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Tonsillar TLR9 expression and efficacy of tonsillectomy with steroid pulse therapy in IgA nephropathy patients

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Abstract

Background. Patients with IgA nephropathy (IgAN) often show aggravation of renal injury with macroscopic hematuria after mucosal infections, especially tonsillitis. We previously demonstrated the important role of mucosal Toll-like receptor 9 (TLR9) activation in the pathogenesis of murine IgAN. Moreover, a single nucleotide polymorphism (SNP) in *TLR9* was significantly associated with pathological severity in human IgAN. In this study, we investigated correlations between tonsillar TLR9 messenger RNA expression, *TLR9* SNP genotypes and clinical outcomes following tonsillectomy with steroid pulse therapy (SPT) in IgAN patients.

Methods. Tonsillar TLR9 expression was examined in IgAN ($n = 49$) and control ($n = 15$) patients who had undergone tonsillectomy. The correlations between tonsillar TLR9 expression level, *TLR9* SNP genotypes and clinical outcomes after tonsillectomy with SPT were examined.

Results. High expression of tonsillar TLR9 was observed in ~23% of IgAN patients. These patients showed stronger and earlier remission of hematuria and proteinuria than those with low TLR9 expression. Patients with the TT genotype of *TLR9* SNP (rs352140) had more severe renal damage than those with other genotypes. Patients whose serum IgA level decreased more than average after tonsillectomy (large ΔIgA) showed higher cumulative remission rates of proteinuria than patients with a smaller decrease in these levels (small ΔIgA). CT/CC genotypes were more dominant and tonsillar TLR9 expressions significantly higher in large ΔIgA patients than in small ΔIgA patients.

Conclusion. In IgAN patients, expression levels of tonsillar TLR9 and *TLR9* SNP were well correlated with the efficacy of tonsillectomy with SPT.

Keywords: IgA nephropathy; SNP; tonsillectomy; TLR9

Introduction

IgA nephropathy (IgAN) is the most common form of glomerulonephritis globally, accounting for 25–50% of patients with primary glomerulonephritis [1]. Long-term follow-up studies have shown that up to 25–30% of patients progress to end-stage kidney disease within 20–25 years [2]. Although the pathogenesis of IgAN remains unclear, IgA or IgA immune complexes are assumed to be a causative factor. IgAN patients often show exacerbation of the disorder with macroscopic hematuria following upper respiratory tract infections such as tonsillitis and pharyngitis, indicating that local immune responses in these mucosal organs may be involved in the pathogenesis of IgAN. In fact, tonsillectomy is effective in improving long-term renal survival in IgAN patients [3]. A combination of tonsillectomy and high-dose methylprednisolone has been reported to have a great impact on clinical remission in IgAN patients [4–6], and tonsillectomy alone decreases serum IgA levels in these patients [7, 8]. Moreover, production of not only IgA-positive cells but also polymeric IgA is increased in tonsils of IgAN patients [9–11], suggesting that tonsils may be a major site of nephritogenic IgA production.

The Toll-like receptor (TLR) is a family of pathogen recognition molecules that discriminate pathogens from self and activate suitable defense mechanisms involving the Th1 immune response [12]. TLRs on antigen-presenting cells also initiate and modulate adaptive immunity during infection [13]. To date, 10 types of human TLRs have been identified [14]. We recently reported that nasal challenge with CpG oligodeoxynucleotide (CpG-ODN), a ligand of TLR9, aggravated renal injury with elevation of albuminuria, serum IgA level and mesangial IgA deposition in an IgAN-prone mouse model [15]. We also found that a single nucleotide polymorphism (SNP) in *TLR9* (TT genotype in rs352140) in IgAN patients was an important risk factor for disease progression [15], indicating involvement of TLR9 in the pathogenesis of human IgAN. These findings led us to speculate

that activation of TLR9 in the mucosa and tonsils by exogenous antigens may contribute to the pathogenesis of IgAN.

The therapeutic validity of tonsillectomy for IgAN remains controversial as its rationale is unclear. In addition, a large, randomized control trial of tonsillectomy with steroid pulse therapy (SPT) undertaken by the Special Study Group on Progressive Glomerular Disease, Ministry of Health, Labor and Welfare of Japan has not been completed. Therefore, it is important to investigate how tonsillar TLR9 expression and *TLR9* SNPs contribute to the outcome of this therapy in IgAN patients. These investigations may provide pathogenic insights and indications for this therapy.

Materials and methods

Patients and treatment protocol

Forty-nine patients with biopsy-proven IgAN (17 males) who had undergone tonsillectomy at the Department of Otorhinolaryngology of Juntendo University Faculty of Medicine were analyzed (Table 1). Fifteen patients with chronic tonsillitis ($n = 10$), obstructive sleep apnea syndrome ($n = 4$) and orpalmoplantar pustulosis ($n = 1$) were enrolled as controls.

Using the prognostic criteria for IgAN published by the joint committee of the Special Study Group on Progressive Glomerular Disease of the Ministry of Health and Welfare of Japan and the Japanese Society of Nephrology [16], the renal lesions were classified into four grades according to light microscopy findings of renal specimens based on the percentage of glomerular sclerosis, adhesion of glomerular tufts to Bowman's capsules and formation of crescents or tubulointerstitial lesions. The renal damage grades of the 49 study patients were as follows: Grade I (good prognosis, $n = 1$), II (relatively good prognosis, $n = 6$), III (relatively poor prognosis, $n = 18$) and IV (poor prognosis, $n = 14$). The pathological grades of 10 patients were unknown (missing information). At least 2 weeks after tonsillectomy, the patients received three courses of SPT (methylprednisolone 0.5 g/day for 3 days) at intervals of 2 months. During therapy, prednisolone at 0.5 mg/ideal body weight (kg) was also administered once every 2 days. Before and after treatment, the patients were evaluated for the following clinical outcomes: proteinuria (g/g·creatinine; g/g·Cr), hematuria (mean number of erythrocytes/high power field; HPF) and serum IgA level (mg/dL). Clinical remission of hematuria was defined as $\leq$5 erythrocytes/HPF during the observation period. Similarly, clinical remission of proteinuria was defined as $\leq$0.15 g/g·Cr.

Our hospital's Institutional Review Board approved this study, and all subjects gave prior informed consent.

Table 1. Patients' profile just before treatment

Age (years old)	31.9 ± 7.9
Male:female	14:35
Duration until tonsillectomy (years)	5.6 ± 6.3
Prognostic criteria for IgAN[a] (cases)	
Good prognosis Grade I	1
Relatively good prognosis Grade II	6
Relatively poor prognosis Grade III	18
Poor prognosis Grade IV	14
Unknown	10
Chemistries	
sCr (mg/dL)	0.76 ± 2.7
BUN (mg/dL)	12.9 ± 2.7
eGFR[b] (mL/min/1.73m^2)	86.5 ± 23.2
Proteinuria (g/g·Cr)	0.75 ± 0.8
Hematuria (RBC/HPF)	25.1 ± 9.0

[a]Patients are divided clinically into four groups at the time of renal biopsy according to clinical guidelines for IgAN in Japan, second version 16.
[b]Estimated glomerular filtration rate (eGFR) is calculated by the next formula; eGFR (mL/min./1.73m^2) = $194 \times Cr^{-1.094} \times Age^{-0.287}$ (male), $194 \times Cr^{-1.094} \times Age^{-0.287} \times 0.739$ (female) (Japanese Association of Chronic Kidney Disease Initiative, 2008).

Real-time reverse transcription polymerase chain reaction (PCR) analysis

Tonsil samples were snap frozen in liquid nitrogen immediately after resection and stored at −80°C before use. Total RNA was extracted using the RNeasy mini kit (QIAGEN, Hilden, Germany) according to the manufacturer's instructions. RNA quantity was assessed using the NanoDrop ND-1000 spectrophotometer (NanoDrop Products, Wilmington, DE). A 16-μL reaction mixture containing 1 μg of RNA, 4 μL of 2.5 mmol/L dNTP mixture (Takara Biochemicals, Ohtsu, Japan) and 2 μL of Random Decamers RETROscript (Ambion Inc., Austin, TX) in RNase-free water was inactivated by heating at 80°C for 3 min. The product was added to 2 μL of 10× polymerase chain reaction (PCR) buffer (Takara Bio Inc., Shiga, Japan), 1 μL of Protector RNase Inhibitor (Roche Diagnostics Corp., Mannheim, Germany) and 0.5 μL of M-MLV Reverse Transcriptase (Invitrogen Corp., Carlsbad, CA), followed by incubation at 42°C for 60 min. The complementary DNA (cDNA) product was used for real-time PCR. A 3-μL aliquot of diluted cDNA, 1.25 μL of each TaqMan Gene Expression Assay (Applied Biosystems, Carlsbad, CA) (Table 2), 12.5 μL of TaqMan Gene Expression Master Mix (Applied Biosystems) and 8.25 μL of cDNA-free double-distilled (dd) H_2O were then mixed to obtain a final reaction mixture of 25 μL according to the manufacturer's instructions. The mixture was denatured and amplified using the 7500 Real-Time PCR system (Applied Biosystems) under the following conditions: 15 s at 95°C and 60 s at 60°C for 40 cycles. Negative controls (cDNA-free dd H_2O) were included in each reaction. For quantification of the PCR product, the samples were standardized with the PCR product for glyceraldehyde-3-phosphate dehydrogenase (GAPDH). The results of TLR9 and related subjects' messenger RNA (mRNA) expressions were obtained by three repeated independent experiments.

Determination of TLR9 *genotype*

The SNP of *TLR9* (rs352140) was examined in the 44 enrolled patients as we have previously shown that this polymorphism is associated with prognosis based on histological severity in Japanese IgAN patients [15]. Genomic DNA was collected from blood samples using the DNA extraction kit GENOMIX® (Talent SRL, Trieste, Italy). The SNP probes used for genotyping were purchased from Applied Biosystems. PCR was performed using Applied Biosystems' 7500 Real-Time PCR system and TaqMan genotyping PCR Master Mix (Applied Biosystems). The PCR program was 50°C for 2 min, 95°C for 10 min and 40 cycles at 95°C for 15 s and at 60°C for 1 min.

Tonsillar cell preparation

Tonsil samples were obtained from the patients who had undergone tonsillectomy in our hospital. These tissue samples were immediately dissected into small pieces and incubated for 40 min at 37°C with 1 mg/mL collagenase IV (Worthington Biochemical Corporation, Lakewood, NJ). Subsequently, pan tonsillar cells were dissociated with a 100-μm cell strainer, followed by three washes to remove the collagenase. These cells were suspended in BAMBANKER® (Nippon Genetics, Tokyo, Japan), kept at −80°C overnight and after a cell count were stored at −196°C before use.

Fluorescence imaging

Approximately 1×10^6 pan tonsillar cells were stained to obtain fluorescence images. In brief, the cell surface staining was performed using Phycoerythrin (PE)-conjugated anti-human BDCA2 antibody (Miltenyi Biotech, Tokyo, Japan), followed by incubation with Fc receptor-blocking reagent (Miltenyi

Table 2. TaqMan® gene expression assay

Gene symbol	Gene name	Assay ID	Ref seq
TLR9	Toll-like receptor 9	Hs00370913_s1	NM_017442.2
IFN-α	Interferon alpha	Hs00256882_s1	NM_024013.1
IFN-γ	Interferon gamma	Hs99999014_m1	NM_138880.2
IL-12	Interleukin-12	Hs00233688_m1	NM_002187.2
GAPDH	Glyceraldehyde-3-phophate dehydrogenase	Hs02786624_g1	NM_002046.3

Biotech) or anti-human CD19 antibody (Beckman Coulter, Tokyo, Japan). Intracellular staining was performed using FITC-conjugated anti-human TLR9 antibody (Santa Cruz Biotechnology, Santa Cruz, CA), with IntraPrep (Beckman Coulter) being used for fixation and permeabilization of the examined cells. The images were taken using Biozero BZ-8100 (Keyence, Osaka, Japan) with BZ Viewer™ (Keyence).

Flow cytometric analyses

Approximately 1×10^6 pan tonsillar cells were stained for flow cytometry.

To confirm TLR9 expression in B cells, these cells were stained using PE-conjugated anti-human CD19 antibody (Beckman Coulter) or PE-conjugated isotype-matched mouse IgG1 (BD Bioscience, Flankline Lakes, NJ). Intracellular staining was performed using FITC-conjugated anti-human TLR9 antibody (Santa Cruz Biotechnology). IntraPrep (Beckman Coulter) was used for fixation and permeabilization of the examined cells, following FACScan analysis using CellQuest™ (Becton Dickinson Immunocytometer System, San Jose, CA). To confirm TLR9 expression in plasmacytoid dendritic cells (pDCs), the cells were analyzed using the Human Plasmacytoid Dendritic Cell/TLR9 Kit (IMGENEX, San Diego, CA) according to the manufacturer's instructions. To exclude B cells, T cells and macrophages, populations negative for FITC-conjugated mixed antibodies (anti-human CD3, CD14, CD16, CD19, CD20 and CD56 antibodies) and positive for PerCP Cy-5.5-conjugated anti-human HLA-DR were collected from tonsillar cells. Alexa-Fluor 647-conjugated anti-human CD123 antibody was used for further purification of the pDC population. These cells were permeablilized and stained using the PE-conjugated anti-human TLR9 antibody and analyzed by flow cytometry with CellQuest™ (Becton Dickinson Immunocytometer System).

Statistical analysis

Data are expressed as mean ± SD. Differences between the groups were examined for statistical significance using Student's *t*-test. A P-value of 0.05 was considered statistically significant. Using the Fisher's exact test, we compared CT/CC and TT genotype patients results session.

Results

Patients with high TLR9 expression in tonsils showed good therapeutic outcomes

The mean values of tonsillar TLR9/GAPDH expression of the IgAN patients and controls were 4.75 ± 2.83 and 4.45 ± 2.26, respectively. Relatively high expression of TLR9 (>mean + SD) in tonsils was observed only in 11 of 49 IgAN patients (22.4%). The mean values were calculated for the IgAN and control samples. These 11 patients were designated as the high TLR9 group and the remaining IgAN patients as the low TLR9 group (Figure 1a).

Cumulative remission rates of hematuria and proteinuria in the high TLR9 group were higher than those in the low TLR9 group. However, statistically significant differences were observed in the remission rate of proteinuria between the high TLR9 and low groups before the second SPT ($P = 0.925$), before the third SPT ($P = 0.196$) or after the therapy ($P = 0.371$). In addition, no statistically significant differences were observed between these two groups in remission rate of hematuria before the second SPT ($P = 0.596$), before the third SPT ($P = 0.343$) or after the therapy ($P = 0.632$). Despite similar clinical backgrounds just before tonsillectomy (Table 3), clinical remission of proteinuria and hematuria appeared earlier in the TLR9 high-group than in the low TLR9 group (Figure 1b). In addition, the TLR9 expression level did not correlate significantly with duration from estimated onset to tonsillectomy, age or

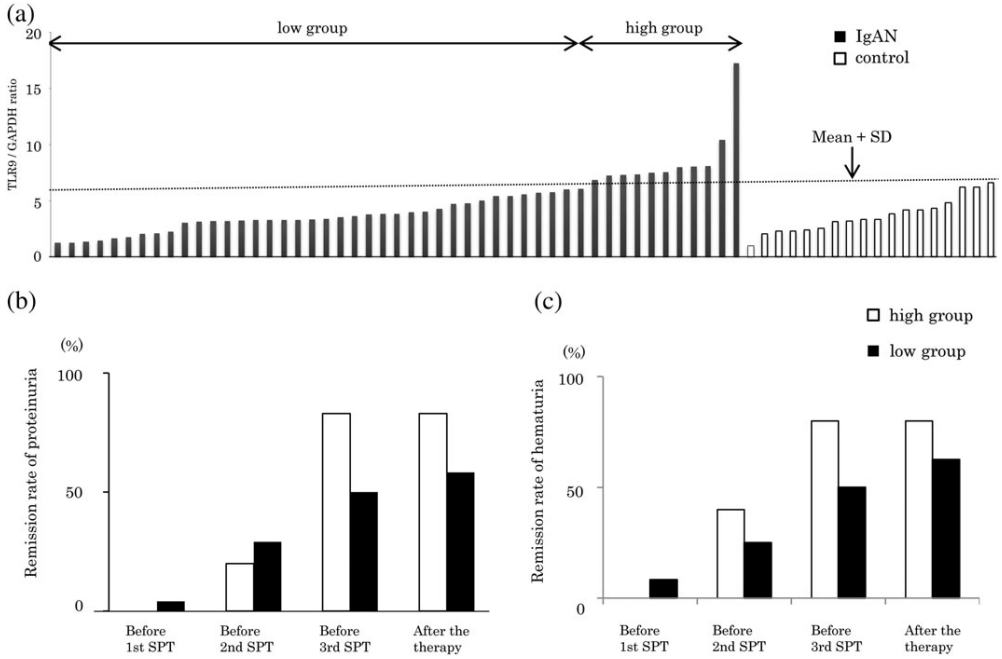

Fig. 1. (**a**) mRNA expression of TLR9. Total RNAs isolated from tonsillar tissues of IgAN patients and controls were reverse transcribed, and the mRNA levels of TLR9 determined by real-time PCR. Each sample dataset was standardized with mRNA expression of GAPDH. Of the 49 patients, 11 showed marked expression of TLR9 (>mean + SD). We defined these 11 patients as the high TLR9 group and the other patients as the low TLR9 group. We obtained the average value and SD from the IgA nephropathy patients and controls. The broken line represents the average + SD. (**b**, **c**) Comparison of cumulative remission rates of hematuria and proteinuria between the high TLR9 group and low group. In the high TLR9 group, designated by the open bar, the cumulative remission rates of hematuria and proteinuria were higher than those in the low TLR9 group. This response occurred at an earlier phase of this therapy than in the low TLR9 group, designated by the filled bar.

levels of proteinuria and hematuria at tonsillectomy (data not shown).

Patients with TT genotype TLR9 SNP *(rs352140) showed severe pathological damage and poor therapeutic outcomes*

We divided the patients into two groups according to their *TLR9* SNP (rs352140) genotype, TT or CT/CC, and then evaluated the clinical features and treatment responses in the two groups. The frequency of the TT genotype was ~23% in the 44 IgAN patients. No patient with a TT genotype was classified as Grades I/II (Grades I + II/III + IV: 0/9 in TT, 7/23 in CT/CC) (Table 4), suggesting that patients with this genotype tend to have more severe histological damage. In addition, expression of inflammatory cytokines such as IFN-α, IFN-γ and IL-12 in tonsils of TT genotype patients was higher than those in tonsils of CT/CC genotype patients (Table 4). Moreover, the remission rates of hematuria and proteinuria in TT genotype patients were lower than in CT/CC genotype patients (Table 4). At the last observation point, both TT and CT/CC genotype patients showed almost equal remission rates in urinary findings. We analyzed the correlation between TLR9 expression and *TLR9* SNP and showed that there was no significant difference in the tonsillar TLR9/GAPDH expression ratio [(CT/CC ($n = 30$) versus TT genotypes ($n = 10$); 2.86 ± 1.17 versus 2.45 ± 1.41; $P = 0.64$)]. In addition, the Fisher's exact test did not show a significant correlation between *TLR9* SNP (CT/CC and TT groups) and the expression level (TLR9 high-group and low-group) ($P = 0.232$).

Table 3. Clinical data of TLR9 high- and low-groups

	TLR9 high-group ($N = 11$)	TLR9 low-group ($N = 38$)
Duration until tonsillectomy (years)	5.9 ± 4.5	6.1 ± 7.2
sCr (mg/dL)	0.8 ± 0.2	0.7 ± 0.2
BUN (mg/dL)	12.4 ± 2.2	13.0 ± 2.8
eGFR (mL/min/1.73m^2)	79.0 ± 22.0	88.6 ± 23.4
Serum IgA (mg/dL)	338.5 ± 116.9	296.4 ± 98.2
Proteinuria (g/g·Cr)	0.9 ± 0.4	0.7 ± 0.7
Hematuria (RBC/HPF)	12.0 ± 0.1	26.3 ± 7.6

Patients whose serum IgA decreased more than the average by tonsillectomy alone had good therapeutic outcomes

To elucidate the relationship between the decrease in serum IgA level after tonsillectomy and treatment response, IgAN patients were divided into two groups according to their reduction in serum IgA levels. Both serum IgA levels before and after therapy could be evaluated in 30 patients. The levels decreased from 308.4 ± 103.0 to 279.2 ± 97.0 mg/dL, with the mean reduction rate in serum IgA being ~8.1%. The large ΔIgA group included patients whose serum IgA decreased more than the average (8.1%) following tonsillectomy alone, with the remaining 16 patients being designated as the small ΔIgA group. Patients in the large ΔIgA group showed higher cumulative remission rates of proteinuria and hematuria than those in the small ΔIgA group (Figure 2a and b). The expression levels of TLR9 mRNA in tonsils of the large ΔIgA group were significantly higher than those in the small ΔIgA group ($P < 0.05$) (Figure 2c). In addition, the percentage of TT genotype patients was obviously small in the large ΔIgA group (Table 5).

TLR9 expression in B cells or plasmacytoid dendritic cells

Figure 3a shows that parts of $CD19^+$ B cells or $BDCA2^+$ pDCs from tonsils of IgAN patients expressed TLR9. To further confirm these results, we examined the flow cytometric analyses. Figure 3b shows certain parts of $CD19^+$ B cells expressed TLR9 (FL-1; TLR9, FL2; CD19). Lineage negative (anti-human CD3, CD14, CD16, CD19, CD20 and CD56 antibodies) and HLA-DR-positive cells were gated as R1 for detection of DC (Figure 3c). Nextly, we

Table 4. Patient profiles, TLR9 and related cytokine expressions in tonsil and therapeutic efficacy of TT and CT/CC patients

Patients profiles	TT	CT/CC
Number	10	34
Gender (% female)	70.0	75.0
Duration until tonsillectomy (years)	3.5 ± 3.8	6.2 ± 7.1
Histopathological prognostic criteria (Grades I + II versus Grades III + IV)	0 versus 9	7 versus 24
Proteinuria before tonsillectomy (g/g·Cr)	1.1 ± 1.1	0.6 ± 0.5
Hematuria before tonsillectomy (RBC/HPF)	27.1 ±8.4	24.5 ± 9.2
Tonsillar expression of TLR9 and related cytokines		
TLR9	2.4 ± 1.3	3.0 ± 1.4
IFN-α	36.0 ± 58.8	30.2 ± 54.6
IFN-γ	7.3 ± 3.3	5.6 ± 4.8
IL-12	22.5 ± 21.0	12.2 ± 12.9
Therapeutic efficacy		
Remission rate after first SPT (%)		
Hematuria	12.5 (1/8)	31.8 (7/22)
Proteinuria	37.5 (3/8)	52.6 (10/19)
Remission rate 1 year after the start of treatment (%)		
Hematuria	62.5 (5/8)	63.6 (14/22)
Proteinuria	75.0 (6/8)	78.9 (15/19)

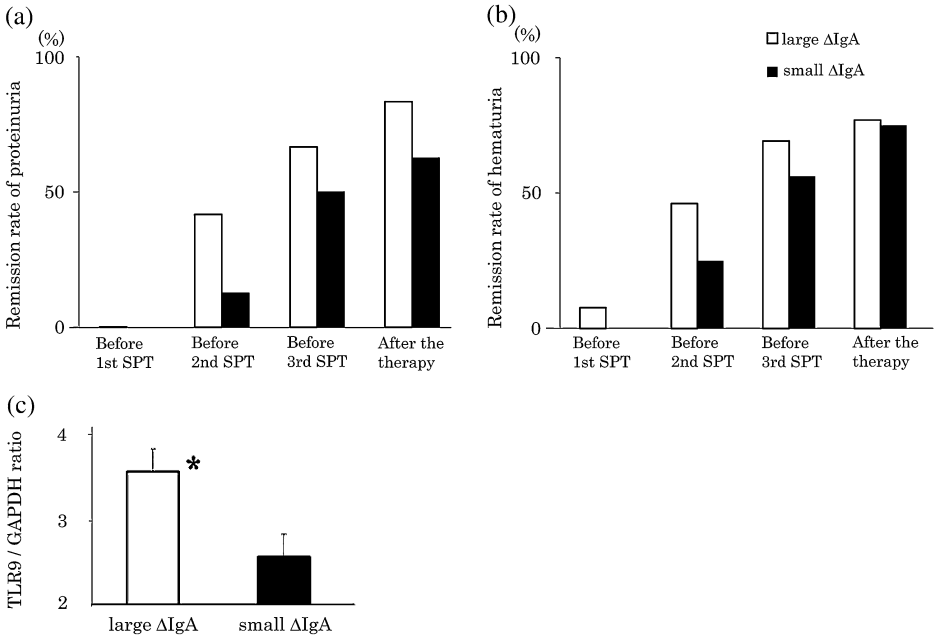

Fig. 2. (**a**, **b**) Clinical distinction between patients whose serum IgA decreased after tonsillectomy alone and patients whose serum IgA did not decrease. The IgAN patients were divided into two groups. The large ΔIgA group included patients whose serum IgA decreased more than the average (8.1%) following tonsillectomy alone (open bars). The remaining 16 patients were designated as the small ΔIgA group (filled bars). Comparison of the cumulative remission rates of proteinuria (a) and hematuria (b) showed the large ΔIgA group had higher remission rates than the small ΔIgA group. However, the remission rate of hematuria did not show the same tendency. (**c**) The expression levels of TLR9 in tonsils of the large ΔIgA group (open bars) was significantly higher than that of the small ΔIgA group (filled bars) ($P < 0.05$).

Table 5. Distribution of TLR9 SNP in large and small ΔIgA groups

	TT	CT/CC
Large ΔIgA	3	13
Small ΔIgA	4	8

further gated R1 using the pDC marker (anti-CD123 antibody) as R2 (Figure 3d). As shown in Figure 3e, certain parts of the R2 pDC population also expressed TLR9.

Discussion

A previous Japanese study on the clinical prognosis of IgAN patients with tonsillectomy concluded that tonsillectomy had a favorable effect on long-term renal survival [3]. Hotta *et al.* [4] reported that tonsillectomy combined with SPT had a significant impact on clinical remission, with histopathological improvements in IgAN patients after a median follow-up period of 75 months. The remission rate of hematuria associated with combination therapy reached ~80% regardless of the severity of the glomerular lesions. To avoid future aggravation of renal injury, these studies recommended this therapy for patients in the early stage of the disease before a 'point of no return' [17]. However, the indication for tonsillectomy in IgAN is controversial, even in Japan. Although tonsillectomy in certain patients can be an effective treatment, 7–10% of IgAN patients show spontaneous clinical remission [18]. Therefore, rationale and reasonable clinical markers are needed for indication of this therapy. Recent studies have shown that predictive factors for resistance to tonsillectomy with SPT are age at onset, severity of proteinuria and hematuria and pathological grade [19]. Although there is an ongoing randomized control trial concerning the effect of tonsillectomy on this disease, carried out by the Special Study Group on Progressive Glomerular Disease of the Ministry of Health, Labor and Welfare of Japan and the Japanese Society of Nephrology, the results are not yet available. Therefore, the present study aimed to evaluate the correlation between the efficacy of this therapy, tonsillar TLR9 expression and *TLR9* SNP in order to provide evidence for indications for tonsillectomy.

Our group recently reported a novel spontaneous IgAN-prone animal model. We found that human and murine IgAN are regulated, at least in part, by the same genes [20]. An association study using an IgAN-prone mouse showed that the progression of murine IgAN was linked to signaling molecules of TLR. We then examined the relationship between the TLRs mRNA expression levels in splenocytes and disease activity and found that the severity of glomerular injury in this model was clearly linked only to the degree of TLR9 expression in splenocytes [15]. In fact, intranasal immunization with CpG-ODN, an established ligand for TLR9, aggravated glomerular damage with an elevation in serum IgA levels. The present study demonstrates that some IgAN patients exhibit relatively high expression of tonsillar TLR9 mRNA (TLR9 high-group) and have an earlier more complete clinical remission than those with low expression (TLR9 low-group). These findings raise the possibility that TLR9 expression

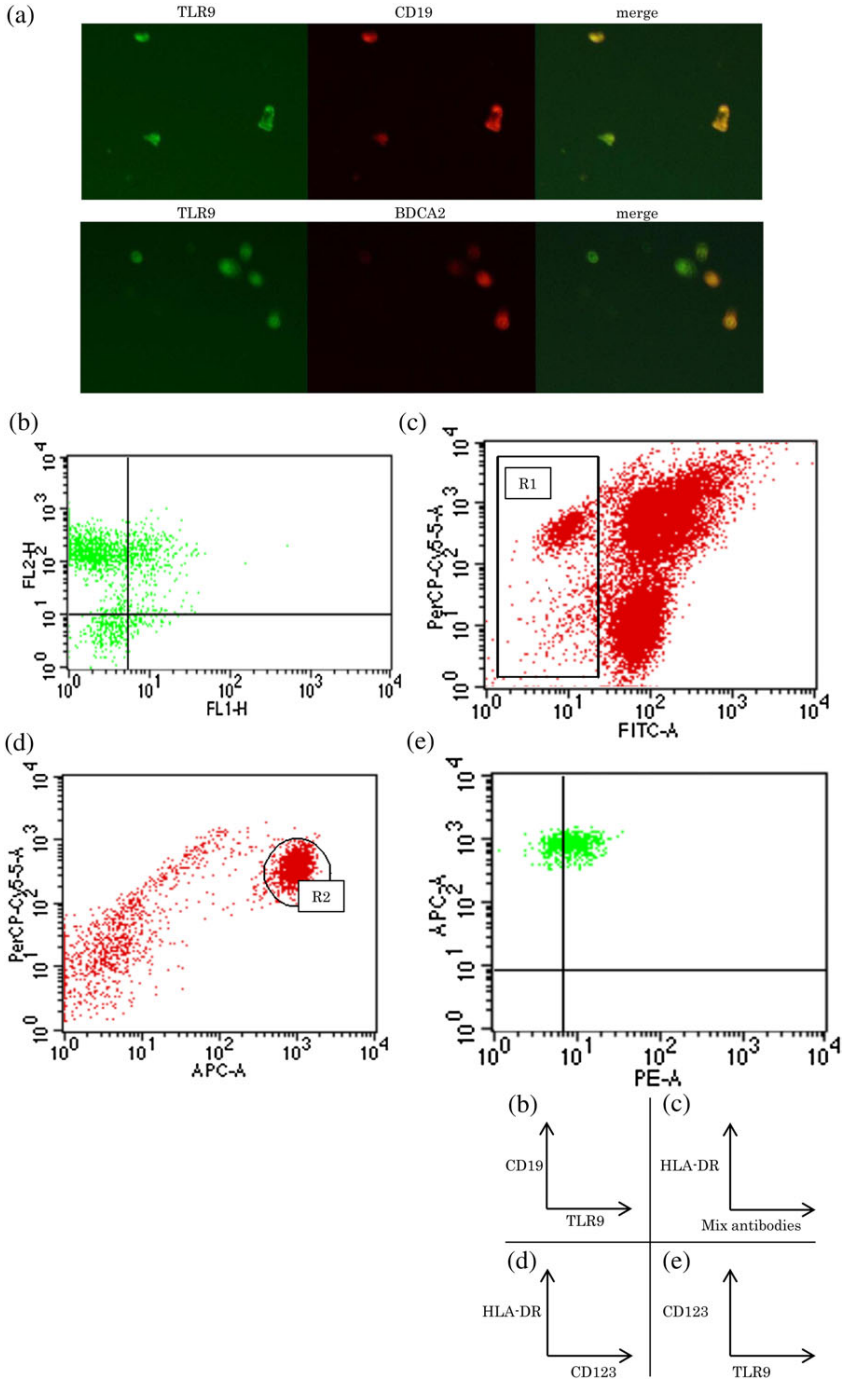

Fig. 3. TLR9 expressed on tonsillar B cells and pDC. (**a**) Fluorescence images and flow cytometric analysis confirmed TLR9 expression in B cells and plasmacytoid dendritic cells. (**b**) Certain parts of $CD19^{+}$ B cells expressed TLR9. (**c**) Lineage-negative (anti-human CD3, CD14, CD16, CD19, CD20 and CD56 antibodies) and HLA-DR-positive cells were gated as R1. (**d**) Further gating of R1 by the pDC marker (anti-CD123 antibody) as R2. (**e**) Certain parts of R2 (pDC population) also expressed TLR9.

is involved in the pathogenesis of not only murine but also human IgAN. Recent studies revealed that immunocompetent cells primed at mucosal sites may be recruited to other tissues by specific engagement of chemokines, chemokine receptors and adhesion molecules, suggesting that immunocompetent cells responsible for IgAN may also be primed in tonsils and disseminated to other systemic lymphoid tissues or bone marrow [21–23]. Therefore, surgical removal of tonsils may directly decrease the number of responsible cells and the chance of new priming in tonsils. Additional SPT may further eliminate the responsible disseminated cells. We consider that the mechanism

mediated by TLR9 may not be limited to the specific IgAN population. Although we also have an interest in the contribution of TLRs in kidneys and therefore examined this possibility in a previous study [15], we could not find a clear contribution, at least in murine IgAN. At present, we speculate that exogenous antigen may contribute mainly to the production of nephritogenic underglycosylated IgA (GdIgA) via TLR9 and subsequent immune complex formation with anti-glycan IgG but has no direct influence on renal resident cells in IgAN [21, 24]. Our previous study demonstrated that two genotypes of *TLR9* have a strong association with the progression of IgAN, further indicating the involvement of TLR9 in the pathogenesis of human IgAN [15]. We demonstrated that the CT/CC genotype in rs352139 and TT genotype in rs352140 could be risk factors for the progression of IgAN. As logistic regression analysis showed that *TLR9* SNP (rs352140) has a statistically strong association with the severity of pathological findings, we investigated *TLR9* SNP (rs352140) in IgAN patients in the present study. The histological prognostic criteria for the patients in the study tended to be similar to those reported previously [15]. The cumulative remission rate of proteinuria in patients with the CT/CC genotype of *TLR9* SNP (rs352140) was greater than in patients with the TT genotype. Moreover, in patients with the TT genotype, tonsillar inflammatory cytokine expression tended to be higher than in those with the CT/CC genotype. Although remission rates in patients with the TT genotype were lower than those in patients with the CT/CC genotype after the first SPT, cumulative remission rates of hematuria and proteinuria were almost the same on completion of this therapy. Therefore, early diagnosis and adequate treatment are important, particularly in the TT genotype patients, as their renal prognosis tends to become worse than that of the CT/CC genotype patients. Accordingly, *TLR9* SNP (rs352140) may be a good marker for aggressive treatment, including tonsillectomy with SPT.

The cumulative remission rate of proteinuria was higher in the large ΔIgA group than in the small ΔIgA group. Moreover, tonsillar TLR9 expression in the large ΔIgA group was significantly higher than in the small ΔIgA group. Snapshot evaluation of serum IgA level has limited clinical value for estimating disease activity. In fact, it is well known that only 30% of patients have high serum IgA levels. However, the findings of the present study suggest that the degree of reduction in serum IgA level after tonsillectomy may be related to the efficacy of this treatment and that tonsillar TLR9 may determine the serum IgA level in IgAN. GdIgA is thought to be involved in the pathogenesis of this disease [2, 25–27]. Moreover, IgA1 produced by tonsillar lymphocytes may be aberrantly glycosylated in IgAN patients [28, 29]. Therefore, tonsils may play an important role in the production of GdIgA1. On the other hand, the frequency of the CT/CC genotype was higher in the large ΔIgA group, which suggests that *TLR9* SNP (rs352140) may influence the amount of GdIgA production. However, as *TLR9* SNP (rs352140) is a non-coding lesion, further investigations are required.

In conclusion, the cells responsible for IgAN may exist in palatine tonsils and participate in the production of nephritogenic IgA mediated by the activity of TLR9. Therefore, tonsillar TLR9 expression level and *TLR9* SNP variation may influence the efficacy of tonsillectomy with SPT.

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