High Notch1 Expression Correlates with Tumor Stage and Size in Clear Cell Renal Cell Carcinoma

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Purpose: Although the influence of Notch signaling on several types of malignancies has been studied, the role of Notch signaling in clear cell renal cell carcinoma (ccRCC) remains unclear. In this study, we evaluated the levels of Notch1 and Jagged1 and their significance in ccRCC.

Materials and Methods: Tumor tissue and matched normal adjacent kidney tissue from 49 ccRCC cases were obtained. The expression of Notch1 and Jagged1 was analyzed using real-time polymerase chain reaction (PCR) and Western blotting. Tissue samples were divided into several groups according to clinicopathological features, and the relative expression of Notch1 and Jagged1 was assessed.

Results: Real-time PCR revealed increased Notch1 expression in tumor tissues compared with that in adjacent normal tissues (p=0.044). Based on the pathological stage, a significant difference in Notch1 expression was observed between tumor and normal kidney tissues in pT2 and pT3 ccRCC (pT2, p=0.041; pT3, p=0.001). Notch1 expression in ccRCC relative to that in normal tissue was higher in later-stage ccRCC and larger ccRCC. Notch1 expression showed significant positive correlation with the maximal diameter of the primary renal tumor (mRNA, p<0.001; protein, p=0.001). High Notch1 expression was associated with recurrence and disease-specific death, although the difference was not significant. Jagged1 level was not significantly correlated with any of the factors examined.

Conclusions: Notch1 may play a significant role in the tumorigenesis and progression of ccRCC. Notch signaling may be a potential target for chemopreventive or adjuvant therapeutics for ccRCC. (Korean J Urol Oncol 2016;14:130-137)

Key Words: Renal cell carcinoma • Notch1 • Jagged1 • Biomarkers • Tissue array analysis

INTRODUCTION

Renal cell carcinoma (RCC) is a common cause of death in urological cancer, and the most common histologic type is clear cell renal cell carcinoma (ccRCC).1 Although several target agents have shown a significant survival benefit, effective targeted therapy for patients with advanced ccRCC still have several limitations.2 Therefore, a comprehensive understanding of the genetic and molecular biology is required to identify additional signaling pathways and develop new targeted molecules in ccRCC.3

Notch signaling is a widely expressed signal pathway that functions as a diversity of cellular processes, including proliferation, differentiation, and apoptosis through direct cell to cell interactions in normal development and tumorigenesis.4
Contradictory effects of the Notch signaling have been researched in several malignancies, and Notch may act as a suppressor as well as a promoter depending on the type of tumor.\(^5\)\(^7\) Although there were some researches about the effect of Notch1 and Jagged1 as the most studied molecules in ccRCC, the pathological roles of the Notch pathway in ccRCC are not clearly identified.\(^8\)\(^9\)

To identify the pathological roles of Notch signaling in ccRCC, we determined whether differences in Notch1 and Jagged1 expression between tumor tissue and normal adjacent kidney tissue vary across relevant clinicopathological features of ccRCC.

**MATERIALS AND METHODS**

1. Patients and Samples

This study was approved by the Ethics Committee of Kyungpook National University Medical Center (KNUMCBIO_13-1005). Between May 2012 and April 2014, 49 patients with ccRCC that were pathologically confirmed by radical nephrectomy were included. All patients had no evidence of synchronous metastasis. Tumor tissue and normal kidney tissue were obtained from each patient’s kidney specimen after radical nephrectomy and were analyzed using histological and molecular biological approaches. Normal kidney tissue was excised in the specimen from the most distant part of the cancer tissue. Clinicopathological characteristics are shown in Table 1. Clinical data, including age, sex, follow-up duration, disease recurrence, and disease-specific death, were recorded. Pathological data included TNM stage, tumor size, nuclear grade, lymphovascular invasion, and margin status. The stage was classified according to the TNM classification system 2010,\(^10\) and nuclear grade was assessed according to Fuhrman system.\(^11\)

2. Quantitative Real-Time PCR Analysis

Total RNA was extracted using an RNeasy-kit (Qiagen, Hilden, Germany) according to the manufacturer’s instructions. A total of 2 \(\mu\)g of RNA was used for cDNA synthesis using cDNA reverse transcription kits (Applied Biosystems, Foster City, CA, USA). The primers were designed using Primer Express Software (Applied Biosystems). The assay was performed using the ABI Prism Sequence Detection System 7500 with SYBR Green Polymerase Chain Reaction Master Mix (Applied Biosystems). To analyze the data, the \(2^{-\Delta\Delta Ct}\) method of relative quantification was adapted to estimate the copy numbers. The primer sequences were: for Jagged1, 5'-CTCCT GTCGGGATTTGTAGTA-3' and 5' -GCATAGCCAGGTTGAC AGAT-3'; for Notch, 5’-TCCACCCAGTTGAAGTGCA-3' and 5’-GCAGAGGGTTGTATTTGTTT-3'; for \(\beta\)-actin, 5’-ACTGTCACCAGCAATGCT-3' and 5’-AAGCCATGCCCAATCTCATCTTG-3'.

3. Western Blot Analysis

Paired tumor and normal tissues were solubilized in lysis buffer (50 mM Tris, pH 7.4, 1% NP-40, 150 mM NaCl, 1 mM EDTA, 1 mM PMSF, 1 mM Na\(_2\)VO\(_4\), 1 mM NaF, 1 g/mL peptatin, and 1 g/mL aprotinin) on ice. All lysates were centrifuged at 4°C at 10,000 \(\times\)g for 10 minutes. Protein concentration was determined using the Bradford protein assay (Bio-Rad Laboratory, Hercules, CA, USA). A total of 20-\(\mu\)g protein from each sample was electrophoresed by 8% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and blotted onto a nitrocellulose membrane. The membrane was blocked with 5% skim milk in Tris-buffered saline containing Tween-20 (20 mM Tris, pH 7.6, 137 mM NaCl, and 0.1% Tween 20) at room temper-

**Table 1. Clinicopathological characteristics of 49 patients with clear cell renal cell carcinoma**

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (y)</td>
<td>60.0±11.4</td>
</tr>
<tr>
<td>Sex</td>
<td>Male/Female</td>
</tr>
<tr>
<td></td>
<td>34 (69.4)/15 (30.6)</td>
</tr>
<tr>
<td>T stage</td>
<td>T1a/T1b/T2/T3/T4</td>
</tr>
<tr>
<td></td>
<td>20 (40.8)/12 (24.5)/9 (16.3)/9 (18.4)/0 (0)</td>
</tr>
<tr>
<td>Tumor size (mm)</td>
<td>50.8±27.6</td>
</tr>
<tr>
<td>Grade</td>
<td>I/II/III/IV</td>
</tr>
<tr>
<td></td>
<td>3 (6.1)/21 (42.9)/23 (46.9)/2 (4.1)</td>
</tr>
<tr>
<td>Lymphovascular invasion</td>
<td>Yes/No</td>
</tr>
<tr>
<td>Nx or N0/N(+)(+)</td>
<td>48 (98.0)/1 (2.0)</td>
</tr>
<tr>
<td>M0/M(+)(+)</td>
<td>49 (100)/0 (0)</td>
</tr>
<tr>
<td>Margin positive</td>
<td>Yes/No</td>
</tr>
<tr>
<td></td>
<td>0 (0)/49 (100)</td>
</tr>
<tr>
<td>Follow-up duration (mo)</td>
<td>12.6±8.1</td>
</tr>
<tr>
<td>Range</td>
<td>3-25</td>
</tr>
<tr>
<td>Local/distant recurrence</td>
<td>Yes/No</td>
</tr>
<tr>
<td>Cancer-specific death</td>
<td>Yes/No</td>
</tr>
</tbody>
</table>

Values are presented as mean±standard deviation or number (%) unless otherwise indicated.
Fig. 1. Notch1 and Jagged1 expression in clear cell renal cell carcinoma (ccRCC) tissues and normal kidney tissues. (A) Notch1 Jagged1 mRNA expression in ccRCC. (B) Notch1 Jagged1 protein expression in ccRCC. Real-time polymerase chain reaction showed higher Notch1 expression in ccRCC tissues compared to in normal kidney tissue (p=0.044). However, the Jagged1 mRNA expression and Notch1 and Jagged1 protein levels did not show significant difference between ccRCC and normal kidney tissue. *p < 0.05, significant differences between RCC tissue and normal tissue.
Fig. 2. Relative molecular expressions of ccRCC tissue compared to normal kidney tissue at different stages. (A) Notch1, Jagged1 mRNA expression in ccRCC. (B) Notch1, Jagged1 protein expression in ccRCC. Increased Notch1 expression was more pronounced in patients with higher pathological stage. Significantly higher Notch1 expression was observed in T3 stage compared to T1 stage tumors. Differences according to pathological stage were not significant for Jagged1 expression. *p < 0.05, significant differences between T1 and T3.

different between ccRCC and normal kidney tissues in both the real-time PCR and western blotting results (p=0.061 on the mRNA level, p=0.221 on the protein level) (Fig. 1).

In real-time PCR, Notch1 expression did not differ between ccRCC and normal kidney tissue in pT1 ccRCC (p=0.768). However, Notch1 expression was significantly elevated in pT2 and pT3 ccRCC tissues compared to in matched nontumor tissues (p=0.041 on pT2 and p=0.001 on pT3). Notch1 expression in ccRCC tumor versus normal kidney was higher in patients with a higher pathological stage (pT1, 114.3%; pT2, 159.5%; and pT3, 161.7% on the mRNA level; pT1, 104.6%; pT2, 131.0%; and pT3, 149.3% on the protein level). The relative ratio of Notch1 expression in ccRCC to normal kidney tissue in pT3 was significantly higher than that in pT1 (p=0.031) (Fig. 2).

The relationship between Notch1 and Jagged1 expression and tumor size is illustrated in Fig. 3. Notch1 expression in tumor tissue relative to that in normal kidney tissue was higher in large tumors (>4 cm) than in small tumors (≤4 cm) (large tumors 151.5% and small tumors 99.8% on the mRNA level, p<0.001; large tumors 134.2% and small tumors 92.3% on the protein level, p=0.002). In addition, we evaluated the correlation between Notch1 and Jagged1 relative expression and the maximum diameter of the primary tumor. Notch1 expression was positively correlated with the maximum diameter of the primary tumor (R=0.514, p<0.001 on the mRNA level and R=0.457, p=0.001 on the protein level), while Jagged1 expression had no correlation with tumor size (p=0.657 on the mRNA level and p=0.539 on the protein level). Other clinico-pathological features, including age, sex, grade, lymphovascular invasion, and nodal state, were not correlated with the expression of Notch1 and Jagged1.

Although a significant difference in Notch1 and Jagged1 was not found to be related to disease recurrence and disease-specific death, Notch1 expression was much higher in patients with recurrence and cancer-specific death than in those with cancer-free status (mRNA level [recurrence (+) group, 160.4%; recurrence (-) group, 127.0%; p=0.183], protein level, [recurrence (+) group, 143.8%; recurrence (-) group, 114.1%; p=0.193], mRNA level [cancer-specific deaths (+) group, 163.6%; cancer-specific deaths (-) group, 127.4%; p=0.193], protein level [cancer-specific deaths (+) group, 158.8%; cancer-specific deaths (-) group, 113.4%; p=0.069]) (Fig. 4).

DISCUSSION

In this study, we evaluated the level of Notch1 and Jagged1 in ccRCC and matched normal kidney tissues based on clinico-pathological features to determine the pathological role of Notch signaling in ccRCC. We observed that Notch1 expression was significantly elevated in ccRCC tissues compared to in adjacent normal kidney tissues. In addition, Notch1 expression was higher in patients with higher pathological stage and was positively correlated with primary tumor burden. Thus, Notch1 may be associated with tumorigenesis and aggressive features in ccRCC.
Fig. 3. Analysis of tumor diameter and levels of Notch1 and Jagged1 in clear cell renal cell carcinoma (ccRCC). (A, B) Notch1 expression was higher in large tumors (>4 cm) than in small tumors (≤4 cm). (C, D) Positive correlation between Notch1 expression and primary tumor diameter in ccRCC. (E, F) No correlation between Jagged1 expression and tumor size in ccRCC. *p < 0.05, significant differences between ≤4 cm and >4 cm.
Fig. 4. Analysis of oncological outcome and molecular expression. Notch1 expression was much higher in patients with recurrence (A, B) and cancer-specific death (C, D) than in those with recurrence and cancer free status, although the differences were not significant. ccRCC: clear cell renal cell carcinoma, CSD: cancer specific death.

The Notch signaling is known to be involved in the determination of cell fate by regulating proliferation, differentiation, and apoptosis. From an oncological perspective, Notch has suppressive as well as oncogenic role depending on the type of tumor and the influence of other signaling pathways. The oncogenic role of Notch signaling is thought involved the prevention of differentiation and the maintenance of unlimited proliferation. In addition, several studies have reported that the Notch pathway is crucial for tumor angiogenesis, which is essential for tumor formation and progression. The overexpression of Notch receptors, ligands, and targets has been observed in many cancer types. In head and neck squamous cell carcinoma, Notch signaling was found to be related to the aggressiveness of the cancer. However, contrasting effects of Notch signaling have been reported in other cancers. Some studies found that the Notch pathway was a predictive factor of oncological outcome in breast cancer and prostate cancer.

Although several studies showed that Notch1 is a pro-metastatic molecule in various cancers, the role of Notch signaling in ccRCC remains unclear. In this study, to identify the pathological roles of Notch signaling in ccRCC, we analyzed the levels of Notch1 and Jagged1 in 49 ccRCC tumors and adjacent normal kidney tissues. Compared to normal kidney tissue, Notch1 mRNA expression was significantly elevated in ccRCC, similar to previous studies. In high-stage ccRCC compared with low-stage ccRCC and was significantly correlated with tumor burden in ccRCC. Although the difference was not statistically significant, high relative expression of Notch1 was associated with recurrence and disease-specific death in this study. However, some studies showed the opposite results, in which the expression of Notch
receptors was down-regulated and Notch signaling functioned as a tumor suppressor in RCC progression. Because Notch signaling is a complex pathway, further studies including extensive evaluation of all receptors, ligands, and downstream target genes are required to investigate the detailed mechanism of Notching signaling in ccRCC.

The tumor size of the primary lesion has been recognized as a risk factor of synchronous and metachronous metastasis in ccRCC. Therefore, we analyzed the correlation between the tumor diameter of the primary lesion and the relative expression of Notch1 and Jagged1 mRNAs in 49 ccRCC samples to evaluate the relationship between tumor burden and Notch signaling. Our results showed that the expression of Notch1 was higher in large (>4 cm) tumors than in small (≤4 cm) tumors. In addition, Notch1 expression was significantly positively correlated with the maximum diameter of the primary tumor, while Jagged1 expression had no correlation with ccRCC size. Similar studies also reported a correlation between Notch signaling and the tumor burden of ccRCC. Ai et al. reported that the average size of metastatic tumors was significantly larger than that of localized tumors in T1 stage ccRCC and that elevated Notch1 expression was significantly positively correlated with tumor diameter. Wu et al. suggested that the expression level of Jagged1 was strongly associated with tumor size, nuclear grade, and TNM stage, and high level of Jagged1 expression was statistically correlated with poor prognosis in ccRCC, especially at the early stage. In addition, Sjölund et al. showed that Notch1 and the Notch ligand Jagged1 were expressed at significantly higher levels in ccRCC tumors than in normal kidney tissue, and the inhibition of Notch signaling weakened the growth of primary ccRCC cells. Although additional study for detailed mechanism of Notch signaling should be needed, Notch signaling may involve the aggressive feature in ccRCC.

However, some investigators showed contrasting results to the negative effect of Notch signaling on ccRCC. Sun et al. suggested that Notch signaling may act as a suppressor in ccRCC because of deregulated expression of Notch receptors. Previous results showed that the levels of Notch1 and Notch4 were significantly decreased in ccRCC tissues compared with in adjacent normal kidney tissues and that Notch1 was negatively correlated with tumor stage and not correlated with tumor size. The oncological effect of Notch signaling on RCC is difficult to determine because Notch signaling is a complicated and comprehensive pathway.

Over the past few decades, numerous studies have shown that Notch signaling plays an important role in tumor biology. These studies suggested therapeutic approaches for treating cancers associated with abnormal Notch signaling involving pharmacologic blocking of the Notch pathway. Although their widespread use has been limited by severe adverse effects, gamma secretase inhibitors, the blocker of Notch signaling by restraining the proteolytic step, are introduced and may be effective in restraining cancer cell proliferation. Recently, several studies have focused on the development of antibodies against specific Notch receptors and ligands to reduce side effects while ensuring therapeutic efficacy. By understanding the various cellular events and signaling pathway interactions that contribute to tumor progression, the targeting Notch signaling may provide a novel and effective strategy for treating ccRCC.

There were several limitations to our study. This study was based on a retrospective analysis with small subjects and a short follow-up period and included the problem of the selection bias including the presence of a resectable tumor and the patient’s race. Although the central necrosis is considered to be prognostic factor of ccRCC, this factor was not available on this retrospective study. In addition, this study estimated the expression of the some molecules (Notch1 and Jagged1) in Notch signaling, not all molecules known to engage in Notch signaling, further studies including extensive and complete study of all receptors, ligands, and even downstream target genes are necessary.

CONCLUSIONS

In this study, we found that Notch1 expression was significantly elevated in ccRCC tissues compared to in adjacent normal kidney tissues, was higher in patients with higher pathological stage, and was positively correlated with primary tumor burden. Therefore, Notch1 may have a significant role in the tumorigenesis and progression of ccRCC. Notch signaling is a potential target for chemopreventive or adjuvant therapeutics for ccRCC.

CONFLICT OF INTEREST

The authors claim no conflicts of interest.
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