

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, TLR11, 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 TLR2 and TLR4 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

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

Abstract

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

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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 TLRs (11,12). However, the expression patterns of TLRs in human cancer tissues are largely unknown. To our knowledge, there is no previous study of TLRs (TLR1-10) mRNA expression in ccRCC.

Thus, the aim of this study was to investigate TLR1-10 expression in non-tumoral kidney tissue and tumoral tissue in patients with RCCs and to evaluate the prognostic significance of TLRs expression profile in ccRCCs.

Materials and Methods

Study Samples

Twenty-three tumoral ccRCC and 23 non-tumoral kidney tissue nephrectomy specimens were provided by Istanbul Gaziosmanpaşa Hospital. Patients were histopathologically diagnosed as having ccRCC at our hospital between 2007 and 2017. Cases with cystic ccRCC 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 FFPE 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 TaqMan gene expression analysis (5®3’) assays that were prepared using TaqMan gene expression analysis (5®3’) assays that were conducted on a LightCycler 480 platform (Roche Diagnostics). Primer sequences and UPL probes used for real-time gene expression analysis (5®3’) are presented in Table 1. The final reaction volume for the analysis of TLRs expression was 20 μL; 1 μL from each primer and probe set, 4 μL of 5 LightCycler TaqMan Master Mix, 2 μL cDNA sample, and 13 μL 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     | UPL number                      |
| TLR1                            | CCTAGCAGTATCTACAAGCTCAAA (Forward) | #79 (04689020001) |
| TLR2                            | CCTTGGGGCCATTCCAATA (Reverse)    | #56 (04688538001) |
| TLR3                            | GGCAGCAAAATTACCTGTGTG (Forward)  | #56 (04688538001) |
| TLR4                            | GTGGCCCTTTAAAAATTGTGA (Forward)  | #151 (04694376001) |
| TLR5                            | TCATTGTCTGACAGGAGGT (Forward)    | #62 (04688619001) |
| TLR6                            | TGAGGAGCTTTTCTCATCTCAAGT (Forward) | #31 (04687647001) |
| TLR7                            | TTGGATTTACACTATAACCTTGC (Forward) | #121 (04693558001) |
| TLR8                            | GATCTAAATGGCTAGATGCTAC (Reverse) | #102 (04692209001) |
| TLR9                            | CAGATAGCGTCTAACAACATCA (Forward) | #59 (04688562001) |
| TLR10                           | CTGGGAACCTTGTGACTGCT (Forward)   | #98 (04692152001) |
| TLR11                           | GTCAACCACTGCTGACTGCT (Forward)   | #76 (04688996001) |
| TLR12                           | ATGGCGACATGCGCGTTCC (Forward)    | #11 (04685105001) |
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>
<thead>
<tr>
<th>Characteristics</th>
<th>Number of patients (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gender</td>
<td></td>
</tr>
<tr>
<td>Female</td>
<td>6 (26)</td>
</tr>
<tr>
<td>Male</td>
<td>17 (74)</td>
</tr>
<tr>
<td>Affected side</td>
<td></td>
</tr>
<tr>
<td>Right</td>
<td>12 (52)</td>
</tr>
<tr>
<td>Left</td>
<td>11 (48)</td>
</tr>
<tr>
<td>Pathological grade</td>
<td></td>
</tr>
<tr>
<td>Grade I</td>
<td>2 (8)</td>
</tr>
<tr>
<td>Grade II</td>
<td>7 (30)</td>
</tr>
<tr>
<td>Grade III</td>
<td>13 (56)</td>
</tr>
<tr>
<td>Grade IV</td>
<td>1 (6)</td>
</tr>
<tr>
<td>pT stage</td>
<td></td>
</tr>
<tr>
<td>pT1a</td>
<td>7 (30.4)</td>
</tr>
<tr>
<td>pT1b</td>
<td>8 (34.7)</td>
</tr>
<tr>
<td>pT2a</td>
<td>1 (4.5)</td>
</tr>
<tr>
<td>pT3a</td>
<td>7 (30.4)</td>
</tr>
<tr>
<td>Tumor size</td>
<td></td>
</tr>
<tr>
<td>&lt;5</td>
<td>8 (34.7)</td>
</tr>
<tr>
<td>≥5</td>
<td>15 (65.3)</td>
</tr>
<tr>
<td>LN involvement</td>
<td></td>
</tr>
<tr>
<td>Nx</td>
<td>5 (21.7)</td>
</tr>
<tr>
<td>N0</td>
<td>17 (74)</td>
</tr>
<tr>
<td>N1</td>
<td>1 (4.3)</td>
</tr>
<tr>
<td>Capsular infiltration</td>
<td></td>
</tr>
<tr>
<td>Negative</td>
<td>15 (65.3)</td>
</tr>
<tr>
<td>Positive</td>
<td>8 (34.7)</td>
</tr>
<tr>
<td>Lymphovascular infiltration</td>
<td></td>
</tr>
<tr>
<td>Negative</td>
<td>20 (87)</td>
</tr>
<tr>
<td>Positive</td>
<td>3 (14)</td>
</tr>
<tr>
<td>Perirenal infiltration</td>
<td></td>
</tr>
<tr>
<td>Negative</td>
<td>19 (82.6)</td>
</tr>
<tr>
<td>Positive</td>
<td>4 (17.4)</td>
</tr>
<tr>
<td>Renal sinus infiltration</td>
<td></td>
</tr>
<tr>
<td>Negative</td>
<td>19 (82.6)</td>
</tr>
<tr>
<td>Positive</td>
<td>4 (17.4)</td>
</tr>
<tr>
<td>Necrosis</td>
<td></td>
</tr>
<tr>
<td>Negative</td>
<td>16 (69.5)</td>
</tr>
<tr>
<td>Positive</td>
<td>7 (30.5)</td>
</tr>
</tbody>
</table>

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

Discussion

It is believed that TLRs play important roles in innate immunity; and chronic inflammation is one of the vital events in carcinogenesis. TLRs are expressed in macrophages, natural killer cells (NK), dendritic cells (DCs) and T cells. Today, it is known that TLRs 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 TLR1 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). TLR5 overexpression in ccRCC tissue samples showed a significant association with tumor grade III (p = 0.028). In addition, a negative correlation was found between TLR1-4-7-9 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).
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 TLR2 and TLR6 expressions were increased in hypoxia (23). Morikawa et al. showed that TLR3 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 TLR3 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 Z, 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, TLR3 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 TLR4 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 TLR9 was associated with good prognosis and that low TLR9 expression was associated with short-term survival (1). In the present study, TLR1-4-7-9 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**


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**References**


