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ORIGINAL ARTICLE

Differential expression and cross-talk of peroxisome proliferator-activated receptor γ and retinoid-X receptor α in urothelial carcinomas of the bladder

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Summary

Purpose: *The peroxisome proliferator-activated receptor γ (PPARγ), known to play a key role in homeostatic biological pathways, is also implicated in the process of carcinogenesis. Ligands for PPARγ and its heterodimeric partner, retinoid-X receptor (RXR), have exhibited anticancer effects both in vitro and in vivo. Unexpectedly, some studies suggested that PPARγ ligands may stimulate cancer formation. This study aimed to estimate the signaling of PPARγ-RXRα heterodimer in bladder urothelial carcinomas (BUC).*

Methods: *We studied PPARγ and RXRα expression in specimens obtained from 97 patients with BUC of various grades and stages using immunohistochemistry.*

Results: *PPARγ expression was significantly downregulated with BUC stage and grade progression, and the dynamics of this phenomenon was significantly influenced by RXRα's level of expression.*

Conclusion: *The positive association of PPARγ expression in BUC with more differentiated, non-invasive tumors is strengthened by the presence of RXRα. This knowledge could probably be of use in the development of new chemotherapeutic agents.*

Key words: bladder urothelial carcinomas, immunohistochemistry, PPARγ, RXRα

Introduction

PPARs comprise an important subfamily of the nuclear hormone receptor (NHR) superfamily. The most intensively studied PPAR isoform is PPARγ. During the past decade it has been shown that PPARγ is a transcription factor that participates in biological pathways of great importance [1-5] and, like other members of the NHR superfamily, controls the expression of a large number of genes relevant to the process of carcinogenesis [6].

Loss of PPARγ expression could be an important risk factor for the development of carcinoma, as animal studies have shown that PPARγ heterogeneous (+/−) mice are at enhanced risk for colon carcinogenesis after exposure to the colon carcinogen azoxymethane [7]. On the other hand, other studies have shown that PPARγ signaling increases the risk of breast cancer in mice already susceptible to the disease [8]. It has been proposed that this paradox could be explained by different levels of PPARγ gene expression and signaling in different tissues at risk for carcinogenesis [9].

The ability of PPARγ to modulate gene activity requires the presence of RXR. RXRs are members of the steroid hormone receptor superfamily. Three RXR isotypes (α, β, γ) have been identified. Like other members of this family, RXRs act as ligand-activated DNA-binding transcription factors through binding -as heterodimers with RARs, the other type of retinoid receptors- to cis-acting RA-response elements present in cognate genes [10]. They also play a central role as heterodimeric partners for other nuclear receptors (subfamily1 of nuclear receptors), including the PPARs [11]. The RXR/PPARγ heterodimer represents a permissive bifunctional transcription factor [12], that allows integration of two independent hormone signaling pathways by a single functional unit [13]. Moreover, there is strong evidence that RXRα is mainly responsible for intranuclear distribution of PPARγ [14]. All-trans-retinoic acid (a retinoid acid receptor ligand) has been used successfully to induce remission of acute promyelocytic leukemia [15]. Retinoids are able to reverse premalignant lesions and prevent recurrence of head and neck cancers

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[16] and also to suppress carcinogenesis in a variety of tissue types in many animal models [17].

To our knowledge the present study is the first morphological evaluation of PPARγ and RXRα differential expression and cross-talk in a series of urothelial carcinomas of the bladder.

Methods

Patients and histopathological classifications

For the present study we used a total of 97 archived human paraffin-embedded sections from the Pathology Department of the University hospital of Patras. These specimens were obtained from patients who had undergone transurethral biopsy or cystectomy from 2000 to 2002. The mean patient age was 72.2 ± 10.52 years (range 23-90). Thirty-seven (38.1%) were 70 years old or less and 60 (61.9%) were over 70 years. Twenty-four out of the 97 patients were females (24.7%) and 73 males (75.3%). Tissue specimens were fixed in 10% buffered formalin and embedded in paraffin. Serial 4-μm sections were obtained for hematoxylin-eosin staining and for immunohistochemical study. The histopathological and stage classification of the tissue specimens used is summarized in Table 1.

Immunohistochemistry

Four-micrometer sections were mounted on Super Frost Plus slides, baked at 60 ºC for 60 min, deparaffinized and rehydrated through graded alcohol rinses. Heat-induced antigen retrieval was performed by immersing the slides in EDTA-NaOH buffer (pH 8.0) and microwaving at 550W for 2.5 min, at 750W for 2.5 min and finally at 350W for 10 min. The slides were then cooled at room temperature for 20 min. Antigen was detected by 2-h incubation at room temperature with the relevant primary antibody, followed by an appropriate secondary antibody conjugated to a peroxidase complex (EnVision+ poly-HRP system, DAKOCytomation). Negative controls were processed by substituting the primary antibody with nonimmune mouse serum. Color development was done using DAB+ Chromogen (DAKOCytomation). The primary antibodies used in the present study were: Sc 7273, mouse polyclonal (dilution 1:50) for PPARγ and Sc 553, rabbit polyclonal (dilution 1:100) for RXRα, (both Santa Cruz Biotechnology). The semiquantitative method based on a four-point scale was chosen for the assessment of the intensity of immunostaining: - (negative); + (weak positivity); ++ (moderate positivity); +++ (strong positivity)

Statistical analysis

The 97 tissue specimens were categorized by grade and by stage of BUC. Grade I BUC were classified as low grade (n=29), whereas grade II and grade III composed the high grade (n=68) BUC group. Stages pTa and pT1 were grouped together as early stage cancer (n=63) and stages pT2 or higher as advanced cancer (n=34).

The relationship between each molecular target expression and the different patient and tissue characteristics [age (>70/≤70 years old), sex (male/female), grade (low/high) and stage (early/advanced)] was evaluated for its strength and direction (positive vs. negative) using the Spearman's rho correlation coefficient. The possibility of statistically significant different levels of expression of the examined transcriptional factors among the different patient and tissue parameters was evaluated by nonparametric Mann-Whitney analysis.

Results

The immunohistochemical results are summarized in Tables 2 and 3.

Expression of PPARγ

The staining reaction was predominantly localized to nuclei and was more intense in the superficial urothelial cells than in the basal cells.

Among the 29 specimens with low grade BUC 9 (31%) showed moderate positivity for PPARγ, while 20 (69%) showed strong positivity for PPARγ (Figure 1a). Eight out of 68 (11.8%) specimens with high grade

Table 1. Histopathological and stage classification of the 97 specimens

Grade	*Ta*	*T1*	*T2*	*T3*	*T4*	*Total, n (%)*
I	14	15				29 (29.9)
II	6	18	7			31 (32)
III		10	20	3	4	37 (38.1)
Total, n (%)	20 (20.6)	43 (44.4)	27 (27.8)	3 (3.1)	4 (4.1)	97 (100)

Table 2. Immunohistochemical expression of peroxisome proliferator-activated receptor (PPAR) γ and retinoid-X-receptor (RXRα) in low and high grade urothelial carcinomas of the bladder (BUC)

Antibodies	*BUC grade*	*Negative n (%)*	*+1 n n (%)*	*+2 n n (%)*	*+3 n n (%)*
PPARγ	Low grade (n=29)	–	–	9 (31.0)	20 (69.0)
	High grade (n=68)	4 (5.9)	8 (11.8)	36 (52.9)	20 (29.4)
RXRα	Low grade (n=29)	–	–	11 (37.9)	18 (62.1)
	High grade (n=68)	–	2 (2.9)	20 (29.4)	46 (67.6)

Table 3. Immunohistochemical expression of peroxisome proliferator-activated receptor (PPAR) γ and retinoid-X-receptor (RXRα) in early and advanced stage urothelial carcinomas of the bladder (BUC)

Antibodies	*BUC grade*	*Negative n (%)*	*+1 n n (%)*	*+2 n n (%)*	*+3 n n (%)*
PPARγ	Early stage (n=63)	–	–	28 (44.4)	35 (55.6)
	Advanced stage (n=34)	4 (11.8)	8 (23.5)	17 (50.0)	5 (14.7)
RXRα	Early stage (n=63)	–	–	18 (28.6)	45 (71.4)
	Advanced stage (n=34)	–	2 (5.9)	13 (38.2)	19 (55.9)

BUC showed weak positivity (Figure 1e), 36 (52.9%) were moderately positive (Figure 1c) and 20 (29.4%) were strongly positive for PPARγ, while 4 (5.9%) specimens were immunonegative.

The expression of PPARγ was shown to be significantly different among the two histopathological groups of BUC (Mann-Whitney $p<0.0001$). Specifically, a statistically significant downregulation of PPARγ was depicted (Spearman's rho=–0.392, $p<0.0001$) with loss of differentiation in BUC (Figure 2a).

As for disease stage, 28 out of 63 (44.4%) specimens with early-stage cancer showed moderate positivity (Figure 1c), while the rest 35 (55.6%) exhibited strong positivity for PPARγ (Figure 1a). Among the 34 tissue specimens with advanced-stage BUC 4 (11.8%) were immunonegative, 8 (23.5%) were weakly positive (Figure 1e), 17 (50%) moderately positive and 5 (14.7%) strongly positive for PPARγ.

The comparison of PPARγ expression between early and advanced cancer showed a statistically significant difference (Mann-Whitney $p<0.0001$) among these two groups and more specifically a statistically significant downregulation (Spearman's rho=–0.513, $p<0.0001$) as cancer became invasive (Figure 2b).

Expression of RXRα

In 18 of 29 (62.1%) specimens with low grade, strong positivity for RXRα was seen (Figure 1b), whereas the rest 11 (37.9%) showed moderate positivity.

Most specimens with high grade BUC (46 out of 68; 67.6%) showed strong positivity (Figures 1d, 1f), 20 out of 68 (29.4%) were moderately positive and only 2 (2.9%) were weakly positive. RXRα expression did not show any statistically significant differentiation between the two histopathological groups of BUC ($p>0.05$).

Eighteen out of 63 (28.6%) specimens in the early-stage group showed moderate positivity for RXRα, while the rest 45 (71.4%) were strongly positive (Figures 1b, 1d). Among 34 specimens of advanced stage, 2 (5.9%) were weakly positive, 13 (38.2%) moderately positive and 19 (55.9%) strongly positive (Figure 1f).

No statistically significant difference in RXRα expression was detected between the two groups of BUC stages ($p>0.05$).

Statistical correlations between PPARγ and RXRα expression

Importantly, the level of RXRα expression influenced the strength of PPARγ's downregulation and was related with loss of BUC differentiation (Spearman rho = –0.371, p = 0.040 for RXRα expression = +2 and Spearman's rho = –0.393, p = 0.001 for RXRα expression = +3). RXRα expression = +1 was found only in 2 specimens with high grade BUC, both of which were immunonegative for PPARγ (hence variable PPARγ and variable grade were constant). Inversely, PPARγ's contribution in differentiation is enhanced by the presence of RXRα.

Likewise, the level of RXRα expression was shown to significantly influence the strength of PPARγ downregulation as BUC stage advanced (Spearman rho = –0.432, p=0.015 for RXRα expression = +2 and Spearman's rho = –0.519, $p<0.0001$ for RXRα expression = +3). RXRα expression = +1 was found only in 2 speci-

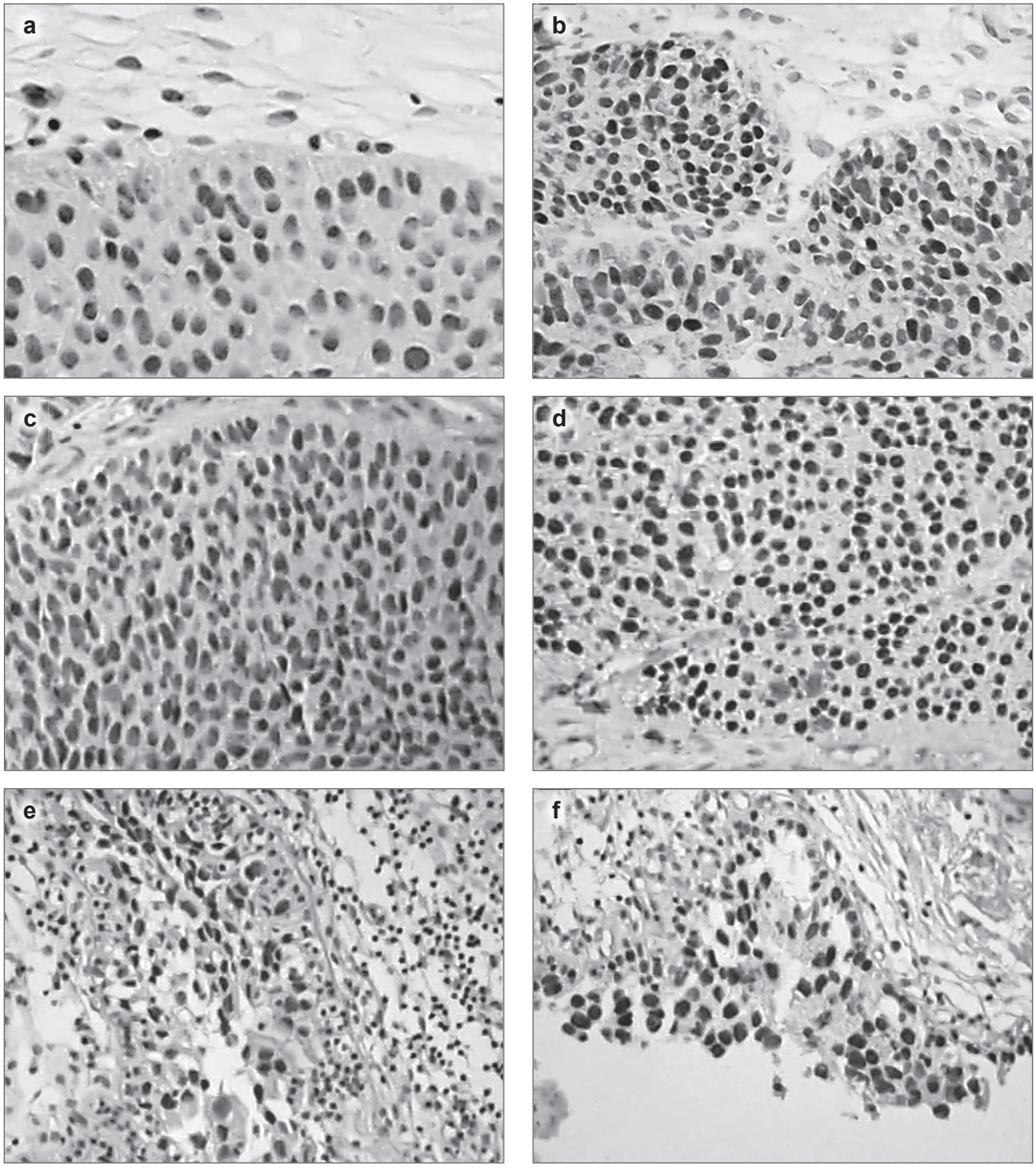

Figure 1. Peroxisome proliferator-activated receptor (PPAR) γ and retinoid-X-receptor (RXRα) expression in three different urothelial carcinomas of the bladder (BUC) cases: low grade early-stage BUC with strong positivity for PPARγ (**a**, ×400) and strong positivity for RXRα (**b**, ×400). High grade early-stage BUC with moderate positivity for PPARγ (**c**, ×400) and strong positivity for RXRα (**d**, ×400). High grade advanced-stage BUC with weak positivity for PPARγ (**e**, ×400) and strong positivity for RXRα (**f**, ×400).

mens with advanced stage BUC, both of which were immunonegative for PPARγ (hence variable PPARγ and variable stage were constant).

These results indicate that as RXRα expression became stronger, the relation between PPARγ expression and early-stage BUC became more prominent.

Finally, PPARγ and RXRα level of expression had no statistically significant correlation with patients' sex (male vs. female) or age group (>70 vs. ≤70 years old; $p>0.05$).

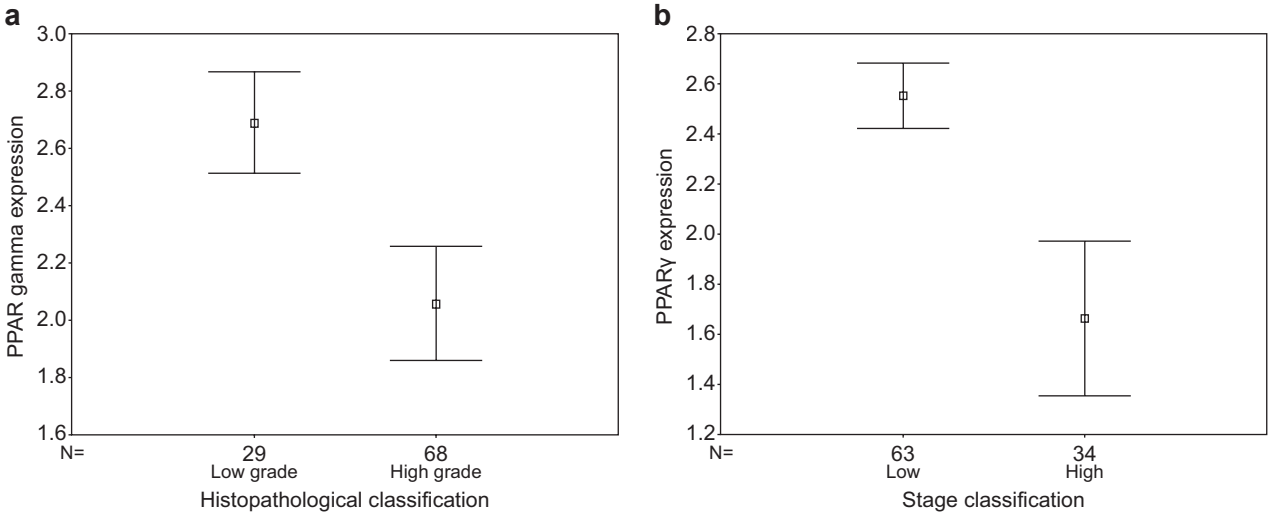

Figure 2. PPARγ downregulation in (**a**) grade and (**b**) stage progression in BUC.

Discussion

During the last years PPARγ has become object of extended research in order to develop new agents for chemoprevention [18] and chemotherapy [19] of cancer.

It is already known that PPARγ plays a critical role in the differentiation of preadipocytes into adipocytes [4,5] and that PPARγ ligands induce apoptosis of macrophage and endothelial cells [20]. Based on this observation, a series of studies showed that PPARγ ligands reduced the growth rate in various carcinoma cell lines [21-23]. Moreover, studies with cell cultures showed that PPARγ signaling promotes differentiation in normal human urothelium [24] as well as in urothelial carcinomas [25]. In agreement with these results, we observed a statistically significant downregulation of PPARγ along with loss of differentiation in BUC. Similar observations were reported by Nakashiro et al. [26] and Mylona et al. [27]. We also noted that loss of PPARγ expression correlates significantly with increasing invasiveness in BUC, as PPARγ was more frequently and more intensely immunodetected in early-stage BUC than in advanced ones. Other studies in BUC also reported similar observations [27] whereas PPARγ ligands reportedly decreased the invasion potential of normal and cancer cells [28].

Despite the observations mentioned above, there is also evidence that PPARγ signaling might promote colon [7] or breast [8] tumor progression under certain circumstances. Moreover, it was shown that dual-acting PPAR alpha and gamma agonists caused urothelial cancers in rodents [29] and rosiglitazone, a PPARγ agonist currently used as antidiabetic drug, promoted the carcinogenic effect of OH-BBN, a urinary bladder- specific carcinogen [30]. It has also been proposed that nonneoplastic urothelial cells, which proved to express PPARγ, could be more sensitive to the cytocidal effects of PPARγ ligands than carcinoma cells which have low transcriptional activity or even failure to express PPARγ [26].

In order to further elucidate PPARγ's signaling in BUC, we also studied the expression of its heterodimeric partner, RXRα, and the cross-talk between them. This study showed that RXRα expression enhances the differentiating role of PPARγ in urothelium and strengthens PPARγ's relationship with early-stage BUC compared to advanced stages. The PPARγ-RXR heterodimer can be activated by ligands of either PPARγ or RXR (permissive type) to cause a synergistic activation [12,13]. Retinoids enhance apoptosis caused from PPARγ ligands when given to preneoplastic lesions [31] or carcinoma cells [32-34].

Based on the observations from our study and the previous relevant knowledge, we propose that PPARγ ligands could be useful chemotherapeutic agents in low grade and early-stage BUC, but probably not as effective in less differentiated and invasive ones. Additional administration of RXR ligands may enhance the anticancer and differentiating effect of PPARγ activation and probably reduce the side effects caused on nonneoplastic cells by PPARγ ligands, as smaller amounts will be needed to achieve the therapeutic result. Nevertheless, additional studies are needed to confirm *in vivo* the applicability of this hypothesis.

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