9.37 years vs 6.88 years, $p=0.001$, and mean RFS: 9.30 years vs 6.70 years, $p=0.000217$).

We then looked at the association between cytoplasmic PPARγ expression and OS in different subgroups by stratifying the cohort, according to parameters mentioned in Table 4. Compared to the correlation of cytoplasmic PPARγ expression with OS in the whole cohort ($p=0.027$, Fig. 2a), the correlation was stronger in the subgroup of luminal A tumors ($p=0.005$ Fig. 2c), and lost in the luminal B subgroup (Fig. 2d). Similarly, the correlation was very strong in the subgroup of N-Cadherin low expressing tumors ($p=0.007$, Fig. 2e) and absent in the N-Cadherin high expressing tumors (Fig. 2f).

We then focused on subgroups of patients according to Cox expression in their tumors. As demonstrated in Fig. 3, expression of cytoplasmic PPARγ was still clearly related to a worse prognosis in the subgroup of tumors expressing no Cox-1 ($p=0.001$, Fig. 3a), as observed in the whole cohort ($p=0.027$, Fig. 2a). On the contrary, no correlation of cytoplasmic PPARγ existed with the OS of patients with tumor expressing Cox-1, and the trend, although not significant, was even inverted with an apparently better prognosis for group with high cytoplasmic PPARγ expression (Fig. 3b).

In the subgroup of patients with low Cox-2 expression (using a cut-off IRS of 7), expression of cytoplasmic PPARγ was still related to a poor prognosis ($p=0.009$, Fig. 3c) while no correlation of cytoplasmic PPARγ and OS existed for the patients with high Cox-2 expression (Fig. 3d).

Cytoplasmic PPARγ expression as an independent prognostic parameter of OS in N-cadherin low and Cox-1 negative tumors

We then performed multivariate analyses for the whole cohort and for the subgroups of patients described above, using the Cox regression model with cytoplasmic PPARγ expression and various clinicopathological features (age at time of diagnosis, tumor size, ER, and HER2 status). As shown in Table 5, data demonstrated that in the whole cohort, only age, tumor size and ER were independent prognostic markers of OS. Very interestingly, cytoplasmic PPARγ appeared as an independent prognosis marker in the N-cadherin low (IRS < 3.5) and Cox-1 negative subgroups ($p=0.044$ and $p=0.014$ respectively), with hazard ratios of 1.996 and 2.047 indicating a much higher risk of death for the patients with tumors expressing high levels of cytoplasmic PPARγ.

On the opposite, cytoplasmic PPARγ had no independent prognostic value in the N-cadherin high or Cox-1 positive expressing subgroups, in the subgroups with low or high Cox-2 expression (IRS cut-off of 7) or even in the Luminal A subgroup (data not shown). The same analysis

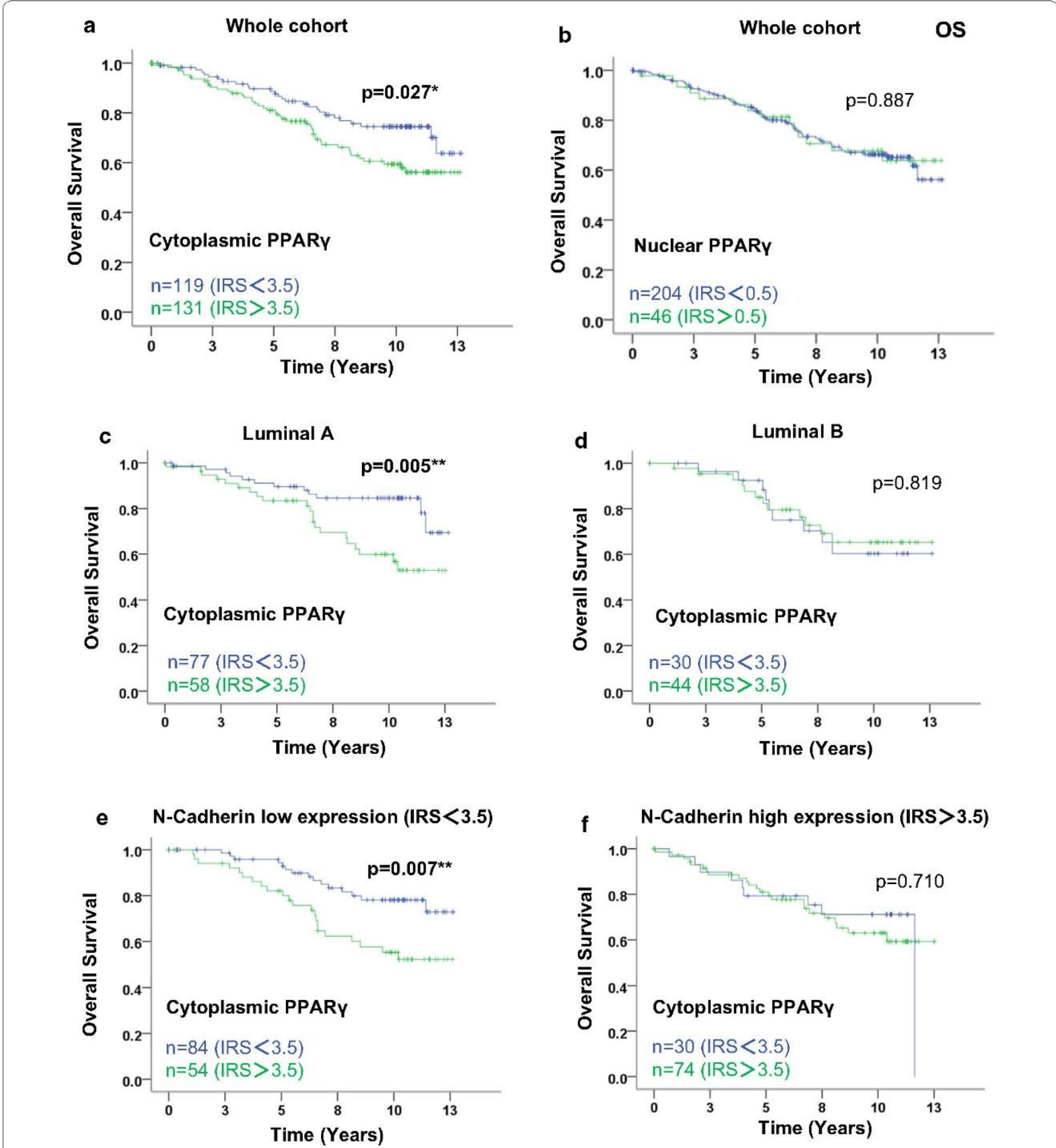

Fig. 2 Kaplan–Meier analysis of patient overall survival according to nuclear and cytoplasmic PPARγ expression in the whole cohort, and to cytoplasmic PPARγ expression in subgroups. In the whole cohort, overall survival (OS) curves are presented according to cytoplasmic PPARγ (**a**) and nuclear PPARγ (**b**) status. In luminal (**c**, **d**) and N-Cadherin (**e**, **f**) subgroups, overall survival curves are presented according to cytoplasmic PPARγ status. The IRS cut-off values with the number of cases for each group are indicated in each graph. Statistical significance is shown as p-value from log-rank test (*p < 0.05; **p < 0.01)

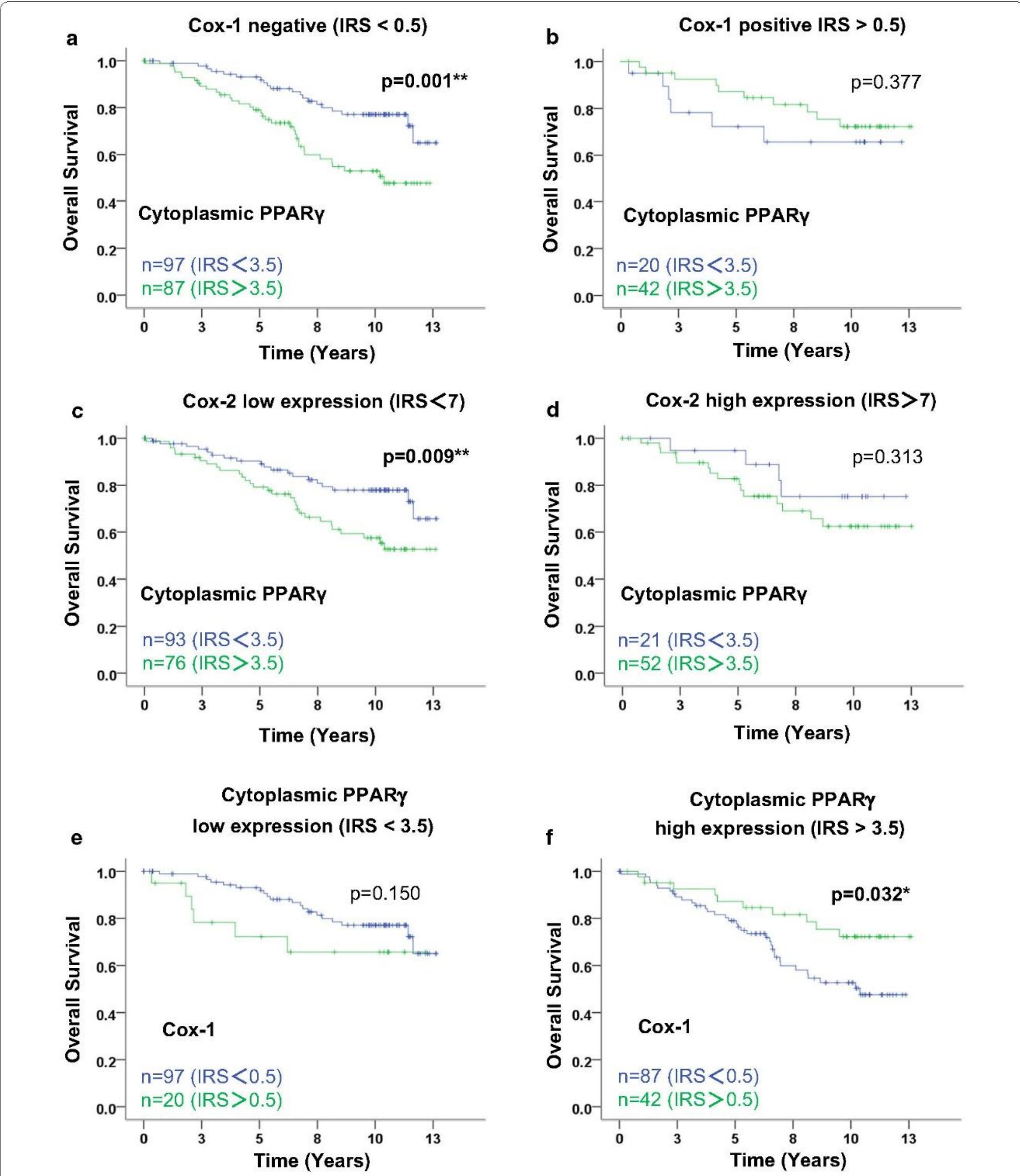

Fig. 3 Kaplan–Meier analysis of patient overall survival according to cytoplasmic PPARγ and of Cox-1 expression in subgroups. Overall survival (OS) curves are presented according to cytoplasmic PPARγ status in Cox-1 (**a**, **b**) and Cox-2 (**c**, **d**) subgroups. OS of Cox-1 is then presented according to cytoplasmic PPARγ (**e**, **f**). The IRS cut-off values with the number of cases for each group are indicated in each graph. Statistical significance is shown as p-value from log-rank test (*p < 0.05; **p < 0.01)

Table 5 Multivariate analysis of significant clinicopathological variables and of cytoplasmic PPARγ regarding OS in the whole cohort and in various subgroups

	Age	pT	ER	HER2	Cytoplasmic PPARγ
Whole cohort					
p	0.00001***	0.00000007***	0.008**	0.154	0.129
HR	1.040	3.769	0.508	1.616	1.457
N-cadherin low					
p	0.002**	0.00037***	0.015*	0.528	0.044*
HR	1.041	3.370	0.420	1.341	1.996
N-cadherin high					
p	0.000733***	0.000032***	0.174	0.035*	0.902
HR	1.045	6.121	0.583	3.437	1.052
Cox-1 negative					
p	0.000023***	0.000008***	0.162	0.307	0.014*
HR	1.045	3.835	0.655	1.598	2.047
Cox-1 positive					
p	0.017*	0.017*	0.023*	0.253	0.454
HR	1.051	3.574	0.284	1.907	0.670
Cox-2 low					
p	0.000002***	0.00008***	0.015*	0.969	0.102
HR	1.058	3.272	0.440	0.983	1.665
Cox-2 high					
p	0.112	0.000343**	0.801	0.021*	0.545
HR	1.027	7.681	0.867	5.369	1.427

HR, hazard ratio; p, p-value

In the sub-groups, the same cut-off as in Figs. 2 and 3 have been used, namely 3.5 for N-Cadherin and 7 for Cox-2. Correlations are statistically significant for $p<0.05$ (*) or $p<0.01$ (**), $p<0.001$ (***)

performed with nuclear or total PPARγ, with Cox-1 or Cox-2, did not reveal any independent prognostic value as seen with cytoplasmic PPARγ.

Cox-1 expression is associated with favorable OS only in tumors with high cytoplasmic PPARγ expression

We then checked in the whole cohort that neither Cox-1 nor Cox-2 expression was related to OS (Additional file 1: Figure S2A, B respectively). In order to strengthen the link between PPARγ, Cox1, and survival, we analyzed the prognostic value of Cox1 according to PPARγ levels. By selecting patients with tumors expressing high levels of cytoplasmic PPARγ (Fig. 3f), Cox-1 expression appeared statistically correlated to a better OS of patients ($p=0.032$). For patients with tumors expressing low levels of cytoplasmic PPARγ (Fig. 3e), no correlation with OS appeared although we observed again an opposite trend, with Cox-1 expression numerically correlated with a poor OS. Altogether, these data strengthened our results demonstrating that the relative expression of cytoplasmic PPARγ and Cox-1 is linked to prognosis in primary BC, with a high cytoplasmic PPARγ/Cox-1 ratio being a marker for poor prognosis, and that Cox-1 expression correlated with longer OS in an unselected cohort.

Discussion

The aim of this study was to characterize the intracellular expression and possible interplay of PPARγ and the Cox (Cox-1 and Cox-2) expression in a wide range of BC specimens, in relation with the clinicopathological parameters as well as patient survival. We already demonstrated that cytoplasmic PPARγ is overexpressed in BRCA1 mutated BC compared to sporadic cases, but without correlation to survival [27]. In previous surveys, either nuclear PPARγ or cytoplasmic PPARγ had a correlation with an improved clinical outcome of BC patients [8, 36, 37], but fewer specific subgroups of patients were analyzed.

Our data demonstrated that PPARγ expression was detected in a majority of BC tissues and that it is predominantly localized in cytoplasm (92.3% vs 18.7%). This is in accordance with previous studies [8, 27, 38]. However, positive PPARγ immunoreactivity was previously described as mainly nuclear in normal cells from benign samples; in malignant cells, a decreased expression was shown which was related to a favorable survival for patients [37, 39]. In addition, it was demonstrated that casein-kinase-II-dependent phosphorylation of PPARγ leads to subcellular translocation of PPARγ from cytoplasm to nucleus regulated by CRM1 and that urokinase-type plasminogen activator promoted atherogenesis in hepatocytes by downregulating PON1 gene expression via PPARγ nuclear export mechanism [9, 40]. Intracellular distribution of PPARγ was observed in BC tissues and cell lines [41], suggesting that poorly differentiated samples and highly invasive cell lines displayed mainly cytoplasmic PPARγ expression. Moreover, cytoplasmic localization of PPARγ was described as being mediated by Skp2 upon MEK1-dependent mechanism indicating cytoplasmic translocation of PPARγ promoted tumorigenesis in BC. In another study [17], α-ESA, considered as a PPARγ agonist like rosiglitazone, as well as GLA [38], suppressed cell growth in BC cell lines by activating PPARγ nuclear compartmentalization, which suggested that nuclear localization of PPARγ plays a role in anti-cancer functions in BC. Besides the predominant cytoplasmic localization of PPARγ, our data demonstrate a significant correlation between total and cytoplasmic PPARγ and an inverse relationship between cytoplasmic and nuclear PPARγ (Table 3), supporting the hypothesis of the translocation mechanism of PPARγ in the carcinogenic process.

Concerning the correlation between PPARγ expression and clinicopathological features or aggressiveness markers, our data demonstrated that nuclear PPARγ expression was inversely correlated with tumor grade, HER2 and N-cadherin expression, whereas total and cytoplasmic PPARγ were positively related with HER2, CD133, and N-cadherin (Fig. 1 and Table 4). These correlations strongly suggest that only cytoplasmic PPARγ was associated with the more aggressive tumors, namely ER negative, HER2 positive, CD133 (as a CSC marker [33, 34]) positive and NCAD (as an EMT marker [35]) positive sub-groups. Nonetheless, cytoplasmic PPARγ expression being much higher (15 fold) than nuclear one, total PPARγ expression exhibited similar association as cytoplasmic one with tumor aggressiveness. Several authors also found, as we did, a negative correlation between nuclear PPARγ and histological grade [36, 37, 39], and one paper indicated that nuclear PPARγ was negatively associated with HER2 [39]. Interestingly, PPARγ protein was expressed in both transfected MCF-7/Neo and MCF-7/HER2, but with higher levels of expression in the MCF-7/HER2 cells [42]. Moreover, HER2 up-regulated PPARγ expression, causing BC cells to become resistant to PPARγ ligand response [43]. Both CD133 and N-cadherin play a critical role in cancer migratory and invasive properties. Indomethacin could decrease CD133 expression, which means reducing CSCs via inhibiting Cox-2 and NOTCH/HES1 and activating PPARγ [44]. According to our previous work [29], N-cadherin-positive tumors without LNM had a significantly shorter survival time. Enhanced activity of PPARγ had an inhibition on TGF-β induction of N-cadherin promoter in lung carcinoma cell lines [45].

Overall, nuclear PPARγ possess a possible protective role against BC development, whereas cytoplasmic PPARγ was defined as a promoter during BC progression. Our data emphasize this hypothesis of opposite correlation of nuclear PPARγ with antioncogenic parameters and of cytoplasmic PPARγ with oncogenic or aggressive parameters. Survival analysis in the whole cohort demonstrated that only cytoplasmic PPARγ expression had a strong correlation with poor OS (Fig. 2), whereas both total and cytoplasmic PPARγ expression had a strong correlation with poor RFS (Additional file 1: Figure S1). As described earlier, PPARγ activation has been shown to exert antiproliferative and pro-apoptotic effects in BC cell lines [16–18, 46]. Moreover, cell death has been shown to be triggered in BC cell lines through the localization of PPARγ into the nucleus followed by the induction of Fas ligand [19]. The analysis of apoptosis markers will be necessary to give more insight in the molecular mechanisms underlying the differential effects of cytoplasmic and nuclear PPARγ.

Analysis of Cox in our cohort of primary BC substantiated that both Cox-1 and Cox-2 were dominantly localized in cytoplasm with a predominant negative or low expression for Cox-1 and a high expression for Cox-2 (Table 2). However, they were both significantly and positively correlated with total and cytoplasmic PPARγ, whereas only Cox-2 expression was negatively correlated with nuclear PPARγ (Table 3). Additionally, similarly to cytoplasmic PPARγ, Cox-1 was positively associated with HER2, CD133, and N-cadherin. Nonetheless, it was inversely related to LN involvement (Table 4), suggesting the hypothesis that Cox-1 expression may be related to the evolution of the tumor, especially expressed during the early non-metastatic stages of BC. Moreover, Cox-2 was positively related to Ki-67, CD133, and N-cadherin. In breast CSCs deprived from tumor cells of HER2/Neu mice, both Cox-1 and Cox-2 genes, belonging to a set of genes representing possible molecular targets correlated with BC survival, are overexpressed [47]. Compared to Cox-2, less attention was paid to Cox-1 in tumors and fewer data elucidated that Cox-1 selective inhibitors, such as SC-560 [48], catechin [49] and FR122047 [50], suppressed cell growth in BC. More interestingly, corticotropin-releasing factor, a hypothalamic neuropeptide, promoted cell invasiveness in MCF-7 BC cell line via induction of Cox-1 expression but not of Cox-2, as well as the production of prostaglandins [51].

Cox was officially known as an enzyme responsible for the synthesis of PGs from arachidonic acid. The role of Cox-2 and PPARγ in pro-apoptosis and tumor regression was explored in lung cancer cell lines, demonstrating that cannabidiol induced the upregulation of Cox-2 and PPARγ following a nuclear translocation of PPARγ by Cox-2 dependent PGs [52]. Modulation of 15d-PGJ_2, a natural ligand of PPARγ, may influence the development of BC progress [53]. Cox-1 could lead to the activation of PPARγ [54]. Our finding of a strong correlation between Cox-1 and cytoplasmic PPARγ highlight their possible interaction in BC cells. Furthermore, Cox-1 and Cox-2 expression has been shown to be strongly associated in BC to the expression of the aromatase (CYP19A1) [55] which has been shown to be associated with a poor survival of ER positive BC patients [56]. As a consequence, the link of cytoplasmic PPARγ with poor survival might involve the dysregulation of CYP19A1 expression through Cox activity. Obviously, other mechanisms might participate and further work will be needed to decipher the precise underlying mechanisms.

In our study, although neither Cox-1 nor Cox-2 were related to OS in the whole cohort (Additional file 1: Figure S2A, B), high cytoplasmic PPARγ expression was significantly associated with poor OS in the Cox-1 negative subgroup and in the Cox-2 low expression subgroup

(Fig. 3a, c). In addition, we also observed that the trend was inverted with an apparent, although not significant, better prognosis for the patients with high cytoplasmic PPARγ expression in the Cox-1 positive subgroup. Moreover, the data we generated demonstrate that cytoplasmic PPARγ expression is an independent prognostic marker in the Cox-1 negative subgroups, related to a twofold higher risk of death for those patients. Interestingly, positive Cox-1 expression (inversely related to the LN status) was defined as a favorable outcome marker for the patients with high cytoplasmic PPARγ expression (Fig. 3f), and tended to be a bad outcome marker for the patients with low cytoplasmic PPARγ expression. Our data suggest that the expression of Cox-1 and cytoplasmic PPARγ are interdependent, with the ability for Cox-1 to rescue the negative impact of cytoplasmic PPARγ on patient outcome. A hypothesis could be a potential role of Cox-1 in nucleocytoplasmic translocation of PPARγ, thereby suppressing tumor growth.

Conclusions

In our primary BC cohort, PPARγ was predominantly expressed in cytoplasm of BC cells and may perform different roles in tumorigenesis according to its subcellular localization. Cytoplasmic PPARγ was strongly correlated with Cox-1 mainly, as well as with other bad prognosis markers (HER2, CD133, N-cadherin), contributing to explore their interactions during BC progression. High cytoplasmic PPARγ expression was correlated with short OS in the whole cohort and in several subgroups with good prognosis. A major conclusion is that this bad prognostic impact of cytoplasmic PPARγ depends on Cox-1 expression, as it is worse when Cox-1 is negative and lost when Cox-1 is expressed. Altogether, this leads to the strengthening that the intracellular PPARγ localization might be involved in tumorigenesis, and to the conclusion that cytoplasmic PPARγ may be defined as a potential therapeutic target and a prognostic marker in BC. Further analyses are now needed to decipher the molecular mechanisms underlying PPARγ interplay with Cox-1 and Cox-2 to modulate BC aggressiveness through the control of cell proliferation and/or apoptosis.

Supplementary information

Supplementary information accompanies this paper at https://doi.org/10.1186/s12967-020-02271-6.

Additional file 1: Figure S1. Kaplan–Meier analysis in the whole cohort of patient overall survival according to Total PPARγ expression and patient relapse-free survival according to total, cytoplasmic and nuclear PPARγ expression. Overall survival (OS) curves are presented according to total PPARγ (A) status. Relapse-free survival (RFS) curves are presented according to total (B), cytoplasmic (C) and nuclear (D) PPARγ status. The IRS cut-off values with the number of cases for each group are indicated in each graph. Statistical significance is shown as p-value from log-rank test (*: $p<0.05$; **: $p<0.01$). **Figure S2.** Kaplan–Meier analysis in the whole cohort of patient overall survival according to Cox-1 or Cox-2 expression. Overall survival (OS) curves are presented according to Cox-1 (A) or Cox-2 expression. The IRS cut-off values with the number of cases for each group are indicated in each graph. Statistical significance is shown as p-value from log-rank test (*: $p<0.05$; **: $p<0.01$).

Abbreviations

BC: Breast cancer; Cox: Cyclooxygenase; CSC: Cancer stem cell; DCIS: Ductal carcinoma in situ; EMT: Epithelial mesenchymal transition; ER: Estrogen receptor; FISH: Fluorescence in situ hybridization; HER2: Human epidermal growth factor receptor 2; HR: Hazard ratio; IHC: Immunohistochemistry; IRS: Immunoreactive score; LCoR: Ligand-dependent corepressor; LMU: Ludwig Maximilians University; LNM: Lymph node metastasis; NR: Nuclear receptor; NST: Non-special type; OS: Overall survival; PBS: Phosphate buffered saline; PG: Prostaglandin; pN: Primary lymph node; PPARs: Peroxisome proliferator-activated receptors; PPARγ: Peroxisome proliferator-activated receptor γ; PPREs: Proliferator-activated receptor response elements; PR: Progesterone receptor; pT: Primary tumor size; RAR: Retinoic acid receptor; RFS: Relapse-free survival; RIP140: Receptor interacting protein of 140 kDa; ROC-curve: Receiver operating characteristic curve; RXR: Retinoid X receptor; TNBC: Triple-negative breast cancer; TPA: Tetradecanoyl phorbol acetate.

Acknowledgements

Not applicable.

Authors' contributions

SS, VC and UJ conceived and supervised the project. DM, ND and SM provided the samples and the related clinical data. MK and VW performed the stainings with the help of CK and the supervision of UJ. WS performed most analysis and wrote the first draft of the paper. SS, VC, UJ and NH contributed to manuscript writing and editing. All authors read and approved the final manuscript.

Funding

SS salary was supported by the University Paul Sabatier in Toulouse (France). WS was supported by the China Scholarship Council (CSC) with a 3-year stipend (No. 201606170096) at the University of Munich. The project was supported by the "Centre de Coopération Universitaire Franco-Bavarois" (CCUFB) or "Bayerisch-Französishes Hochschilzentrum" (BFHZ), Project funding FK19-15.

Availability of data and materials

All data generated or analysed during this study are included in this published article and its Additional file.

Ethics approval and consent to participate

This study was approved by the Ethical Committee of the Medical Faculty, Ludwig-Maximilian-University, Munich, Germany (approval number 048-08) and informed consent was obtained from all patients.

Consent for publication

Not applicable.

Competing interests

The authors declare no competing interests.

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Received: 30 August 2019 Accepted: 14 February 2020
Published online: 21 February 2020

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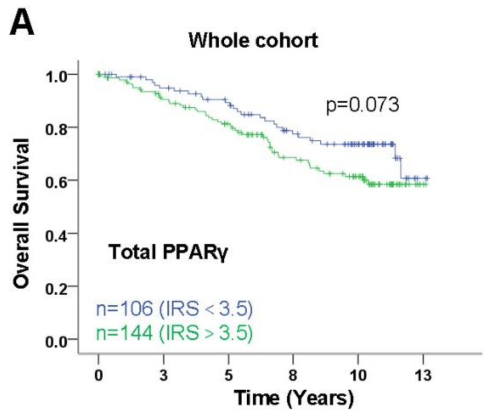
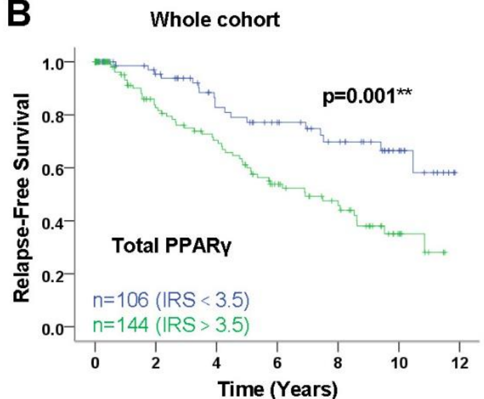
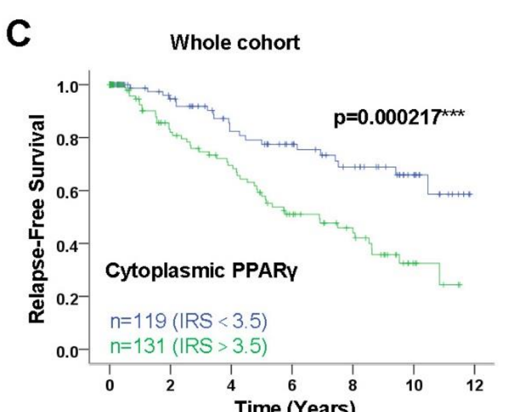






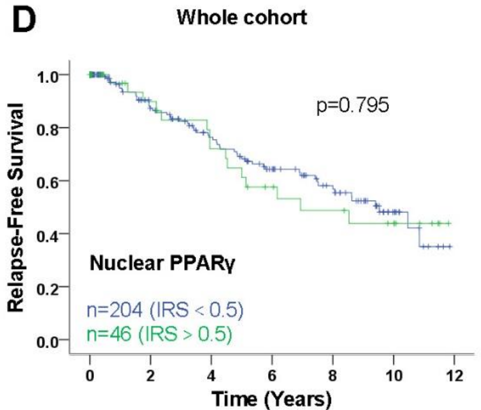
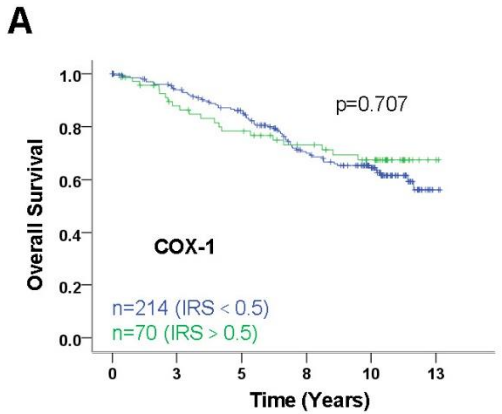


Figure S1



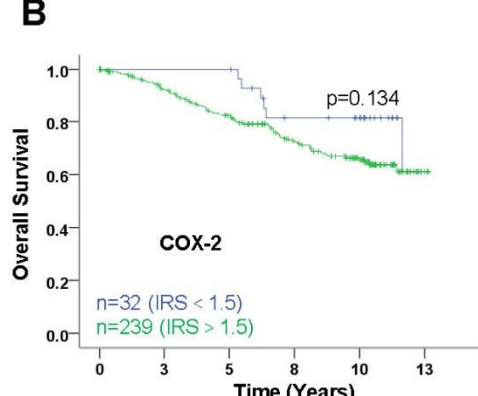


Figure S2

6. Publication II

Int J Mol Sci. 2020 Jan 3;21(1). pii: E330. doi: 10.3390/ijms21010330.

Cytoplasmic and nuclear forms of thyroid hormone receptor β1 are inversely associated with survival in primary breast cancer

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Article

Cytoplasmic and Nuclear Forms of Thyroid Hormone Receptor β1 Are Inversely Associated with Survival in Primary Breast Cancer

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Received: 25 November 2019; Accepted: 31 December 2019; Published: 3 January 2020

Abstract: The aim of this study was to investigate the expression of thyroid hormone receptor β1 (THRβ1) by immunohistochemistry in breast cancer (BC) tissues and to correlate the results with clinico-biological parameters. In a well-characterized cohort of 274 primary BC patients, THRβ1 was widely expressed with a predominant nuclear location, although cytoplasmic staining was also frequently observed. Both nuclear and cytoplasmic THRβ1 were correlated with high-risk BC markers such as human epidermal growth factor receptor 2 (HER2), Ki67 (also known as MKI67), prominin-1 (CD133), and N-cadherin. Overall survival analysis demonstrated that cytoplasmic THRβ1 was correlated with favourable survival ($p = 0.015$), whereas nuclear THRβ1 had a statistically significant correlation with poor outcome ($p = 0.038$). Interestingly, in our cohort, nuclear and cytoplasmic THRβ1 appeared to be independent markers either for poor ($p = 0.0004$) or for good ($p = 0.048$) prognosis, respectively. Altogether, these data indicate that the subcellular expression of THRβ1 may play an important role in oncogenesis. Moreover, the expression of nuclear THRβ1 is a negative outcome marker, which may help to identify high-risk BC subgroups.

Keywords: thyroid hormone receptor beta 1; subcellular localization; overall survival; breast cancer

1. Introduction

Breast cancer (BC), the most frequent cause of cancer death worldwide [1], is highly heterogeneous, leading to great complexity for diagnosis and therapy selection [2,3]. So far, only few diagnostic markers are well recognized in invasive BC, including expression of the two nuclear receptors (NR), the estrogen receptor (ER) and progesterone receptor (PR), and overexpression of human epidermal

growth factor receptor 2 (HER2). Although therapies targeting ER and HER2 (e.g., tamoxifen and trastuzumab) have been very successful, some tumors ultimately develop resistance to single or even combination therapies [4]. Thus, the identification of other biomarkers is essential for optimal and personalized BC management.

Links between BC and expression of other NR have already been outlined by our lab and others [5–10]. Thyroid hormone receptors (THR) are members of the NR superfamily that mediate the classical genomic actions of thyroid hormone (TH) signaling in numerous tissues and regulate important physiological and developmental processes [11,12]. THR primarily act as ligand-dependent transcription factors, after heterodimerization with retinoid X receptor (RXR). Various factors influence TH activity, including THR mutations, interactions with heterodimerization partners and coregulators, and expression of various THR subtypes and their related intracellular localization [13–15]. Indeed, rapid shuttling of various THR isoforms between the nucleus and cytoplasm has been described, and such dynamic transport pathways may be linked to specific TH signaling activities in nucleus, cytoplasm, or mitochondria [11,12]. These properties have even led to a new classification scheme with four TH signaling pathways; the canonical pathway, in which liganded THR binds directly to DNA (type 1), is tethered to chromatin-associated proteins (type 2) or functions without recruitment to chromatin either in the nucleus or cytoplasm (type 3). Finally, in the type 4 pathway, TH acts at the plasma membrane or in the cytoplasm without binding THR [16].

A significant association between thyroid hormone (TH) signaling and BC has already been demonstrated [17–19]. High TH levels are correlated with advanced clinical stages of BC [20]. A negative relationship between the presence of nuclear saturable high affinity binding sites of TH and lymph node (LN) status of BC patients has also been known for decades. These binding sites have been named thyroid hormone receptors (THRs) [21]. In a study performed at mRNA level in 116 breast samples, both THRα and THRβ mRNA levels were decreased in BC compared with normal tissues; yet, only THRβ expression, and not that of THRα, was negatively associated with histological grade [22]. Literature regarding the clinical significance of THR at the protein level is still limited. A recent study performed in 41 invasive BC tissues suggested that nuclear THRα is down-regulated during breast carcinogenesis [23]. Other studies highlighted the role of THRβ as a tumor suppressor in BC. For instance, low THRβ expressing tumors were associated with poor outcome in triple negative BC [24]. Lack of nuclear THRβ1 staining was reported in early stage BC and explained not only by loss of heterozygosity, but also by THRβ1 promotor hypermethylation [25]. More recently, a study in early BC demonstrated that THRβ1 expression is associated with long survival and is an independent prognosis marker [26].

BC signaling and progression is also influenced by the complex interplay between NR, their transcriptional coactivators, and corepressors that also have prognostic significance [27]. Among the transcriptional coregulators, RIP140 (receptor-interacting protein of 140 kDa) and LCoR (ligand-dependent corepressor) play major roles in BC cell proliferation [7]. Moreover, we recently analyzed RIP140 and LCoR expression at the protein level in BC biopsies, showing that expression of these two proteins was highly correlated in more than 80% of tumors and that cytoplasmic RIP140 expression was significantly correlated with a poor patient survival [9]. By sharing the same heterodimerization partner and/or coregulators, other NRs such as peroxisome proliferator-activated receptor γ (PPARγ) or vitamin D receptor (VDR) [6,28] may indirectly impact THR signaling.

While THRβ1 clearly appears to be a key player in BC carcinogenesis, the importance of its subcellular localization remained to be elucidated. Therefore, purpose of this study was to analyze the nuclear and cytoplasmic localization of THRβ1 in a well-defined cohort of 274 primary BC patients, and to correlate the results with clinicopathological parameters and clinical outcome.

2. Results

2.1. *THRβ1 Expression in Primary Breast Cancers*

The total cohort consisted of 274 samples from 271 primary BC patients (Table 1). Approval by the Ethical Committee of the Medical Faculty, Germany had been granted and informed consent was obtained from all patients, as described in Section 4.1. Median age at initial diagnosis was 57.0 years (range 34.8–94.6 years); median follow-up between first diagnosis and last follow-up was 126 months (range 4–153 months). During this period, 39 (14.2%) and 54 (19.7%) cases experienced either local recurrence or distant metastases, respectively; 15 experienced both (5.7%); and 75 (27.4%) women died.

Table 1. Clinical and pathological characteristics of all patients.

Clinical and Pathological Characteristics [a]		%
Median age (years, $n = 274$) [b]	57.00	range 34.79–94.62
Median follow up (months, $n = 274$) [b]	126	range 4–153
Histology [c] ($n = 260$)		
No Special Type (NST)	139	53.46%
NST with DCIS	74	28.46%
Other invasive	47	18.08%
ER status ($n = 272$)		
Positive	219	80.51%
Negative	53	19.49%
PR status ($n = 272$)		
Positive	160	58.82%
Negative	112	41.18%
HER2 status ($n = 273$)		
Positive	27	9.89%
Negative	246	90.11%
Molecular subtype ($n = 273$)		
Luminal A (Ki-67 ≤ 14%)	152	55.68%
Luminal B (Ki-67 > 14%)	60	21.98%
HER2 positive luminal	20	7.33%
HER2 positive non luminal	7	2.56%
Triple negative	34	12.45%
Grade ($n = 152$)		
I	13	8.55%
II	95	62.50%
III	44	28.95%
Tumor size ($n = 261$)		
pT1	169	64.75%
pT2	78	29.89%
pT3	4	1.53%
pT4	10	3.83%
Lymph node metastasis ($n = 256$)		
Yes	112	43.75%
No	144	56.25%
Distant metastases [d] ($n = 261$)		
Yes	54	20.69%
No	207	79.31%
Local recurrence ($n = 261$)		
Yes	39	14.94%
No	222	85.06%

[a] All information refers to the primary tumor; [b] 3 of 271 patients have bilateral primary breast cancer (BC); here, we consider each tumor as an individual one ($n = 274$); [c] NST include the formerly called "invasive ductal" and "other" types; [d] distant metastasis was detected during the follow-up in 53 patients (1 of them is bilateral BC, so $n = 54$). DCIS, ductal carcinoma in situ; ER, estrogen receptor; PR, progesterone receptor; HER2, human epidermal growth factor receptor 2; Ki67 (also known as MKI67) is a cellular marker for proliferation.

Expression of THRβ1 was analyzed by immunohistochemistry (IHC), using immunoreactive scores (IRS) as described in Material and Methods. Distribution of staining intensities and percentages of stained cells are presented in Supplemental Figure S1 (panels A and B). THRβ1 was widely expressed and detected in 67.3% of the samples with predominantly nuclear location. Cytoplasmic staining also

occurred and was quite strong in some cases. Distribution of IRS obtained either for nuclear (C) or cytoplasmic (D) THRβ1 staining (n = 263 tumors stained) is presented in Supplemental Figure S1. It is noteworthy that, for cytoplasmic THRβ1 staining, the highest IRS was 8. This was observed for only two patients (exemplified in Figure 1A, enlarged in B); next to these two cases, 4 was the maximum IRS observed. Consequently, panel C of Figure 1 shows one of the high cytoplasmic THRβ1 IRS (IRS 4). In Figure 1, THRβ1 staining is illustrated for four patients with examples of absent or high expression, and the respective nucleo–cytoplasmic IRS ratio. For extreme nuclear–cytoplasmic ratios (i.e., 0:0 and 12:8), enlarged photos are added (panels B and F).

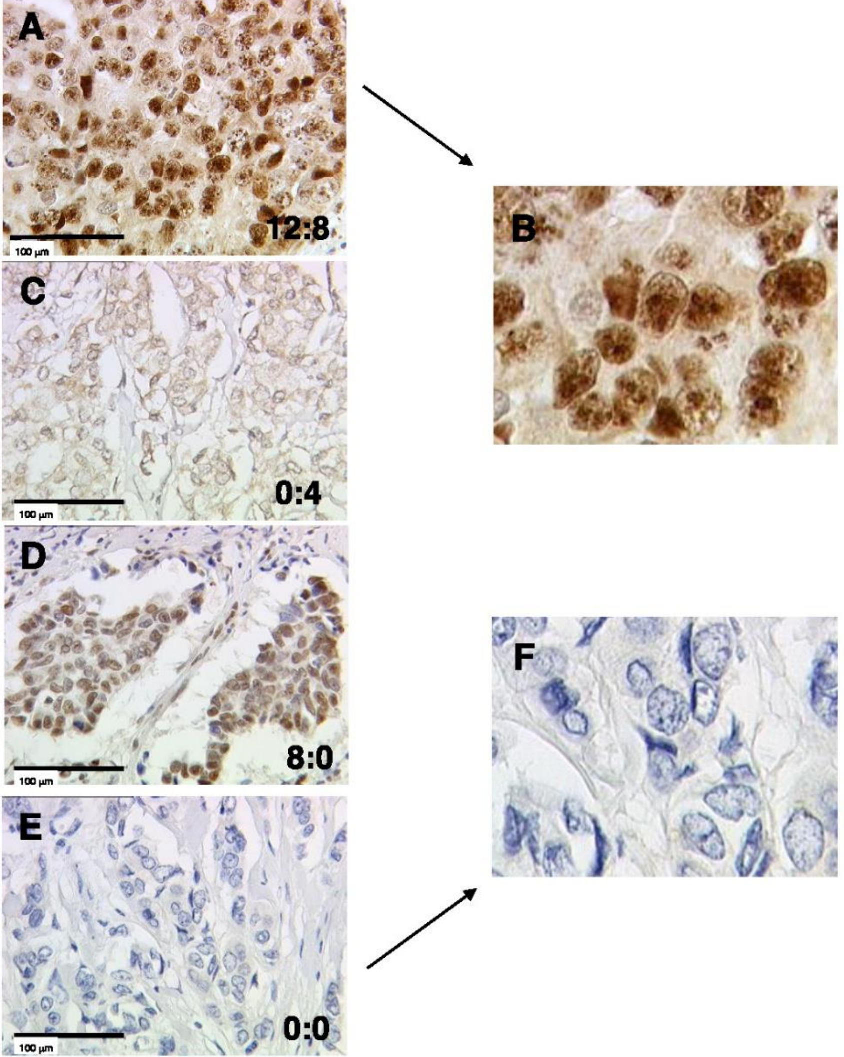

Figure 1. Immunohistochemical staining of thyroid hormone receptor β1 (THRβ1) in breast cancer samples. THRβ1 staining is illustrated for four patients (**A**,**C**–**E**) with examples of absent or high expression. Samples (**A**,**E**) are enlarged in panels (**B**,**F**), respectively. Nucleo–cytoplasmic IRS (immunoreactive score) ratios are indicated in each photomicrograph (25× magnification) and the scale bar equals 100 μm.

THRβ1 distribution was then analyzed both in nucleus and in cytoplasm, and total expression (sum of nuclear and cytoplasmic IRS) was calculated (Table 2). Nuclear staining was significantly stronger than the cytoplasmic one ($p < 0.05$), although both means were quite low (1.41 and 1.30, respectively). Nuclear THRβ1 staining was present in 60.5% of the tumors, and cytoplasmic THRβ1 in 43.3%. Interestingly, nuclear and cytoplasmic THRβ1 was significantly and positively correlated with each other (r = 0.440 $p < 0.01$ using Spearman–Rho test).

Table 2. Distribution of thyroid hormone receptor β1 (THRβ1) expression.

	Nuclear	Cytoplasmic
Mean IRS ± SE	1.41 ± 0.11	1.30 * ± 0.11
Median IRS	1	0
IRS range	0–12	0–8
Number of samples with negative expression **	104 (39.54%)	149 (56.65%)
Number of samples with positive expression **	159 (60.46%)	114 (43.35%)

* Correlations were statistically significant for $p < 0.05$ (*), using Spearman–Rho test using mean bilateral analysis; ** negative defined as immunoreactive score (IRS) = 0, and positive expression as IRS > 0; SE = standard error of means.

Distribution of tumors with negative or positive nuclear, or cytoplasmic, THRβ1 staining was analyzed for all 263 tumors stained (Supplemental Table S1). It appeared that almost one-third of the tumors were either negative (32.7%) or positive (36.5%) for both nuclear and cytoplasmic THRβ1 localizations. Regarding the nucleo–cytoplasmic ratio, 115 tumors (43.7%) had a ratio of 1, 80 tumors (30.4%) had a ratio greater than 1 (i.e., more expression in the nuclear compartment), and 68 tumors (25.9%) a ratio less than 1 (i.e., more expression in cytoplasm).

2.2. *Correlation with Nuclear Receptor and Related Coregulators*

Using pairwise analysis, we first analyzed the correlation of THRβ1 expression with expression of other NR and coregulators (Table 3) with previously reported expression data [6,28–30]. We observed that both nuclear and cytoplasmic expression of THRβ1 was strongly positively correlated with expression of its heterodimerization partner RXR. We also searched for correlation with other NRs, namely, ER, PR, PPARγ, and VDR, and the coregulators LCoR and RIP140. We found no correlation with ER and PR, but saw a strong correlation between nuclear and cytoplasmic THRβ1 and PPARγ. In contrast, only cytoplasmic THRβ1 was significantly correlated with VDR.

Table 3. Correlation between THRβ1 expression and nuclear receptors and related coregulators.

	n	References	Nuclear	Cytoplasmic
RXR	246	[28,30]	0.256 **	0.186 **
ER	262		0.043	−0.115
PR	262		0.085	−0.014
PPARγ	247	[28,30]	0.315 **	0.247 **
VDR	248	[28]	−0.097	−0.155 *
LCoR	257	[9]		
Nuclear			0.011	−0.060
Cytoplasmic			0.110	0.221 **
RIP140	258	[9]		
Nuclear	262		0.027	−0.046
Cytoplasmic	262		−0.009	0.029

Correlations are statistically significant for $p < 0.05$ (*) or $p < 0.01$ (**), using Spearman–Rho test. RXR, retinoid X receptor; PPARγ, peroxisome proliferator-activated receptor γ; VDR, vitamin D receptor; LCoR, ligand-dependent corepressor; RIP140, receptor interacting protein of 140 kDa.

We previously investigated the expression of two NR transcriptional coregulators, namely RIP140 and LCoR, and demonstrated that their sub-cellular localization may define their association with BC

aggressiveness and survival [9]. Regarding THRβ1, only cytoplasmic expression of the transcriptional coregulator LCoR was positively correlated with cytoplasmic THRβ1. No significant association was observed with RIP140.

2.3. Correlation with Clinicopathological Parameters

Correlations between THRβ1 expression and known clinicopathological characteristics, besides ER and PR, were also analyzed. CD133, a widely used marker for isolating cancer stem cells [31,32], and N-cadherin, a well-known marker for epithelial-to-mesenchymal transition [33], are associated with BC aggressiveness; we previously reported quantification of their expression in the same BC cohort [34]. As shown in Table 4, both nuclear and cytoplasmic THRβ1 expression were significantly and positively correlated with CD133 and N-cadherin (NCAD). Nonetheless, only cytoplasmic THRβ1 expression was positively correlated with proliferation marker Ki67 and HER2, but negatively with tumor size. No further significant correlation between the clinicopathological characteristics mentioned in Table 1 and THRβ1 expression was found.

Table 4. Correlation between THRβ1 expression and clinicopathological markers. HER2, human epidermal growth factor receptor 2; NCAD, N-cadherin.

	n	Nuclear	Cytoplasmic
pT	251	−0.023	−0.151 *
pN	247	0.044	−0.066
Grade	145	0.128	0.101
HER2 status	262	0.080	0.131 *
Triple negative	263	−0.052	0.031
Ki67	204	0.089	0.225 **
CD133	240	0.183 **	0.178 **
NCAD	244	0.342 **	0.327 **

Correlations are statistically significant for $p < 0.05$ (*) or $p < 0.01$ (**), using Spearman–Rho test; CD = cluster of differentiation.

2.4. Correlation between THR Expression and Patient Outcome

In order to analyze the correlation between THRβ1 and patient outcome, we performed Kaplan–Meier analyses (Figure 2). Instead of the simple negative/positive cut-off (Table 2), we determined optimal IRS cut-off values for overall survival (OS) using receiver operating characteristic (ROC)-curve analysis, thus allowing maximum difference between sensitivity and specificity parameters. We then divided the tumors in low or high expressing subgroups for all survival analyses.

As shown in Figure 2A,B, neither nuclear nor cytoplasmic THRβ1 had any significant correlation with relapse-free survival (RFS), for various IRS cut-off values tested. Nonetheless, there was an opposite trend regarding RFS; nuclear THRβ1 expression was related to poor outcome and cytoplasmic expression to favourable outcome. Analyzing OS (Figure 2D,E), we found that the nuclear THRβ1 was significantly correlated with poor outcome, while cytoplasmic THRβ1 was significantly correlated with favourable outcome ($p = 0.038$ and 0.015, respectively). Analyzing total THRβ1 expression of (sum of nuclear and cytoplasmic IRS), no correlation with RFS (Figure 2C) nor OS (Figure 2F) was found.

To determine the specificity of this result (opposite correlations of nuclear and cytoplasmic THRβ1 expression with OS), we performed the identical analyses with another THR, namely THRα2, in the same cohort (staining in Supplemental Figure S2; distribution in Supplemental Table S2). Analyzing OS according to nuclear and cytoplasmic THRα2 expression (Supplemental Figure S3), we could demonstrate, as expected, a significant beneficial effect of nuclear THRα2 on OS (panel A). Yet, cytoplasmic THRα2 expression did not have any significant correlation with OS (panel B). These additional analyses suggest that the opposite impact on outcome observed for cytoplasmic and nuclear THRβ1 expression may not be true for all THR isoforms.

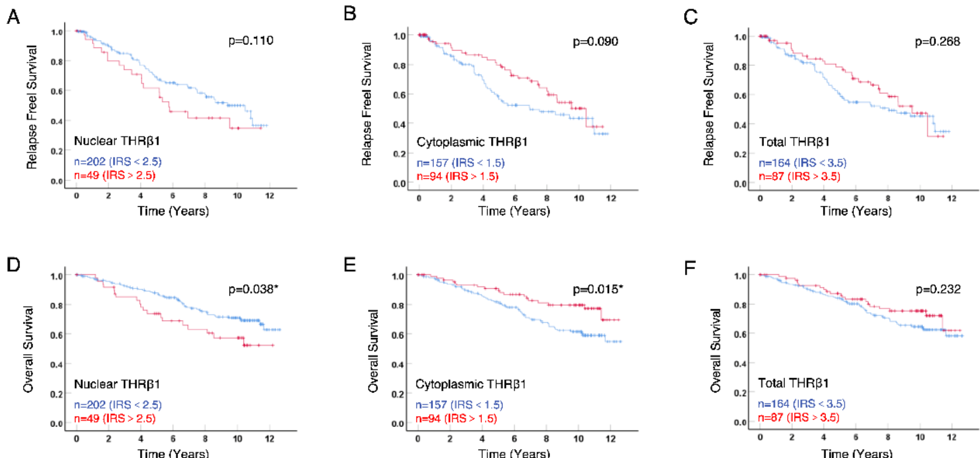

Figure 2. Kaplan–Meier survival analyses according to nuclear, cytoplasmic, and total THRβ1 expression. Relapse free survival (RFS) (**A**–**C**) and overall survival (OS) (**D**–**F**) curves are presented according to THRβ1 expression—either nuclear (**A**,**D**), cytoplasmic (**B**,**E**), or total (**C**,**F**) expression. Optimal IRS cut-off values and number of cases for each group are indicated in each graph. Statistical significance is shown as p-value from log-rank test (* $p < 0.05$).

2.5. Nuclear and Cytoplasmic THRβ1 Expression as Independent Prognostic Parameters for OS

Finally, we performed multivariate analyses using the Cox regression model with cytoplasmic and nuclear THRβ1 expression and four relevant clinicopathological features (age at time of diagnosis, tumor size, ER-, and HER2 status). As shown in Table 5, we found that age, tumor size, and ER were independent prognostic markers for OS. As expected, the cytoplasmic form of THRβ1 expression appeared to be an independent prognostic marker for OS with a hazard ratio of 0.545, confirming its correlation with favorable outcome. Interestingly, nuclear THRβ1 expression was shown as an independent prognostic marker for poor OS; with a hazard ratio of 2.860 indicating a higher risk of death for patients whose tumors express high levels of nuclear THRβ1.

Table 5. Multivariate analysis (OS, overall survival) of clinicopathological variables and THRβ1.

Variable	*p*-Value	HR (95% CI)
Age	0.000007 ***	1.042 (1.023–1.061)
pT	0.0000002 ***	3.701 (2.256–6.073)
ER	0.001 **	0.408 (0.242–0.687)
HER2	0.209	1.566 (0.778–3.153)
Cytoplasmic THRβ1	0.048 *	0.545 (0.299–0.995)
Nuclear THRβ1	0.0004 **	2.860 (1.597–5.119)

Hazard ratios (HRs) are indicated with 95% confidence intervals (CIs). Correlations are statistically significant for $p < 0.05$ (*), $p < 0.01$ (**), or $p < 0.001$ (***).

3. Discussion

The aim of this study was to characterize THRβ1 expression in a wide range of primary BC tissues, taking into account its intracellular expression, and to correlate the results with clinicopathological parameters and patient outcome.

Our study confirmed that THRβ1 is expressed with a predominantly nuclear location, as previously described for most THR isoforms. Nonetheless, our results also demonstrate cytoplasmic localization of THRβ1 in BC. THs are able to modulate gene expression by binding to THRα either in the cytoplasm or in the nucleus of the cells [35]. It is also known that THR can be present not only in the nucleus,

but also in the cytoplasm and in the mitochondria [12]. T3 can also be associated to plasma membrane structural α5β3 integrin, thereby regulating cell–cell and cell–extracellular matrix interactions and changing the morphology of BC cells [36]. Our results are supported by a previous study reporting cytoplasmic expression of THRβ1. In a large cohort of early BC patients, THRβ1 expression was predominantly found in the cytoplasm [26]. In most studies, however, including ours, THRβ1 expression is predominantly nuclear. We are aware that the different antibodies used in each study may explain substantial differences in expression. Nonetheless, in another study, THRβ was described as being expressed in nuclei of proliferative cells, in in situ carcinoma, and in the cytoplasm in normal breast and in infiltrative BC cells [37].

The second major observation provided by our study is that nuclear and cytoplasmic forms of THRβ1 may exhibit opposite roles in breast tumorigenesis. Indeed, considering the correlation with patient survival (Figure 2), cytoplasmic expression consistently behaved opposite to nuclear expression. These correlations are strengthened by the fact that nuclear THRβ1 is an independent prognostic marker for poor outcome in multivariate analysis, whereas cytoplasmic THRβ1 is an independent prognostic marker for favorable outcome (Table 5). The only other study that took the subcellular localization of THRβ1 expression into account (n = 796) [26] focused solely on cytoplasmic THRβ1, but did not consider nuclear expression. It should be noted that, in our study, both nuclear and cytoplasmic THRβ1 expression correlate with the heterodimerization partner, RXR, but only cytoplasmic THRβ1 correlates with VDR and cytoplasmic LCoR. Consequently, mere analysis of nuclear THRβ1expression, although this is the predominant expression, does not allow a complete understanding of the relevance of both expression types. Considering the subcellular THRβ1 localization seems to be essential for further analysis of its impact on patient outcome. A recent in vitro study suggested a novel role of THRβ, namely THRβ1, in the biology of cancer stem cells that could explain its action as a tumor suppressor in BC [38]. In our study, both nuclear and cytoplasmic THRβ1 strongly correlate positively with CD133 and N-cadherin, without any differential effect according to their subcellular location.

Concerning the link with ER, the study by Jerzak et al. [26] reported a correlation of cytoplasmic THRβ1 with favorable outcome only in ER-positive BC. Although we did not see a significant correlation between ER expression and nuclear or cytoplasmic THRβ1 expression (Table 3), we confirmed that cytoplasmic THRβ1 expression was correlated with good outcome in ER-positive tumors (p = 0.021), but not in ER-negative ones (p = 0.161) (Supplemental Figure S4A,B). Consequently, we demonstrated that cytoplasmic THRβ1 expression was also correlated with good outcome in luminal tumors (p = 0.035), but not in non-luminal ones (p = 0.142) (Supplemental Figure S4C,D). Yet, when we stratified our cohort according to ER expression, nuclear THRβ1 was no longer correlated with OS in either subgroup (data not shown). Further investigations are needed to define the link between cytoplasmic THRβ1 and estrogen signaling in BC cells at the molecular level.

Our results also suggest that the differential impact on outcome depending on nuclear or cytoplasmic THRβ1 localization is not a common feature for all THRs. In the present study, we also analyzed THRα2 expression. Previously, we had demonstrated that nuclear THRα2 expression tends to be an independent and favorable prognostic marker for survival in a small cohort of 82 invasive BC cases [39]. This was confirmed in another cohort of 130 invasive BC samples, where THRα2 (nuclear and cytoplasmic) negatively correlated with HER2 status, and positively with ER/PR and favorable OS [40]. In the present work, we confirmed that nuclear THRα2 was significantly correlated with a favorable prognosis. Interestingly, we did not find any inverse correlation of cytoplasmic THRα2 expression with OS (Supplemental Figure S3). Taken together, our data suggest a specific role of each subcellular expression only for THRβ1.

In summary, the present study confirms the complexity of the links between subcellular localization of the THRβ1 protein and its association with patient outcome. To our knowledge, it is the only study supporting the fact that the nuclear form of THRβ1, probably acting as a classical ligand-dependent transcription factor, may have tumor-promoting effects in BC. Our results emphasize the importance

of more precise investigations of the subcellular localization of THRs in order to define their impact as potential biomarkers in breast cancer.

4. Materials and Methods

4.1. Patient Cohort

A total of 274 formalin-fixed paraffin-embedded primary BC tissues were collected from 271 patients (3 of them with bilateral BC) who underwent surgery between 2000 and 2002 at the Department of Obstetrics and Gynecology of the Ludwig-Maximilians-University Munich, Germany. All patient data and clinical information from the Munich Cancer Registry were fully anonymized and encoded for statistical analysis. Research was approved by the Ethical Committee of the Medical Faculty, Ludwig-Maximilian-University (LMU), Munich, Germany (approval number 048-08; 18 March 2008) and informed consent was obtained from all patients. Union for International Cancer Control (UICC) TNM classification was performed to evaluate the size and extent of the primary tumor (pT), lymph node involvement (pN), and distant metastasis (M). Tumor grade was determined by an experienced pathologist (Dr D. Mayr) of the LMU Department of Pathology, according to a modification of Elston and Ellis grading proposed by Bloom and Richardson [41]. ER, PR, HER2, Ki67, and histological status were determined by an experienced pathologist (LMU Department of Pathology), as described below. HER2 2+ scores were further evaluated through fluorescence in situ hybridization (FISH) testing.

4.2. Immunohistochemistry (IHC)

Expression of ERα and PR was determined at diagnosis in all BC samples of this cohort at the LMU Department of Pathology, Germany. ERα and PR expression were evaluated by IHC, as previously described [6,30]. 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 analysis (2000–2002). HER2 expression was later analyzed using an automated staining system (Ventana; Roche, Mannheim, Germany), according to the manufacturer's instructions. Ki67 was stained using an anti-Ki67 monoclonal antibody (Dako, Hamburg, Germany) at a dilution of 1:150 on a VENTANA®-Benchmark Unit (Roche, Mannheim, Germany) as previously described [28]. The Ki67 cut-off used to differentiate luminal A from luminal B tumors (all HER2 negative) was 14%, as this was commonly used at the time of the analysis, although 20% is now preferred [42]. We performed paired-analysis, and used data on N-cadherin and CD133 expression in these BC samples extracted from a previously published study [34], as well as RXR, VDR and PPARγ [28,30], and RIP140 and LCoR [9]. For THRα and THRβ1 analysis by IHC, samples were processed as previously described [9,10,30,34,39,43]. All sections were first cut and prepared from paraffin-embedded BC samples using standard protocols. Phosphate buffered saline (PBS) was used for all washes and sections were incubated in blocking solution (ZytoChem Plus HRP Polymer System Kit, ZYTOMED Systems GmbH, Berlin, Germany) before incubation with primary antibodies. All primary antibodies were rabbit IgG polyclonal: anti- both THRα1 and THRα2 (immunogen being a synthetic peptide corresponding to a region within internal sequence amino acids 246–295 of human thyroid hormone receptor alpha 1 and 2, Abcam, ab 105003, Cambridge, UK) and anti-THRβ1 (immunogen being a synthetic peptide within amino acids 1–100 of the N-terminus of human TR-beta protein, Zytomed, 520-4074, Berlin, Germany). Isoform specific antibodies against THRα2 have been used for Supplemental Figure S2, namely a monoclonal mouse one against the N-terminus region of THRα2 (MCA 2842, AbD Serotec, Oxford, UK).

After incubation with a biotinylated secondary anti-rabbit or anti-mouse IgG antibody, and with the associated avidin–biotin–peroxidase complex (both Vectastain Elite ABC Kit; Vector Laboratories, Burlingame, CA, USA), visualization was performed with substrate and chromogen 3, 3-diamino-benzidine (DAB; Dako, Glostrup, Denmark). Negative and positive controls were used to assess the specificity of the immunoreactions. Negative controls (colored in blue) were performed in BC tissue by replacement of the primary antibodies by species-specific (rabbit/mouse) isotype

control antibodies (Dako, Glostrup, Denmark). Appropriate positive controls (placenta samples) were included in each experiment. Sections were counterstained with acidic hematoxylin, dehydrated, and immediately mounted with Eukitt (Merck, Darmstadt, Germany) before manual analysis with a Diaplan light microscope (Leitz, Wetzlar, Germany) with 25× magnification. Pictures were obtained with a digital Charged Coupled Device (CCD) camera system (JVC, Tokyo, Japan). All slides were analyzed by two or three independent examiners.

4.3. Immunoreactive Score (IRS)

Expression of THRβ1 and THRα2 was assessed according to IRS, determined by evaluating the proportion of positive tumor cells, scored as 0 (no staining), 1 (≤10% of stained cells), 2 (11%–50% of stained cells), 3 (51%–80% of stained cells), and 4 (≥81% of stained cells); as well as their staining intensity, graded as 0 (negative), 1 (weak), 2 (moderate), and 3 (strong) (IRS = percentage score × intensity score), as presented in Supplemental Figure S1 (panels A and B). Thus, IRS values range from 0 to 12. As previously described for LCoR and RIP140 [9] and for AhR [10], cytoplasmic and nuclear staining of THRβ1 and THRα2 were evaluated in parallel, with a separate determination of cytoplasmic IRS and nuclear IRS. For all other markers, staining and IRS were determined in the whole cells, without differentiation of nuclear and cytoplasmic staining. A total of one hundred cells (three spots with around thirty cells each) was analyzed for each sample and the IRS corresponded to the mean of the IRS determined on the three spots. The intensity and distribution pattern of the immunochemical staining reaction was evaluated by two independent blinded observers. In five cases (2% of the total), the evaluation of the two observers differed. These cases were re-evaluated by both observers together. After the re-evaluation, both observers agreed on the result. The concordance before the re-evaluation was 98.0%.

4.4. Statistical and Survival Analysis

Statistical analyses were performed using software package used for interactive, or batched, statistical analysis (SPSS) 24 (IBMSPSS Statistics, IBM Corp., Armonk, NY, USA). For all analyses, p values below 0.05 (*), 0.01 (**), or 0.001 (***) were considered statistically significant. Differences in Table 2 were calculated using mean or percentage bilateral analysis. Receiver operating characteristic (ROC) curve analyses were performed to calculate the optimal cut-off values between low and high THRβ1 and THRα2 expressions, based upon the maximal differences of sensitivity and specificity. The threshold determined regarding OS was an IRS ≥ 2.5 for nuclear THRβ1, ≥1.5 for cytoplasmic THRβ1, ≥1.5 for cytoplasmic THRα2, and ≥0.5 for nuclear THRα2.

Correlation analyses presented in Tables 3 and 4 were performed by calculating the Spearman–Rho correlation coefficient (p values of Spearman–Rho test presented), using pairwise analysis. Survival times were compared by Kaplan–Meier graphics and differences in RFS and OS were tested for significance using the chi-square statistics of the log rank test. Data were assumed to be statistically significant in the case of p-value <0.05 or <0.01. Kaplan–Meier curves and estimates were then provided for each group and each marker. The p value and the number of patients analyzed in each group are given for each chart.

The multivariable analysis for outcome (OS) presented in Table 5 was performed using the Cox regression model and included nuclear and cytoplasmic of THRβ1 expressions and relevant clinicopathological characteristics as independent variables. Variables were selected based on theoretical considerations and forced into the model. p values and hazard ratios were indicated, knowing that the hazard ratios of covariates are interpretable as multiplicative effects on the hazard, and holding the other covariates constant.

5. Conclusions

Although THRβ1 was predominantly expressed in tumor cell nuclei in our primary BC cohort, cytoplasmic expression was also detected; its correlation with patient survival was inverse to that

of nuclear THRβ1. Our results demonstrate that THRβ1 may have different roles in tumorigenesis according to its subcellular localization. A major conclusion is also that THR, particularly nuclear THRβ1, can exhibit tumor-promoting activities in the mammary gland, as demonstrated by its independent prognostic value.

Supplementary Materials: The following are available online at http://www.mdpi.com/1422-0067/21/1/330/s1.

Author Contributions: S.S., V.C., and U.J. conceived and supervised the project. D.M., N.D., and S.M. provided the samples and the clinical data. M.K. and V.W. performed the IHC staining with the help of C.K. and under supervision of U.J. W.S. performed most analysis and wrote the first draft of the paper. S.S., V.C., U.J., and N.H. contributed to manuscript writing and editing. All authors have read and agreed to the published version of the manuscript.

Funding: S.S. salary was supported by the University Paul Sabatier in Toulouse (France). W.S. was supported by the China Scholarship Council (CSC) with a 3-year stipend (No. 201606170096) at the University of Munich. The project was supported by the "Centre de Coopération Universitaire Franco-Bavarois" (CCUFB) or "Bayerisch-Französishes Hochschulzentrum" (BFHZ), project funding FK19-15.

Acknowledgments: We thank Karine Gauthier-Vanacker (IGFL/ENS Lyon) for helpful discussions.

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

Abbreviations

AhR	aryl hydrocarbon receptor
BC	breast cancer
CI	confidence interval
DCIS	ductal carcinoma in situ
ER	estrogen receptor
FISH	fluorescence in situ hybridization
HER2	human epidermal growth factor receptor 2
HR	hazard ratio
IHC	immunohistochemistry
IRS	immunoreactive score
LCoR	ligand-dependent corepressor
LMU	Ludwig-Maximilians-University
LN	lymph node
LXR	liver X receptor
NR	nuclear receptor
NST	non-special type
OS	overall survival
PBS	phosphate buffered saline
pN	primary lymph node
PPARγ	peroxisome proliferator-activated receptor γ
PR	progesterone receptor
pT	primary tumor size
RFS	relapse free survival
RIP140	receptor interacting protein of 140 kDa
ROC-curve	receiver operating characteristic curve
RXR	retinoid X receptor
TH	thyroid hormone
THR	thyroid hormone receptor
VDR	vitamin D receptor

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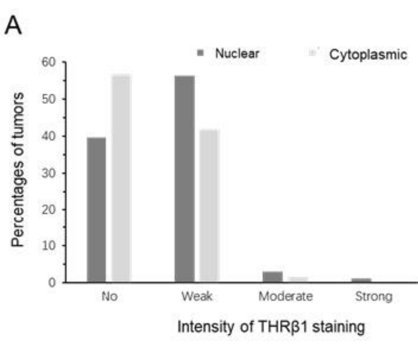
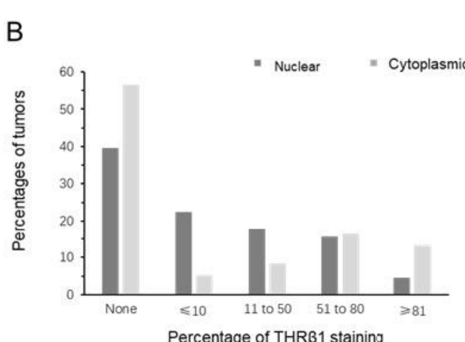
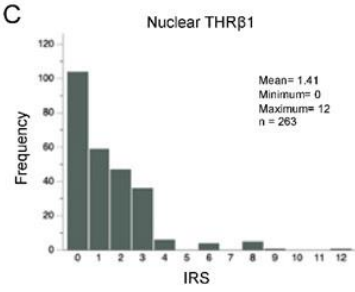
Supplementary materials







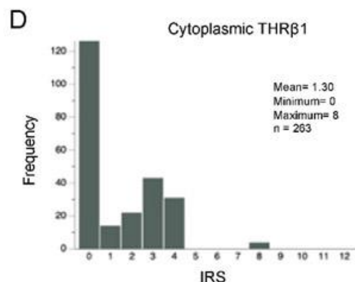


Figure S1. Distribution of intensity and percentages, and IRS frequency, of the nuclear and cytoplasmic THRβ1 stainings. The percentage of tumors with each intensity category (**A**) and percentage range (**B**) used for the IRS calculation are presented according to either the nuclear or cytoplasmic stainings of THRβ1. Distribution of IRS is represented according to either the nuclear (**C**) or cytoplasmic (**D**) stainings of THRβ1.

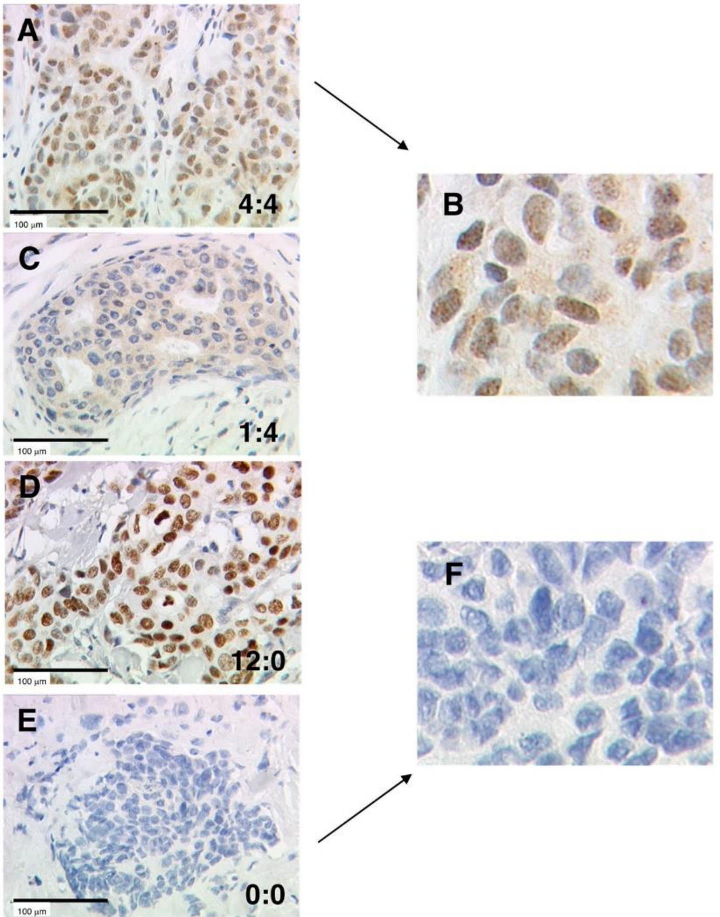


Figure S2. Immunohistochemical staining of THRα2 in BC samples. THRα2 staining is illustrated for 4 patients with examples of null or high expression. Samples A and E are enlarged in panels B and F respectively. Nucleo:cytoplasmic IRS (immunoreactive score) ratios are indicated in each photomicrograph (25× magnification) and scale bar equals 100 µm.

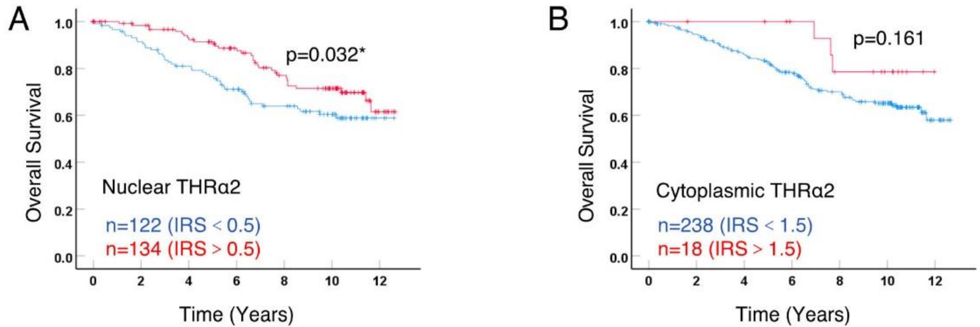


Figure S3. Kaplan-Meier analysis according to nuclear and cytoplasmic THRα2 expression. Overall survival (OS) curves are presented according to THRα2, with either the nuclear (**A**) or cytoplasmic (**B**) expression. The optimal IRS cut-off values with the number of cases for each group are indicated in each graph. Statistical significance is shown as *p*-value from log-rank test (*: $p < 0.05$).

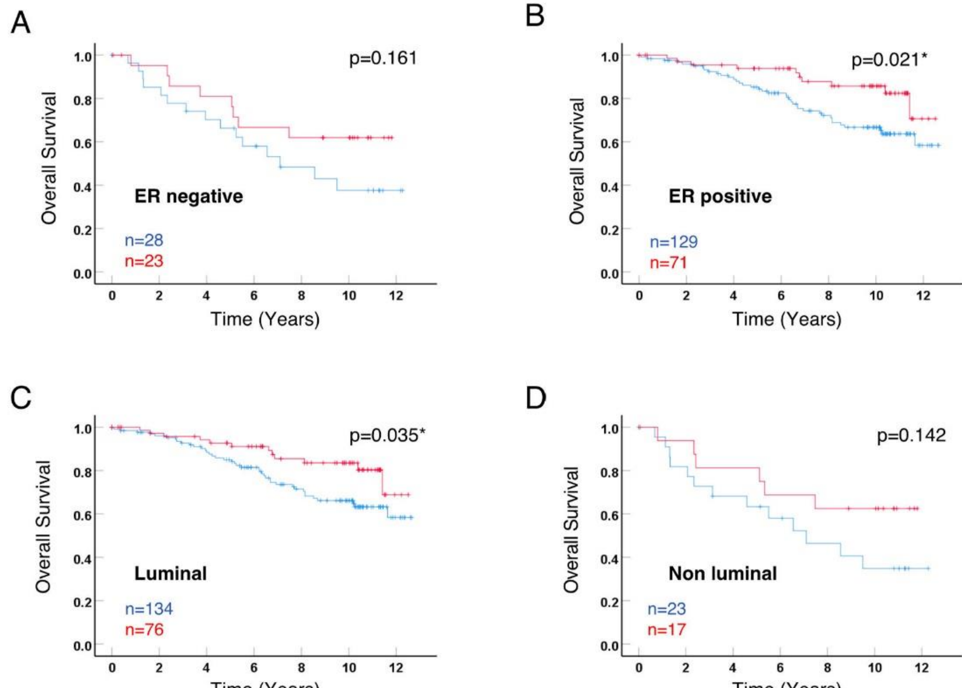


Figure S4. Kaplan-Meier analysis according to cytoplasmic THRβ1expression in ER positive vs negative and luminal vs non luminal sub-groups of patients. Overall survival (OS) curves are presented according to cytoplasmic THRβ1, for either ER negative (**A**) or positive (**B**) sub-groups of patients, and for either luminal (**C**) or non-luminal (**D**) sub-groups. The optimal IRS cut-off value of 1.5 was used and the number of cases for each group are indicated in each graph. Statistical significance is shown as p-value from log-rank test (*: $p < 0.05$).

Table S1. Distribution of the tumors with nuclear and/or cytoplasmic positive THRβ1 expression.

Nuclear	Cytoplasmic	n	%
Negative	Negative	86	32.7
Positive	Negative	63	23.9
Negative	Positive	18	6.8
Positive	Positive	96	36.5

Table S2. Distribution of THRα2 expression.

	Nuclear	Cytoplasmic
Mean IRS±SE	1.67±1.47	0.21*±0.04
Median IRS	1	0
IRS range	0-12	0-4
Negative expression**	120 (45.63%)	236 (89.73%)
Positive expression**	143 (54.37%)	27 (10.27%)

* Correlations were statistically significant for p<0.05 (*), using Spearman-Rho-Test using mean bilateral analysis; ** Negative and positive expression were respectively defined as IRS = 0 and IRS >0.

3

7. Summary

Dysfunction of NR signaling pathway lead to numerous diseases including cancers. NRs regulate cellular processes by classical genomic or nongenomic mechanism. In BC, ER and PR, two members of NR superfamily, are of particular importance in tumorigenesis and prognosis and drugs targeting these two receptors achieve great success. However, it is still a tremendous challenge to make relevant therapies for advanced or metastatic cases and TNBC disease. More study of NR-related signaling pathway may provide novel therapeutic targets for BC. Therefore, we investigated subcellular expression of another two NRs, PPARγ and THRβ1, in the same cohort of BC tissues and analyzed correlation with several clinicopathological characteristics and patient survival.

Publication I: Cytoplasmic PPARγ is a marker of poor prognosis in patients with Cox-1 negative primary breast cancers

The aim of this study was to investigate the subcellular expression of PPARγ and related Cox-1 and Cox-2 in a cohort of 308 BC tissues and correlate them to survival. Immunohistochemistry was performed for PPARγ, Cox-1 and Cox-2 nuclear and cytoplasmic expression, clearly exhibiting that PPARγ was expressed in most BC samples with predominantly cytoplasmic location, Cox-1 and Cox-2 being only cytoplasmic. Cytoplasmic PPARγ had a positive correlation with Cox-1, Cox-2, and other high-risk markers of BC (HER2, CD133, and N-cadherin), whereas inversely with nuclear PPARγ and ER expression. Kaplan Meier analysis demonstrated that cytoplasmic PPARγ was a significant unfavorable predictor of overall survival in the whole cohort, as well as in the subgroup of patients with no Cox-1 expression where it appeared as an independent marker of poor prognosis. In addition, to examine the relationship between PPARγ and Cox-1, we identified that Cox-1 was associated with good prognosis only in patients with high cytoplasmic PPARγ expression. In conclusion, our results suggest that the relative expression of cytoplasmic PPARγ and Cox-1 may be essential in BC physiopathology and that both could be defined as potential targets for BC personalized therapeutic strategies.

Publication II: Cytoplasmic and nuclear forms of thyroid hormone receptor β1 are inversely associated with survival in primary breast cancer

This study aimed then to assess the subcellular distribution and prognostic roles of THRβ1 in the same cohort (with 274 primary BC). Nuclear THRβ1 was detected in 60.46% of all samples by immunohistochemistry, with frequent cytoplasmic location too. In addition, nuclear and cytoplasmic THRβ1 were positively associated with each other and both had a strong correlation with high-risk markers of BC, as performed in Publication I. Overall survival performed by Kaplan Meier analysis demonstrated that high level of cytoplasmic THRβ1 was strongly correlated with long-term survival, whereas nuclear THRβ1 had an inverse statistically significant correlation with long-term survival. Cox regression model showed that nuclear THRβ1 served as an independent marker for unfavorable prognosis, whereas cytoplasmic THRβ1 served as an independent marker for favorable one. In conclusion, these data indicate that the subcellular expression of THRβ1 may determine specific effects on BC physiopathology. Finally, nuclear THRβ1 expression is another negative predictive biomarker which may play a role for BC personalized therapeutic strategies.

In conclusion, cytoplasmic PPARγ and nuclear THRβ1 are both regarded as negative survival markers to identify high-risk BC subgroups. The cross-talk between genomic and nongenomic actions of NRs may play different roles in BC development. Thus, the further study of the intracellular distribution of NRs may give new insights to identify novel therapy for BC.

8. Zusammenfassung

Eine Funktionsstörung des Kernrezeptor- (NR) Signalwegs führt zu zahlreichen Krankheiten, einschließlich Brustkrebs (BC). NRs regulieren zelluläre Prozesse durch klassische genomische oder nichtgenomische Mechanismen. In BC sind Estrogenrezeptoren (ER) und Progesteronrezeptoren (PR), zwei Mitglieder der NR-Superfamilie, von besonderer Bedeutung für die Tumorentstehung und –prognose. Deshalb sind Arzneimittel, die auf diese beiden Rezeptoren abzielen, bei Hormonrezeptor-positiven Patienten erfolgreiche Behandlungsoptionen. Es ist jedoch immer noch eine enorme Herausforderung, relevante Therapien für fortgeschrittene oder metastatische Fälle und Hormonrezeptor negative und HER2-negative (TNBC)-Erkrankungen zu entwickeln. Weitere Untersuchungen des NR-bezogenen Signalwegs könnten neue therapeutische Ziele für diese Patientinnen liefern. Daher untersuchten wir die subzelluläre Expression von zwei weiteren NRs, PPARγ und THRβ1, in derselben Kohorte von BC-Geweben und analysierten die Korrelation mit mehreren klinisch-pathologischen Merkmalen und dem Überleben des Patienten.

Veröffentlichung I: Zytoplasmatisches PPARγ ist ein Marker für eine schlechte Prognose bei Patienten mit Cox-1-negativem primären Brustkrebs

In einer gut charakterisierten Kohorte von 308 primären BC-Gewebeschnitten wurden die zytoplasmatische und nukleare Expression von PPARγ, Cox-1 und Cox-2 mittels Immunhistochemie untersucht. Korrelationen mit klinisch-pathologischen- und weiteren Merkmalen sowie das Überleben der Patientinnen wurden mit Hilfe statistischer Methoden und letzteres unter Verwendung der Kaplan-Meier-Analyse erhoben. PPARγ wurde in fast 58% der Proben mit einer vorherrschenden zytoplasmatischen Lokalisation exprimiert. Cox-1 und Cox-2 waren ausschließlich zytoplasmatisch. Zytoplasmatisches PPARγ war invers mit der nuklearen PPARγ- und ER-Expression korreliert, jedoch positiv mit Cox-1, Cox-2 und anderen Hochrisikomarkern von BC, z.B. HER2, CD133 und N-Cadherin. Die Gesamtüberlebensanalyse zeigte, dass zytoplasmatisches PPARγ in der gesamten Kohorte eine starke Korrelation mit einer schlechten Überlebensrate aufwies und in der Untergruppe der Patienten ohne Cox-1-Expression, bei denen die zytoplasmatische PPARγ-Expression als unabhängiger Marker für eine schlechte Prognose auftrat, noch stärker war. Zur Unterstützung dieses Zusammenhanges zwischen PPARγ

und Cox-1 stellten wir fest, dass Cox-1 nur dann zu einem Marker für eine gute Prognose wurde, wenn zytoplasmatisches PPARγ mit einem hohen Score exprimiert wurde. Insgesamt lassen diese Daten darauf schließen, dass die relative Expression von zytoplasmatischem PPARγ und Cox-1 eine wichtige Rolle bei der Onkogenese spielt und als potenzieller Prognosemarker zur Identifizierung spezifischer hochriskanter BC-Untergruppen definiert werden könnte.

Veröffentlichung II: Zytoplasmatische und nukleäre Formen des Schilddrüsenhormonrezeptors β1 sind invers mit dem Überleben bei primärem Brustkrebs assoziiert

In einer gut charakterisierten Kohorte von 274 primären BC-Gewebeschnitten wurde THRβ1 hauptsächlich im Zellkern der Tumorzellen exprimiert, obwohl auch häufig eine zytoplasmatische Färbung beobachtet wurde. Sowohl das nukleäre als auch das zytoplasmatische THRβ1 wurden mit Hochrisiko-BC-Markern wie HER2, Ki67, CD133 und N-Cadherin korreliert. Die Gesamtüberlebensanalyse zeigte, dass das zytoplasmatische THRβ1 mit einem günstigen Überleben korrelierte, wohingegen das nukleare THRβ1 eine statistisch signifikante Korrelation mit einer schlechten Überlebensrate aufwies. Interessanterweise erwiesen sich in unserer Kohorte, dass nukleäres und zytoplasmatisches THRβ1 als unabhängige Marker für schlechte bzw. gute Prognosen angesehen werden können. Insgesamt deuten diese Daten darauf hin, dass die subzelluläre Expression von THRβ1 eine wichtige Rolle bei der Onkogenese spielen könnte. Darüber hinaus ist die Expression von nukleärem THRβ1 ein negativer Marker, der zur Identifizierung von BC-Untergruppen mit hohem Risiko beitragen kann.

Zusammenfassend werden sowohl zytoplasmatisches PPARγ; als auch nukleäres THRβ1 als negative Überlebensmarker angesehen, um BC-Untergruppen mit hohem Risiko zu identifizieren. Der Zusammenhang von genomischen und nichtgenomischen Wirkungen von NRs kann bei der BC-Entwicklung eine wesentliche Rolle spielen. Daher könnte die weitere Untersuchung der intrazellulären Verteilung von NRs neue Erkenntnisse liefern, um eine neuartige Therapien für BC zu identifizieren.

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10. Acknowledgements

I would like to dedicate my thesis to all those who I meet in Munich, my friends and family in China and China Scholarship Council (CSC) with the financial supports.

Firstly, I would like to extend my deep appreciation to Prof. Dr. Udo Jeschke and Prof. Dr. med. Nadia Harbeck, who gave me the precious opportunity to study in the Department of breast cancer, Klinik und Poliklinik für Frauenheilkunde und Geburtshilfe, as a doctoral student. Special acknowledgement is given to my respectable supervisor, Prof. Dr. Udo Jeschke, whose excellent scientific knowledge, patient guidance and useful suggestions are beneficial to my research a lot.

Secondly, I express my great gratitude to Prof. Sophie Sixou, who gave me tremendous support and encouragement both in my academic and daily life. Her insightful comments on every draft of thesis, publications and projects, which provided me with many enlightening ideas, have inspired me to a great extent. Without her supervision, these thesis and publications would not come into being.

Moreover, sincere thanks go to my technicians, Christina Kuhn and Simone Hofmann. They taught me all the basic experiment techniques carefully and answered all my questions patiently. Same thanks to Dr. Zhang Dongmei (charge of LMU-CSC Program) from the international office of LMU, providing me a well-organized orientation course and living assistance when I arrived in Munich.

Particular thanks to Zhang Xi. With her great help, I could adjust to the new life in Munich so quickly and keep my research going so smoothly at the beginning. Great thanks also to my other colleagues, Ye Yao, Zhu Junyan and Peng Lin. We shared experimental experiences and helped each other. I'm grateful to be a member of such a supportive and considerate working group. I'll remember all the happy time and talks in the lab!

Finally, I would like to thank to my family for their infinite love and support during my life and my friends (Kelly, Haijing, Lawson, Mu Nan, Fanfan, Liying and Ziqian…) for the greatest joy of their company in Germany.

My parents are the source of my courage and strength. Thanks for their unconditional love.

I love you, Mum. And, I miss you, Dad (RIP, 2019)!

感谢那些曾经帮助过我，爱过我，安慰过我的所有人。