Significant association of interleukin 10 receptor mRNA levels with renal cell carcinoma metastasis

Hideyuki Abe, Tomonori Yamanishi, Tomoko Mashidori, Kyoko Arai and Takao Kamai
Department of Urology, Dokkyo Medical University, Tochigi, Japan
(Received 15 October 2007; and accepted 11 November 2007)

ABSTRACT
Immunosuppressive cytokine, interleukin 10 (IL-10), is associated with progression of the renal cell carcinoma (RCC). However, the roles of its cell surface receptor, interleukin 10 receptor (IL-10R), remain elusive. We quantified IL-10R mRNA expression in paired tumor and non-tumor samples from the surgical specimens of 71 consecutive patients with RCC using a real-time reverse transcription polymerase chain reaction (RT-PCR). The absolute level of IL-10R mRNAs in tumor and non-tumor tissues did not correlate with the malignant and metastatic profiles. The relative yields of the PCR product from the tumor tissue to that from the corresponding non-tumor tissue (T/N) for the expression of IL-10R mRNAs were calculated. A high T/N ratio of IL-10R correlated with poor differentiation ($P<0.001$) and metastasis ($P<0.0001$). By univariate analysis, a high T/N ratio of IL-10R predicted a shortened overall survival in all cases ($P<0.01$). These findings suggest that IL-10R is associated with the progression of RCC.

Renal cell carcinoma (RCC) is increasing steadily, and accounts for 2–3% of all adults malignancies and < 40% of patients who die due to metastasis (15,18), because of its high frequency of metastases at diagnosis or relapse following nephrectomy. Patients with distant metastases have a poor prognosis, with a 5-year survival rate of less than 10% (18). RCC is notoriously resistant to chemotherapy and radiotherapy (5, 18, 27). RCC has been considered to be an immunogenic tumor (10). Cytotoxic T lymphocytes recognize and selectively kill autologous RCC cells, and tumor-specific T cells are detectable in the blood of RCC patients (10). Immunosuppressive factor, interleukin-10 (IL-10), prevents or limits efficient antigen presentation and activation of tumor-specific T cells. IL-10 is released by living tumor cells, including renal carcinoma cells (15), by tumor cells undergoing apoptosis (8), and by peripheral leukocytes (16). The release of IL-10 is initiated by the phagocytosis of apoptotic cells by macrophages (25). IL-10 interferes with T-cell activation and co-stimulation and with the functional maturation of antigen-presenting dendritic cells (6, 18). It has been reported that elevated serum levels of IL-10 correlated with unfavorable prognosis in RCC (26), and that the failure in IL-10 secretion by engulfing macrophages of responding metastatic RCC patients may exalt the immunogenicity of dying tumor cells, contributing to the success of immunotherapy (2). Thus, IL-10 may be involved in the progression of RCC. IL-10 binds to the cell surface IL-10 receptor (IL-10R), however, the roles of IL-10R in RCC remain elusive.

We compared IL-10R mRNA expression in RCC tissues with non-neoplastic portions of the same resected specimen using a real-time reverse transcription-polymerase chain reaction (RT-PCR). The relationship between IL-10R expression and selected pathologic features of the tumors was examined. We also assessed whether IL-10R expression could pre-
dict the survival of the patients with these tumors.

MATERIALS AND METHODS

Patients and tissue preparation. We studied 71 consecutive Japanese patients (48 men, 23 women), 33 to 81 years old (mean age 61.5 years), with newly diagnosed clear cell RCC, from 2000 to 2006. All patients routinely underwent imaging studies (CT and/or MRI) for preoperative staging prior to radical nephrectomy. The postoperative follow-up ranged from 2 to 78 months (median, 31 months). Patients underwent surgery before receiving any other therapy.

For every case, three different tumor sites and varying portions of the non-neoplastic kidney were resected for the study. The resected tissues were stored at −80°C as described previously (12, 13, 23). Staging were carried out according to the criteria of the TNM classification (22). The study was conducted in accordance with the Helsinki Declaration. Institutional Review Board approval was obtained for this investigation. Each patient signed a consent form approved by the Committee on Human Rights in Research of our institution.

Real-time RT-PCR assay. Total RNA was purified from all 71 sets of tumor and non-tumor RCC tissue samples using the RNA preparation kit “High Pure RNA Kit” (Roche Diagnostic Ltd, Germany). Total RNA was used as a template for cDNA synthesis. A 100 μL reaction mixture containing 1 μg of random hexamers and 100 units of MMLV-reverse transcriptase was incubated at 25°C for 10 min, 42°C for 30 min, and then 99°C for 5 min. The expression profiles of the IL-10R genes were analyzed with an ABI PRISM 7700 Sequence Detection System (Applied Biosystems, Foster City, CA) using the SYBR Green method. The following specific primers were designed to amplify their respective genes in all of the primary carcinoma tissues after confirming their specificities; IL-10R, sense: 5'-AGGATGGAGTGAGATGGGTGAGATGCTGGG-3', anti-sense: 5'-ACAAGAGAAATGGACACGAC-3'; β2-microglobulin, sense: 5'-AACCCCACTGAAAGATGTA-3', anti-sense: 5'-ATCTTCAAACCTCCATGATG. A real-time RT-PCR assay was performed on a 25 μL reaction mixture containing 20 ng of sample cDNA, 100 nM sense primer, 100 nM anti-sense primer, and 12.5 μL of SYBR Green PCR Master Mix (Applied Biosystems). The PCR was carried out for 50 cycles of 95°C for 15 sec and 60°C for 1 min. To normalize the amplified products in each sample, we used β2-microglobulin as a quantitative internal control (12, 23). A standard curve for each mRNA expression was generated using five-fold dilutions of a control RNA sample (25 ×, 5 ×, 1 ×, 0.2 ×, 0.04 ×). The mRNA expression levels of IL-10R gene were presented as a ratio to that of β2-microglobulin, and the relative expression levels were calculated (12, 23). The mean values from the real-time RT-PCR data for the three samples of the resected tissues were used for the analysis according to a method described previously (12, 23).

Statistical analysis. The results of real-time RT-PCR were analyzed statistically using the Mann-Whitney U test for two groups as described previously (12), and the Kruskal-Wallis test for over three groups. The expression levels of mRNAs for IL-10R, as well as tumor grade and stage, were assessed in terms of survival by the Cox proportional hazards model using univariate and multivariate analyses. The Kaplan-Meier method was used to estimate survival as a function of time, and survival differences were assessed by the log-rank test. P values less than 0.05 were considered significant. Data were analyzed using commercially available software.

RESULTS

IL-10R mRNA expression and pathologic characteristics

IL-10R mRNAs were detected in tumor and non-tumor kidney specimens. The absolute level of IL-10R mRNAs ranges widely due to inter-individual variations. The absolute level of mRNA expression for IL-10R in the tumor and non-tumor tissues did not correlate with the malignant and the metastatic profiles of the RCC tumors. Since inter-individual variations in the expression of IL-10R mRNAs may be important, the relative yield of the PCR product from the tumor to that from the corresponding non-tumor tissue (T/N) for expression of IL-10R was calculated as previously (12, 23). The mean values for the T/N ratios of IL-10R mRNAs in the samples (mean ± S.D.) were 1.03 ± 2.16.

An increased T/N ratio of IL-10R mRNA was associated with poorer differentiation: grade 1 tumors had a T/N (mean ± S.D.) = 0.26 ± 0.51; grade 2, 0.65 ± 1.24; and grade 3, 2.06 ± 3.32, P = 0.0032 (Fig. 1A). The T/N ratio of IL-10R mRNA did not correlate with stage: pT1-2 tumors had a T/N ratio = 1.23 ± 1.13, while the comparable value for pT3-4 tumors was 0.72 ± 1.41, P = 0.6422 (Fig. 1B).

The T/N ratio of IL-10R in tumors (that included a
Fig. 1  T (tumor)/N (non-tumor) ratio of IL-10R mRNA expression in kidney. A: In Grade 1 to 3 tumors. B: In pT1-2 and pT3-4 tumors. C: In cell-type. D: In metastasis (+) M1 and (−) M0. The data show the 95% confidential interval.

Fig. 2  T (tumor)/N (non-tumor) ratio of IL-10R mRNA expression in kidney. A: In classification in stage/metastasis. B: In classification in stage/metastasis/cell-type. The data show the 95% confidential interval.
sarcomatoid component which confers a worse prognosis) was increased compared to the conventional acinar type (3.19 ± 2.76 vs. 0.66 ± 1.46, \( P < 0.0001 \); Fig. 1C). The T/N ratio of IL-10R was higher in M1 tumors compared to those in M0 tumors (0.37 ± 0.84 vs. 2.18 ± 3.11, \( P = 0.0006 \); Fig. 1D).

In these findings, a high T/N ratio of IL-10R mRNAs expression was not related to local invasion (pT classification). We analyzed what factors influenced on this finding. Eight patients included a sarcomatoid component (all patients with grade 3), thus we also analyzed a T/N ratio of IL-10R mRNAs expression regarding histological grade within conventional acinar typed RCCs (\( n = 63 \)). A T/N ratio of IL-10R mRNAs expression in grade 3 (\( n = 14 \)) was 1.18 ± 2.41, showing that a higher T/N was associated with poorer differentiation among conventional acinar typed RCCs. We also analyzed the relationship between a T/N ratio of IL-10R mRNAs expression and local-invasion/metastasis. As shown in Fig. 2A, a T/N ratio of IL-10R mRNAs expression is higher in pT1-2/M1 than in pT3-4/M1 group (\( P = 0.0236 \)). For further characterization of sarcomatoid, we examined a T/N ratio of IL-10R mRNAs expression in subgroups of local-invasion/metastasis/cell-type (Fig. 2B). As expected, there was no difference of a T/N ratio between pT1-2/M1/sarcomatoid and pT3-4/M1/sarcomatoid group. M1 cases showed a tendency toward a higher T/N ratio of IL-10R mRNAs expression in sarcomatoid than in conventional acinar typed carcinomas in both pT1-2 and pT3-4 groups, however, there was no statistical difference. In contrast, pT1-2/M1/acinar subgroup showed a increased T/N ratio of IL-10R mRNAs expression than pT3-4/M0/acinar subgroup (\( P = 0.0400 \)), and pT2-1/M1/sarcomatoid subgroup also showed a higher T/N ratio than pT3-4/M1/acinar subgroup (\( P = 0.0017 \)).

**IL-10R mRNA expression and survival**

The mean values for the T/N ratios of IL-10R mRNAs in the samples were 1.03 (± 2.16). As described previously (12, 23), the cases were divided into two groups (high and low) based upon whether the expression was above or below this mean. Comparison of the Kaplan-Meier survival rate plots in patients with low vs. high expression of IL-10R mRNAs suggested that a high T/N ratio of IL-10R mRNAs expression was associated with a shortened overall survival (\( P = 0.0007 \), Fig. 3A). By univariate analysis according to the Cox proportional hazards model, overall survival was influenced significantly by grade, stage, cell type, metastasis, and IL-10R (Table 1). By multivariate analysis, grade, cell type, and metastasis were independent prognostic factors (Table 1).

Cases of tumors without distant metastasis at nephrectomy (M0; 45 patients) were divided into those with the T/N ratio of IL-10R mRNAs above or below, a mean of 0.37 (± 0.84). In these localized tumors, Kaplan-Meier plots showed that a high T/N ratio of IL-10R mRNAs had no influence on disease-free survival (\( P = 0.1845 \), Fig. 3B). Although grade and stage predicted disease-free survival in this group in the univariate analyses, stage remained significant in the multivariate analysis (Table 1).

**DISCUSSION**

IL-10 is an immunosuppressive factor, and it has been reported that an elevated serum level of IL-10
is an independent predictor of unfavorable prognosis in RCCs. We studied the role of its surface receptor, IL-10R. To take into account the possibility of inter-individual variations in the expression of mRNAs for IL-10R, we compared the mRNA expressions between paired tumor (T) and non-tumor (N) kidney samples, and the relative yield of the PCR product from the tumor to that from the corresponding non-tumor tissue (T/N) for the expression of IL-10R mRNAs was calculated as described previously (12, 23). Impressively, a high T/N ratio of IL-10R mRNA expression in tumors was associated with poor differentiation, metastasis, and shortened survival. To our knowledge, this is the first report analyzing the relationship between IL-10R and renal cancer.

In the current study, a high T/N ratio of IL-10R mRNAs expression was associated with poor differentiation, metastasis, and poor survival, but not with local invasion. According to our well known conventional pathological analysis (20), we will predict that pT3-4 shows a higher T/N ratio of IL-10R mRNAs expression than pT1-2. Indeed, local invasion of pT3-4 cases showed unfavorable prognosis than pT1-2 cases in this study, as well as poor differentiation, distant metastasis, and sarcomatoid component. For further characterization of prognostic factor, we classified the cases, in which classification of local-invasion/metastasis/cell-type showed interesting findings. As well as in pT1-2/pT3-4 groups, pT1-2 showed a higher T/N ratio of IL-10R mRNAs expression than pT3-4 in M1 cases ($P = 0.0263$, Fig. 2A). However, there was no difference between pT1-2 and pT3-4 subgroups in M1/sarcomatoid group (Fig. 2B). In addition, M1 cases showed a tendency toward a higher T/N ratio of IL-10R mRNAs expression in sarcomatoid than in conventional acinar typed carcinomas in both pT1-2 and pT3-4 groups, however, there was no statistical difference. In contrast, within acinar typed carcinomas, pT1-2/M1 cases showed a high T/N ratio of IL-10R mRNAs expression than pT3-4/M0 cases. Taken together, therefore, a higher T/N ratio of IL-10R mRNAs expression in pT1-2 than in pT3-4 might be considered to reflect the involvement of sarcomatoid. Since our series included relatively small subgroup (n = 3 in pT3-4/M1/sarcomatoid, n = 5 in pT1-2/M1/acinar, and n = 5 in pT1-2/M1/sarcomatoid), we may not draw definitive conclusions. However, our findings that the cases with distant metastasis but non-invasive (pT1-2/M1) had a high T/N ratio of IL-10R mRNAs expression and were associated with unfavorable prognosis suggest-

Table 1  Cox regression analysis for various potential prognostic factors in survival

<table>
<thead>
<tr>
<th>Variable</th>
<th>Overall survival (71 patients)</th>
<th>Disease-free survival (45 patients) in M0 at nephrectomy</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Unfavorable/favorable characteristics</td>
<td>No. of Patients</td>
</tr>
<tr>
<td>Grade</td>
<td>3/2/1</td>
<td>22/39/10</td>
</tr>
<tr>
<td>pT</td>
<td>4,3,2,1</td>
<td>27/44</td>
</tr>
<tr>
<td>Cell type</td>
<td>sarcoma-toid/acinar</td>
<td>8/63</td>
</tr>
<tr>
<td>Metastasis (+)/(-)</td>
<td>26/45</td>
<td>0/45</td>
</tr>
<tr>
<td>IL-10R</td>
<td>high/low</td>
<td>18/53</td>
</tr>
</tbody>
</table>
ed that IL-10R played a significant role in metastatic potential of RCCs. Moreover, eight patients with sarcomatoid component presented with metastasis at diagnosis, and their prognosis is extremely poor in comparison to conventional acinar type. These sarcomatoid cases showed an increased T/N ratio of IL-10R mRNAs expression, indicating that high IL-10R may be associated with highly metastatic profiles of sarcomatoid, however, the relation between the possible role of IL-10R and the sarcomatoid should be examined in a large number of sarcomatoid RCCs in the future. Furthermore, although it is unknown how a T/N ratio of IL-10R mRNAs can predict for poor survival at present, Cox analysis showed that a higher T/N ratio of IL-10R mRNAs was associated with unfavorable prognosis. Therefore, cases with a higher T/N ratio of IL-10R mRNAs need to be followed closely.

It has been reported that an elevated circulating C-reactive protein concentration, indicating systemic inflammatory response, is associated with a poor survival in metastatic RCCs (3, 4). IL-6 and IL-10 might be stimulant and suppressive on systemic inflammatory response, respectively, on immune cells (7, 11). IL-6 is an autogrowth factor for tumors, but also plays suppressive roles (24). IL-10 is immunosuppressive in anti-tumor response (17). Negrier et al. stated that circulating IL-6 level appeared to be an important independent prognostic factor in patients with metastatic RCCs (19). Thus, IL-6 and IL-10 may be important factors in progression of RCCs. Heterogeneity in the ability to release immunosuppressive factors related to polymorphism of IL-10 gene promoter has been well known (9). Therefore, we should analyze the polymorphisms of IL-10R in the RCC patients. The relationship between serum IL-10 and IL-10R in RCCs will be our forthcoming study.

We recently reported that interferon alpha receptor 2 (IFNAR2) is associated with renal cell carcinoma metastasis (14). It has been known that interferon receptor and IL-10R form gene cluster (21). Therefore, IFNAR2 and IL-10R might cooperatively play a role in the progression of RCC. This relation should be studied in more large patients in the future.

Acknowledgements

The authors are grateful to Dr Ken-Ichiro Yoshida, Chairman of Department of Urology, for his thoughtful and constructive suggestions, Yoshiaki Yanai for his contribution to the design, and Aki Yanagibayashi and Hitomi Yamazaki for their excellent technique in this study.

REFERENCES

IL-10 receptor in renal cancer


