Renal cell carcinoma (RCC) is one of the 10 most frequent malignancies in developed countries accounting for ~3% of all the malignancies. Incidence and mortality rates for RCC are steadily increasing in the United States (1) and Japan (2). Whereas RCC encompasses a group of at least five histologic subtypes, the vast majority (80%) are classified as clear cell RCC (CCRCC), followed by papillary RCC (10-15%) and chromophobe RCC (5%; ref. 3). Because CCRCC has the worst prognosis among the three major histologic subtypes of RCC (4), improvement of the prognosis in patients with CCRCC is of great importance.

At present, surgical resection is the most effective therapy for localized CCRCC, but no satisfactory treatment is available for patients with advanced-stage CCRCC. Systemic chemotherapies are nearly of no effect (5). Cytokines, such as IFNα and interleukin-2, are commonly used for metastatic CCRCC, although the response rate is only 10% to 20%, and mostly the response is partial (5). Therefore, a novel therapy against CCRCC is required to be developed.

Therapeutic targets in this highly resistant malignancy have been identified through elucidation of the mechanism for CCRCC tumorigenesis (6). The majority of CCRCCs are characterized by von Hippel-Lindau (VHL) gene inactivation, resulting in the up-regulation of hypoxia-inducible genes through a mechanism involving hypoxia-inducible factor-1α. Vascular endothelial growth factor (VEGF), one of the hypoxia-inducible molecules, plays a critical role in tumor angiogenesis and progression. Therefore, therapeutic targeting of VEGF in CCRCC has a biological rationale and several clinical trials of VEGF-targeting therapies have been conducted. Some of patients with metastatic CCRCC achieved a partial response using these therapies, but none of those therapies have been proven to improve overall survival (7–9). Although VEGF-targeting therapy is under development, it is still important to search for other therapeutic targets.

One therapeutic strategy against malignancies that has been drawing attention lately is immunotherapy targeting Toll-like receptors (TLR; refs. 10, 11). TLRs are key molecules in innate
immune systems, of which 10 are known in humans (12). TLR3 is a receptor involved in antiviral responses by recognition of viral components such as double-stranded RNA polyinosinic-polycytidilic acid [poly(I:C)] and by inducing production of type I IFNs, especially IFNβ (13). Recent studies have also shown that TLR3 signaling induces apoptosis in certain cells, which may be a part of the host innate immune response to viral infections (14). TLRs are expressed on immune cells, such as dendritic cells and macrophages. In addition, recent studies have revealed that some cancer cells also express TLRs in vitro (15), implying the possibility of TLR-based cancer therapy. However, expression patterns of TLRs in human cancer tissues are largely unknown. Moreover, the effects of TLR signaling in cancer cells may vary among cancer cell types. For example, stimulation of TLR9-expressing breast cancer cells with TLR9 agonist increases their in vitro invasion (16). On the other hand, activation of TLR9 in prostate cancer cells induces apoptosis in vitro as well as complete tumor regression in vivo xenograft model (17). Thus, TLRs on tumor cells may act as a ‘double-edged sword’ (18). Appropriate tumor types, receptors, and ways of activation should be selected to realize a novel TLR-based cancer therapy.

In this study, we identified that TLR3 is specifically overexpressed in CCRCC tissues by using genome-wide gene expression analysis and immunohistochemistry. Stimulation of TLR3 on RCC cells with its ligand induced TLR3-dependent growth inhibition. Moreover, TLR3 ligand and IFNαs exerted a synergistic growth-inhibitory effect against Caki-1 RCC cells. Our results suggest that TLR3 is a novel therapeutic target for CCRCC.

Materials and Methods

Tissue samples. Tissue samples were obtained from patients who underwent nephrectomy or renal biopsy (one patient) at The University of Tokyo Hospital (Tokyo, Japan). Tumor lesions and corresponding normal kidney tissues away from the tumor were separated, snap frozen in liquid nitrogen immediately after resection, and stored at −80°C or lower until RNA extraction. Paraffin sections were made from each specimen and classified according to International Union Against Cancer classification (3). Twenty-seven CCRCC tissues and nine corresponding normal kidney tissues were used in this study. The 27 tumors included 18 stage I, 2 stage II, 3 stage III, and 4 stage IV tumors, according to the tumor-node-metastasis classification (19). Institutional review board approval and written informed consent from each patient were obtained.

RNA extraction and microarray analysis. Tissues or cells were lysed directly in Trizol reagent (Invitrogen Corp.) and homogenized. Total RNA was extracted according to the manufacturer’s instructions. Twenty-seven CCRCC tissues and nine corresponding normal kidney tissues along with purchased RNA (TaKaRa) representing 15 different adult normal tissues (including one kidney tissue) were analyzed on HG-U133 Plus 2.0 array (Affymetrix) containing 54,675 probes for human genes. Further information on the source of RNA is available at our Web site. Microarray analysis was done essentially as described previously (20). For global normalization, the average signal in an array was given a value of 100.

Systematic selection of potential therapeutic target genes for CCRCC based on microarray analysis. We systematically explored CCRCC-specific genes by following criteria: (a) more than a 5-fold increase of median signal score across 27 CCRCCs compared with that of 9 adjacent normal kidney tissues; (b) median signal score across 27 CCRCCs of >500; and (c) signal score of <500 in all of normal tissues examined.

Quantitative reverse transcription PCR. After digesting genomic DNA using DNase I (Invitrogen), cDNA was synthesized from 1 μg total RNA using SuperScript III First-Strand Synthesis System for reverse transcription-PCR (Invitrogen). Quantitative reverse transcription-PCR (qRT-PCR) was done using SYBR Green I nucleic acid gel stain (BMA) with an iCycler IQ Detection System (Bio-Rad). Primers were as follows: 5′-GGCGCGGTGTTTTTGCAGA-3′ and 5′-TGAACCTGAT-AGTACCTTGTA-3′ for TLR3; 5′-GACTATGGTGAGAACCTCC-3′ and 5′-GAGACTGTCATGAGTTITTCC-3′ for IFNβ; and 5′-AGAAGGATGACTGCGCTTGCCACC-3′ and 5′-CCCTGCTTGATCACCACACTGCTG-3′ for β-actin. All samples were run in triplicate, and the results were averaged. No amplification of primer dimers was verified by checking the melting curve after PCR and gel electrophoresis. The expression levels of TLR3 and IFNβ were indicated as a relative ratio to that of β-actin.

Immunoblot. Immunoblot analysis was done as described previously (21). Briefly, tissues were lysed by 10 mmol/L Tris (pH 7.4), 150 mmol/L NaCl, 5 mmol/L EDTA, 1.0% Triton X-100, 1.0% sodium deoxycholate, and 0.1% SDS with protease inhibitor cocktail (Sigma) at 4°C. Anti-TLR3 antibody (clone 40C1285.6; 5 μg/mL; Immunex) or anti-β-actin antibody (clone AC-40; 0.1 μg/mL; Sigma) was used as primary antibodies.

Immunohistochemistry. A tissue microarray including 208 primary RCC specimens of three major histologic subtypes (189 CCRCCs, 11 papillary RCCs, and 8 chromophobe RCC) and 8 formalin-fixed, paraffin-embedded lung metastatic CCRCC specimens were prepared from the archives at The University of Tokyo Hospital. The tissue microarray was constructed as described previously (22). Preparations of sections, antigen retrieval, and immunostaining were carried out essentially as described previously (22). The primary antibody, anti-TLR3 antibody (clone 40C1285.6; 50 μg/mL) was applied for 1 h, followed by the secondary reaction with DAKO Envision+ Reagent (DakoCytomation). Immunoreactivity was evaluated by two pathologists (T.M. and S.O.) and scored 0 (negative staining), 1+ (weak staining), 2+ (moderate staining), or 3+ (strong staining).

Cell lines. Eight RCC cell lines were used in this study. OS-RC-2, TUHR10-TKB, and TUHR14-TKB were purchased from RIKEN Cell Bank (Tsukuba Science City, Japan). Caki-2 was purchased from the American Type Culture Collection. Caki-1, ACHN, SW839, and VMRC-TUHR10-TKB, and VMRC-TUHR14-TKB were purchased from RIKEN Cell Bank (Tsukuba Science City, Japan). Cells were cultured as monolayers in McCoy’s 5A medium (Invitrogen) for Caki-1 and Caki-2, or in RPMI 1640 medium (Sigma) for other cells, supplemented with 10% fetal bovine serum (Invitrogen). All cultures were maintained at 37°C in air containing 5% CO2.

Small interfering RNA. Transfection of cells with small interfering RNA (siRNA) targeting TLR3 was carried out using HiPerFect (Qiagen) at a final concentration of 20 nmol/L siRNA according to the manufacturer’s instructions. The target sequence of siRNA was 5′-CCUGAGCUCCUGACACAACACCUU-3′. siRNA targeting TLR3 and negative control siRNA were purchased from Invitrogen.

Cell viability assay. Cells were seeded at 2 × 104 or 4 × 103 cells per well (varied according to the growth speed) in 96-well plates and cultured for 24 h. In experiments examining the effect of IFNα, cells were incubated with or without IFNα (R&D Systems, Inc.) for additional 24 h. After discarding the supernatants, 100 μL of culture medium with or without poly(I:C) (Invitrogen) were added and incubated. Cell viability was determined by modified 3-(4,5-dime-thylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay (23) with Cell Counting Kit-8 (Dojindo Molecular Technologies, Inc.) according to the manufacturer’s instructions. Absorbance was measured at 450 nm and background absorbance measured at 630 nm was subtracted. Experiments were conducted in triplicate and the results were...
averaged. At least two independent experiments were done for each condition, and similar results were obtained.

**Apoptosis assay.** Apoptosis of the cells was visualized by Annexin V staining. TUHR10-TKB cells were seeded at 5 \times 10^5 cells per well in four-well culture slides (BD Biosciences) and cultured for 24 h. After discarding the supernatants, 500 µL culture medium, with or without 50 µg/mL poly(I:C), were added and incubated for 5 h. After discarding the supernatants, cells were incubated with fluorescein-conjugated Annexin V (Roche Diagnostics) in HBSS for 15 min. Apoptotic cells stained by Annexin V-fluorescein were detected by fluorescence microscopy and counted.

**Statistical analysis.** We analyzed the statistical significance of the relationship between the TLR3 immunoreactivity (0 and 1+ versus 2+ and 3+) and clinicopathologic features by \( \chi^2 \) test. Student’s \( t \) test was used to test differences in cell viability assays. Differences were considered significant at \( P < 0.05 \).

**Results**

**Microarray analysis identifies 34 genes specifically up-regulated in CCRCC.** To discover candidate therapeutic target genes for CCRCC, we examined the global gene expression profiles of CCRCC tissues as well as a panel of normal tissues by using microarray. We identified 34 genes whose expression was up-regulated in CCRCC tissues but low in normal tissues by using the criteria described in Materials and Methods (Fig. 1A). Among these genes, the CCRCC-specific overexpression of TLR3 was

![Fig. 1. Overexpression of TLR3 in CCRCC.](image-url)}
intriguing because TLR3 signaling induces IFN production in certain cells (13). Because immunotherapy, including IFNs, is the most common therapy against CCRCC, we expected that TLR3 signaling might be a novel therapeutic target in CCRCC. TLR3 was the only gene involved in such an immune response among the 34 genes selected, and overexpression of TLR3 has not been reported previously in CCRCC; then, we further investigated whether it represents a good molecular target for CCRCC.

**TLR3 mRNA up-regulation in CCRCC compared with normal kidney tissues.** To confirm array data, we did qRT-PCR analysis. Up-regulation of TLR3 mRNA was confirmed in all seven pairs of CCRCC compared with the corresponding normal kidney tissues analyzed (Fig. 1B).

**Frequent expression of TLR3 protein in primary/metastatic CCRCC and papillary RCC but not in chromophobe RCC.** To confirm the overexpression of TLR3 protein in CCRCC tissues, we did an immunoblot analysis. We observed overexpression of TLR3 protein in all four CCRCC tissues compared with corresponding normal kidney tissues for which protein was available (Fig. 2A).

To further examine the frequency and the localization of TLR3 protein expression in primary and metastatic RCC tissues, we carried out an immunohistochemical analysis using a tissue microarray. In normal kidney tissues, most tubules showed limited (1+) TLR3 staining. TLR3 staining was observed in the vast majority of CCRCCs (184 of 189, 97.4%; Fig. 2B and C) and papillary RCCs (9 of 11, 81.8%), but no chromophobe RCC among eight cases showed positive staining. One hundred thirty-nine of 189 (73.5%) CCRCCs showed 2+ or 3+ staining, which is stronger than most of normal tubules. TLR3 staining was observed in the cytoplasm as cytoplasmic vesicles, as reported for human dendritic cells (24), and sometimes clustered. The frequency of TLR3 expression in RCCs is summarized in Table 1. No correlation between TLR3 expression intensity and clinicopathologic features (age, sex, pT, grade, and venous invasion) was observed (Table 2).

TLR3 expression was observed not only in primary CCRCCs but also in lung metastatic CCRCCs. In six of eight (75%) lung metastatic CCRCC cases, >50% of cancer cells showed 2+ or 3+ staining, whereas normal lung or bronchial epithelium showed minimal staining (0-1+; Fig. 2D).

**In vitro anticancer effect of TLR3 ligand.** To examine the effects of TLR3 signaling in proliferation of RCC cells, we transfected OS-RC-2 cells with a synthetic siRNA duplex targeting human TLR3 or negative control siRNA, and in vitro cell viability assays were done. OS-RC-2 cells expressed a readily detectable abundance of TLR3 mRNA, which was markedly reduced on transfection with the TLR3-specific siRNA (Fig. 3A). The knockdown of TLR3 expression, however, did not affect the cell viability significantly (Fig. 3B, left). Next, we stimulated OS-RC-2 cells with poly(I:C), a TLR3 ligand. The cell viability was suppressed by poly(I:C), and this growth-inhibitory effect was halved by the TLR3-specific siRNA (Fig. 3B, right). Poly(I:C) also induced IFNβ expression in OS-RC-2 cells, which was reduced substantially by the knockdown of TLR3 expression (Fig. 3C). Among the eight RCC cell lines examined, five cells showed significant growth inhibition with 10 μg/mL poly(I:C). There was a positive correlation between the expression level of TLR3 and growth-inhibitory effect of poly(I:C) (r = 0.69; Fig. 3D). To evaluate whether apoptosis contributed to the reduced cell viability, TUHR10-TKB cells stimulated with poly(I:C) were stained by fluorescein-conjugated Annexin V. Approximately 10% of apoptotic cells were detected in

### Table 1. Immunohistochemical analysis of TLR3 in RCCs

<table>
<thead>
<tr>
<th>TLR3 Staining</th>
<th>No. cases</th>
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<tbody>
<tr>
<td>0</td>
<td>45</td>
</tr>
<tr>
<td>1+</td>
<td>85</td>
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<td>2+</td>
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**Table 1.** Immunohistochemical analysis of TLR3 in RCCs.
TUHR10-TKB cells stimulated with poly(I:C), whereas <1% of apoptotic cells were detected in control TUHR10-TKB cells (Supplementary Fig. S1).

Combination of IFNα and TLR3 ligand exerts a synergistic growth-inhibitory effect against Caki-1 RCC cells. IFNα is known to enhance TLR3 signaling by up-regulating TLR3 expression in certain cells (25). Thus, we expected that IFNα would reinforce poly(I:C)-induced growth-inhibitory effect in RCC cells. As shown in Fig. 4A, IFNα stimulation up-regulated TLR3 expression in Caki-1 cells but not in Caki-2 cells. Furthermore, poly(I:C)-induced IFNβ expression (Fig. 4B) and growth-inhibitory effect (Fig. 4C) in Caki-1 cells were dramatically enhanced when pretreated with IFNα. Viabilities of cells treated with 10,000 units/mL IFNα or 50 μg/mL poly(I:C) alone were decreased by only 23% and 17% compared with that of untreated cells, respectively. When the two agents were used together, the decrease in cell viability was increased to as much as 76% (Fig. 4C). In other words, IFNα and poly(I:C) exerted a synergistic growth-inhibitory effect against Caki-1 cells. Such an effect was not observed in Caki-2 cells (Fig. 4D).

Discussion

In this study, we examined the global gene expression profiles of CCRCC tissues as well as a panel of normal tissues by using microarray to discover candidate therapeutic target genes for CCRCC. We identified 34 genes, of which 24 were annotated unique genes whose expression was specifically up-regulated in CCRCC. These genes included several reported candidate therapeutic and/or diagnostic target genes for CCRCC (ANGPTL4, NDUFA4L2, EGLN3, HIG2, CP, FLT1, CCND1, VEGF, and EDN1), suggesting robustness of our selection for CCRCC-specific genes. Among these genes, the CCRCC-specific overexpression of TLR3 was intriguing because TLR3 signaling induces IFN production in certain cells (13), suggesting its use as a therapeutic target in CCRCC.

Here, we showed that TLR3 is frequently overexpressed in both primary and metastatic CCRCCs (Fig. 2). Moreover, we showed that poly(I:C), a TLR3 ligand, induced TLR3-dependent growth-inhibitory effect in RCC cells (Fig. 3). These results suggest that a TLR3 agonist may be an effective

Table 2. Correlation between TLR3 expression and clinicopathologic features in CCRCC

| No. cases | TLR3 staining | P  
|-----------|---------------|-----
| Age (y)   | Weak (0, 1+)  | Strong (2+, 3+) |
| <60       | 97            | 28   | 69   | 0.5435 |
| ≥60       | 92            | 22   | 70   |       |
| Sex       |               |      |      |       |
| Male      | 143           | 35   | 108  |       |
| Female    | 46            | 15   | 31   | 0.3699 |
| Grade     |               |      |      |       |
| 1, 2      | 129           | 35   | 94   |       |
| 3, 4      | 60            | 15   | 45   | 0.8941 |
| PT        |               |      |      |       |
| 1, 2      | 157           | 39   | 118  |       |
| 3, 4      | 32            | 11   | 21   | 0.3704 |
| Venous invasion |       |      |      |       |
| Negative  | 138           | 31   | 107  |       |
| Positive  | 51            | 19   | 32   | 0.0627 |

Fig. 3. Poly(I:C)-induced, TLR3-dependent growth-inhibitory effect in RCC cells. A, OS-RC-2 cells were transfected with control or TLR3 siRNA. After 48 h, cells were collected and the expression levels of TLR3 were analyzed by qRT-PCR. Columns, TLR3/ACTB; bars, SD. B, OS-RC-2 cells were transfected with control or TLR3 siRNA. After 72 h, cells were stimulated with or without 50 μg/mL poly(I:C) for 48 h. Cell numbers were determined as described in Materials and Methods. Columns, absorbance; bars, SD. C, OS-RC-2 cells were transfected with control or TLR3 siRNA. After 72 h, cells were stimulated with or without 50 μg/mL poly(I:C) for 6 h. Cell numbers were determined as described in Materials and Methods. Columns, viability; bars, SD. **, P < 0.01, ***, P < 0.001, respectively, statistical difference from respective controls.
anticancer agent in CCRCC with TLR3 overexpression. IFNβ, one of the type I IFNs, has diverse biological functions, including antiviral activity, growth inhibition, and immune cell stimulation (26). Intratumoral transfection of the IFNβ gene has been reported to cause dramatic regression of tumors in a xenograft model (27), although clinical experience with IFNβ protein therapy of solid tumors, including CCRCCs, has been disappointing, probably due to an extremely short half-life in the blood (28, 29). Researchers have attempted IFNβ gene therapy, and methods of IFNβ gene transfection into RCC cells have been investigated (30, 31). Our results suggest that activation of TLR3 on RCC cells is a new way to induce IFNβ expression, resulting in growth inhibition. A recent study by Salaun et al. (32) has also shown that poly(I:C) induced IFNβ production and apoptosis in breast cancer cells in vitro, consistent with our results. In Salaun’s study, however, expression levels of TLR3 in cancer cells were not indicated. In our study, there was a positive correlation between the expression level of TLR3 and growth-inhibitory effect by poly(I:C) (Fig. 3D). Because TLR3 mRNA level was low in all normal tissues examined compared with CCRCC tissues, it is expected that adverse effects of TLR3 agonist may be low.

Immunotherapies targeting TLRs, including TLR3, against human malignancies have been drawing attention lately (10, 33). The accepted rationale for those therapies is that TLR agonists stimulate TLR-expressing immune cells, such as dendritic cells, and lead to anticancer effects. The present study is the first report that RCC cells themselves express TLR3 in vivo and in vitro. Moreover, we showed that TLR3 expressed on RCC cells is functional and may be able to induce apoptosis and a direct anticancer effect. Thus, although further studies using in vivo models are warranted, this study presents a novel rationale for therapeutic targeting of TLR3 in CCRCC.

We also showed that a combination of poly(I:C) and IFNα exerted a synergistic growth-inhibitory effect against Caki-1 RCC cells (Fig. 4C). This synergistic effect was apparently due to up-regulation of TLR3 expression (Fig. 4A) and reinforcing TLR3 signaling as shown by IFNβ expression (Fig. 4B), although we could not exclude the possibility that IFNα also affected the expression of other double-stranded RNA receptors, such as RIG-1 (34). IFNα is commonly used in systemic therapy against metastatic CCRCC, but the response rate is only 10% to 20%, and mostly the response is partial (5). A combination therapy of IFNα and another agent is therefore expected to be established. For example, a randomized phase III trial investigating the addition of bevacizumab to initial IFNα therapy in advanced CCRCC is ongoing (35). Our result suggests that stimulation of TLR3 on RCC cells with TLR3 agonist may improve the anticancer effect of IFNαs. However, a combination of TLR3 agonist and IFNα may also kill normal cells (36). Further examination is therefore necessary before clinical application.

The mechanism for TLR3 overexpression and its role in CCRCC is unclear. The majority of CCRCCs are characterized by VHL gene inactivation (37), which is, however, unlikely to cause TLR3 overexpression, because TLR3 overexpression was observed not only in CCRCCs but also in papillary RCCs (Table 1), where VHL gene abnormalities have not been reported (37). TLR3 is reported to be induced by various kinds of cytokines, such as IFNs, tumor necrosis factor-α, and interleukin-1 (38). The expression analysis in this study revealed overexpression of cytokine-inducible genes, such as ISG20 (39) and TNFAIP6 (40), in CCRCC tissues (data not shown). Therefore, we speculate that some cytokines, possibly produced by cancer cells or infiltrating immune cells, induce TLR3 expression in CCRCCs. Because the knockdown of TLR3 expression on RCC cells alone did not affect cell viability significantly, TLR3 expression itself is unlikely to play an important role in proliferation of CCRCC cells. Further studies are needed to clarify the mechanism and the role of TLR3 overexpression in CCRCC.
Spontaneous cancer regression, although rare, has been observed even in the advanced stage of RCC (41). For example, in a series of 73 patients with metastatic RCC, 5 (7%) patients had “unexplained spontaneous regression” during the period of observation (42). The mechanism for spontaneous regression is unclear, although an involvement of immunologic factors have been implicated. Involvement of TLR3 signaling is suggested by our results that (a) metastatic CRCCs frequently overexpressed TLR3, and (b) stimulation of TLR3 on RCC cells with its ligand resulted in their growth inhibition, and the reported evidence that (c) endogenous mRNA released from or associated with necrotic cells as well as double-stranded RNA produced by viruses could be a ligand for TLR3 (43).

In conclusion, we identified frequent overexpression of TLR3 in both primary and metastatic CRCC tissues. Stimulation of TLR3 on RCC cells with its ligand induced IFNβ expression and growth inhibition. Moreover, TLR3 ligand and IFNexerted a synergistic growth-inhibitory effect against Caki-1 RCC cells. Thus, TLR3 pathway could be a novel therapeutic target for CRCC.

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References

Identification of Toll-Like Receptor 3 as a Potential Therapeutic Target in Clear Cell Renal Cell Carcinoma

Teppei Morikawa, Akira Sugiyama, Haruki Kume, et al.


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