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The effect of RORα expression on the development of biological malignancy of urinary bladder cancer.

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Abstract

Background. Morbidity and mortality relating to urinary bladder cancer have remained largely unchanged for many years. Similarly, five-year survival rate in this disease has not improved considerably. New developments in individualized therapy necessitate the search for novel factors that could predict the development of malignancy in UBC. In this study, we provide the first evidence that the expression of ROR alpha transcription factor influences the development of malignancy in UBC.

Materials and methods. 105 patients with stage pT1-pT4 urothelial bladder carcinoma who underwent cystectomy were included in the study. 4 µm tissue samples were stained immunohistochemically with a polyclonal anti-RORα antibody. The expression of RORα by the tumor cells (TCs) was assessed by counting TCs with a cytoplasmic and/or nuclear staining for RORα per 1000 TCs. The association between the extent of RORα expression and non-classic differentiation, tumor advancement (pT), grade (G) and regional lymph node spread was analyzed.

Results. The cytoplasmic expression of RORα was detected in near all analyzed tumor samples (104/105). The extent of RORα expression was significantly higher in tumors which were more malignant with more propensity for non-classic differentiation and lymph node metastasis. We noted a lower percentage of TCs expressing RORα in poorly differentiated tumors (G3), compared to tumors moderately and higher differentiated (G1/G2).

Conclusions. Our results suggest that RORα may play a significant role in the progression of urinary bladder cancer. RORα has a broad spectrum of regulatory activity relating to cell and tissue differentiation the mechanism of which is not fully understood. This study represents another step in the process of understanding the mechanisms of RORα regulation and highlights its potential role as a therapeutic target in urothelial bladder cancer.
Keywords: RORα; UBC; urothelial bladder cancer; NDN; non-classic differentiation number; ND; non-classic differentiation; lymphnode status

1. Introduction

Urinary bladder cancer (UBC) is one of the most frequently diagnosed cancers in the world [1] with a four-fold higher incidence rate in men [1–4]. UBC is typically diagnosed in women in more advanced stages and appears to be more aggressive [4, 5]. Morbidity and mortality relating to UBC have not changed significantly for many years. Likewise, five-year survival rates have not improved [4, 6]. Urothelial bladder carcinoma constitutes approximately 90% cases of UBC. Early detection of UBC allows for endoscopic removal of the tumor with transurethral resection of bladder tumor (TURBT) [7]. However, cancer recurrence is frequent recurrence rates of 50% one year after surgical treatment [8]. Disease progression in 10-20% of those cases qualifies the patient for radical cystectomy [9]. In order to reduce the number of radical cystectomies and to personalize treatment regimens it is necessary to search for new prognostic and predictive markers of malignancy in UBC. RORα (retinoid acid related orphan receptor alpha; RORA; NR1F1) belongs to the nuclear receptor family of transcription factors called retinoid-related orphan receptors which includes also RORβ and RORγ. Changes in the expression levels of nuclear receptors are observed in different types of cancers including melanoma, breast cancer or liver cancer [10–13]. RORs have a modular structure including a DNA binding domain (DBD) and a ligand binding domain (LBD) which are distinctive for nuclear receptors. RORs regulate transcription through DBD binding to specific regulatory DNA sequences (ROR Response Elements) in target genes. However, ROR Response Elements are not specific only for RORs. Other nuclear receptors including transcription repressors may compete for the binding sites of the regulatory DNA sequences in an antagonistic manner [14–17]. The presence of LBDs allows for the modulation of ROR
activity by various molecules acting as agonists or antagonists which respectively enhance or suppress the effect of RORs on the transcription of target genes [18, 19]. Accordingly, RORs became an important research area in the biology of cancer due to their potential to become new targets for therapeutic strategies. Some studies demonstrate that RORs play critical roles in the regulation of a variety of physiological processes [20]. The orphan nuclear receptor ROR alpha has been shown to be involved in the control of cell growth and differentiation [21]. There are reports of the role of RORs in breast cancer [22], melanoma [10, 23], hepatocellular carcinoma [24], and colorectal cancer [25]. However, we are not aware of studies involving the role of RORs expression in UBC. In this study, we provide the first evidence that the expression of ROR alpha transcription factor influences the development of pT1-pT4 UBC as defined by histological and clinical indicators of malignancy including tumor advancement (pT), grade (G), number of non-classic differentiation types (NDN) and lymph node spread (pN).

2. Materials and methods

2.1 Study group

105 patients with pT1-pT4 urothelial bladder cancer who underwent radical cystectomy/cystoprostatectomy in from Oncology Centre-Prof. Franciszek Łukaszczyk Memorial Hospital in Bydgoszcz between 2011 and 2014 were included in the study. The median age of patients was 65 years. The mean age was 65 years (64 years in women and 65 years in men). The detailed characteristics of the study group is presented in Table 1. The study was approved by the Bioethics Committee at the Nicolaus Copernicus Univeristy in Torun, Collegium Medicum in Bydgoszcz, Poland (No: KB 587/2018).

2.2 The preparation and assessment of H&E stained samples
Tissue sections were fixed in 10% buffered formalin and embedded in paraffin blocks according to a standard protocol. Tumor advancement (pT stage), histological malignancy (G), frequency of non-classic differentiation (ND) and the number of non-classic differentiation types (NDN) was assessed as previously described [26–29]. Staging and histological type of tumors were assessed according to the WHO TNM classification [30].

2.3 The preparation and assessment of immunostained samples

Immunohistochemical staining was performed on 4 µm tissue sections with the use of polyclonal anti-RORα. Antigen retrieval was performed in a high pH buffer (EnVision Flex+ Target Retrieval Solution, High pH: Bufor Tris/EDTA, pH 9; Dako) in PT Link (Dako) pre-treatment module. Following the blocking of intracellular peroxidase (EnVision Flex, Dako) and blocking of non-specific binding sites (Block Surface Blocker, Candor Bioscience GmbH) the tissue samples were incubated with anti-RORα antibody overnight at 4˚C. Afterwards, the samples were incubated with the secondary antibody (EnVision FLEX/HRP, Dako) for 30 minutes. The staining was visualized after a 5-minute incubation with EnVision FLEX DAB+ Chromogen, Dako, followed by a hematoxylin staining. The samples were assessed using a Nikon Eclipse 80i light microscope. A positive and negative sample of a skin section was included in each staining cycle, with the omission of the RORα antibody for the negative control. The expression of RORα by the tumor cells (TCs) was assessed by counting TCs with a cytoplasmic and/or nuclear staining for RORα per 1000 TCs regardless of the intensity of the staining.

2.4 Statistical analysis

The relationships between TCs expression of RORα and variables such as pT, pN, G and NDN were analyzed using Mann-Whitney-Wilcoxon test. The statistical analyses were
performed using STATISTICA data analysis software (version 13, StatSoft, Inc., Tulsa, OK, USA). A p value <0.05 was considered to be significant.

3. Results

Cytoplasmic expression of RORα was detected in close to all tested tumor samples (104/105) while nuclear expression was noted in only 3 cases.

3.1 Expression of RORα and lymph node status (pN)

In the cases of tumors with lymph node involvement (n=43) a higher percentage of TCs expressing RORα was observed (Figure 1CD), compared to non-metastatic tumors (n=62) (Figure 1AB).

Figure 1

A correlation between the presence of lymph node metastases and the extent of RORα positive TCs was observed. The percentage of RORα positive TCs was significantly higher in tumors presenting lymph node metastases (Figure 2).

Figure 2

3.2 Expression of RORα and NDN

The percentage of TCs expressing RORα is higher in tumors with non-classic differentiation (Figure 3).

Figure 3

In tumors with one or more non-classic differentiation types (NDN>0) the extent of RORα positive TCs was significantly higher compared with tumors with classic type of differentiation (NDN=0) (Figure 4).
3.3 Expression of RORα and grading (G)

We noted a lower percentage of TCs expressing RORα in poorly differentiated tumors (G3), compared to moderately and higher differentiated tumors (G1 and G2).

3.4 RORα expression in neoplastic cells and tumor stage (pT)

There was no significant correlation between RORα frequency and tumor staging (not shown).

4. Discussion

A literature review reveals that RORα is a widely distributed nuclear receptor known for its regulatory activity regarding metabolism, inflammation, angiogenesis or circadian rhythm [17, 31]. Increasingly, retinoid acid receptor-related orphan receptors are being studied in the context of cancer-related processes including gastric cancer, melanoma or colorectal cancer [32]. Fu RD et al. observed that a lowered RORα expression was associated with worse survival prognosis in hepatocellular carcinoma [13]. In our study, RORα was weakly expressed (Figure 1D, Figure 3D) in every analyzed urothelial tumor. A downregulation of RORα expression may be related to the process of neogenesis which led us to the analyze the extent of RORα expression within the tumor. We observed that the percentage of TCs expressing RORα was notably higher in metastatic tumors compared to tumors without the involvement of regional lymph nodes (Figure 2). Gain of the ability to metastasize is considered to be the last stage in the progression of urothelial cancer and identifies a highly
malignant tumor capable of forming secondary distant tumor sites [33]. The broad extent of weakly-expressed RORα may indicate a deficit of the transcription factor or perhaps insufficient efficiency of cellular repair systems. We have not found a sufficient explanation of that problem in the available literature. Further research aimed at the identification of RORα-regulated metabolic pathways that result in increased metastasis are necessary.

Considering the established role of RORα in the processes of cell growth and differentiation [34], the role of RORα warrants investigation in the context of its impact on increased malignant potential of UBC and its histological differentiation. Xiong G et al. observed a suppression of the invasive capacity of breast cancer through RORα [22]. In our study, we showed that a large extent of weakly-expressed RORα was associated with tumors displaying non-classic types of differentiation (Figure 3CD, Figure 4) which is a marker of increased malignancy in UBC tumors [27, 28]. Thus, the influence of RORα on non-classic differentiation cannot be dismissed yet it has not been explored in literature to date. Likewise, the results showing a correlation between RORα expression and histological dedifferentiation in UBC are novel finding. The data obtained in this study show a statistically significant difference in RORα expression between moderately differentiated (G2) and poorly differentiated (G3) tumors. We also demonstrated that lower histological malignancy was associated with a higher extent of RORα expression (Figure 5), resulting probably from insufficiency of RORα depended cellular repair systems. The biology of UBC is still poorly understood. Therefore, research on the mechanisms of promotion and progression of UBC is highly necessary. RORα is an interesting potential therapeutic target in various tumors thanks to its ability to be influenced by various agonists and antagonists binding to its ligand binding domain [35, 36]. This study reinforces that notion demonstrating a potential role of RORα in influencing the malignancy of UBC.

5. Conclusions
The observed association between the extent of RORα expression and malignancy of urothelial bladder carcinoma may suggest an important role of that receptor in the progression of UBC. As a transcription factor, RORα displays a broad range of regulatory effects involving cell and tissue differentiation. The mechanisms of regulation by RORα are not yet fully understood. This study represents another step in the process of understanding those mechanisms and highlights a potential role of RORα as a therapeutic target in urothelial bladder cancer.

6. Grant support

This study was supported in part by funds for statutory research from Collegium Medicum, Nicolaus Copernicus University.

7. Conflicts of interest

The authors declare no conflicts of interest.

8. List of abbreviations

TCs – tumor cells

UBC – urothelial bladder cancer

ND – non-classic differentiation

NDN – non-classic differentiation number

9. References


### Table 1. Patient clinical and pathological characteristics

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11. Figures

Figure 1. Atypical morphology of a non-metastatic tumor in H&E staining (A, 100x) is associated with none or few RORα-positive tumor cells (B, 40x). A high percentage of RORα-positive TCs (D, 40x) identifies metastatic tumors with atypical morphology in H&E staining (C, 40x). Arrows indicate RORα+ TCs.
Figure 2. The percentage of RORα positive TCs was about 30% higher in metastatic tumors in comparison with non-metastatic tumors.
Figure 3. A classic differentiation (A, 100x) is associated with a low frequency of tumor cells expressing RORα (B) (case with NDN=0, 40x). A higher frequency of RORα+ tumor cells (D, 40x) can be observed in tumors with at least one non-classic differentiation type (C) (case with NDN>0, 100x).
Figure 4. The percentage of RORα positive TCs was about 30% higher in NDN>0 tumors in comparison with tumors of only classic type of differentiation.
Figure 5. The percentage of RORα positive TCs was about 20% lower in G3 tumors in comparison with tumors less dedifferentiated (G2).