The Association of piR-36707 and piR-36741 with Clear Cell Renal Cell Carcinoma

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Abstract

Background: Clear cell renal cell carcinoma (ccRCC) is a malignant entity that develops from the renal tubular epithelium. piR-36707 and piR-36741 have not previously been associated with ccRCC and their potential roles in the pathogenesis of ccRCC are mystery.

Methods: In the present study, formalin-fixed paraffin embedded (FFPE) specimens were obtained from 19 patients having ccRCC diagnosis. Then, the expression levels of piR-36707 and piR-36741 were analyzed by Real-Time PCR with respect to clinical parameters.

Results: The expression levels of piR-36707 and piR-36741 were found higher in tumor samples than control ones. Also, piR-36707 expression showed significantly different pattern in terms of gender. Moreover, piR-36707 and piR-36741 expressions displayed statistically significant difference with respect to some TNM staging comparisons. piR-36707 and piR-36741 expressions were significantly differed between Fuhrman nuclear grade 2 and 4. Furthermore, piR-36741 expression change was significantly associated with the changes between low and normal RBC counts of ccRCC patients. Similarly, piR-36707 expression was significantly associated with hemoglobin count change of ccRCC patients.

Conclusion: According to the literature, this is the first study that relates ccRCC pathogenesis with piR-36707 and piR-36741. Prospectively, all genes in target of piR-36707 and piR-36741 should be studied for the pathogenesis of ccRCC.

Keywords
ccRCC, piRNA, piR-36707, piR-36741, Expression analysis

Abbreviations
RCC: Renal Cell Carcinoma; ccRCC: Clear Cell RCC; piRNAs: Piwi Interacting RNAs; ncRNA: Non-Coding RNA; FFPE: Formalin-Fixed Paraffin Embedded; ISUP: International Society of Urological Pathology; RT-PCR: Real Time PCR; mt: Mitochondrial; TNM: Tumor Node Metastasis; RBC: Red Blood Cell

Introduction

Renal cell carcinoma (RCC) consists of a group of heterogeneous cancer originating from renal tubular cells. Among the urological cancers, RCC, the third most common cause of death after prostate and bladder cancer, accounts for about 2% of all adult cancer cases. In addition, the clinical course of urological cancers is the most mortal. The incidence of RCC is geographically different in developed countries, especially in males. Although it is not known exactly why men are more prevalent in developed countries, genetic factors may be associated with environmental factors such as smoking and working conditions [1].

Studies aimed at understanding the molecular mechanisms underlying the development of clear cell RCC (ccRCC) contribute to the adoption of more...
rational approaches to treatment. With each passing day, new information on the oncogenesis of ccRCC is introduced and the therapies for these defined molecules are being developed rapidly. Grade and stage are the most important prognostic factors in ccRCC. Apart from these, many anatomical, microscopic, cytogenetic and molecular markers have been studied and these determinants can be used in the future. However, the value of these biomarkers is not proven by adequate clinicopathological studies at present. Detection of genetic disorders in kidney tumors will lead to correct diagnosis. In the near future, combining clinical, anatomical, pathological data with molecular determinants can lead to better prognostic information and better individual treatment [2].

One of the most recent and known silencing mechanisms induced by RNA is silencing by piwi interacting RNAs (piRNAs). The regulation of gene expression through piRNA is a new research topic that is widely available in today’s scientific community. To date, few piRNA studies have been performed. In these studies, the mechanisms of regulation of genes targeted by piRNAs have been investigated and the levels of piRNA expressions have been studied in certain diseases, but information on the regulation of piRNA expression is still insufficient [3].

piRNAs are the largest class of small non-coding RNA molecules expressed in animal cells. piRNA interacts with piwi proteins to form RNA-protein complexes. These complexes are associated with epigenetic and post-transcriptional gene silencing of retrotransposons and other genetic elements in germ line cells, particularly during spermatogenesis. They differ from microRNAs in size, lack of sequence protection, and increased complexity. How piRNAs are produced remains unclear, but biogenesis pathway differs from miRNA and siRNA. piRNAs, which are members of the non-coding RNA (ncRNA) group, consist of intergenic repetitive elements in the genome [3].

piRNAs and the proteins interacted with them have the function of both transcriptional and post-transcriptional gene silencing. Expressions of some piRNAs in specific cancers have been found to have increased oncogenic properties, while others have decreased expression and have tumor suppressor properties. For example, piR-651 and piR-823 have been shown to exhibit oncogenic properties in many types of cancer. Considering our study, piR-36707 and piR-36741 were not previously associated with any type of cancer. According to one study, piR-36707 and piR-36741 were expressed in the blood stably and if they are associated with transparent cell RCC, they may be the ideal molecule to be biomarker due to these properties [4]. In this way, it is aimed to determine the new candidate molecules that may play a role in the pathology and progression of the disease.

The aim of the present project is to measure the expression levels of two piRNAs (piR-36707 and piR-36741), which have not been previously associated with ccRCC, in the tumor and surrounding normal renal biopsy specimens taken from patients with ccRCC, and to investigate the association of the expression levels of them with patients’ clinicopathological parameters.

**Materials and Methods**

**Patient selection**

Nineteen patients applying to Urology Clinic, Faculty of Medicine, Ondokuz Mayis University between June 2016 and June 2017 for the first time and diagnosed with ccRCC were included in the study. The number of patients to be included in the study was detected with 80% test power and 95% confidence interval. The statistically significant number of patients was calculated as at least 19. Demographic and clinicopathological characteristics of patients are presented in Table 1.

Histopathological examination was performed on tumor tissue and some of the healthy kidney tissues around this tumor resected by radical nephrectomy. Formalin-fixed, paraffin embedded (FFPE) tissues were stored as archival tissues and kept in room temperature at Department of Pathology. The results of histopathological and radiological examinations of the patients were used for diagnosis of ccRCC. Vancouver Renal Neoplasia Classification (2012) of the International Society of Urological Pathology (ISUP) was used for the histological classification of renal tumors of the patients [5].

**RNA isolation from FFPE tissues**

Before RNA isolation, an expert kidney pathologist discerned tumor tissue from non-tumor kidney parenchyma based on hematoxylin/eosin (H&E) staining. Excess paraffin over the tumor and surrounding healthy kidney FFPE tissue specimens of each subjects in the archive was removed. Then, 3-4 sections of 5 μm thickness were taken using Microtome (Leica Microsystems, Nussloch, Germany). miRNeasy FFPE Kit (Qiagen GmbH, Hilden, Germany) was used for total RNA isolation from FFPE tissue sections. RNA isolation was performed according to the instructions included in the kit [5]. The ratio of absorbance at 260 nm and 280 nm were measured to assess the purity of RNA. A ratio of ~2.0 was accepted as “pure” for RNA.

**cDNA synthesis and measurement of cDNA concentration**

RNA samples obtained were converted to cDNA by reverse transcription using Ipsogen RT Kit (Qiagen GmbH, Hilden, Germany). cDNAs were stored at -20 °C until real time PCR (RT-PCR) was performed. Spectrophotometric method was used to determine quality and concentration of cDNA samples obtained.

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**Table 1.**

<table>
<thead>
<tr>
<th>Patient Data</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sex</td>
<td>Male/Female</td>
</tr>
<tr>
<td>Age</td>
<td>Minimum/Maximum</td>
</tr>
<tr>
<td>Tumor Size</td>
<td>Small/Large</td>
</tr>
<tr>
<td>Stage</td>
<td>I/II/III/IV</td>
</tr>
<tr>
<td>Location</td>
<td>Cortex/Medulla</td>
</tr>
</tbody>
</table>

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The table above shows the demographic characteristics of the patients included in the study. The data are presented as median (range).

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**Table 2.**

<table>
<thead>
<tr>
<th>piRNA</th>
<th>Expression Level</th>
</tr>
</thead>
<tbody>
<tr>
<td>piR-36707</td>
<td>High/Medium/Low</td>
</tr>
<tr>
<td>piR-36741</td>
<td>High/Medium/Low</td>
</tr>
</tbody>
</table>

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The table above shows the expression levels of piRNAs in the tumor tissue and surrounding normal renal tissue of the patients included in the study. The data are presented as mean ± standard deviation.
before RT-PCR experiments. This analysis was performed using a Microplate Spectrophotometer (Multiscan GO, Thermo Scientific, USA).

**Gene expression analysis**

RT-qPCR method was used for gene expression analysis and Rotor Gene Q (Qiagen GmbH, Hilden, Germany) was used for this purpose. The piRNA-specific primer sequences ([GenBank: DQ598641] (piR-36707), 5′-GTGAGATGGGAGGGCCGG-3′; [GenBank: DQ598675] (piR-36741), 5′-GTGATACGGCGCTCACATC-3′) and universal reverse primer (Qiagen GmbH, Hilden, Germany) were used. The piRNA expression was normalized to the small RNA U6B [7]. RT2 SYBR® Green qPCR Mastermix (Qiagen GmbH, Hilden, Germany) was used as pre-mix for gene expression analysis.

**Statistical analysis**

Tumor samples from patients with RCC were compared to healthy tissue on the periphery of tumor tissue of the same patient. Using Ct values obtained from these tissues, the expression levels of the respective piRNAs were compared statistically. In the method based on partial quantities, the measured expression values of piRNAs were normalized by small RNA U6B transcription. For the comparison, Ct values obtained and $2^{-\Delta\Delta C_t}$ formula were used (Formula 1).

**Formula 1. $2^{-\Delta\Delta C_t}$ calculation**

$$2^{-\Delta\Delta C_t} = 2^{[-(Ct_{Tumor} - Ct_{Reference}) - (Ct_{Control} - Ct_{Reference})]}$$

$2^{-\Delta C_t}$ formula was used to calculate piRNA expression levels separately for the tumor and surrounding healthy kidney tissues (Formula 2). Using this formula, the fold changes in the expression of piRNAs in tumor and surrounding healthy kidney tissue relative to the reference were calculated separately. Then, a statistical analysis of significance of the fold changes in piRNA expression levels obtained from this formula was performed.

**Formula 2. $2^{-\Delta C_t}$ calculation**

$$2^{-\Delta C_t} = 2^{[-(Ct_{Tumor} - Ct_{Reference}) - (Ct_{Control} - Ct_{Reference})]}$$

All statistical analyzes were performed using SPSS 21 program (IBM software, Pointe Claire, Quebec, Canada). Normal distribution of data was evaluated statistically by Kolmogorov-Smirnov test. Wilcoxon Signed Rank Test was used in binary comparisons and Kruskal Wallis Test was applied in multi-comparisons. $P < 0.05$ was accepted as a statistically significant value and the evaluation was made at 0.95 confidence interval.

**Results**

In the present study, firstly, the expression levels of two piRNAs, piR-36707 and piR-36741, compared between tumor and neighboring healthy renal biopsy specimens taken from patients with ccRCC. According to RT-qPCR results, the expression levels of piR-36707 and piR-36741 were detected higher in tumor samples than control ones, but these increases were not statistically significant ($p = 0.136$ and $p = 0.265$, respectively) (Figure 1).

Then, the expression levels of piR-36707 and piR-36741 correlated with age, gender, TNM staging, Fuhrman nuclear grade, RBC count and hemoglobin count (Table 1). As to the results, piR-36707 expression showed statistically significant correlation with the
gender (p = 0.006), but piR-36741 not. piR-36707 was found significantly higher in females than males. Moreover, piR-36707 and piR-36741 expressions displayed statistically significant difference with respect to some TNM staging comparisons (for piR-36741, p = 0.01 between stage I and II, and p < 0.001 between stage I and IV; for piR-36707, p = 0.037 between stage I and IV, p < 0.001 between stage II and IV). Both piR-36707 and piR-36741 expressions were found statistically higher in higher TNM stages. With respect to Fuhrman nuclear grade, piR-36707 and piR-36741 expressions were significantly higher in grade 4 than grade 2 (p < 0.001 and p = 0.036, respectively). Furthermore, piR-36741 expression was significantly higher in ccRCC patients having normal RBC counts when compared to ones having low RBC counts (p = 0.042). Similarly, piR-36707 expression was significantly higher in ccRCC patients having low hemoglobin count when compared to ones having high hemoglobin counts (p < 0.001).

### Discussion

Renal cell carcinoma is one of fifteen most frequent cancer types arising globally. The most aggressive subtype, ccRCC comprises about 70% of all kidney tumors. ccRCC is potentially medicable by resection, however approximately 30% of patients show recurrence after nephrectomy. Unfortunately, ccRCC is often non-symptomatic in the early stages, and is repeatedly stated in advanced phase frequently with metastases. In case of metastasis, ccRCC is radiation- and chemo-resistant and remains irremediable in most cases, resulting in a 95% mortality ratio. Up to now, no effective ccRCC therapy has been created and none of the probable biomarkers have been approved for clinical administration [8].

In our study, tumor tissues of the patients with ccRCC were compared with healthy kidney tissues around the tumor tissues of the same patients in terms of expression levels of piR-36741 and piR-36707, and the possible association between the clinical parameters of the patients and piRNAs’ expression levels were analyzed.

As one of the important study results, the expression levels of piR-36707 and piR-36741 were detected higher in tumor samples than control ones. The mitochondrial genome plays a role in tumorigenesis and 29 piRNA sequences align with regions on mitochondrial (mt) genome [9]. Accordingly, a study investigating presence of PIWI and piRNAs in the mitochondria of mammalian cancer cells found that out of the seven tRNAs matching 12 piRNAs, tRNA-Leu associated with a highly expressed (> 98%) piRNA, piR-36707, in three cancer cell lines (HeLa, HEK293, 143B). The localization of piRNAs in mitochondria opens the possibility of mitochondrial localization of PIWI proteins. PIWIL1 has been detected in mitochondrial fractions of Neuro 2a cells. Moreover, mt piRNA precursors are highly expressed in cancer cell types and tumors and hence, they may be involved in stress responses in cancer [10]. Moreover, a closer look

### Table 1: Demographic and clinicopathological characteristics of patients and the statistical significance of the associations of these data with piR-36741 and piR-36707 gene expressions of the patients.

<table>
<thead>
<tr>
<th></th>
<th>Patients (n = 19)</th>
<th>piR-36741</th>
<th>piR-36707</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Gender</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>9 (47.4%)</td>
<td>0.182</td>
<td>0.006</td>
</tr>
<tr>
<td>Female</td>
<td>10 (52.6%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Age</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>35-44</td>
<td>4 (21%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>45-64</td>
<td>10 (52.7%)</td>
<td>0.093</td>
<td>0.306</td>
</tr>
<tr>
<td>&gt; 65</td>
<td>5 (26.3%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>TNM staging</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Stage I</td>
<td>13 (68.4%)</td>
<td>0.01</td>
<td>0.037</td>
</tr>
<tr>
<td>(between stage I and II)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Stage II</td>
<td>3 (15.8%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Stage III</td>
<td>1 (5.3%)</td>
<td>&lt; 0.001</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>Stage IV</td>
<td>2 (10.5%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(between stage I and IV)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Fuhrman nuclear grade</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Grade 2</td>
<td>13 (68.4%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Grade 3</td>
<td>2 (10.5%)</td>
<td>0.036</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>Grade 4</td>
<td>4 (21.1%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>RBC count</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Low</td>
<td>4 (21%)</td>
<td>0.042</td>
<td>0.06</td>
</tr>
<tr>
<td>Normal</td>
<td>14 (73.7%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>High</td>
<td>1 (5.3%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Hemoglobin count</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Low</td>
<td>7 (36.8%)</td>
<td>0.08</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>Normal</td>
<td>12 (63.2%)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

(Abbreviations. TNM: Tumor Node Metastasis) (RBC count lower than 4 million/mm³ and hemoglobin count lower than 13 g/dl were considered as low).
at the predicted piRNAs revealed that three piRNAs had been reported as potential cancer biomarkers in previous studies: piR-36741, piR-21032 and piR-57125 [10]. This data proves the oncogenic potencies of piR-36741 and piR-36707 for ccRCC pathophysiology.

According to the association analysis between demographic (gender, age) and clinicopathological [Tumor Node Metastasis (TNM) staging, Fuhrman nuclear grade, Red Blood Cell (RBC) count, hemoglobin count] parameters and the expression levels of piR-36741 and piR-36707, interesting results were obtained (Table 1). As to the results, piR-36707 expression showed statistically significant correlation with the gender (p = 0.006), but piR-36741 not. Moreover, piR-36707 and piR-36741 expressions displayed statistically significant difference with respect to some TNM staging comparisons (for piR-36741, p = 0.01 between stage I and II, and p < 0.001 between stage I and IV; for piR-36707, p = 0.037 between stage I and IV, p < 0.001 between stage II and IV). With respect to Fuhrman nuclear grade, piR-36707 and piR-36741 expressions were significantly differed between grade 2 and 4 (p < 0.001 and p = 0.036, respectively). This finding shows the power of the expressions of piR-36741 and piR-36707 for gathering information about the grade of ccRCC patients.

Furthermore, piR-36741 expression change was significantly associated with the changes between low and normal RBC counts of ccRCC patients (p = 0.042). Similarly, piR-36707 expression was significantly associated with hemoglobin count change of ccRCC patients (p < 0.001). Anemia is a condition in which you don’t have enough healthy red blood cells to carry adequate oxygen to the body’s tissues. According to a study retrospectively reviewing 204 patients diagnosed with ccRCC between 1995 and 2008 in a community hospital setting, anemia can precede the diagnosis of ccRCC. Upon hemoglobin levels measurement excluded from the study, the severity of anemia corresponds to poorer overall survival of ccRCC patients [11]. On the contrary, it has been known that kidney cancer cells can make a hormone called erythropoietin. This hormone causes the bone marrow to make too many red blood cells. In other tissues, especially in cancer cells, it inhibits apoptosis, stimulates angiogenesis, and promotes cell proliferation. Many studies have confirmed the overexpression of erythropoietin and its receptor in ccRCC [12].

Consequently, the changes in the expression levels of piR-36741 and piR-36707 were analyzed and RCC-specific parameters are needed to be confirmed in the larger study groups. If verified, the expression changes of these piRNAs may be possible to be used as biomarkers for the prognosis of ccRCC. Yang, et al. reported that piR-36741, piR-21032 and piR-57125 are stably expressed in human serum or plasma samples and can serve as valuable blood-based biomarkers for disease detection and monitoring [4]. Also, according to the literature, piR-36707 and piR-36741 are the first and only studies to correlate the pathogenesis of ccRCC. The results of this study may propose that molecular applications can be designed to change the level of the expression of piR-36741 and piR-36707 to affect the development of ccRCC in future projects.

### Ethics and Consent to Participate

Clinical Investigation Ethics Committee of OMU has approved the study and written informed consent has been taken from the subjects participating in the study (Approval no: 2016/139) (Supplementary File 1). All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards. Informed consent was obtained from all individual participants included in the study.

### Competing Interests

The authors declare that they have no competing interests. All authors equally contributed to the study. All authors read and approved the final manuscript.

### Availability of Data and Materials

The data supporting the conclusions of this article are included within the article and its additional files.

### Funding

This study has been financed by Scientific Research Projects Commission of Ordu University (Project number: AP-1713).

### References


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Bilgilerinize arz/rica ederim.

Prof. Dr. Dursun AYGÜN
Klinik Araştırmalar Etik Kurulu Başkanı

Supplementary File 1: Ethics Committee Approval.