

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

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**Abstract Purpose:** Renal cell carcinoma (RCC) is one of the most drug-refractory cancers. The aim of this study is to discover a novel therapeutic target molecule for clear cell RCC (CCRCC), which accounts for the majority of RCC.

**Experimental Design:** Gene expression profiles of 27 CCRCCs and 9 normal kidney tissues as well as 15 various adult normal tissues were examined by Affymetrix U133 Plus 2.0 arrays. Among the 34 genes specifically up-regulated in CCRCC, overexpression of Toll-like receptor 3 (TLR3) mRNA and its protein was validated by quantitative reverse transcription-PCR, immunoblot, and immunohistochemistry. The effects of TLR3 signaling on *in vitro* cell growth were examined.

**Results:** *TLR3* gene was highly expressed in CCRCC, with only limited expression in a panel of normal tissues. On immunohistochemical analysis using a monoclonal antibody against TLR3, overexpression of TLR3 was observed in 139 of 189 (73.5%) cases of CCRCC as well as in lung metastatic CCRCC (6 of 8), whereas TLR3 expression was entirely absent in chromophobe RCC (0 of 8). Polyinosinic-polycytidilic acid, a TLR3 ligand, exerted a growth-inhibitory effect against RCC cells in a *TLR3*-dependent manner. Moreover, a combination of polyinosinic-polycytidilic acid and IFN $\alpha$  exerted a synergistic growth-inhibitory effect against Caki-1 RCC cells.

**Conclusions:** This is the first report that TLR3 is overexpressed in CCRCC. These observations suggest that TLR3 pathway may represent a novel therapeutic target in CCRCC.

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 IFN $\alpha$  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-1 $\alpha$ . 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

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Received 3/14/07; revised 6/27/07; accepted 7/10/07.

**Grant support:** Program of Fundamental Studies in Health Sciences of the National Institute of Biomedical Innovation, NFAT project of New Energy and Industrial Technology Development Organization (T. Kodama), and Grants-in-Aid for Scientific Research (S) 16101006 and Scientific Research on Priority Areas (C) 17015008 from the Ministry of Education, Culture, Sports, Science and Technology (H. Aburatani).

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**Note:** Supplementary data for this article are available at Clinical Cancer Research Online (<http://clincancerres.aacrjournals.org/>).

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doi:10.1158/1078-0432.CCR-07-0603

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 IFN $\beta$  (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 over-expressed 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 IFN $\alpha$  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  $-80^{\circ}\text{C}$  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.<sup>6</sup> 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  $\mu\text{g}$  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'-GGCGGGTGTGTTTGAAGTACTAGA-3' and 5'-TGAAGTGCATGATGTACCTTGA-3' for *TLR3*; 5'-GACTATTGTTGAGAACCTCC-3' and 5'-AGACTGCTCATGAGTTTCC-3' for *IFN $\beta$* ; and 5'-AGAAGGAGATCACTGCCCTGGCACC-3' and 5'-CCTGCTTGCTGATCCACATCTGCTG-3' for  *$\beta$ -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 *IFN $\beta$*  were indicated as a relative ratio to that of  *$\beta$ -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  $4^{\circ}\text{C}$ . Anti-*TLR3* antibody (clone 40C1285.6; 5  $\mu\text{g}/\text{mL}$ ; Imgenex) or anti- *$\beta$ -actin* antibody (clone AC-40; 0.1  $\mu\text{g}/\text{mL}$ ; 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  $\mu\text{g}/\text{mL}$ ) 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-RCW were obtained from Cell Resource Center for Biomedical Research, Tohoku University (Sendai, Japan). Cells were cultured as monolayers in McCoy's 5A modified 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  $37^{\circ}\text{C}$  in air containing 5%  $\text{CO}_2$ .

**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'-CCUGAGCUGUCAAGCCACUACCUUU-3'. siRNA targeting *TLR3* and negative control siRNA were purchased from Invitrogen.

**Cell viability assay.** Cells were seeded at  $2 \times 10^3$  or  $4 \times 10^3$  cells per well (varied according to the growth speed) in 96-well plates and cultured for 24 h. In experiments examining the effect of IFN $\alpha$ , cells were incubated with or without IFN $\alpha$  (R&D Systems, Inc.) for additional 24 h. After discarding the supernatants, 100  $\mu\text{L}$  of culture medium with or without poly(I:C) (Invivogen) were added and incubated. Cell viability was determined by modified 3-(4,5-dimethylthiazol-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 630nm was subtracted. Experiments were conducted in quadruplicate and the results were

<sup>6</sup> <http://www.lsbm.org/db/index.html>

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  $5 \times 10^4$  cells per well in four-well culture slides (BD Biosciences) and cultured for 24 h. After discarding the supernatants, 500  $\mu$ L culture medium, with or without 50  $\mu$ g/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.

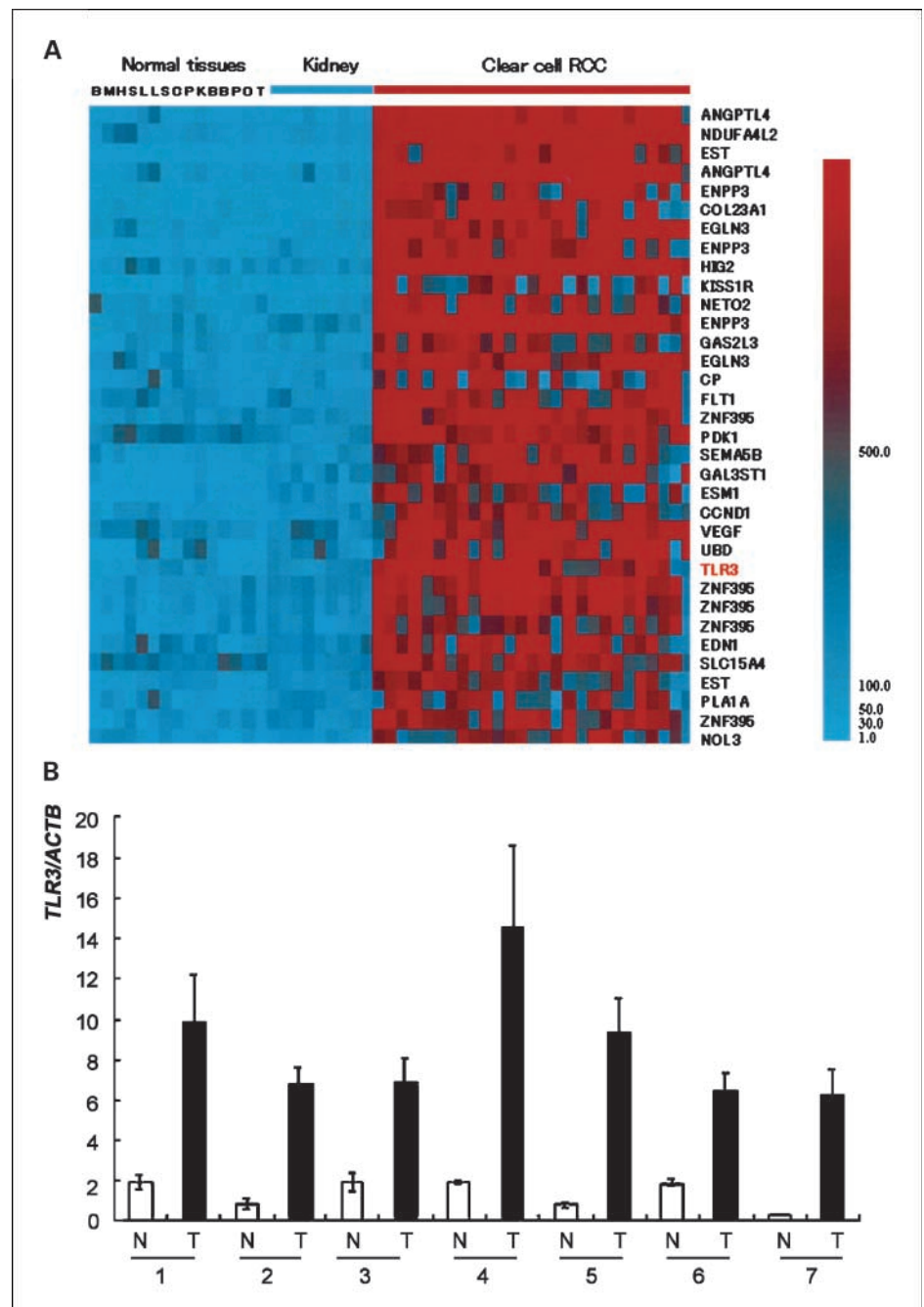
**Statistical analysis.** We analyzed the statistical significance of the relationship between the TLR3 immunoreactivity (0 and 1+ versus 2+ and 3+) and clinicopathologic features by  $\chi^2$  test. Student's *t* test was

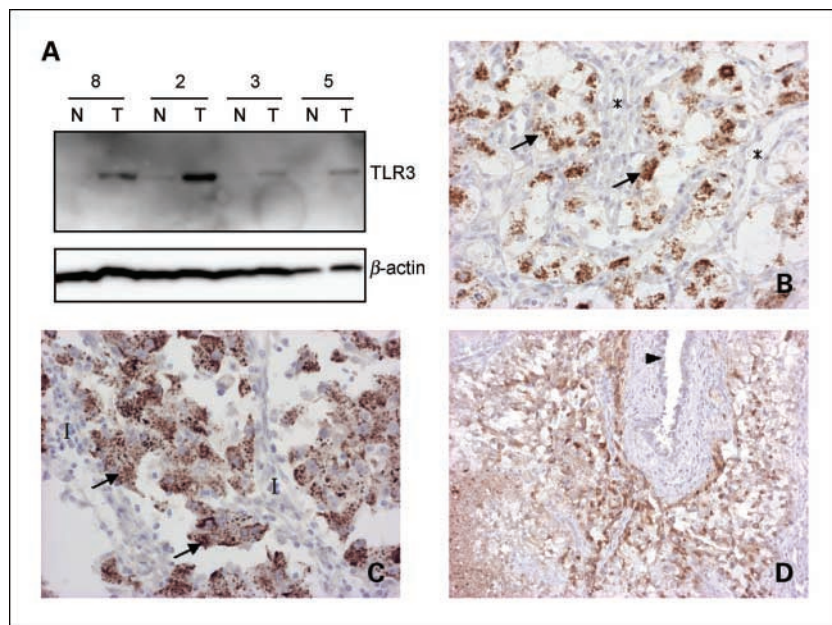
used to test differences in cell viability assays. Differences were considered significant at  $P < 0.05$ .

## 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 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. **A**, gene expression in a panel of 15 normal tissues, 9 normal kidneys, and 27 CCRCCs. Thirty-four CCRCC-specific genes are shown. Each row in the heat map represents a gene, and each column represents a sample. A panel of normal tissues include brain (B), muscle (M), heart (H), skin (S), lung (L), liver (L), stomach (S), colon (C), pancreas (P), kidney (K), bladder (B), bone marrow (B), peripheral blood (P), ovary (O), and testis (T) in order from the left to the right. **B**, qRT-PCR analysis on seven pairs of CCRCC and corresponding normal kidney tissues. Columns, *TLR3/ACTB*; bars, SD.





**Fig. 2.** Expression of TLR3 protein in RCC. *A*, immunoblot analysis of TLR3 protein in four pair samples of CCRCC (*top*).  $\beta$ -Actin was used as an internal control (*bottom*). *B* to *D*, immunohistochemical staining of TLR3. *B*, strong staining in grade 1 CCRCC. Positive staining was observed only in cancer cells (*arrows*) not in blood vessels (\*). Magnification,  $\times 400$ . *C*, strong staining in grade 3 CCRCC. Positive staining was observed only in cancer cells (*arrows*) not in tumor-infiltrating inflammatory cells (*I*). Magnification,  $\times 400$ . *D*, strong staining was also observed in lung metastatic CCRCC, whereas normal bronchial epithelium (*arrowhead*) showed minimal staining. Magnification,  $\times 200$ .

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 *IFN $\beta$*  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  $\mu$ g/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

	No. cases	TLR3 staining			
		0	1+	2+	3+
CCRCC	189	5	45	85	54
Papillary RCC	11	2	3	4	2
Chromophobe RCC	8	8	0	0	0

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

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

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 IFN $\alpha$  and TLR3 ligand exerts a synergistic growth-inhibitory effect against Caki-1 RCC cells.** IFN $\alpha$  is known to enhance TLR3 signaling by up-regulating TLR3 expression in certain cells (25). Thus, we expected that IFN $\alpha$  would reinforce poly(I:C)-induced growth-inhibitory effect in RCC cells. As shown in Fig. 4A, IFN $\alpha$  stimulation up-regulated TLR3 expression in Caki-1 cells but not in Caki-2 cells.

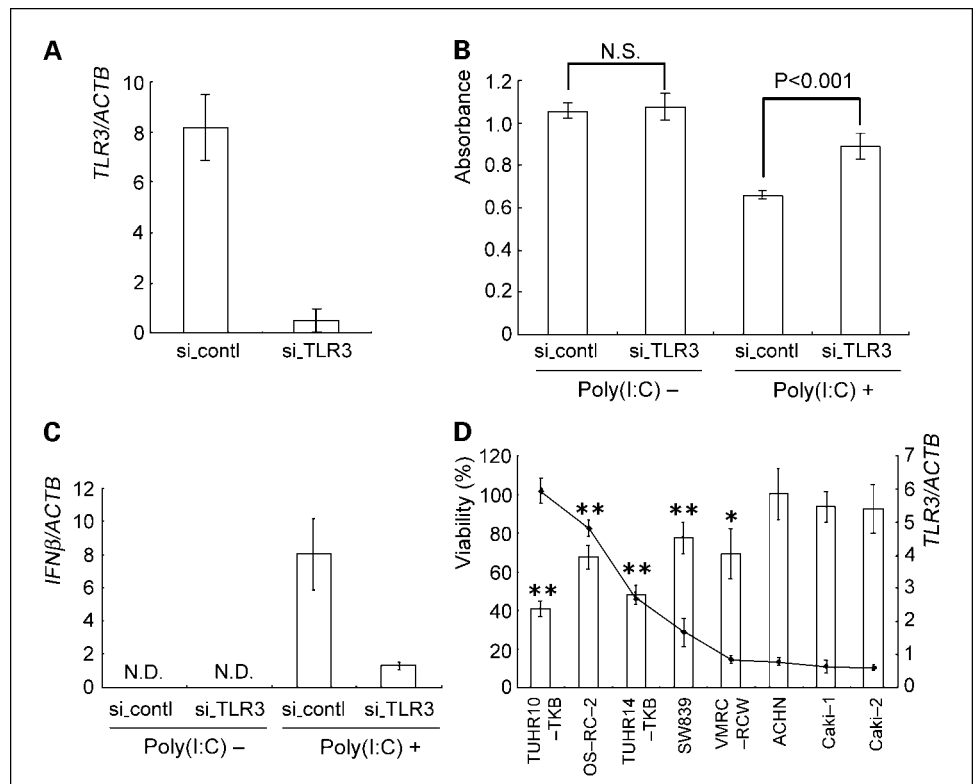
Furthermore, poly(I:C)-induced IFN $\beta$  expression (Fig. 4B) and growth-inhibitory effect (Fig. 4C) in Caki-1 cells were dramatically enhanced when pretreated with IFN $\alpha$ . Viabilities of cells treated with 10,000 units/mL IFN $\alpha$  or 50  $\mu$ g/mL poly(I:C) alone were decreased by only 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, IFN $\alpha$  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).

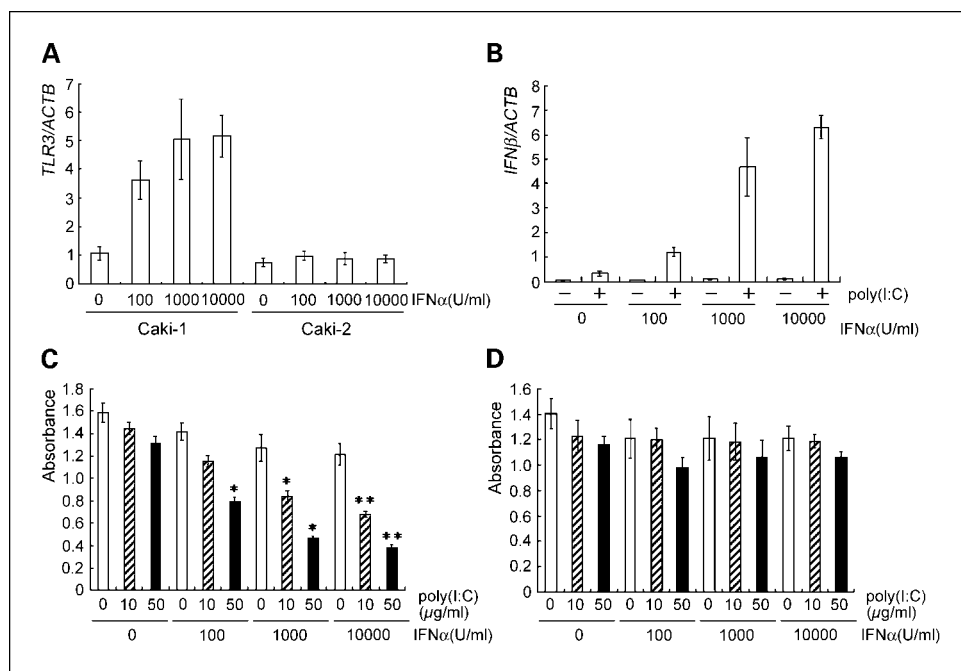
## Discussion

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 IFN $\beta$  expression and growth inhibition in RCC cells (Fig. 3). These results suggest that a TLR3 agonist may be an effective

**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  $\mu$ g/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  $\mu$ g/mL poly(I:C). After 6 h, cells were collected and the expression levels of IFN $\beta$  were analyzed by qRT-PCR. N.D., not detected. Columns, IFN $\beta$ /ACTB; bars, SD. **D**, the expression levels of TLR3 in eight RCC cell lines were determined by qRT-PCR and represented on the line plot. Cells were treated for 72 h with or without 10  $\mu$ g poly(I:C). Cell numbers determined as described in Materials and Methods are represented on the bar graphs as a percentage, with cultures in medium alone considered to be 100%. Columns, viability; bars, SD. \*,  $P < 0.05$ ; \*\*,  $P < 0.01$ , respectively, statistical difference from respective controls.





**Fig. 4.** A combination of IFN $\alpha$  and poly(I:C) exerted a synergistic growth-inhibitory effect in Caki-1 cells but not in Caki-2 cells. **A**, Caki-1 or Caki-2 cells were stimulated without or with increasing doses of IFN $\alpha$ . After 6 h, cells were collected and the expression levels of *TLR3* were analyzed by qRT-PCR. Columns, *TLR3/ACTB*; bars, SD. **B**, Caki-1 cells were stimulated without or with increasing doses of IFN $\alpha$  for 24 h, after which supernatants were discarded and cells were stimulated without or with 50  $\mu$ g/mL poly(I:C). After 6 h, cells were collected and the expression levels of *IFN $\beta$*  were analyzed by qRT-PCR. Columns, *IFN $\beta$ /ACTB*; bars, SD. **C** and **D**, Caki-1 (**C**) or Caki-2 (**D**) cells were stimulated without or with increasing doses of IFN $\alpha$  for 24 h, after which supernatants were discarded and cells were stimulated without or with poly(I:C). After 48 h, cell numbers were determined as described in Materials and Methods. \*,  $P < 0.05$ ; \*\*,  $P < 0.01$ , respectively, statistical difference between the growth-inhibitory effect by a combination of IFN $\alpha$ +poly(I:C) and the sum of the growth-inhibitory effect by the same concentrations of IFN $\alpha$  or poly(I:C) alone. Columns, absorbance; bars, SD.

anticancer agent in CCRCC with TLR3 overexpression. IFN $\beta$ , one of the type I IFNs, has diverse biological functions, including antiviral activity, growth inhibition, and immune cell stimulation (26). Intratumoral transfection of the *IFN $\beta$*  gene has been reported to cause dramatic regression of tumors in a xenograft model (27), although clinical experience with IFN $\beta$  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 *IFN $\beta$*  gene therapy, and methods of *IFN $\beta$*  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 IFN $\beta$  expression, resulting in growth inhibition. A recent study by Salaun et al. (32) has also shown that poly(I:C) induced IFN $\beta$  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 IFN $\alpha$  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 *IFN $\beta$*  expression (Fig. 4B), although we could not exclude the possibility that IFN $\alpha$  also affected the expression of other double-stranded RNA receptors, such as RIG-1 (34). IFN $\alpha$  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 IFN $\alpha$  and another agent is therefore expected to be established. For example, a randomized phase III trial investigating the addition of bevacizumab to initial IFN $\alpha$  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 IFN $\alpha$ . However, a combination of TLR3 agonist and IFN $\alpha$  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- $\alpha$ , 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 CCRCCs 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 CCRCC tissues. Stimulation of TLR3 on RCC cells with its ligand induced *IFN $\beta$*  expression and growth inhibition. Moreover, TLR3 ligand and IFN $\alpha$  exerted a synergistic growth-inhibitory effect against Caki-1 RCC cells. Thus, TLR3 pathway could be a novel therapeutic target for CCRCC.

## Acknowledgments

We thank Drs. Atsushi Kaneda, Akira Watanabe, Takayuki Isagawa, and Hisashi Hasumi for helpful comments, Shogo Yamamoto for data processing, and Hiroko Meguro for excellent technical support.

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