

Enhanced Expression of Peroxisome Proliferate-activated Receptor Gamma (PPAR- γ) in Advanced Prostate Cancer

SEBASTIAN ROGENHOFER¹, JÖRG ELLINGER¹, PHILIP KAHL², CHRISTINE STOEHR³,
ARNDT HARTMANN³, DIRK ENGEHAUSEN⁴, WOLF-FERDINAND WIELAND⁵,
STEFAN C. MÜLLER¹, FERDINAND HOFSTÄDTER⁶ and BERNHARD WALTER⁴

¹Department of Urology, University of Bonn, Germany;

²Institute of Pathology, University of Cologne, Germany;

³Institute of Pathology and ⁴Department of Urology, Friedrich Alexander University, Erlangen, Germany;

⁵Department of Urology and ⁶Institute of Pathology, University of Regensburg, Germany

Abstract. *Background:* Recent studies have underlined the role of nuclear receptors in the involvement of prostate cancer signalling pathways. *Patients and Methods:* A total of 84 benign prostate hyperplasia (BPH), 84 low risk prostate cancer (LPC) and 64 advanced disease (APC) cases were sampled on a tissue microarray (TMA) and stained for retinoic acid receptor (RAR)- α , retinoid X receptor (RXR)- α , liver X receptor (LXR)- α , farnesoid X receptor (FXR) and proliferate-activated receptor gamma (PPAR)- γ and the (pro)-inflammatory molecules cyclooxygenase 2 (COX2), tumor necrosis factor (TNF)- α and inducible Nitric oxide synthase (iNOS) immunohistochemically. *Results:* PPAR- γ expression in APC tissues was found to be significantly higher than that in LPC and BPH specimens ($p < 0.001$). In contrast, RXR- α expression was significantly lower ($p < 0.001$). COX2 staining demonstrated a trend towards overexpression in APC ($p = 0.025$). No significant differences were found for RAR- α , iNOS and TNF- α expression. Staining of FXR and LXR was seen diffusely in the cytoplasm as well as in the nucleus, preventing sufficient evaluation by definition. *Conclusion:* This study provides the basis for applying PPAR- γ ligands clinically in treatment of APC.

Prostate cancer (PCA), the most frequent malignancy in men, is responsible for the second highest number of cancer-related deaths (1). Around half of all patients have metastatic disease at the initial diagnosis, and nearly 50% of those who present with an initial localized disease will develop subsequent metastases. Androgen deprivation therapy leads to a remission of PCA, but after some years nearly all patients evolve

towards an androgen-independent state, resulting in death due to widespread metastasis (2). Despite encouraging advancements in the field of taxane-based chemotherapy regimes (3), it is noteworthy that such therapy does cause severe adverse side-effects and disease control is limited to 12 weeks of duration (4), underlining the need for additional treatment options. Thereby, a phase II study using a modular treatment approach including the cyclooxygenase 2 (COX2) antagonist etoricoxib and the proliferate-activated receptor gamma (PPAR)- γ ligand pioglitazone successfully proved the hypothesis of concerted anti-inflammatory drug action in patients with castration refractory prostate cancer (CRPC) (5). This combined modular therapy approach was able to induce major responses with a minimum of side-effects.

PPAR- γ belongs to the nuclear receptors (NRs), comprising a large family of highly conserved transcription factors, which regulate many key processes in normal and neoplastic tissues. NRs control different aspects of cellular metabolism including growth and differentiation, lipid and glucose homeostasis and inflammation (6, 7). At present, this group of proteins forms the second largest class of drug targets in the current pharmaceutical market (8). Furthermore, NRs are reported to be important key players in the field linking cancer and inflammation (9). As an example, the glucocorticoid receptor (GR) acts as a target for anti-inflammatory and immunosuppressive interference, as well as being a commonly explained co-worker in cancer treatment. Further examples of additive complementation of anti-tumourous effects by primarily anti-inflammatory-acting NRs are well-documented, experimentally and in clinical trials respectively (10-15).

Due to recent advances in the understanding of the biological role in cancer biology, NRs function as attractive treatment targets and might overcome the limitations of current drugs. However, systematic analyses of NR expression profiles in neoplastic tissues, *i.e.* PCA are lacking. The study presented herein based on a tissue microarray analysis (TMA), examines the expression profile of eight inflammatory-acting

Correspondence to: Bernhard Walter, MD, Department of Urology, University of Erlangen, 91054 Erlangen, Germany. Tel: +49 86715091225, e-mail: bernhard.walter@uk-erlangen.de

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receptors, including retinoid X receptor (RXR)- α , retinoic acid receptor (RAR)- α , liver X receptor (LXR)- α , farnesoid X receptor (FXR), PPAR- γ and the pro-inflammatory molecules COX2, tumor necrosis factor (TNF)- α and inducible Nitric oxide synthase (iNOS) in benign prostate tissue, as well as in early PCA and advanced stage cancer specimens. To the best of our knowledge, this is the largest study analyzing the expression of selective inflammatory-acting molecules related to malignant progression in PCA.

Patients and Methods

Patients. The local Institutional Review Boards of the University of Regensburg granted approval for this study. We included a cohort of patients with clinically localized low-risk PCA (LPC; n=84) undergoing radical prostatectomy. Non-malignant prostate tissue samples were collected from patients undergoing transurethral resection of the prostate (BPH; n=84). For comparison, we included tissue of 65 patients with advanced metastatic or CRPC (APC) undergoing a transurethral resection. All specimens were retrieved from the Institute of Pathology, University of Regensburg. A TMA was built using representative areas from formalin-fixed, paraffin-embedded tumour material. Briefly, suitable areas for tissue retrieval were marked on standard haematoxylin/eosin (H&E)-stained sections, punched out of the paraffin block and inserted into a recipient block. The tissue arrayer was purchased from Beecher Instruments (Woodland, CA, USA). The punch diameter was 0.6 mm. The freshly-cut 5- μ m sections of the resulting blocks underwent strictly synchronous treatment throughout all staining procedures.

Immunohistochemistry (IHC). Immunohistochemical studies utilized an avidin-biotin peroxidase method with a 3-amino-9-ethylcarbazole (AEC) chromotogen. After antigen retrieval (steam boiling with citrate-buffer, pH 6.0 for 20 minutes), IHC was carried out applying the ZytoChemPlus HRP Broad Spectrum Kit (Zytomed Systems, Berlin, Germany) according to the manufacturer's instructions.

The following primary antibodies were used: anti-COX2 (mouse monoclonal, dilution 1:200; Cayman Chemical, Ann Arbor, MI, USA;), anti-PPAR- γ (rabbit monoclonal, dilution 1:50; Abcam Inc., Cambridge, MA, USA), anti-LXR- α (mouse monoclonal Abcam Inc., dilution 1:100; Cambridge, MA, USA), anti-FXR (mouse monoclonal, dilution 1:20; US Biological, MA, USA), anti-RXR (rabbit monoclonal, ready to use; Abcam Inc.) and anti-RAR- α (mouse monoclonal, dilution 1:20; Abcam Inc.).

Cytoplasmic COX2, iNOS, TNF- α and nuclear PPAR- γ , RXR, RAR, LXR and FXR immunoreactivity was evaluated using the Remmele scoring system (16). The number of positive by staining epithelial cells was estimated per core and scaled (0, no positive cells; 1, 1-25% positive cells; 2, 26-50% positive cells; 3, 51-75% positive cells; and 4, 76-100% positive cells). These scores were multiplied by the intensity score (0, negative; 1, weak; 2, moderate; and 3, intensive staining). An experienced pathologist (P.K.) performed the evaluation of immunohistochemical staining without knowledge of the clinicopathological parameters. For COX2, iNOS and TNF- α , positivity was considered depending of the percentage of cytoplasmic staining (0, no positive cells; 1, 1-25% positive cells; 2, 26-50% positive cells; 3, 51-75% positive cells; and 4, 76-100% positive cells). For PPAR- γ , RXR, RAR, LXR and FXR immunoreactivity was stained positively depending of the

percentage of positive cell nuclei (0, no positive cells; 1, 1-25% positive cells; 2, 26-50% positive cells; 3, 51-75% positive cells; and 4, 76-100% positive cells).

RNA isolation and quantitative real-time (PCR). Samples for molecular analysis were taken from surgical specimens immediately after resection and flash frozen in liquid nitrogen. H&E stained cryostat sections (5 μ m) were used for histological evaluation. The remaining tissue was formalin-fixed and paraffin-embedded for routine histological evaluation. Total RNA was isolated from ~20 mg tissue using the PureLink RNA Mini Kit (Invitrogen, Carlsbad, CA, USA) according the manufacturers guidelines. RNA concentrations were determined using an ND-1000 spectrophotometer (Peglab, Erlangen, Germany), and 500 ng of total RNA was reverse-transcribed using SuperScript III First-Strand Synthesis Kit (Invitrogen).

mRNA levels were determined using quantitative real-time PCR on an ABI Prism 7900HT (Applied Biosystems, Foster City, CA, USA) in 384-well plates in triplicates. Each 10 μ l reaction consisted of 1 \times SYBR GreenER qPCR SuperMix (Invitrogen), 5 ng of cDNA and 3 pmol primer. The PCR reaction was started with 2 min at 50°C for (UDG) treatment and 10 min at 95°C for polymerase activation. For amplification, we performed 40 cycles with 15 sec at 95°C and 60 sec at 60°C were used. Finally, melting curve analysis was performed to check for specificity. Primer sequences were: *PPARG-II* (Fwd: GAC-CTG-AAA-CTT-CAA-GAG-TAC-CAA-A; Rev: TGA-GGC-TTA-TTG-TAG-AGC-TGA-GTC), nuclear receptor subfamily 1, group H, member 3 (*NR1H3*) (Fwd: CAG-GGC-TCC-AGA-AAG-AGA-TG; Rev: ACA-GCT-CCA-CCG-CAG-AGT), nuclear receptor subfamily 1, group H, member 4 (*NR1H4*) (Fwd: ACC-TGT-GAG-GGG-TGT-AAA-GGT; Rev: GCC-CCC-GTT-TTT-ACA-CTT-G), *RXRA* (Fwd: CAT-CGT-CCT-CTT-TAA-CCC-TGA; Rev: CCA-AGG-ACG-CAT-AGA-CCT-TC) and hypoxanthine phosphoribosyltransferase 1 (*HPRT1*) (Fwd: TGA-CCT-TGA-TTT-ATT-TTG-CAT-ACC; Rev: CAT-CTC-GAG-CAA-GAC-GTT-CA). Relative levels of NR mRNA were determined using the $\Delta\Delta$ CT formula; *HPRT1* was used as reference gene.

Results

Immunohistochemistry. PPAR- γ , RAR- α and RXR- α expression was found to be positive in 81.5% (190/233), 88.8% (207/233) and 87.5% (207/233) of cases (Figure 1).

PPAR- γ staining in BPH tissue was significantly lower than in PCA tissue ($p<0.001$). A significant difference was also seen for PPAR- γ in APC compared to LPC ($p<0.001$).

A significant opposite relation was observed in cases of RXR- α staining: Immunoreactivity in BPH tissues was significantly higher than in PCA tissue ($p<0.001$), as well as in LPC compared to in APC ($p<0.001$). RAR- α staining was evenly distributed through all three different prostate tissues, thereby demonstrating no significant differences.

Staining of FXR and LXR was seen diffusely in the cytoplasm, as well as in the nucleus and nuclear membrane of BPH and PCA respectively, preventing a sufficient evaluation by definition.

COX2, iNOS and TNF- α immunoreactivity was positive in 81.5% (190/233), 89.2% (208/233) and 83.2% (194/233) of cases, respectively. Generally, positive COX2 staining in PCA tissues was detected significantly more often than in BPH

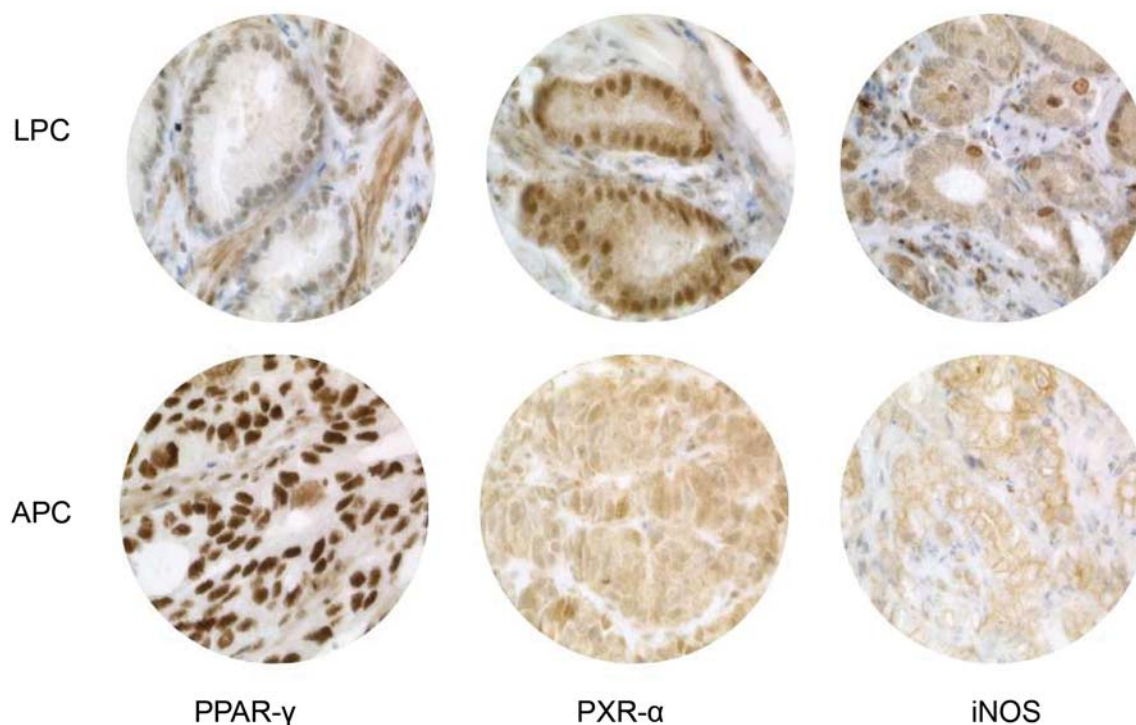


Figure 1. Immunohistochemistry of proliferate-activated receptor gamma (PPAR)- γ , retinoid X receptor (RXR)- α and retinoic acid receptor (RAR)- α in low grade (LPC) and advanced prostate cancer (APC). Magnification $\times 200$. The upper row shows representative examples of a LPC with immunoreactivity for PPAR- γ , RXR- α and RAR- α . The lower row shows representative examples of a APC with immunoreactivity for PPAR- γ , RXR- α and RAR- α .

($p=0.004$). There was a trend towards COX2 overexpression in APC compared to LPC ($p=0.024$). In this context, iNOS and TNF- α showed no significant differences, neither in terms of BPH and PCA overall ($p=0.423$ and $p=0.327$, respectively) nor with respect to APC compared to LPC ($p=0.325$) (Figure 2).

Real-time PCR. Total RNA was purified from samples of 17 patients with PCA and 9 with BPH. With specific primers for *PPARG-II*, *NR1H3*, *NR1H4* and *RXRA* the amplification predicted fragments of 95, 78, 75, and 86 base pairs, respectively, in length. The housekeeping gene *HPRT1* was used for normalization of RNA input. *HPRT1* gene expression was selected as being independent of cell type and external influences in all cells. This led to a comparable cohort of tumour and normal tissue, allowing for relative quantification. *PPARG-II*, *NR1H3*, *NR1H4* and *RXRA* mRNA was detected in samples of BPH, as well as PCA.

NR1H4 and *RXRA* were found to be significantly overexpressed in BPH ($p=0.001$) compared to PCA. Regarding the different histopathological stages, *NR1H4* was expressed significantly more often in locally-advanced prostate cancer specimens (pT3) ($p=0.048$). *PPARG-II* demonstrated a trend for higher expression in aggressive prostate cancer (pT3). *NR1H3* expression was not significantly different between BPH and PCA tissue.

Discussion

Intervening in the NR unit of PPAR- γ enables modulation of various key processes in the tumour microenvironment: Basic research studies demonstrated PPAR- γ ligands to be efficient in control of tumour-progression through their effects on various cellular processes, including proliferation, apoptosis, angiogenesis, inflammation and metastasis (8). However, implementation of this knowledge into clinical practice is just starting: a modular treatment approach, targeting COX2 and the PPAR- γ receptor, successfully proved the hypothesis of concerted anti-inflammatory drug action in patients with APC (8). In contrast, a phase I study using the RXR ligand bexarotene and the PPAR- γ agonist rosiglitazone in patients with refractory cancer, such as APC, failed with regard to objective responses (17). Future treatment strategies in this field will probably incorporate expression status of the related targets in the tumour compartment, predicting individual prognosis as well as therapy response.

PPAR- γ was the only receptor in the context of our study results which demonstrated significant expression in APC disease. This finding is in accordance with former studies, in which PPAR- γ was reported to be overexpressed in PCA tissues (18, 19). Distinction of different PPAR- γ expression between LPC and APC was reported by Nagata *et al.*, who

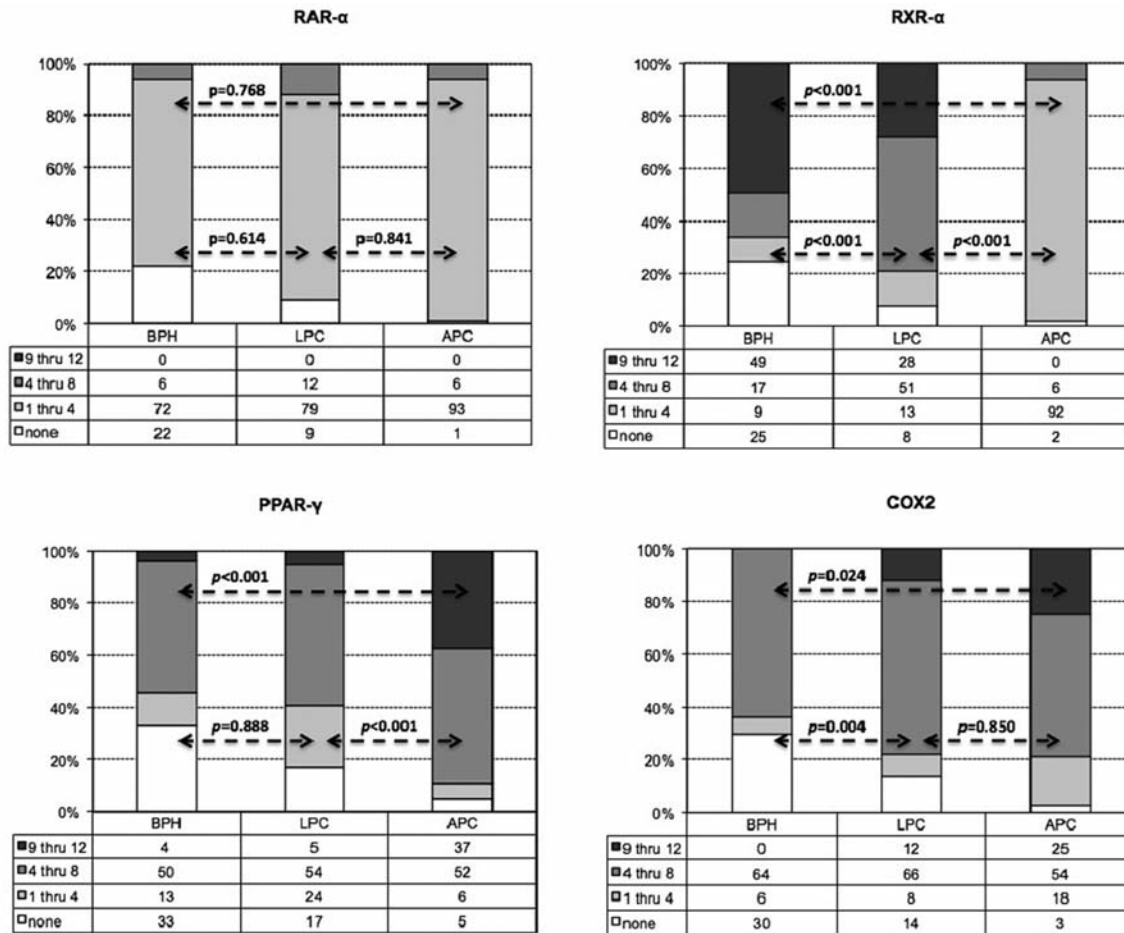


Figure 2. Cumulative bar charts of cyclooxygenase 2 (COX2) and the nuclear receptors retinoic acid receptor (RAR)- α , proliferate-activated receptor gamma (PPAR)- γ , retinoid X receptor (RXR)- α immunoreactivity in BPH, low risk PCA (LPC) and advanced PCA (APC) tissue. Data shown is the percentage of patients in correlation to the Remmele scoring system.

found PPAR- γ to be overexpressed both in APC cell lines and human APC tissues (20).

Significantly reduced RXR- α staining in APC specimens in our study is consistent with the results of an investigation focused on RXR expression in neoplastic prostate tissue (21). The authors argued for a pre-therapeutic RXR receptor status evaluation to avoid diminished therapy responsiveness due to NR underexpression in the target compartment; this might have been helpful in the case of the above mentioned phase I trial, in which bexarotene and rosiglitazone lacked treatment efficacy. In contrast, our findings for RAR- α are in contrast to the results of two studies in which RAR- α was found to be significantly overexpressed in high-grade PCA (22, 23).

COX2 expression is one of the best examined topics regarding the link between pro-inflammatory molecules and cancer. Thus, COX2 has been assessed as an attractive target for cancer therapy (24, 25). We found COX2 to be similarly expressed as described in the literature, although differences

between the prostate specimens occasionally were not as pronounced (26, 27).

Overall, our data provide a “mix” of over-, suppressed or unaffected receptors in the prostate cancer compartment. Possible explanations for this paradox are multifaceted and complex. A variety of soluble agents in the tumour microenvironment, such as chemokines, growth factors, lipids, angiogenic factors, proteinases and proteinase inhibitors, are involved in an extensive crosstalk between tumour and tumour stroma (28). Given the complexity of mechanisms underlying PCA progression, research into the relationship between NR responsible for binding steroids, retinoids and secosteroids and the involvement of apoptosis-regulating proteins is just beginning (29).

Consistent with previously published data (30), our immunohistochemical analysis demonstrated a significant increase of PPAR- γ expression due to malignant transformation in PCA and a trend towards significance for COX2, thereby supporting the necessity for a clinical trial (5).

The combination of both drugs in patients with CRPC is a logical consequence of experimental studies indicating that COX2 and PPAR- γ signalling pathways are multiply intertwined (29).

To fully evaluate and understand the potential of NR modulation, further studies using tissue microarrays of patients with CRPC participating in trials on antiinflammatory drugs, are needed to determine whether NR may be useful as prognostic markers which correlate responsiveness to biomodulatory stroma-targeted therapy.

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