Original Article

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Prognostic Value of the mRNA Expression of Members of the Toll-like Receptor Family in Clear Cell Renal Cell Carcinoma

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Abstract

Objective: The aim of this study was to examine *TLRs* expression in tumoral and non-tumoral kidney tissue in patients with clear cell renal cell carcinoma (ccRCC) and to evaluate the prognostic significance of *TLRs* expression profile in ccRCC.

Materials and Methods: *TLR* 1-10 mRNA expressions were measured by real-time polymerase chain reaction (RT-PCR) in formalin-fixed paraffin-embedded (FFPE) 23 ccRCC tumoral tissue samples and 23 non-tumoral kidney tissue samples.

Results: A total of 46 individuals were included in the study. None of the patients had rhabdoid or sarcomatoid features. Lymphovascular invasion was observed in only three patients. RT-PCR analyses revealed *TLRs* mRNA expressions in 23 ccRCC samples and 23 non-tumoral FFPE kidney tissue samples. TLR (*TLR1*-10) mRNA expression was significantly increased in FFPE ccRCC tissues according to RT-PCR results ($p<0.05$).

Conclusion: The results demonstrated that *TLRs* might have function in ccRCC pathogenesis. This present study will shed light on research to understand the role of the *TLR* gene family expression in tumor progression of ccRCC.

Keywords: RCC, *TLR*, mRNA expression

Introduction

Renal cell carcinoma (RCC) is the most common type of kidney cancer worldwide with an increasing incidence. During the diagnosis process, one third of the patients have metastasized and half of the remaining patients will experience a recurrence after treatment (1). Clear cell RCC (ccRCC) is the most frequent pathological subtype, representing approximately 70% of RCC cases (2). ccRCC has a poor prognosis with low response rates to conventional therapies such as chemotherapy (3).

Studies have shown that the most important genetic alteration in ccRCC is the loss of function of *von Hippel-Lindau tumor suppressor* (*VHL)* gene. In 90% of sporadic ccRCC, one copy of *VHL* is mutated, while another copy is lost through 3p deletions (4). According to The Cancer Genome Atlas, ccRCC is characterized by recurrent mutations in *PI3K/AKT/MTOR* (5). Tumor microenvironment has an important role in many processes observed in tumor progression, such as immune-escaping, chemotherapy resistance and metastasis. Recently, studies related to genetic changes in Toll-like receptors (*TLRs*) that recognize danger-associated molecular patterns derived from cancer cells in tumor microenvironment are increasing rapidly.

TLRs are a conserved family of receptors capable of recognizing pathogenic structures known as pathogen-associated molecular patterns (6). Until today, 13 *TLR* analogues have been identified in mammals, *TLR*11, 12 and 13 are not expressed in humans but are functional only in mice (7). They are located on the cell surface or on endosomes within the cell. Although endosomal *TLRs* primarily detect viral and bacterial nucleic acids, surface *TLRs* such as *TLR*2 and *TLR*4 primarily recognize bacterial proteins (8). *TLRs* are mainly expressed in immune system cells such as macrophages and DCs, and are key sensors of pathogen invasion (9). Recent data suggest that functional *TLRs* are

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expressed not only in immune system cells but also in cancer cells (10). Damage-associated molecular patterns derived from damaged normal epithelial cells and necrotic cancer cells are found in the tumor microenvironment, and these patterns are thought to stimulate chronic inflammation by inducing specific *TLR*s (11,12). However, the expression patterns of *TLR*s in human cancer tissues are largely unknown. To our knowledge, there is no previous study of *TLR*s (*TLR*1-10) mRNA expression in ccRCC.

Thus, the aim of this study was to investigate *TLR*1-10 expression in non-tumoral kidney tissue and tumoral tissue in patients with RCCs and to evaluate the prognostic significance of *TLR*s expression profile in ccRCCs.

Materıals and Methods

Study Samples

Twenty-three tumoral ccRCC and 23 non-tumoral kidney tissue nephrectomy specimens were provided by İstanbul Gaziosmanpaşa Hospital. Patients were histopathologically diagnosed as having ccRCC at our hospital between 2007 and 2017. Cases with cystic RCC were excluded from our study since this subtype of RCC is composed of hypocellular tumor areas.

All patients were staged based on the Union for International Cancer Control Tumor-Node-Metastasis classification. The retrospective study design was approved by the Institutional Review Board (2017-KAEK-189_2018.10.10_02).

Tumor Selection

The histopathological slides stained with Hematoxylin-Eosin (H&E) were microscopically examined to select paraffin embedded blocks with preserved, viable tumor tissue comprising over 90% of the block. The tumor area was marked and cut. Areas containing necrosis and hemorrhage were excluded from the study. Two pieces of 10-µm-thick sections were cut from each selected paraffin block.

RNA Extraction and cDNA Synthesis

Total RNA from 10 µm Formalin-Fixed Paraffin-embedded (FFPE) sections was isolated using High Pure FFPET RNA isolation kit according to the manufacturer's instructions (Roche Diagnostics, Mannheim, Germany). Total RNA concentrations were measured and 1 µg RNA was used as a template for the synthesis of complementary DNA (cDNA) using Transcriptor First Strand cDNA Synthesis Kit (Roche Diagnostics, Mannheim, Germany). The cDNAs were stored at -80°C until used as a template in real-time quantitative PCR.

Real-time Quantitative PCR

Real-time PCR analyses were performed using quantitation of *TLR*1-10 genes and an internal reference gene (*β-actin*) at mRNA level using the LightCycler 480 platform (Roche Diagnostics). PCR primers and Universal Probe Library probes for *TLR*1-10 and internal reference gene (*β-actin*) are presented in Table 1. The final reaction volume for the analysis of *TLR*s expression was 20 µ; 1 µL from each primer and probe set, 4 µ of ×5 LightCycler TaqMan Master Mix, 2 µ cDNA sample, and 13 µ PCRgrade water. The cycle conditions were 95°C for 10 minutes, followed by 45 cycles at 95°C for 10 seconds, 60°C for 30 seconds, and 72°C for one second. All runs included one negative cDNA control consisting of DNase- and RNase-free water. The housekeeping β-actin gene was used as a control to normalize expression of each gene and the final results were obtained with LightCycler 480 software.

Statistical Analysis

SPSS 18 package program was used for statistical analysis. Non-parametric statistical methods were used to determine the differences between the groups. Mann-Whitney U test was used for variables with two groups and Kruskal-Wallis H test was used when the number of groups was more than two. Values were expressed as mean ± standard deviation. $p< 0.05$ was considered statistically significant.

Table 1. Primers and UPL probes used for real-time gene expression analysis (5′®3′)

Primer sequences	UPL number
TLR1	
CCTAGCAGTTATCACAAGCTCAAA (Forward) CCTTGGGCCATTCCAAATA (Reverse)	#79 (04689020001)
TLR2	
GGCCAGCAAATTACCTGTGTG (Forward) AGGATCAGCAGGAACAGAGC (Reverse)	#56 (04688538001)
TLR3	
GTGGCCCTTAAAAATGTGGA (Forward) GTGTTTCCAGAGCCGTGCTAA (Reverse)	#151 (04694376001)
TLR4	
TCATTGTCCTGCAGAAGGTG (Forward) TCC CAC TCC AGG TAA GTG TT (Reverse)	#62 (04688619001)
TLR5	
TGAGGGACTTTCTCATCTTCAAGT (Forward) CCTTAATGCAGTCAGATGGCTA (Reverse)	#31 (04687647001)
TLR6	
TTTGGATTTATCTCATAATCAGTTGC (Forward) GATCTAAATGCCTGAAACTCACAA (Reverse)	#121 (04693558001)
TLR7	
GTCTAAAGAACCTGGAAACTTTGG (Forward) TCTCAGGGACAGTGGTCAGTT (Reverse)	#102 (04692209001)
TLR8	
CAGAATAGCAGGCGTAACACATCA (Forward) TGTTGTCATCATCATTCCACAA (Reverse)	#59 (04688562001)
TLR9	
CTGGGACCTCTGGTACTGCT (Forward) CTGCGTTTTGTCGAAGACCA (Reverse)	#98 (04692152001)
TLR10	
TGTCACCATTGTGGTTATTATGC (Forward) GCAGATCAAAGTGGAGACAGC (Reverse)	#76 (04688996001)
β-actin	
ATTGGCAATGAGCGGTTC (Forward) CGTGGATGCCACAGGACT (Reverse)	#11 (04685105001)
UPL: Universal ProbeLibrary	

Results

A total of 46 individuals were included in the study. The mean age of the ccRCC group (six female and 17 male) and the control group (nine female and 14 male) was 58.4±7.5 years (range, 48-72 years) and 56.3±6.9 years (range, 45-70 years), respectively. None of the patients had rhabdoid/sarcomatoid features. Lymphovascular invasion was observed in only three patients. The tumor characteristics are summarized in Table 2.

Table 2. Tumor characteristics of ccRCC patients

Characteristics	Number of patients (%)
Gender	
Female	6 (26)
Male	17 (74)
Affected side	
Right	12 (52)
Left	11 (48)
Pathological grade	
Grade I Grade II Grade III Grade IV	2 (8) 7 (30) 13 (56) 1 (6)
pT stage	
pT1a	7 (30.4)
pT1b	8 (34.7)
pT2a	1 (4.5)
pT3a	7 (30.4)
Tumor size	
<5	8 (34.7)
≥5	15 (65.3)
LN involvement	
Nx	5 (21.7)
N0 N1	17 (74) 1 (4.3)
Capsular infiltration	
Negative	15 (65.3)
Positive	8 (34.7)
Lymphovascular infiltration	
Negative	20 (87)
Positive	3 (14)
Perirenal infiltration	
Negative	19 (82.6)
Positive	4 (17.4)
Renal sinus infiltration	
Negative	19 (82.6)
Positive	4 (17.4)
Necrosis	
Negative	16 (69.5)
Positive	7 (30.5)

ccRCC: clear cell renal cell carcinoma, LN: Lymph node

TLR (*TLR*1-10) mRNA expression was significantly increased in FFPE ccRCC tissues according to real time PCR results ($p<0.05$) (Figure 1). There was no significant relationship between *TLR* mRNA expression and tumor localization (right vs left kidney), tumor size, pT-class, capsular invasion, renal sinus invasion and necrosis ($p>0.05$). *TLR*5 overexpression in ccRCC tissue samples showed a significant association with tumor grade III ($p=0.028$). In addition, a negative correlation was found between *TLR*1-4-7-9 expression and perirenal invasion, respectively ($p=0.023$, $p=0.041$, $p=0.041$, $p=0.031$). *TLR2* overexpression in ccRCC tissue samples showed a significant association with Nx category ($p=0.044$).

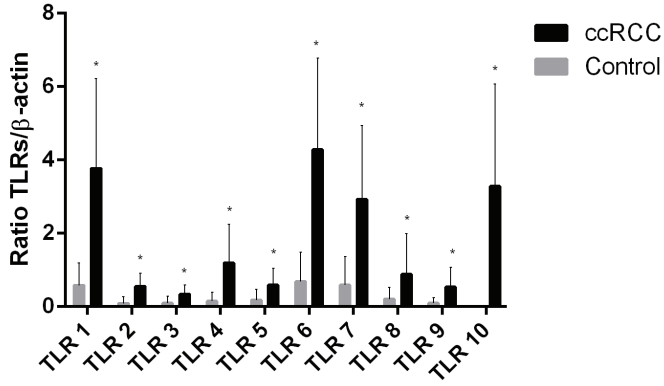

Figure 1. mRNA expression level of TLRs in FFPE kidney samples. All TLR mRNA expression was followed by real-time PCR and the results were normalized to β-actin mRNA. Values were expressed as mean ± standard deviation. Asterisks (*) denote significant differences ($p<0.05$)

ccRCC: Clear cell renal cell carcinoma

Discussion

It is believed that *TLR*s play important roles in innate immunity; and chronic inflammation is one of the vital events in carcinogenesis. *TLR*s are expressed in macrophages, natural killer cells (NK), dendritic cells (DCs) and T cells. Today, it is known that *TLR*s are also expressed in cancer cells (13). For this reason, it is considered that *TLR* gene expression profiles may be important markers in cancer development and progression.

Here we demonstrated for the first time that *TLR*1-10 mRNA is frequently expressed in FFPE kidney tissues in patients with ccRCCs. The expression of *TLR* 1-10 mRNA was significantly increased in RCC patients compared to the control group. There were differences in *TLR* expression in many cancers (14). *TLR* expression studies in ccRCC are quite limited in the literature. In the current study, *TLR*5 overexpression in ccRCC tissue samples showed a significant association with tumor grade III. Similarly, it was suggested that *TLR*5 expression had become more diffuse during the progression to dysplasia (15). Further studies are needed to clarify the true role of *TLR*5 expression in ccRCC.

Immunogenic processes are effective in the pathogenesis of RCC and RCC may affect different effector cells of the natural and adaptive immune system including NK cells, DCs and various T cells (16). Failure in desired full response to

target-specific therapies has revived the interest in immune modulation in RCC treatment. The importance of the role of the immune response to RCC was understood when it was shown that metastatic lesions might regress spontaneously (17). Additionally, it was confirmed that there was a complex interaction between the tumor and host immune response with demonstration of increase in cytokines (18) and chemokines (19) as well as tumor-infiltrating lymphocytes (20) in circulation in patients with RCC.

Low levels of oxygen in the cellular environment occur in many pathophysiological conditions such as infection, inflammation, and solid tumor development (21). The relation of tumor microenvironment with RCC development can be explained especially with the production of proangiogenic factors, which end up with the hyperactivation of Hypoxia-Inducible Factor 1 (*HIF-1*) in lesions with VHL mutations (22). In one study, it was reported that *TLR*2 and *TLR*6 expressions were increased in hypoxia (23). Morikawa et al. showed that *TLR*3 expression was increased in ccRCC patients compared to the control group (24). For this reason, probably, some cytokines produced by cancer cells or by infiltrating immune cells may induce the *TLR*3 expression in ccRCCs.

TLR expression profiles have been investigated in many types of cancer. Some studies have shown that TLRs inhibit tumor growth, while others have indicated that they enhance tumor progression. In a study, Bednarczyk found that three proteins namely Dual specificity protein phosphatase 2, Interferon gamma and Eukaryotic initiation factor 4A-I (DUSP2, IFNγ, EIF4A1) were associated with TLR system, which differentiate early stages of colorectal cancer from healthy tissue (25). TLRs also play a critical role in the induction of colitis, which in consequence can lead to cancer. One study reported that chronic stress could increase the expression of TLR in the colonic mucosa (26). Furthermore, ovarian cancer cells showed overexpression of TLR2, TLR3, TLR4, and TLR5 (27,28), while there was a high expression of TLR5 and TLR9 in human cervical cancer (29). The results underline the role of pathways associated with TLR activation in the pathogenesis of several cancers.

In line with these studies, *TLR*3 mRNA expression was found to be higher in ccRCC patients compared to the control group in our study. In a study that was conducted with the immunohistochemical technique by Wang et al.(30), it was reported that *TLR*4 expression was increased in RCC tissues compared to neighboring normal tissues. In another study that was conducted on RCC FFPE tissues, it was shown that *TLR*9 was associated with good prognosis and that low *TLR*9 expression was associated with short-term survival (1). In the present study, *TLR*1-4-7-9 expressions were increased in patients with no perirenal invasion, which is an aggressive clinicopathological parameter for ccRCC.

Study Limitations

The main limitation of our study was the small number of patients. In addition to the expression of mRNA, it would be appropriate to show protein expression in these tissues.

Conclusion

As a result, *TLR* mRNA expressions were significantly increased in ccRCC FFPE tissues compared to non-tumoral tissue samples. It is important to elucidate the potential mechanisms underlying the formation and progression of ccRCC to facilitate the identification of new prognostic markers and development of promising targeted strategies. For this reason, determining *TLR* protein expressions as well as mRNA expression and comparing these data with clinicopathological data in more patients will reveal the role of the changes in expression of *TLR* genes in ccRCC pathogenesis.

Ethics

Ethics Committee Approval: The retrospective study design was approved by the Institutional Review Board (no: 2017-KAEK-189_2018.10.10_02).

Informed Consent: Retrospective study.

Peer-review: Internally and externally peer-reviewed.

Authorship Contributions

Concept: S.S.Ö., P.H., S.Y., Design: S.S.Ö., Data Collection or Processing: S.S.Ö., P.H., Analysis or Interpretation: S.S.Ö., P.H., S.Y., Literature Search: S.S.Ö., Writing: S.S.Ö.

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