in vivo *26*: 87-92 (2012)

Retinoid X Receptor Alpha (RXRα) and Peroxisome Proliferator-activated Receptor Gamma (PPARγ) Expression in Breast Cancer: An Immunohistochemical Study

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Abstract. *Background/Aim: The role of retinoid X receptor alpha (RXRα) and peroxisome proliferator-activated receptor gamma (PPARγ) in breast cancer has been well studied in vitro. The aim of the study was to assess the presence of these molecules in human breast cancer specimens and correlate them with major clinicopathological features. Patients and Methods: Tissue sections from 82 breast cancer cases clustered according to histological grade, lymph node (LN) and hormone receptor (HR) status were assessed by immunohistochemistry for RXRα and PPARγ. Results: RXRα was found to be strongly and moderately expressed in 11 (14.10%) and 33 (42.31%) cases, respectively. PPARγ was found to be strongly and moderately expressed in 33 (41.25%) and 25 (31.25%) cases, respectively. Only RXRα expression was inversely correlated with histological grade. Surprisingly, significantly elevated PPARγ expression was found in cases with positive LN status. Survival analysis did not yield significant results. Conclusion: Our data support the current thesis of RXRα being a potential target for feature molecular interventions.*

Retinoid X receptor alpha (RXR) and peroxisome proliferator-activated receptor-γ (PPARγ) are members of the nuclear hormone receptor (NHR) superfamily (1). Activation of these receptors is achieved by binding with their ligands. After forming heterodimers (2, 3), they act as transcription factors by translocating to the nucleus and bind to specific response elements upon promoters of specific genes (4). Furthermore, this transcription regulation involves the recruitment of other coactivators adjusting transcriptional activity. Different ligands bound to these receptors seem to recruit different coactivators thus regulating different genes and biological functions (4).

Both RXR and PPARγ have been shown to be expressed by breast cancer cells (5, 6), with a higher expression of RXRα being seen more in breast cancer rather than benign breast tissue (7). The same pattern of expression was also observed for PPARγ (8). Both receptors are reported to induce growth arrest and differentiation in breast cancer cells *in vitro* and in animal models (9, 10). Although well studied *in vitro*, few reports exist in the literature regarding the expression of RXRα and PPARγ in human breast cancer specimens.

In the present study the presence of these molecules in human breast cancer specimens was assessed by immunohistochemistry and possible correlations with clinicopathological characteristics were investigated.

Materials and Methods

Population. Cases with primary non-metastatic breast cancer operated on between 1990-2000 in the First Department of Obstetrics and Gynecology of the Ludwig Maximilians University in Munich, Germany, were randomly selected to be included in the study. The randomization was performed by clustering cases according to their lymph node (LN) status, histological grade of the primary tumour and hormone receptor (HR) Estrogen receptor (ER)/ Progesterone receptor (PR)- status. In each cluster, a random selection of a maximum ten cases –when more than ten were available – was included. Selection clustering is presented in Figure 1. All included cases were reviewed by an expert pathologist for verifying the initial diagnosis and the specific histological characteristics.

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Key Words: RXRα, PPARγ, breast cancer, positive lymph nodes.

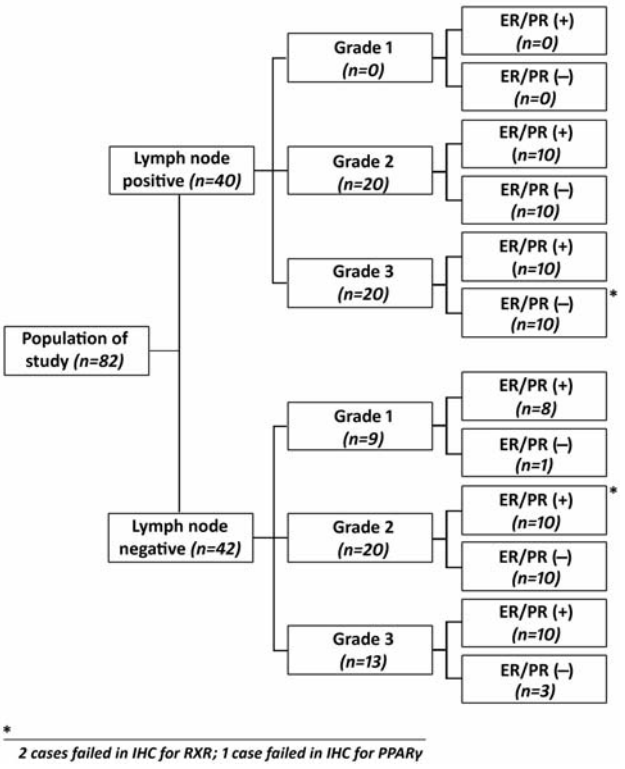

Figure 1. *Presentation of the clustering applied, regarding inclusion of breast cancer cases in the current study.*

The current study was approved by the Research Ethics Committee of the Ludwig Maximilians University of Munich.

Immunohistochemistry. Formalin-fixed paraffin-embedded tissue sections (4 μm thick) were de-paraffinized, rehydrated in a descending series of alcohol and subjected to epitope retrieval in a pressure cooker using sodium citrate buffer (pH 6.0). After returning to room temperature, sections were washed twice in phosphate-buffered salin (PBS) and blocked with 3% hydrogen peroxide (Merck, Darmstadt, Germany) in methanol for endogenous peroxidase activity. Non-specific binding of the primary antibodies was inhibited by incubating the sections with diluted normal serum (10 ml PBS containing 150 μl horse serum; Vector Laboratories, Burlingame, CA, USA). All primary antibodies were then incubated for 60 min in room temperature (salient features of the antibodies used in this study are presented in Table I). Reactivity was then detected with the mouse IgG-Vectastain Elite ABC kit (Vector Laboratories), according to the manufacturer's protocol. Substrate and chromagen (3,3'-diaminobenzidine DAB; Dako, Glostrup, Denmark) were finally added. The slides were then counterstained with Mayer's acidic haematoxylin, dehydrated in an ascending series of alcohol and covered. Placental tissue served as positive control for this study, while negative controls were obtained by incubating placental tissue with mouse IgG (for RXRα) and rabbit IgG (for PPARγ).

The intensity and distribution patterns of the specific immunocytochemical staining were evaluated using a semi-quantitative method (immunoreactivity score, IRS) as was previously described (11). Briefly, the IRS was calculated as the product of the optical staining intensity (0: no staining; 1: weak staining; 2: moderate staining and 3: strong straining) and the graded staining extent (0: no staining; 1: <10% staining; 2: 11-50 % staining; 3: 51-80 % staining and 4: >80 % staining).

Statistical analysis. The correlation between RXRα and PPARγ IRS was evaluated by Spearman test, while correlations between IRS and histological grade were assessed by gamma correlation coefficient. Differences to RXRα and PPARγ IRS according to ER, PR and LN status were evaluated by Mann-Whitney test. For evaluating survival, the cases were grouped according to their RXRα and PPARγ IRS as low (score 0-1), medium (score 2-4) and high (score 6-12); the log-rank test was applied. Each observation with $p<0.05$ was considered significant.

Results

Population characteristics. Eighty-two cases were enrolled in this study. All the cases, diagnosed as invasive ductal breast carcinoma, underwent primary breast surgery along with sentinel LN detection accompanied with/without axillary LN dissection. Forty cases (48.78%) were identified as being LN positive. The differentiation of the primary tumour was high in 9 (G1-10.98%), moderate in 40 (G2-48.78%) and low in 33 (G3-40.24%) cases. HR status was positive in 48 (58.54%) cases (Table II).

Immunohistochemistry. Immunohistochemistry was successful in 78 and 80 cases for RXRα and PPARγ, respectively. In the remaining cases, immunohistochemistry was not feasible due to section detachment from the slides. The RXRα immunoreactivity was revealed mainly with a nuclear pattern, while PPARγ reactivity was both nuclear and cytoplasmic (Figure 2). All cases were reviewed by two observers (with consensus) in order to be graded according to the IRS. RXRα was found to be strongly expressed in 11 (14.10%) cases, while another 33 (42.31%) were categorized as moderate expression; the median value was IRS 4 (range 0-8) (Table II). Additionally, PPARγ was found to be strongly and moderately expressed in 33 (41.25%) and 25 (31.25%) cases, respectively (Table II). The median PPARγ IRS was 2 (range 0-12).

Correlations between RXRa, PPARγ and clinicopathological features. The mean IRS for RXRα and PPARγ did not differ significantly between HR-positive and -negative cases. Indeed, the mean IRS for RXRα was 4.10±0.448 *vs* 4.96±0.388 ($p=0.15$), while the mean IRS for PPARγ was 3.13±0.645 *vs* 2.98±0.488 ($p=0.767$), for HR-negative *vs* HR-positive cases respectively (Figure 3).

LN status was significantly correlated with an increased PPARγ IRS (mean IRS=1.97±0.445 for negative LN status *vs* 4.16±0.597 for positive LN status, $p=0.001$), but not with

Table I. *Salient features of the primary antibodies used in this study.*

Antibody, clone	Source	Concentration	Positive control	Negative control
Anti-RXRα, mouse monoclonal IgG, K8508	Perseus Proteomics, Tokyo, Japan	5 μg/ml	Placenta	Mouse IgG
Anti-PPARγ, rabbit polyclonal IgG, ab27649	Biozol Diagnostica GmbH, Eching, Germany	0.33 μg/ml	Placenta	Rabbit IgG

the corresponding RXRα IRS (4.51±0.385 *vs* 4.70±0.457, respectively, p=0.799).

RXRα positivity was inversely related to histological grade (gamma correlation=–0.302, p=0.030), while PPARγ correlation was proven of borderline significance (gamma correlation=0.251, p=0.055).

No correlation was found between RXRα and PPARγ expression (Spearman test, p=0.511).

Disease-free (DFS) and overall survival (OS). Patients were under follow-up for a median of 12 years (range 10-20 years). For eleven patients, follow-up data was not available. Overall and disease-free survival did not differ significantly between groups with different IRS for RXRα and PPARγ (Figure 4).

Discussion

Nuclear receptors, as well as their cognate ligands, serve as potent regulators of development, cell differentiation, and normal physiology. Moreover, they may have important implications for different pathologies, such as breast cancer (12). As previously shown, RXR and PPAR were detected as forming functional PPAR/RXR heterodimers in human breast cancer cell lines (12). Both were able to mediate selective responses, namely growth inhibition and apoptosis, supporting initially a protective role as far as breast cancer development is concerned (4).

Taking this RXR/PPAR interaction for granted and as these molecules are considered both to be potential targets for molecular therapy (9, 13, 14), we decided to evaluate the RXR/PPAR status of a rather small sample of breast cancer patients and to correlate it with major clinicopathological characteristics such as LN and HR status. The decision to use a unified approach regarding HR status, considering it as positive when either estrogen receptor or progesterone receptor was found positive, was based on the molecular classification of breast cancer cases where luminal A and B breast cancer cases are either ER or PR positive or both (15). IRS evaluation was used, because both RXRα and PPARγ belong to the group of nuclear receptors for which the IRS is commonly used (11).

Table II. *Main clinicopathological features of the breast cancer cases enrolled in the current study.*

	n	(%)
Lymph node status (n=82)		
Positive	40	(48.78)
Negative	42	(51.22)
Grade (n=82)		
1	9	(10.98)
2	40	(48.78)
3	33	(40.24)
Hormone receptor status (n=82)		
Positive	48	(58.54)
Negative	34	(41.46)
RXRα Immunostaining (n=78)		
Strong (IRS 6-12)	11	(14.10)
Medium (IRS 2-4)	33	(42.31)
Weak-negative (IRS 0 – 1)	34	(43.59)
PPARγ Immunostaining (n=80)		
Strong (IRS 6-12)	33	(41.25)
Medium (IRS 2-4)	25	(31.25)
Weak-negative (IRS 0-1)	22	(27.50)

IRS: Immunoreactive score; RXRα: retinoid X receptor alpha; PPARγ: peroxisome proliferator-activated receptor gamma.

Although PPAR/RXR dimers have been well studied in cell culture and in animal models, there are few studies performed by immunohistochemical detection of human breast cancer cases. In two large series (16, 17), it was reported that PPARγ expression was inversely correlated with tumour grade. PPARγ was also found to be a favorable factor for overall survival (17) but not a factor affecting relapse. Interestingly, quite the opposite finding was reported by Papadaki *et al*. (16), with PPARγ being considered a factor affecting disease-free but not overall survival. Despite the protective role implied by these reports, the role for PPARγ remains controversial since animal experiments have shown that PPARγ expression, once the tumourigenesis is complete, can act as a tumour promoter in the mammary gland (18). Our results are closer to this thesis, supporting a positive effect of PPRAγ on breast cancer tumour progression, since the IRS for PPARγ is marginally

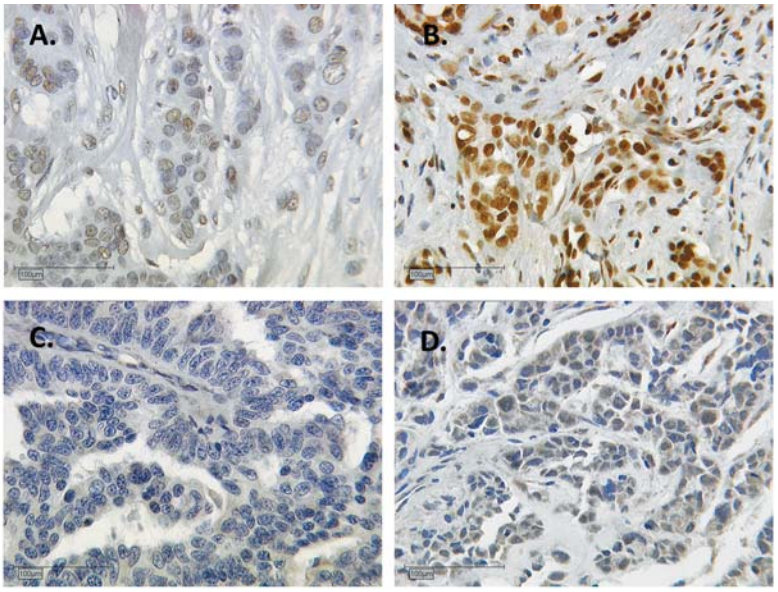

Figure 2. *Representative microphotographs of low (A) and high (B) expression of RXRα, as well as of low (C) and high (D) expression of PPARγ in tissue sections of breast cancer cases, as this was revealed by immunohistochemistry.*

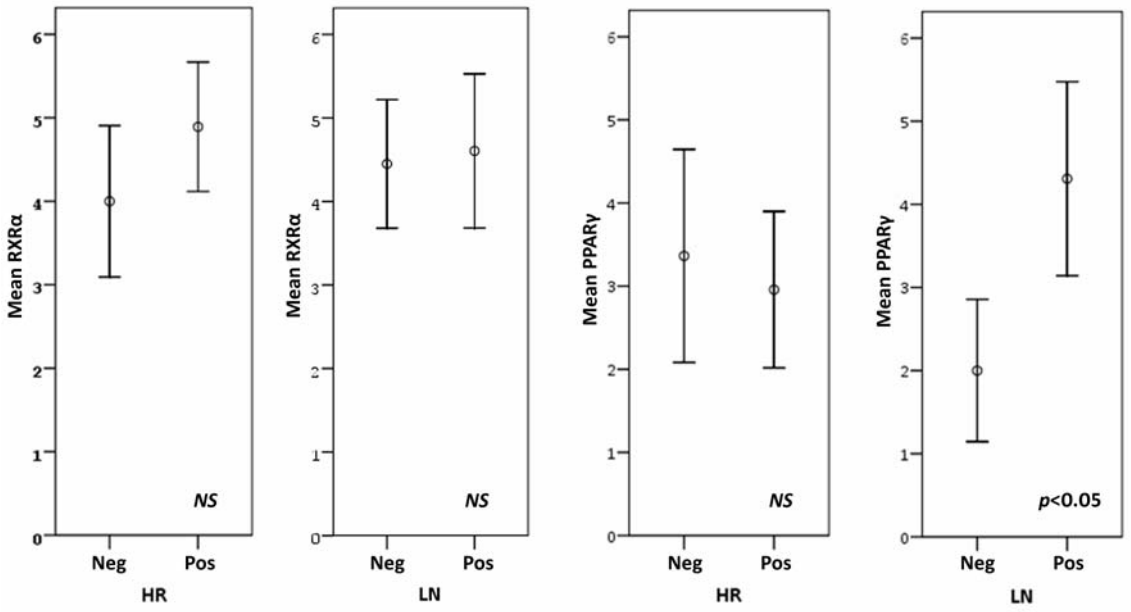

Figure 3. *Presentation of the mean IRS of both RXRα and PPARγ staining. Breast cancer cases were grouped according to hormone receptor (HR) and lymph node (LN) status. PPARγ expression seems significantly elevated in lymph node positive cases ($p<0.05$). Margins represent 95% confidence intervals of the means. Neg: negative, Pos: positive.*

correlated with tumour grade. In line with this finding, higher PPRAγ expression was more observed in LN-positive than in LN-negative breast cancer cases (Figure 3), being also in disagreement with a previous report inversely correlating LN status with PPARγ positivity (17).

However, our study was performed on a cluster of cases according to their LN status, histological grade of the primary tumour and HR (ER/PR) status; as such, our results represent PPARγ and RXRα expression according only to grading, LN involvement and dependence on steroid hormones. Based on

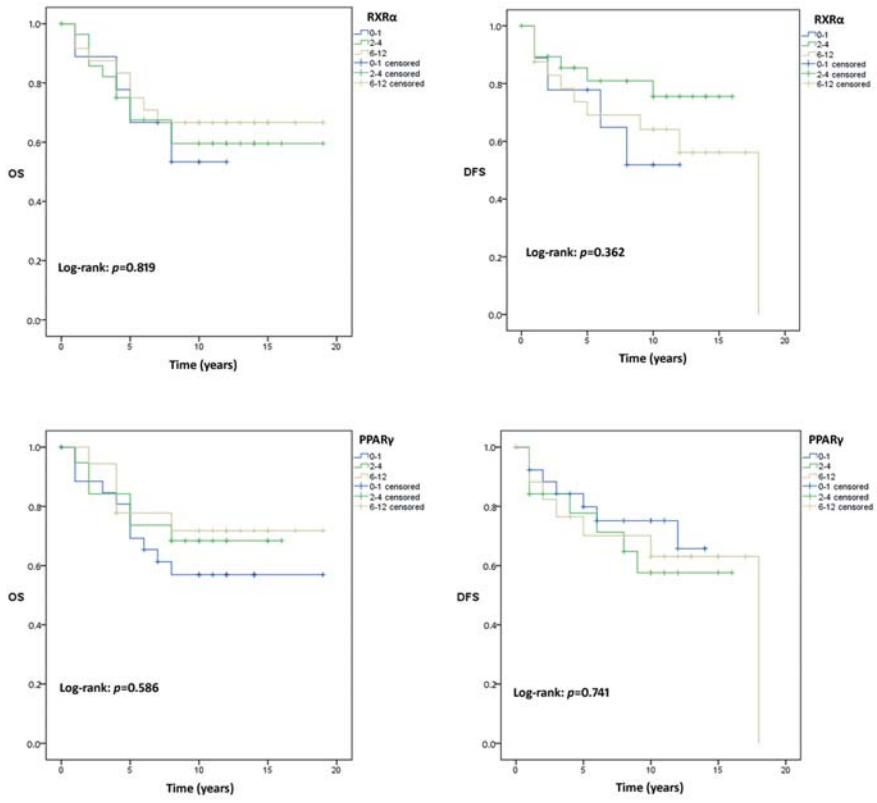

Figure 4. *Presentation of the overall (OS) and disease-free survival (DFS) of the cases included in the current study. Neither RXRα nor PPARγ were found to significantly affect survival.*

our results on elevated PPARγ expression in LN-positive cases, we could thus assume that this transcription factor could be involved in dissemination of breast cancer cells.

The role of RXR in breast cancer biology has been well studied *in vitro*. RXR ligands or rexinoids are reported to induce apoptosis in BCL2-positive human cancer cells (13), while a selective RXR agonist suppressed mammary tumourigenesis in transgenic mice (19). RXR activation was shown to down-regulate COX-2 expression in breast cancer cells (20), and block the breast cancer cell cycle at the G1 phase (21). Further experiments revealed that combined activation of RXR and PPARγ may induce apoptosis *via* p53/p21(WAF1/Cip1) pathways (22). Our results cannot clearly support the tumour-inhibiting role of RXR, since no significant difference was noted in survival analysis. However, the significant inverse correlation found in the present study, between RXRα expression and histological grade may imply also that RXRs may protect breast cancer cells from de-differentiation. Such a finding, if proven by larger series, could further strengthen the thesis of using RXR agonists as potential therapeutic regimes.

In summary, the current study demonstrates that in breast cancer cases, RXRα expression is inversely related to histological grade, verifying existing data regarding its antitumour effects. Despite previous histological data supporting an equivalent role for PPARγ, the findings of this study seem to rather support the opposite. Such a finding is considered with great caution, due to the specific clustering of the samples in the current study and the application of the IRS. If however this holds true, taking also under consideration the interaction between RXR and PPARγ, a potential contradictory role of the RXR-PPARγ heterodimer would make it rather difficult for an *in vivo* therapeutic effect to be predicted. Perhaps this is a possible explanation for the PPARγ activation to be considered of minor clinical value in several clinical trials (23-25).

Acknowledgements

We thank M. Rübekeil, S. Kunze, and Ch. Kuhn for their excellent technical assistance.

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Received August 12, 2011
Revised October 4, 2011
Accepted October 6, 2011