RESEARCH NOTE

Association of *CTLA4*, *CD28* and *ICOS* gene polymorphisms with clinicopathologic characteristics of childhood IgA nephropathy in Korean population

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Introduction

Primary IgA nephropathy (IgAN) is the most common glomerular disease in children and adolescents who undergo renal biopsy because of isolated microscopic haematuria or haematuria associated with proteinuria (Coppo 2008). Some evidence suggests the importance of the abnormal T-cell response in the pathogenesis of IgAN, and co-stimulatory molecules such as cytotoxic T-lymphocyte antigen 4 (CTLA4), CD28 molecule and inducible co-stimulator (ICOS) have been found to be vital for naïve T-cells to initiate and terminate immune responses (Carreno and Collins 2002; Carreno *et al.* 2005). In this study, we tested single nucleotide polymorphisms (SNPs) of *CTLA4*/*CD28*/*ICOS* genes, and they were found to be associated with pathophisipology of paediatric IgAN in Korean population.

The best-characterized T-cell co-stimulatory pathway involves receptors such as the CD28 and CTLA4 (also known as CD152). In addition, the ICOS has been also defined recently as a new pathway (Keir and Sharpe 2005). The *CD28*/*CTLA4*/*ICOS* genes lie within the 300-kb region on human chromosome 2q33 and their expressions are differentially regulated; although CD28 is constitutively present on naïve T-cells, whereas CTLA4 and ICOS are present only after activation (Collins *et al.* 2005). Moreover, all of these molecules were found to be expressed by regulatory T-cells for the development and maintenance (Keir and Sharpe 2005).

CTLA4 is a member of the immunoglobulin superfamily and works as a negative regulator of CD28 and T-cell receptor (TCR) signalling. CD28 is a surface molecule expressed by most T-cells, which promotes T-cell differentiation and proliferation. It was found to enhance antibody production by B-cells as well (Collins *et al.* 2005; Keir and Sharpe 2005). ICOS shows regulating roles in the T-cell activation and tolerance, and it is important for activating preactivated T-cells and generating effector T-cell cytokine responses (Carreno and Collins 2002; Khoury and Sayegh 2004).

Abnormal expression of *CTLA4/CD28/ICOS* genes is suggested to increase the risk of several autoimmune diseases (Carreno and Collins 2002; Khoury and Sayegh 2004; Collins *et al.* 2005; Keir and Sharpe 2005). Moreover, recent studies also implicate the polymorphisms of *CTLA4*/*CD28*/*ICOS* in the pathogenesis of autoimmune diseases (Gough *et al.* 2005; Kavvoura *et al.* 2007).

However, no study has yet reported on the associations between these genes and IgAN patients, especially in pediatric ages. In this study, we investigated the association between the SNPs of the *CTLA4*/*CD28*/*ICOS* genes, and childhood IgAN in the Korean population.

Materials and methods

Subjects

We examined a total of 172 Korean paediatric patients with biopsy proven IgAN (97 boys, 12.55 ± 5.65 years (mean age±s.d.); 75 girls, 11.04 ± 5.16 years) and 399 healthy controls (176 men, 38.57 ± 10.03 years; 223 women,

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39.13 ± 9.20 years). Patients were detected by school screening urinalysis and most of them showed no symptom of glomerulo nephritis (GN) other than abnormalities in urinalysis. Accordingly, they are supposed to have relatively early stage disease. All the patients who had haematuria unexplained, prolonged, or concomitant with proteinuria underwent kidney biopsy and all proteinuric patients have received angiotensin converting enzyme (ACE) inhibitors. Healthy controls were also recruited based on routine screening findings. Screening included the completion of a questionnaire, which addressed the presence of symptoms and medical history, in addition to blood pressure, electrocardiography, abdominal sonography, and laboratory findings including several tests for blood and urine. Candidates with an abnormal result for any item were excluded.

IgAN patients were divided into several subsets according to the presence of proteinuria and nephrotic range proteinuria (proteinuria > 4 and > 40 mg/m^2/h). Further subgroups were made according to the presence of podocyte foot process effacement and the degree of mesangial proliferation; mild (normal to mild focal mesangial proliferation) versus advanced (mild diffuse to severe diffuse mesangial proliferation) groups. In addition, to access the association between the candidate SNPs and pathologic disease progression, we have subgrouped the IgAN patients according to the presence of advanced pathologic markers. Members of the advanced disease group had any of the following: interstitial fibrosis, tubular atrophy, and/or global sclerosis found by kidney biopsy as pathologic markers. These pathologic lesions have been known to decrease pressure autoregulation of glomeruli and are the most important findings eventually indicating progression of chronic renal diseases. All the demographic characteristics of the IgAN patients are shown in table 1.

This study was approved by the ethics review committee of the Medical Research Institute, Kyung Hee University Medical Center, Seoul, Korea. Written informed consent was obtained from all subjects.

SNP selection and genotyping

Genomic DNA was extracted from the whole blood of each subject using a commercially available Qiagen DNA Extraction kit (Qiagen, Tokyo, Japan). The SNPs of the *CD28*/*CTLA4*/*ICOS* genes were selected on the basis of extensive database searching (http://www.ensemble.org, http://ncbi.nlm.nih.gov/SNP). SNPs with low heterozygosity (below 0.1) and low minor allele frequency (below 0.05) were excluded. The SNPs were also validated by HapMap database. Finally, we selected 14 SNPs; four SNPs of *CD28* (rs1879877, rs3181097, rs2140148, and rs3116496); two SNPs of *CTLA4* (rs231779 and rs231777); eight SNPs of *ICOS* (rs4270326, rs11571314, rs1365828, rs4404254, rs1559931, rs768180, rs6726035, and rs10183087); see figure 1 in electronic supplementary material at http://www.ias.ac.in/jgenet/). Genomic DNA was amplified using the specific primers. The information for the candidate SNPs and specific primers used for DNA amplification are shown in table 1 of electronic supplementary material (see figure 1 in electronic supplementary material). PCR products were sequenced using the ABI Prism BigDye Terminator Cycle Sequencing system (PE Applied

Table 1. Demographics of the IgAN patients (n = 172).

Subgroup	n (%)	M : F	Age (years) mean±SD
Proteinuria (mg/m^2/h)*			
>4	104 (60.5)	70 : 34	13.26 ± 6.18
≤4	68 (39.5)	27 : 41	10.01 ± 3.07
>40	18 (10.5)	11 : 7	12.61 ± 7.25
≤40	154 (89.5)	86 : 68	11.90 ± 5.18
Podocyte foot process effacement			
(+)	75 (43.6)	48 : 27	12.61 ± 6.44
(−)	97 (56.4)	49 : 48	11.48 ± 4.42
Mesangial proliferation**			
Advanced	59 (34.3)	39 : 20	13.07 ± 5.68
Mild	113 (65.7)	58 : 55	11.41 ± 5.20
Advanced disease markers†			
(+)	20 (11.6)	16 : 4	15.98 ± 9.06
(−)	152 (88.4)	81 : 71	11.45 ± 4.52

*Proteinuria indicates the level of proteinuria observed at the kidney biopsy. **The degree of mesangial proliferation was accessed according to the degree of mesangial proliferation; mild (normal to mild focal mesangial proliferation) versus advanced (mild diffuse to severe diffuse mesangial proliferation) groups. †Members of the advanced disease group had any of followings: interstitial fibrosis, tubular atrophy, and/or global sclerosis found by kidney biopsy as pathologic markers.

Biosystems, Foster City, USA) and an ABI Prism 377 automatic sequencer (PE Applied Biosystems, Foster City, USA). Sequence data were analysed using the SeqManII software (DNASTAR, Madison, USA).

Analysis of the transcription factor bindings for the promoter SNPs

To examine the transcription binding activity of two promoter SNPs (rs1879877 and rs3181097), we investigated whether these genetic variants influence transcription factor binding sites. The transcription factor binding sites were compared using online program AliBaba 2.1 (http://www.gene-regulation.com/pub/programs/alibaba2).

Statistical analysis

For the case–control association study, Hardy–Weinberg equilibrium (HWE) for all SNPs was assessed using SNPstats (Sole *et al.* 2006) in both cases and controls, and the SNPs not in HWE ($P < 0.05$) were excluded from the analysis. For the logistic regression analysis, we used SNPstats, HapAnalyzer version 1.0 and SNPanalyzer (ISTECH, Goyang, Korea). To show alternative effects of the variants, logistic regression analysis was performed in three analysis models (codominant, dominant and recessive models for rare allele). For the SNPs that do not have all three genotypes present in the study population, only allele frequencies were compared by the chi-square test.

A linkage disequilibrium (LD) block of polymorphisms and haplotype analysis was tested using Haploview version 4.1 (Daly Lab, Cambridge, USA). We examined Lewontins's $|D'|$ (Hedrick 1987) and the correlation coefficient r^2 between all pairs of bi-allelic loci; the haplotypes and their frequencies were inferred using the expectation-maximization algorithm (Stephens *et al.* 2001).

Results

Genotyping data showed no significant difference of SNPs between IgAN subjects and controls (data not shown). In order to investigate the relationship between the haplotypes within the *CD28*/*CTLA4*/*ICOS* and IgAN susceptibility, LD data were calculated for all SNP pairs between the IgAN and healthy control groups. Three LD blocks were observed between 14 SNPs within *CD28*/*CTLA4*/*ICOS* by the method of Gabriel *et al.* (2002) (see figure 2 in electronic supplementary material). Although the SNPs revealed strong LDs during pair-wise comparisons among the SNPs of genes, we could find no significant difference between IgAN subjects and controls in the haplotype analysis (data not shown).

In terms of proteinuria, no SNP showed significant association with patients proteinuria > 4 mg/m^2/h. However, patients with nephrotic range proteinuria (> 40 mg/m^2/h) were found to be associated with *CD28* rs1879877 (codominant model, OR = 2.15, 95% CI = 1.06–4.36, P = 0.032) and *CTLA4* rs231779 (codominant model, OR = 2.85, 95% CI = 1.28–6.33, P = 0.009; recessive model, OR = 4.96, 95% CI = 1.33–18.52, P = 0.028) (see table 2 in electronic supplementary material). *CD28* rs3181097, *CTLA4* rs231777, *CTLA4* rs231779, *ICOS* rs4270326, *ICOS* rs11571314, *ICOS* rs10183087, *ICOS* rs4404254, and *ICOS* rs1559931 were significantly associated with the presence of podocyte foot process effacement in IgAN group (table 2). In addition, advanced mesangial proliferation was associated with *CD28* rs3181097 (codominant model, OR = 1.70, 95% CI = 1.06–2.73, P = 0.025; dominant model, OR = 2.48, 95% CI = 1.13–5.43, P = 0.018), *CD28* rs2140148 (dominant model, OR = 2.36, 95% CI = 1.05–5.33, P = 0.039), and *CTLA4* rs231779 (dominant model, OR = 2.26, 95% CI = 1.15–4.45, P = 0.016) (data not shown). Notably, *CTLA4* rs231779 was significantly associated with the presence of proteinuria, podocyte foot process effacement, and advanced mesangial proliferation. However, in terms of associations between SNPs and the presence of pathologically advanced disease markers by renal biopsy, we could not find any significant genotypic differences.

Of the SNPs related to the proteinuria, podocyte foot process effacement, and/or advanced mesangial proliferation, rs3181097 and rs1879877 of *CD28* are located in the promoter region. We have examined whether these SNPs influence transcription factor binding sites. In the rs3181097 SNP site, two transcription factors (SP1 and CEBPA) could bind to the A-containing sequence, and only one (SP1) to the T-containing sequence. In the rs1879877 SNP site, one transcription factor (HNF1A) could bind to the A, and different one (GATA1) to the C.

Discussion

T-cell co-stimulatory signalling molecules have been found to be associated with several autoimmune diseases such as experimental autoimmune encephalomyelitis, multiple sclerosis, and type 1 diabetes (Gough *et al.* 2005). In terms of GN, the blockade of CD28 and/or ICOS had therapeutic effectiveness in both serological and histopathological findings of anti-glomerular basement membrane GN models (Reynolds *et al.* 2000; Okano *et al.* 2004). With lupus nephritis and crescentic GN models, ICOS signalling inhibited all subclasses of IgG autoantibody and mesangial hypercellularity, and/or reduced the deposition of IgG and C3 in glomeruli, even showing the preventive effect on the both of clinical and pathological progression (Iwai *et al.* 2003; Odobasic *et al*. 2006).

In terms of genetic variability, association studies for the *CTLA4*/*CD28*/*ICOS* locus polymorphisms have been performed showing genetic susceptibility in several different autoimmune diseases (Ihara *et al.* 2001; Gough *et al.* 2005; Kavvoura *et al.* 2007). In patients with Graves' disease and myasthenia gravis, *CTLA4* polymorphisms were found to be associated with T-cell proliferation in patients carrying

Table 2. Logistic regression analysis of *CD28*, *CTLA4* and *ICOS* polymorphisms in IgAN patients with or without podocyte foot process effacement after adjustment for gender and age.

Gene symbol	SNPs	Genotype	Noneffacement *n* (frequency)	Effacement* *n* (frequency)	Model	OR (95% CI)	*P* value
CD28	rs1879877	A/A	43 (44.3%)	27 (36.5%)	Codominant	1.33 (0.85–2.08)	0.220
	Promoter	A/C	43 (44.3%)	35 (47.3%)	Dominant	1.40 (0.75–2.63)	0.290
		C/C	11 (11.3%)	12 (16.2%)	Recessive	1.53 (0.63–3.74)	0.350
	rs3181097	A/A	35 (36.1%)	16 (21.9%)	Codominant	1.33 (0.86–2.06)	0.200
	Promoter	A/G	40 (41.2%)	42 (57.5%)	Dominant	**2.11 (1.04–4.27)**	**0.034**
		G/G	22 (22.7%)	15 (20.6%)	Recessive	0.96 (0.45–2.03)	0.900
	rs2140148	T/T	80 (83.3%)	60 (80%)	Codominant	1.26 (0.59–2.69)	0.550
	Intron 1	T/G	15 (15.6%)	15 (20%)	Dominant	1.36 (0.62–3.01)	0.450
		G/G	1 (1%)	0 (0%)	Recessive	NA (0.00–NA)	0.370
	rs3116494	A	179 (92%)	141 (88%)		0.76 (0.32–1.79)	0.533**
	Intron 2	G	15 (8%)	9 (12%)			
CTLA4	rs231777	C/C	77 (80.2%)	49 (67.1%)	Codominant	**2.12(1.08–4.16)**	**0.026**
	Intron 1	T/C	19 (19.8%)	22 (30.1%)	Dominant	2.03 (1.00–4.14)	0.049
		T/T	0 (0%)	2 (2.7%)	Recessive	0.00 (0.00–NA)	0.059
	rs231779	T/T	51 (53.1%)	27 (36.5%)	Codominant	**1.67 (1.01–2.76)**	**0.044**
	Intron 1	T/C	39 (40.6%)	40 (54%)	Dominant	**2.00 (1.07–3.76)**	**0.029**
		C/C	6 (6.2%)	7 (9.5%)	Recessive	1.47 (0.47–4.63)	0.510
ICOS	rs4270326	C/C	81 (83.5%)	52 (69.3%)	Codominant	**2.00 (1.02–3.95)**	**0.041**
	Intron 3	G/C	15 (15.5%)	21 (28%)	Dominant	**2.19 (1.05–4.60)**	**0.036**
		G/G	1 (1%)	2 (2.7%)	Recessive	2.05 (0.18–23.60)	0.550
	rs11571314	A/A	78 (80.4%)	49 (65.3%)	Codominant	1.88 (1.00–3.54)	0.046
	Intron 3	A/G	17 (17.5%)	24 (32%)	Dominant	**2.26 (1.11–4.58)**	**0.023**
		G/G	2 (2.1%)	2 (2.7%)	Recessive	0.99 (0.13–7.40)	0.990
	rs10183087	A/A	78 (80.4%)	49 (65.3%)	Codominant	1.88 (1.00–3.54)	0.046
	Exon 5-UTR	A/C	17 (17.5%)	24 (32%)	Dominant	**2.26 (1.11–4.58)**	**0.023**
		C/C	2 (2.1%)	2 (2.7%)	Recessive	0.99 (0.13–7.40)	0.990
	rs4404254	T/T	78 (81.2%)	49 (66.2%)	Codominant	**1.92 (1.01–3.65)**	**0.043**
	Exon 5-UTR	T/C	16 (16.7%)	23 (31.1%)	Dominant	**2.33 (1.13–4.80)**	**0.020**
		C/C	2 (2.1%)	2 (2.7%)	Recessive	0.99 (0.13–7.41)	1.000
	rs1559931	G/G	78 (80.4%)	49 (65.3%)	Codominant	1.88 (1.00–3.54)	0.046
	Exon 5-UTR	A/G	17 (17.5%)	24 (32%)	Dominant	**2.26 (1.11–4.58)**	**0.023**
		A/A	2 (2.1%)	2 (2.7%)	Recessive	0.99 (0.13–7.40)	0.990
	rs1365828	C/C	42 (43.3%)	32 (42.7%)	Codominant	1.02 (0.64–1.60)	0.940
	Three-near gene	A/C	43 (44.3%)	34 (45.3%)	Dominant	1.06 (0.56–1.99)	0.860
		A/A	12 (12.4%)	9 (12%)	Recessive	0.94 (0.37–2.41)	0.900
	rs768180	C/C	28 (28.9%)	26 (35.6%)	Codominant	1.04 (0.66–1.65)	0.850
	Three-near gene	T/C	57 (58.8%)	31 (42.5%)	Dominant	0.71 (0.37–1.37)	0.310
		T/T	12 (12.4%)	16 (21.9%)	Recessive	1.98 (0.86–4.56)	0.110
	rs6726035	T/T	25 (25.8%)	21 (28.4%)	Codominant	1.03 (0.67–1.58)	0.900
	Three-near gene	T/C	50 (51.5%)	34 (46%)	Dominant	0.88 (0.44–1.75)	0.710
		C/C	22 (22.7%)	19 (25.7%)	Recessive	1.24 (0.61–2.54)	0.560

The statistically significant single nucleotide polymorphisms (SNPs) are shown, of a total of 14 SNPs genotyped from *CD28*, *CTLA4* and *ICOS* in 172 patients with IgAN. Genotype distributions are shown as numbers (%), odds ratios (OR), 95% confidence intervals (CI). *P* values were obtained by logistic regression analysis using codominant, dominant and recessive models after controlling for gender and age. Total numbers of SNPs differ, because the genotypes of some SNPs were unreadable. *Effacement group was defined as IgAN patients with the effacement of podocyte foot process on renal biopsy. **The allele frequencies of *CD28* rs3116494 that did not have all three genotypes present in the study population, only allele frequencies were compared by the chi-square test.

susceptible genotype and mRNA stability and expression of *CTLA4* (Huang *et al.* 2000; Kouki *et al.* 2000).

To our knowledge, this is the first report that studied the association between SNPs of *CTLA4*/*CD28*/*ICOS* locus and human IgAN, especially in paediatric patients. Similar with reports of other workers, *CD28*/*CTLA4*/*ICOS* polymorphisms seem to be associated with pathogenesis of IgAN. In this study, they were found to be related to clinical variables such as proteinuria, effacement of podocyte foot process, and mesangial proliferation in IgAN patients although the genes were not related to the disease susceptibility; especially *CTLA4* rs231779 was found to be associated with all these clinical variables. As proteinuria is known to be resulted from podocyte phenotypic changes, and proteinuria is one of the aggravation factors of pathologic progression including mesangial proliferation, this result suggests that

CTLA4 may play key roles in the pathogenesis of IgAN. However, *CTLA4* rs231779 in an intronic SNP and it is very difficult to explain the expected effects on the pathogenesis of the intron. Some introns have been found to affect the efficiency of normal splicing with various degrees, and structurally stabilize pre-mRNA to protect it against degradation, control the expression of exons, and enhance protein production (Malisic *et al.* 2010; Ying *et al.* 2010).

Further, promoter SNPs, rs3181097 and rs1879877, of *CD28* were found to influence the transcription factor binding sites in the test with AliBaba 2.1. It implicates that these SNPs in changing the expression of *CD28* directly or indirectly inducing the increase or decrease in cellular responses related to *CD28* and supports the possibility of these SNPs to be involved in the pathogenesis of IgAN. In conclusion, we have demonstrated that polymorphisms of three major genes involved in the regulation of immune response, *CTLA4*, *CD28* and *ICOS* might be involved in development of the clinical variables of IgAN such as the proteinuria, effacement of podocyte foot process, and mesangial proliferation in IgAN patients. Our results reinforce the need for the biological evidence that the risk variants impact on the pathogenesis of IgAN.

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