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Microarray analysis of tonsils in immunoglobulin A nephropathy patients

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ABSTRACT

Background: Recently, combination of tonsillectomy and steroid pulse therapy was reported to be effective as the treatment of the immunoglobulin A nephropathy (IgAN). However, the gene expression difference between the tonsils in patients with IgAN and those in control patients is not established.
Methods: We performed tonsillectomy combined with steroid pulse as a treatment to IgAN, analyzed the gene expression in the tonsils ($N = 23$) using microarray, compared with those with patients suffering from chronic tonsillitis ($N = 22$). From some candidate genes related with IgAN, we confirmed the apolipoprotein B messenger RNA-editing enzyme catalytic polypeptides 2 (APOBEC2) gene expression in the tonsil and we also analyzed its expression levels and clinical features.
Results: Up-regulated genes seem to be categorized into two groups. One group belongs to the muscle related genes which might be caused by structural differences. The other group includes the immune system-related genes, such as APOBEC2, CALB2, DUSP27, and CXCL11. APOBEC2 was positively stained in the epithelium and the peripheral region of the germinal center in both tonsils. APOBEC2 expression level was negatively related with serum igg level, but did not correlate with clinical course after tonsillectomy.
Conclusion: We confirmed gene expression differences related with immune system and muscle structure. The APOBEC2 was confirmed to be elevated in the tonsils with IgAN patients, and the gene expression level was negatively related with serum igg level in overall patients. These results might be helpful to reveal the mechanism of IgAN.

Introduction

Immunoglobulin A nephropathy (IgAN) is the most common form of primary glomerulonephritis in the world [1]. It is characterized by IgA deposition to glomerular mesangial cells in pathological point of view and sometimes macroscopic hematuria after upper respiratory infection in clinical point of view. Since it was firstly reported by Berger in 1968, the etiology and cause of the disease has been an ultimate mystery. Recently, polymorphisms related with progression of IgAN were reported in a comprehensive manner from our group, but the origin of the disease has remained unclear [2]. In 1997, Koyama et al. reported that 39% of the patients with IgAN end up in dialysis or death in 20 year, which turned out to be of poor prognosis [3]. But there is no specific therapy to the disease, although anti-platelet drug, cortcosteroid, angiotensin converting enzyme inhibitor or angiotensin II receptor blocker are clinically used.

In consideration of the high frequency in general and especially in the young and the poor renal prognosis of the disease, some strong therapeutic strategy had been urgently needed. It is also a well known fact that in upper respiratory infection such as tonsillitis, IgAN patients often manifest the deterioration of urinary findings; macroscopic hematuria. Tonsil is one of the important immune organs, constituting the ring of Waldeyer whose function is considered to be closely related to mucosal immune system. Therefore, recently, tonsillectomy is a focus of much attention in treating these diseases.

In the wake of this trend, recently, combination of tonsillectomy and steroid pulse therapy was reported to be effective [4–6] and tonsillectomy alone was reported to have a favorable effect to the IgAN [7], while there is some controversial opinion against tonsillectomy in IgAN [8]. However, the gene expression difference between the tonsils in patients with IgAN and those in control patients is not established.

We performed tonsillectomy combined with steroid pulse as a treatment to IgAN, analyzed the gene expression in the tonsils using microarray, and compared them with those with patients

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suffering from chronic tonsillitis. From some candidate genes related with IgAN, we confirmed the apolipoprotein B messenger RNA-editing enzyme catalytic polypeptides 2 (APOBEC2) gene expression in the tonsil and we also analyzed its expression levels and clinical features.

Materials and methods

Patients enrollment. Tonsil tissues of the IgAN patients were obtained from 73 consecutive patients who were over 17 years old, undergoing tonsillectomy at Osaka university hospital from 2004, April to 2007, May. The diagnosis of the IgAN in these patients was confirmed by the renal biopsy prior to the tonsillectomy. From these 73 patients, we picked up all patients whose proteinuria and hematuria were perfectly ameliorated within 2 years after tonsillectomy and steroid pulse therapy, because the tonsils in these patients might be related with IgAN. Twenty-three patients fulfilled the criteria. The 22 control tonsil tissues were obtained from the patients suffering from chronic tonsillitis at tonsillectomy. One sample of tonsil tissues in IgAN patients group was eliminated because of the small amounts of RNA. Patients characteristics are described in Table 1.

Written informed consent was obtained from all participating subjects. This study was approved by the ethical committee of Osaka University Graduate School of Medicine, which abides by the Helsinki Declaration on ethical principles for medical research involving human subjects.

DNA microarray analysis. The quality of the RNA samples was examined using the RNA 6000 Nano LabChip Kit (p/n 5065–4476) on the Agilent 2100 Bioanalyzer (G2940BA; Agilent Technologies, Inc., Palo Alto, CA, USA). Total RNA (500 ng) from 23 IgAN patients or 22 control patients was reverse-transcribed using oligo-dT primers containing the T7 RNA polymerase promoter sequence, and the cDNAs were then subjected to in vitro transcription using T7 RNA polymerase to label cDNAs with Cy3 or Cy5 (CyDye, GE healthcare, Bio-sciences Corp., Piscataway, NJ, USA). Cy-labeled cRNA from IgAN patients (1 mg) was mixed with the same amount of reverse color Cy-labeled product from an equal amount of pooled cRNA from control patients. Labeled cRNAs were fragmented to an average size of approximately 50–100 nt by heating at 60 °C in the presence of 10 mM $ZnCl_2$, and the samples were then added to a hybridization buffer. Hybridizations with the Agilent's whole human genome microarray (Hu44K) (Agilent Technologies, Inc. Santa Clara, CA, US) were conducted at 40 °C. After hybridization, slides were washed and scanned using an Agilent confocal laser scanner (G2565BA). Fluorescence intensities on scanned images were quantified, corrected for background noise and normalized. Fluorophore reversal (dye swap) duplicates were used in two-color DNA microarray experiments.

Quantitative real-time PCR. In order to quantify the expression of mRNA, real-time SYBR-Green PCR analysis was performed. Briefly, 0.4 μg of RNA was converted to single-strand DNA using random primers and SuperScript II (Invitrogen, Carlsbad, CA, US). Each cDNA was mixed with 0.2 μM forward and reverse primers, and 10.0 μl of GYBR Green Master Mix (Applied Biosystems, Foster city, CA, US). PCR was performed with an Applied Biosystems 7900 real-time PCR system. The primer sets had the following sequences: Human CALB2 forward, 5′-acattgacgagcatgagctg-3′; Human CALB2 reverse, 5′-ggtag agcttccctgcctct-3′; Human DUSP27 forward, 5′-aaggacacagaggagga gca-3′; Human DUSP27 reverse, 5′-ctgcatctgagaccatcgaa-3′; Human PIGR forward, 5′-aacgggagagaaggaagtgg-3′; Human PIGR reverse, 5′-gtggagatggctgggaaga-3′; Human APOBEC2 forward, 5′-accatttgga gctggacaac-3′; Human APOBEC2 reverse, 5′-ccttgggtgagcaattagg a-3′; Human CXCL11 forward, 5′-agtcctggaaaagggcatct-3′; Human CXCL11 reverse, 5′-tcacccacctttcatccttc-3′; GAPDH forward, 5′-gagtc aacggatttggtcgt-3′; GAPDH reverse, 5′-ttgattttggagggatctcg-3′.

Quantitative real-time PCR of APOBEC2 using large IgAN patient samples. To confirm the up-regulation of APOBEC2 in IgAN patients, we analyzed the APOBEC2 expression level using more tonsil samples from 73 IgAN patients. This extended IgAN patients includes the original IgAN patients and the patients whose proteinuria and hematuria did not ameliorated after tonsillectomy or whose observation periods was not sufficient after tonsillectomy. There was no significant difference of clinical parameters between the original IgAN patients group and the extended IgAN patients group except period to remission of proteinuria and hematuria (shown in Table 1). The quantitative real-time PCR analysis was performed using the same primers.

Histological analysis of APOBEC2 in human tonsil tissue. For histological analysis of human tonsil, the tissues were fixed in 4% paraformaldehyde (PFA)/PBS just after tonsillectomy. Immunostaining was done using anti-human APOBEC2 rabbit antibody (Abcam plc, Cambridge, UK). Sections were incubated at 4 °C overnight with APOBEC2 antibody, washed three times with PBS and incubated at room temperature for 30 min together with the biotinylated secondary antibody. After another washing with PBS, the sections were incubated at room temperature for 30 min with VECTASTAIN elite ABC Reagent (Vector Laboratories, Burlingame, CA, US), and in peroxidase substrate solution for 40 s.

Analysis of APOBEC2 gene expression levels and clinical features. The relationship between the APOBEC2 expression level and clinical features were analyzed in IgAN patients and in overall patients including control patients. Clinical course after tonsillectomy between the IgAN patients with high APOBEC2 level and those with low APOBEC2 level were analyzed by Kaplan–Meier method.

Table 1
Patient characteristics.

	IgAN group ($N = 23$)	Extended IgAN group ($N = 73$)	Control group ($N = 22$)
Age	32 (24–46)	32 (24–45)	31 (20–40)
Sex (female)	11/23	41/73	12/22
GFR**	92.3 ± 23.2	91.1 ± 25.8	124.2 ± 41.5
Proteinuria	0.38 (0.10–0.51)	0.50 (0.24–0.95)	0
IgA	320 (225–392)	300.5 (230.3–371.8)	261 (211–394)
ASO**	80 (60–320)	46 (31–97)	320 (160–2560)
ASK*	53 (34.5–100.5)	80 (40–320)	119.9 (49.5–234.4)
C3	119.1 ± 18.3	125.7 ± 22.6	104 (86–122.5)
C4	19 (18–23.3)	21.5 (18.0–26.0)	22 (20–25.5)
CH50	41.2 ± 8.5	43.1 ± 9.2	45.8 (38.2–49.4)
History of macrohematuria	10/23	32/73	0/22
Period to remission of proteinuria (month)	3.9 (0.6–6.5)	–	–
Period to remission of hematuria (month)	6.5 (5.1–10.7)	–	–

* $P < 0.05$.
** $P < 0.01$.