Low Expression of Toll-like Receptor 4 Is Associated With Poor Prognosis in Bladder Cancer

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Abstract. *Background/Aim:* The aim of this study was to elucidate the relationship between the progression of bladder cancer (BCa) and TLR4 expression. *Materials and Methods:* The relationship between TLR4 expression and prognosis of BCa patients was analyzed using a publicly available database and immunohistochemical staining of clinical samples. The effect of TLR4 knockdown was also examined on the invasive capabilities of BCa cells. Finally, to investigate the biological function of TLR4, the gene expression profile of TLR4-depleted BCa cells was analyzed by microarray analysis. *Results:* Expression of TLR4 was inversely associated with prognosis of patients with invasive BCa, and depletion of TLR4 significantly enhanced the invasive capability of BCa cells. Gene expression profiling revealed that depletion of TLR4 led to high expression of epithelial differentiation genes. Furthermore, expression of TLR4 was found to be extremely low in areas of squamous differentiation. *Conclusion:* Low TLR4 expression was correlated with tumor progression.

Bladder cancer (BCa) is one of the most common malignancies of the urinary tract worldwide. In 2016, 76,960 new cases of BCa and 16,390 BCa-related deaths were estimated in the United States (1). Approximately 30% of patients with BCa present with muscle-invasive BCa (MIBC) (2), however, effective treatments for MIBC have not yet been established. Elucidating the underlying mechanisms of BCa progression is likely to facilitate the development of new therapeutic approaches to the treatment of MIBC.

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Key Words: Bladder cancer, Toll-like receptor 4, squamous differentiation.

Many gene mutations are involved in the development of BCa, and BCa is thought to be an immunogenic tumor (3). Though various kinds of immune cells are induced via recognition of these tumor neoantigens, BCa cells skillfully survive attacks from these cells and further develop. While inflammation driven from tumor-specific immune reaction can suppress cancer development, non-cancer specific inflammation may even boost cancer cell proliferation (4).

Toll-like receptors (TLRs) are involved in inflammatory cytokine production and are expressed in a variety of epithelial and cancer cells (5-7). More than 10 human TLRs have been identified, and current evidence indicates that the TLR signaling cascade is functionally associated with tumor growth, progression, and invasion (8). Previous studies have reported that TLR expression levels differ between cancer types (9, 10), and in BCa, Olbert *et al.* showed the potential involvement of TLR-dependent immune responses in BCa progression (11). However, the precise role of TLRs in BCa pathology remain unclear.

This study, aimed to clarify the contribution of TLRs, especially TLR4, expression to BCa progression and analyze its function, using a panel of human BCa cell lines.

Materials and Methods

Cell lines and patient specimens. UM-UC-3, 5637, J82 and T24 cell lines were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). RT112 was purchased from Cosmo Bio Co. (Japan). All cells were cultured as recommended by respective sources. A total of 95 patients with BCa diagnosed and operated (transurethral bladder tumor resection) at Tokushima University hospital. Surgically resected patient samples and their clinicopathological information were acquired from Tokushima University Hospital (Tokushima, Japan) after obtaining written informed consent. This study and the use of all clinical materials described above were approved by the ethical review board of Tokushima University Hospital (permission number 709).

Immunohistochemical analysis (IHC). Sections of paraffin-embedded BCa tissue were deparaffinized in xylene and then rehydrated in a graded ethyl alcohol series (100%, 95%, and 90%), before being incubated for antigen retrieval using citrate buffer (10 nM, pH 6.0) for 10 min. To increase specificity and sensitivity, tissues were autoclaved for 10 min to retrieve antigen. After cooling and rinsing in distilled water, endogenous peroxide activity was blocked with 3% H₂O₂ for 10 min. Then, samples were rinsed in 0.01 mol/l phosphate-buffered saline (PBS, pH 7.4) for 10 min and pre-incubated with a protein blocking solution for 5 min. Sections were incubated with a mouse monoclonal antibody against TLR4 (Abcam, ab22048; diluted 1:200 in PBS) overnight at 4°C. They were then washed three times in PBS and incubated with Dako REAL™ EnVision™/HRP, Rabbit/Mouse (DAKO) 1-2 drops for 1 h at room temperature, before being again washed with PBS and incubated with diaminobenzidine (DAB) and substrate chromogen system for 5 min at room temperature, which resulted in brown colored precipitates at the antigen site. Sections were then counterstained with hematoxylin for 1 min and mounted. Sections with more than 10% positive cancer cells were regarded as positive samples.

Evaluation of immunostaining. Three researchers, blinded to clinical features and outcomes, evaluated each slide independently. The IHC expression intensity of TLR4 was scored on a scale of 0 (no staining), 1 (low), 2 (intermediate) and 3 (high), and the percentage of TLR4 positive cells was also evaluated. Multiplication of the TLR4 intensity score and percentage was used to divide patients into two groups for outcome analysis (values ≤60 representing low-expression, and >60 representing high expression).

Quantitative and semi-quantitative reverse transcription-PCR. Total RNA was extracted from cultured cells using the RNeasy Mini Kit (QIAGEN, Valencia, CA, USA) as previously described (12). We prepared the appropriate dilutions of each single-stranded cDNA for subsequent PCR amplification and monitored reactions using β -actin as a quantitative control. Each sample was analyzed in triplicate for each primer pair. The relative expression levels of target genes were obtained using Light Cycler Software Ver.3.5 (Roche Diagnostics, Madison, WI, USA), and calculated using the $2^{-\Delta\Delta C_t}$ method.

Western blot analysis. Cells were lysed with Mammalian Cell Extraction kit (Bio Vision, Mountain View, CA). After centrifugation at 14,000 g for 10 min, the supernatant was collected as the total protein extract and stored at -80°C until use. Equal amounts of protein were separated by SDS-PAGE and transferred to a polyvinylidene difluoride membrane. The membrane then was blocked with Block Ace (Dainippon Pharmaceutical, Osaka, Japan) for 60 min and incubated with anti-TLR4 (Abcam, ab22048, dilution 1:100) or anti- β -actin (Sigma-Aldrich, A5441, dilution 1:1,000) primary antibodies at 4°C overnight. After incubation with an HRP-conjugated secondary antibody (Thermo Fisher Scientific, Waltham, MA, USA, dilution 1:1,000) for 60 min, blots were developed with an enhanced ECL Plus Kit (GE Healthcare, Piscataway, NJ, USA), and scanned using an Image Reader LAS-500 (Fujifilm, Tokyo, Japan).

RNA interference. siRNA oligonucleotides were used to knock down TLR4 in bladder cancer cell lines. Non-silencing RNA duplex was used as a negative control. Transfection with 10 nM of each siRNA

to each cell line was performed using Lipofectamine RNAiMAX (Thermo Fisher Scientific) with the reverse transfection method.

Microarray analysis. Cyanine-3 (Cy3) labeled cRNA was prepared from 150 ng RNA using the One-Color Low Input Quick Amp Labeling kit (Agilent, Santa Clara, CA, USA) according to the manufacturer's instructions, followed by RNeasy column purification (QIAGEN). Dye incorporation and cRNA yield were checked with the NanoDrop ND-1000 Spectrophotometer.

The amount of 600 ng of Cy3-labelled cRNA (specific activity >6 pmol Cy3/ μ g cRNA) was fragmented at 60°C for 30 min in a reaction volume of 25 μ l containing 25 $\times$ Agilent fragmentation buffer and 10 $\times$ Agilent blocking agent following the manufacturer's instructions. On completion of the fragmentation reaction, 25 μ l of 2 $\times$ Agilent hybridization buffer was added to the fragmentation mixture and hybridized to Agilent SurePrint GE Unrestricted Microarrays (G2519F) for 17 h at 65°C in a rotating Agilent hybridization oven. After hybridization, microarrays were washed 1 minute at room temperature with GE Wash Buffer 1 (Agilent) and 1 min with 37°C GE Wash buffer 2 (Agilent), then dried immediately.

Slides were scanned immediately after washing on the Agilent DNA Microarray Scanner (G2505C) using one color scan setting for 8 $\times$ 60K array slides (Scan Area 61 $\times$ 21.6 mm, Scan resolution 3 μ m, Dye channel was set to Green and Green PMT was set to 100%).

The scanned images were analyzed with Feature Extraction Software 10.7.1.1 (Agilent) using default parameters (protocol GE1_107_Sep09 and Grid: 028282_D_F_20110531) to obtain background subtracted and spatially detrended processed signal intensities. Features flagged in Feature Extraction as Feature Non-uniform outliers were excluded.

MTT assay. The 5637 cells were seeded into 96-well plates at 1.0×10^4 cells (100 μ l) per well and maintained in medium containing 10% fetal bovine serum (FBS). After a 72, 96 and 120 h incubation, cell proliferation was evaluated using the Cell Counting Kit-8 (Dojindo, Kumamoto, Japan). At the indicated time points, a 1:10 dilution of the CCK-8 solution was added and incubated for 30 min. Then, the absorbance at 450 nm was measured to calculate the number of vital cells/well using VARIOSKAN FLASH (Thermo Fisher Scientific).

In vitro invasiveness and migration assays. Invasion and migration assays of 5637 cells, with and without TLR4 expression, were performed using the xCELLigence RTCA Analyzer (Roche Applied Science, Mannheim, Germany) in a humidified atmosphere of 5% CO₂ and 95% air at 37°C. Cell invasion and migration (CIM) plates (ACEA Biosciences, San Diego, CA, USA) initially contained cells in transfection solution at a density of 4×10^4 cells per well; this media was replaced with fresh RPMI1640 medium. The lower wells were filled with 160 μ l of RPMI1640 medium with 10% FBS. Invasion capability was tested using a tumor invasion system (BD Biosciences, Bedford, MA, USA), in Matrigel diluted 1:40 with RPMI1640 medium (as detailed by Roche in the application notes) coated onto 16-well plates. Measurement of changes in the cell index, which shows cell invasion or migration status based on the measured electrical impedance, were made over time intervals between 0 to 60 h.

Bioinformatic analyses. PrognoScan database (<http://www.abren.net/PrognoScan/>) analysis was conducted. The expression levels of TLR genes, SPRRs and keratins were analyzed with the dataset of BCa

Figure 1. Relationship between TLR4 expression and prognosis of patients with BCa. (A) Kaplan-Meier analysis of overall survival of BCa patients according to TLR4 expression (based on outcome data provided by Prognoscan). (B) IHC with anti-TLR4 antibody, with each image scored from 0 to 3 for expression level. (C) Kaplan-Meier analysis of overall survival of BCa patients according to TLR4 expression. (D) TLR4 expression in TUR specimens by IHC. Arrows indicate areas of squamous differentiation.

patients (GSE 13507). Gene datasets from microarray analyses were submitted to DAVID (Database for Annotation, Visualization, and Integrated Discovery; <http://david.abcc.ncifcrf.gov/>) to identify significantly enriched biological process (BP), molecular function (MF), and cellular component (CC) terms based on the GO (Gene Ontology) database.

Statistical analysis. Cancer-specific survival curves were estimated using the Kaplan-Meier method, with significance assessed by log-rank test. High and low TLR4-expressing patients were defined as

those with greater than, or less than the median level of TLR4 expression, as described in the evaluation of immunostaining section, respectively. Cox proportional hazards analysis was used to identify significant prognostic clinical factors and to test for independent contributions to cancer-specific survival. Statistical significance in regard to cell proliferation or gene expression analysis was calculated by Student's *t*-test. Significance levels of $p < 0.05$ (*) and $p < 0.001$ (**) were defined, and analyses were performed with SPSS Statistics® 25 (SPSS, IBM Corp, Armonk, NY, USA).

Results

Relationship between TLR expression and BCa prognosis. To identify which TLRs might contribute to progression of BCa, the relationship between their levels of expression and prognosis was analyzed using the public database, Prognoscan (<http://www.prognoscan.org/>). This revealed that low expression of TLR4 was significantly associated with poor prognosis in BCa patients (Figure 1A), and that the relationship was stronger for TLR4 than for any of the other TLRs (Table I).

Clinicopathological characteristics and TLR4 expression in BCa. To confirm the relationship between TLR4 expression and prognosis of BCa patients at the protein level, transurethral resection (TUR) BCa specimens were analyzed by IHC. This analysis confirmed the association with prognosis (Figure 1B, C), and indicated that the expression of TLR4 was significantly associated with T stage and presence of metastasis (Table II). In addition, IHC staining with an TLR4 antibody revealed that the expression of TLR4 was especially low in areas where squamous differentiation was observed (Figure 1D).

TLR4 expression in BCa cell lines. The expression of TLR4 in BCa cell lines was analyzed by western blotting. This analysis revealed that three of five BCa cell lines, 5637, RT112 and J82 cells, had relatively high expression of TLR4 (Figure 2A).

To assess whether TLR4 expression might affect cell proliferation or the invasion capability of BCa cells, MTT, invasion and migration assays were performed using cell lines with endogenously high expression of TLR4, with or without depletion of that expression by siRNA knockdown (Figures 2B-F). The results revealed that although knockdown of TLR4 expression suppressed cell growth (Figure 2D), the invasive and migration abilities of BCa cell lines were dramatically increased in TLR4 depleted cells (Figures 2E, F). Furthermore, the morphology of TLR4-depleted cells changed from a rounded to a polygonal or spindle shape (Figure 2G).

Genome-wide analysis of the influence of TLR4 depletion in BCa cells. To elucidate the molecular basis for the observed morphological changes in BCa cells, the gene expression profiles of the cell lines with/without depletion of TLR4 were compared. GO analysis revealed that many genes functionally involved in epithelial development or differentiation, were dramatically up-regulated in TLR4-depleted cells (Table III). Among these genes, the keratins and small proline-rich proteins (SPRRs) were found to be inversely associated with the expression of *TLR4* (Figure 3A). These findings were confirmed using quantitative RT-PCR in both 5637 and RT112 BCa cell lines (Figure 3B, C). Finally, the expression of epithelial-mesenchymal transition (EMT)-related genes was examined in these cells. However,

Table I. Relationship between TLRs expression and prognosis of patients with bladder cancer.

Gene symbol low vs. high	Cox p-Value	Hazard ratio	HR 95%CI	
TLR1	0.872	0.96	0.58	1.59
TLR2	0.22	0.43	0.11	1.66
TLR3	0.16	0.62	0.32	1.22
TLR4	<0.001	0.15	0.06	0.39
TLR5	0.27	1.17	0.88	1.57
TLR6	0.88	0.95	0.44	2.03
TLR7	0.32	0.75	0.43	1.32
TLR8	0.74	0.78	0.18	3.41
TLR9	0.29	1.64	0.64	4.19
TLR10	0.03	2.22	1.04	4.74

Table II. Univariate analyses of clinical factors associated with TLR4 expression.

	High	Low	p-Value
Age			
≥65	48	15	0.803
65>	23	9	
Gender			
Male	57	19	1
Female	14	5	
Grade			
1 or 2	41	13	0.814
3	30	11	
T			
≤T1	50	11	0.047
≥T2	21	13	
N			
0	65	21	0.688
1	6	3	
M			
0	69	20	0.034
1	2	4	

inconsistent results were obtained regarding the levels of these molecules following TLR4 knockdown (Figure 3D).

To further verify our findings, the association between BCa patient prognosis and expression of keratins and SPRRs was investigated using data from the Prognoscan database. This analysis revealed that high expression of these genes was significantly associated with poor prognosis of patients with BCa (Figure 3E).

Discussion

In this study, TLR4 was identified as an important molecule in the progression of BCa. This conclusion is based upon four key

Figure 2. The relationship between expression of TLR4 and cancer cell viability. (A) Western blot analysis of the expression of TLR4 in BCa cell lines. (B, C) Knockdown effects of si-TLR4s at the mRNA level (B) and protein level (C). (D-G) Effect of TLR4 depletion on cell growth (D), invasion (E), migration (F) and cell morphology (G). * $p < 0.05$, ** $p < 0.001$.

findings. First, there was a significant association between low expression of TLR4 and poor clinical outcome in BCa patients. Second, depletion of TLR4 expression in those cell lines enhanced their invasive ability. Third, gene expression profiling revealed that depletion of TLR4 in these low stage BCa cell

lines increased the expression of cell morphology related genes, including keratin proteins and SPRRs. Fourth, TLR4 expression was found to be lower in areas of squamous differentiation, suggesting that TLR4 controls squamous differentiation of BCa by regulating the expressions of keratins and SPRRs.

Table III. Top-ranking gene set signatures from Gene Ontology (GO) analysis of 5637 cells, after treatment for 72 h with siTLR4.

	GO term	Fold enrichment	FDR
GOTERM_BP_ALL	Epidermis development	8.1	0.0000005
GOTERM_BP_ALL	Ectoderm development	7.5	0.000002
GOTERM_CC_ALL	Cornified envelope	30.4	0.000006
GOTERM_CC_ALL	Extracellular region	2.2	0.00004
GOTERM_BP_ALL	Anatomical structure development	2	0.0001
GOTERM_BP_ALL	System development	2.1	0.0001
GOTERM_CC_ALL	Extracellular region part	2.9	0.0001
GOTERM_BP_ALL	Tissue development	3.4	0.0002
GOTERM_BP_ALL	Developmental process	1.8	0.0002
GOTERM_BP_ALL	Multicellular organismal process	1.7	0.0002
GOTERM_BP_ALL	Multicellular organismal development	1.9	0.0006
GOTERM_CC_ALL	Extracellular space	3.2	0.0008
GOTERM_BP_ALL	Keratinization	16.3	0.0008
GOTERM_MF_ALL	Cytokine activity	5.9	0.003
GOTERM_MF_ALL	Receptor binding	2.8	0.003
GOTERM_BP_ALL	Organ development	2.1	0.005
GOTERM_BP_ALL	Epithelial cell differentiation	7	0.006
GOTERM_BP_ALL	Keratinocyte differentiation	10.6	0.016
GOTERM_BP_ALL	Epidermal cell differentiation	9.7	0.028

It has been reported that TLR4 is expressed in urothelial epithelium (6), especially in response to urinal tract infection (13-16). However, no studies have described the relationship between TLR4 expression and clinical factors, and only a few studies have described the role of TLR4 in BCa. One report showed that a TLR4 polymorphism that results in lower protein expression, contributes to BCa susceptibility by suppressing the immune system (17), but this report examined the TLR4 expression levels in immune cells rather than in the tumor cells themselves. In our study, IHC staining in TUR specimens revealed that the expression of TLR4 is lower in BCa tumors associated with poor prognosis which was consistent with the results from Prognoscan data. These results indicated that the association between TLR4 expression and clinical outcome may be related not only to the functional impairment of immune-related cells, but also to the aggressiveness of cancer cells themselves.

To verify our hypothesis, the association between TLR4 expression and cell proliferation was examined. However, this revealed that depletion of TLR4 expression slightly suppressed proliferation of BCa cell lines. Interestingly, however, depletion of TLR4 was found to dramatically promote the invasive ability of BCa cell lines, and that this was associated with a dramatic change in cell morphology. Subsequent microarray analysis revealed that the expression of many epithelial cell differentiation genes, including cytoskeletal-related keratin and SPRR genes, were significantly upregulated in TLR4 depleted cells.

More than 50 functional keratin genes have been identified, and it has been reported that keratin filaments are abundantly expressed in epithelial cells (18). The expression

pattern of keratins differs between the various kinds of epithelial cells, but our microarray analysis revealed that it was squamous cell related keratin genes, such as *KRT16* and *KRT17* (19, 20), that were upregulated in TLR4 depleted cells. SPRRs on the other hand, encode a class of cornified envelope precursor proteins that are reported to have antioxidant potential and provide tissues with antioxidant barriers (21-23). It has been reported that these genes are up-expressed in squamous cell carcinoma (24) and may contribute to tumor progression through their essential role in wound healing and tissue remodeling following stress and injury (22). Taken together, the changes in keratin and SPRR expression suggest that TLR4 may affect cell morphology by regulating the expression of these squamous differentiation-related genes.

As squamous differentiation is well-known as a poor prognosis factor in BCa (25, 26), these results encouraged us to verify our findings using Prognoscan data. As expected, keratin and SPRR expression was significantly associated with poor prognosis of BCa patients in the cohort and inversely associated with the expression of TLR4. Furthermore, IHC staining in our cohort of clinical BCa specimens revealed that the expression of TLR4 disappeared in areas where squamous differentiation was observed. Taken together, our study suggests that low TLR4 expression may contribute to BCa aggressiveness by controlling cell differentiation.

A limitation of our study is that it did not clarify how TLR4 controls keratin, SPRR, or other cytoskeletal-related genes to regulate squamous differentiation in BCa. Further experiments are thus required to clarify these underlying mechanisms.

Figure 3. The relationship between expressions of TLR4 and epithelial cell differentiation-related genes. (A) Clustered heatmap of microarray data from 5637 cells after 72 h of treatment with si-TLR4, relative to treatment with siRNA-control. (B, C) Quantitative RT-PCR results for keratins and SPRRs in 5637 cells (C) after 72 h of treatment with/without si-TLR4. (D) Expression of EMT-related genes with or without TLR4 depletion. (E) Kaplan-Meier analysis of overall survival in BCa patients, according to the expression of keratins and SPRRs (based on outcome data provided by Prognoscan). * $p<0.05$.

In conclusion, our study revealed that low TLR4 expression is significantly associated with BCa progression, and that TLR4 signaling may regulate invasiveness and differentiation of BCa cells through suppression of expression of keratin or SPRR.

Conflicts of Interest

The Authors have no financial relationships to disclose.

Authors' Contributions

Conception and design: Kusuhara Y, Daizumoto K, Fukawa T, Nakatsuji H; Acquisition of data: Kusuhara Y, Daizumoto K, Kawai K, Hirayama K, Shintani T, Dondoo TO, Ozaki K, Tsuda M, Fukuhara Y, Fukumori T, Takahashi M; Analysis and interpretation of data: Kowada M, Kusuhara Y, Daizumoto K; Writing and revision of the manuscript: Kusuhara Y, Daizumoto K, Fukawa T; Administrative, technical, or material support: Fukuhara Y; Study supervision: Kanayama H; Histological analysis: Bando Y, Uehara H.

Acknowledgements

The Authors wish to thank Ms. Yoshiko Shimada and Ms. Aya Shinomiya for their assistance with experiments and extend our special thanks to Mr. Hideaki Horikawa. We would also like to express our gratitude to the Graduate School of Tokushima University, Institute of Biomedical Sciences, Support center for Advanced Medical Science, for their technical support in microarray analysis.

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Received January 7, 2019

Revised January 20, 2019

Accepted January 22, 2019