Association of interleukin-10 gene –592 A/C polymorphism with the clinical and pathological diversity of lupus nephritis

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Abstract

Objective

To investigate the interleukin-10 (IL-10) gene –592 A/C polymorphism in Chinese patients with lupus nephritis (LN) and evaluate the role of IL-10 in the pathogenesis and clinical/pathological diversity of LN.

Methods

Polymerase chain reaction (PCR) and restriction fragment length polymorphism (RFLP) analyses were used to detect the IL-10 gene –592 A/C polymorphism in 265 LN patients and 100 ethnically matched controls. Frequencies of the genotypes were compared between LN patients and controls and among LN patients with different pathological classes. The clinical and pathological characteristics of the patients with different genotypes were also analyzed. Serum IL-10 levels of the patients were determined by ELISA.

Results

No significant differences were found in the distribution of the polymorphism between healthy controls and LN patients. The parameters of disease activity index (DAI), percentage of positive serum anti-dsDNA antibodies, proteinuria and hematuria, and frequency of glomerular thrombi were all higher in patients with –592 AC/CC genotypes than those with AA genotype. AC/CC genotypes were more frequent in patients with LN-IV than in those with LN-II and Va. There was no significant difference in the serum IL-10 levels in patients with these three genotypes.

Conclusion

The IL-10 gene –592 A/C polymorphism appears to be associated with disease activity and renal pathology of LN, but not associated with LN susceptibility or serum IL-10 levels. Patients carrying the –592 C allele had a higher risk of diffuse proliferative glomerulonephritis, indicating the genetic influence of the IL-10 gene polymorphism in the renal lesions of LN.

Key words

Systemic lupus erythematosus, lupus nephritis, interleukin-10, gene polymorphism.

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Introduction

Lupus nephritis (LN), a major cause of morbidity and mortality in systemic lupus erythematosus (SLE), is the most frequent secondary glomerular disease in China. Despite the general autoimmune nature of its pathogenesis, LN appears to be a heterogeneous glomerulonephritis. The histological lesions in the glomeruli of patients with LN are diverse and LN is divided into six distinctive classes pathologically (1). Patients with each pathological class respond differently to immunosuppressive agents and their prognosis also varies accordingly. The clinical and pathological evidence may indicate different mechanisms underlying these pathological classes of LN, and many investigations have been undertaken to explore the molecular mechanisms underlying the diverse clinical and histopathological manifestations of LN but still much remains unknown.

As a potent stimulator for B cell survival, activation, differentiation, and immunoglobulin secretion (2), interleukin-10 (IL-10) has been implicated to participate in the immune dysregulation of SLE, which is characterized by B cell hyperactivity and autoantibody overproduction. Continuous administration of IL-10 accelerated the development of autoimmunity in the SLE model of NZW/B mice and treatment with anti-IL-10 antibodies substantially delayed the onset of autoimmunity (3). Circulating IL-10 levels were significantly increased in SLE patients with active disease compared with normal controls and correlated with disease activity (4-6), while the treatment with anti-IL-10 antibody could ameliorate human lupus symptoms (7). Although the association of higher IL-10 level with susceptibility (8, 9) and activity of SLE have been widely studied and much progress has been made on IL-10-targeted therapy, the role of IL-10 in lupus glomerulonephritis, one of the major manifestations of SLE, has not been elucidated. Whether IL-10 plays a role in the local immune reactions in the glomeruli of lupus patients is unclear, but is an essential question for understanding the pathogenesis of LN and developing effective therapeutic strategies.

Among individuals, the production of IL-10 often varies remarkably both at the constitutive level and after stimulation *in vitro* (10-12), and about 50% or over 70% of this inter-individual difference in IL-10 production can be attributed to genetic factors (13, 14). The variation of IL-10 expression is thought to be at the transcriptional level (15), since mutations in the IL-10 gene promoter sequence may alter specific transcriptional activation and cytokine production (11). Among identified polymorphisms in the IL-10 promoter, three linked single nucleotide polymorphisms (SNPs) of –1082 G/A, –819 T/C, and –592 A/C have been shown to influence the IL-10 gene expression (16). Allele –1082 G, which is quite rare in the Chinese population (17), is associated with higher IL-10 production (10) compared with –1082 A. The –819 T/C and –592 A/C polymorphisms are completely linked (10). The position –592 is in an area containing putative binding sites for NF-IL-6 and STAT-1 (18, 19). Compared with –592 C allele, the –592 A is associated with lower IL-10 production *in vitro* (20). In a study by Mok *et al.*, the –592 A is implicated to be associated with kidney involvement in Hong Kong Chinese patients with SLE (17), but the authors did not relate it to systemic IL-10 levels and the number of patients with renal pathological diagnosis was relatively small. In this study, we investigated the –592 A/C polymorphism in a large population of Chinese patients with LN, assessing its influence on IL-10 secretion *in vivo* and its role in the pathogenesis and clinico-pathological characteristics of LN.

Materials and methods

Patients and controls

The patient group consisted of 265 unrelated patients with active SLE, who were hospitalized and underwent renal biopsies at the clinical unit of this nephrology center from Feb, 1998 to Dec, 2004. They were 233 females and 32 males from around China mainland (Hong Kong, Macao or Taiwan not included), with the mean onset age of 28.0 ± 9.8 years, ranging from 9 to 51. All the patients fulfilled the 1982

revised criteria of American College of Rheumatology for SLE and were diagnosed lupus nephritis pathologically. They had not received any strong immunosuppressive treatment of high-dose oral prednisone (over 1mg/kg per diem), pulse methylprednisolone or cyclophosphamide at least two months before renal biopsy. According to the World Health Organization morphologic classification of LN (1995 revised version), the patients were divided into five pathological groups: class II (pure mesangial alterations, n = 53), class III (focal segmental glomerulonephritis, n = 7), class IV (diffuse glomerulonephritis, n = 114), class Va (pure membranous glomerulonephritis, n = 34) and Vb (membranous glomerulonephritis with mesangial alterations, n=57). Peripheral blood samples were obtained with informed consent for genotyping from all patients and 100 unrelated and ethnically matched healthy controls (57 females and 43 males, aged 31.6 ± 7.6 years). The study was approved by the local ethics committee.

Clinical and pathological parameters of patients with LN

All relevant data on history, physical and pathological findings of the patients were obtained from the medical records and standard questionnaires in this hospital. The clinical manifestations associated with the disease activity included hematuria, proteinuria, serum levels of autoantibodies, complements, immunoglobulin G and cryoglobulin, and extrarenal organ involvements such as rash, arthritis, hematologic disorder, serositis, lymphadenopathy and neuropsychiatric disorder. The specimens obtained from renal biopsies were routinely stained with hematoxylin-eosin, periodic acid-Schiff, Masson's trichrome and silver stains. Special attention was paid to the occurrence of cellular crescent, karyorrhexis, glomerular thrombi, loop necrosis, glomerular sclerosis, and acute tubulo-interstitial injuries. The method of peroxidase-antiperoxidase complex (PAP)-diaminobenzidine (DAB) was used to detect CD68 for infiltrating monocytes and proliferating cellular nuclear antigen (PCNA) for proliferative cells in the glomerulus and interstitum. Disease activity index (DAI) and pathological activity index (AI) were calculated according to the SLE-DAI scoring criteria (21) and activity and chronicity index in lupus nephritis (22), respectively.

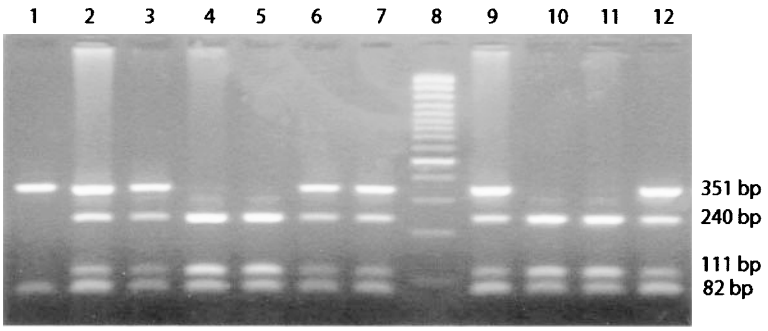

Fig. 1. IL-10 gene –592 A/C polymorphism in LN patients analyzed by PCR-RFLP: electrophoresis on a 2.5% agarose gel. Lane 1: genotype CC; Lane 2, 3, 6, 7, 9 and 12: genotype AC; Lane 4, 5, 10 and 11: genotype AA; Lane 8: 100 bp DNA ladder.

Determination of –592 A/C polymorphisms in the IL-10 gene promoter

Genomic DNA was extracted from whole blood using a phenol-chloroform extraction method. The IL-10 gene polymorphism of –592 A/C was determined by polymerase chain reaction (PCR) and restriction fragment length polymorphism (RFLP) analyses. The oligonucleotide primers were sense 5' TCC AGC CAC AGA AGC TTA CAA C 3' and antisense 5' AGG TCT CTG GGC CTT AGT TTC C 3' (20). PCR was performed on a GeneAmp PCR System 2700 (Applied Biosystems, USA) in the following conditions: 94˚C for 5 minutes; 35 cycles of 94˚C for 30 seconds, 62˚C for 30 seconds, and 72˚C for 30 seconds; followed by a final extending step at 72˚C for 5 minutes. The PCR product was digested for 4 hours at 37˚C with the restriction enzyme *Rsa* I (New England BioLabs, USA). The genotypes of IL-10 –592 A/C were distinguished by separation of the fragments on a 2.5% agarose gel and visualized under ultraviolet light (Fig. 1).

Detection of serum IL-10 levels

Serum samples from 105 cases of the patient group were collected one day before renal biopsy and stored at –70˚C. IL-10 concentration was determined by ELISA using an IL-10 ELISA kit (DIACLONE, France). Each sample was assayed in duplicate. The optical density (OD) value of each sample was determined in an EL x 800 automated microplate reader (Bio-Tek Instruments, USA). The amount of IL-10 in each sample was calculated by extrapolating OD values to IL-10 concentrations using a standard curve, the slope of which was over 0.99. The assay range of this IL-10 ELISA kit was 5 to 400 pg/ml.

Statistical analysis

The statistical analyses were performed using the SPSS 10.0 package. Data were expressed as percentages or mean ± standard deviation. Continuous variables were tested using the Student t-test or Mann-Whitney *U* test. Categorized data were compared using Pearson's Chi-square or Fisher's exact test. Bonferroni correction was applied in multiple comparisons by multiplying the observed *P* value by the number of the pairwise comparisons. All tests were two-sided and statistical significance was defined as $P < 0.05$.

Results

Distribution of the IL-10 gene –592 A/C polymorphism in the Chinese population

The –592 A allele was more prevalent in both patient and control groups, compared with the C allele. In each group, the observed distributions of homozygotes and heterozygotes conformed to expectations based on Hardy-Weinberg equilibrium analyses. There were no statistical differences in the distribution

of the –592 genotypes, or the allele frequencies between LN patients and controls (Table I), suggesting that the –592 polymorphism in the IL-10 gene may not be associated with LN susceptibility in the Chinese population. The allele frequencies were not significantly different in the female subjects compared with male subjects, either in the LN patients or in the control group.

Clinical characteristics of patients with different –592 genotypes

There was no significant difference in sex ratio, onset age and extra-renal involvement, between patients with AA genotype and those with AC/CC genotypes (we put AC and CC together as genotypes having C allele for statistical comparison) (Table II). The SLE-DAI, quantitation of urinary protein and sediment erythrocytes, and percentage of positive serum anti-dsDNA antibodies were all significantly higher in patients with AC/CC genotypes than those with AA genotype.

Pathological characteristics of patients with different –592 genotypes

With respect to the pathological heterogeneity of LN, we compared the IL-10 –592 polymorphism among patients in 4 distinctive pathological types II, IV, Va and Vb (type III not included because of its small sample size n= 7) and observed a significant difference in the IL-10 –592 genotypes among them (Fig. 2). The LN-IV group had the highest frequency of the AC/CC genotypes and the AC/CC genotypes were significantly more frequent in the LN-IV group than in the LN-II (slightly out of the range of significance after Bonferroni correction) or Va group. Patients with AC/CC genotypes had a higher pathological AI and a higher frequency of glomerular thrombi than those with AA genotype (Table III). There were no significant differences in the number of renal CD68 or PCNA positive cells among LN patients with different IL-10 genotypes (Table III).

Serum IL-10 levels of patients with different IL-10 –592 genotypes

Serum IL-10 concentrations of patients with active LN displayed remarkable inter-individual variation, ranging from 0–225.13 pm/ml. However, their difference did not reach a statistical significance among patients with the three genotypes (data not shown).

Table I. Distribution of IL-10 –592 A/C polymorphism in LN patients and healthy controls.

–592 A/C	Genotype frequency (%)			Allele frequency (%)	
	AA	AC	CC	A	C
Controls (n = 100)	41 (41.0)	47 (47.0)	12 (12.0)	64.5	35.5
LN patients (n = 265)	125 (47.2)	119 (44.9)	21 (7.9)	69.6	30.4

The differences in genotype and allele frequencies between LN patients and controls were not significant.

Table II. Clinical and laboratory parameters of LN patients with different IL-10 –592 genotypes.

	AA genotype n = 125	AC/CC genotypes n = 140	Significance
Gender (female / male)	112/13	124/16	NS
Age at the disease onset (years)	28.4 ± 9.9	27.7 ± 9.7	NS
Disease activity index	10.2 ± 4.5	12.5 ± 4.5	* P < 0.0001
Percentage of extrarenal involvement (%)			
Skin rash	54 (43.2)	80 (57.1)	NS
Arthritis	69 (55.2)	54 (38.6)	NS
Hematopoietic disorder	63 (50.4)	70 (50.0)	NS
Serositis	6 (4.8)	11 (7.9)	NS
Lymphadenopathy	5 (4.0)	5 (3.6)	NS
Neuropsychiatric disorder	2 (1.6)	5 (3.6)	NS
Urinary parameters			
Urine protein (g/24h)	3.6 ± 3.4	4.9 ± 4.6	¥ P = 0.019
Urine sediment of erythrocyte (x10^4/ml)	157.0 ± 358.9	330.0 ± 879.1	¥ P = 0.038
Serological parameters			
Percentage of ANA-positive cases (%)	103 (82.4)	118 (84.3)	NS
Percentage of anti-dsDNA-positive cases (%)	35 (28.0)	57 (40.7)	$ P = 0.030
Immunoglobulin G (g/L)	11.6 ± 7.7	12.4 ± 8.1	NS
Complement 3 (g/L)	0.617 ± 0.288	0.549 ± 0.288	NS
Complement 4 (g/L)	0.145 ± 0.091	0.137 ± 0.097	NS
Cryoglobulin (mg/L)	388.1 ± 414.1	353.8 ± 317.3	NS

* Independent samples *t*-test; ¥ Mann-Whitney U test.
$ Pearson's Chi-square test: OR 1.766, 95% CI 1.054 – 2.958 (AC/CC compared with AA.)
Only parts of the patients had their serum IgG (n = 221) and cryoglobulin (n = 157) values determined.
NS represents no significance.
ANA and dsDNA represent antinuclear antibody and double stranded DNA, respectively.

Discussion

Interleukin-10, first recognized for its ability to inhibit the activation and function of Th1 cells, monocytes, and macrophages (23, 24), is a multifunctional cytokine that plays a crucial role in inflammation and the immune system. Various studies have confirmed the association of IL-10 with autoimmune and infectious diseases (26), among which SLE has drawn the most attention. High IL-10 expression and the corresponding IL-10 alleles (27) have been suggested to play a causal or exacerbating role in SLE. A 40-fold increased risk for developing SLE was identified in individuals with particular alleles of both the IL-10 and bcl-2 genes (28), and it has been considered that polymorphisms of IL-10 contribute, at least in part, to the genetics involved in SLE. Although the kidney is one of the major target organs involved in SLE immunological damage, there have

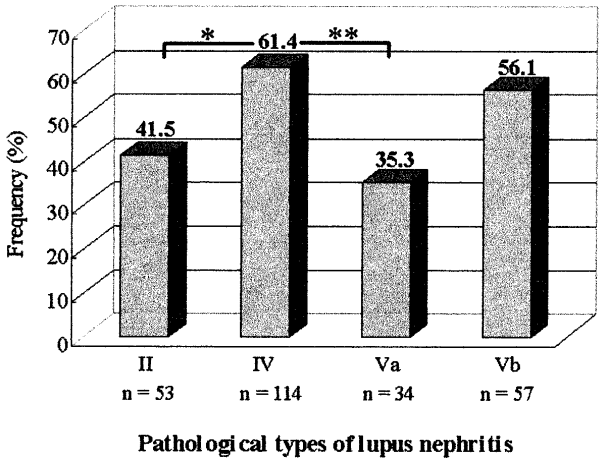

Fig. 2. Distribution of IL-10 –592 AC/CC genotypes in LN patients with different pathological types. Bars represent the frequency of the AC/CC genotypes in each group. Chi-square test shows significant difference in the frequencies of –592 AC/CC genotypes among the four pathological types of LN patients (P = 0.015). The AC/CC genotypes are significantly more frequent in the LN-IV group than in the LN-II (out of the range of significance after Bonferroni correction) or Va group.

* IV versus II: P = 0.016, OR 2.242 (95% CI: 1.154 – 4.354), P_{corr} = 0.096.
** IV versus Va: P = 0.007, OR 2.917 (95% CI: 1.313 – 6.479), P_{corr} = 0.042.

Table III. Histological manifestations of LN patients with different IL-10 –592 genotypes.

		AA genotype n = 125	AC/CC genotypes n = 140	Significance
Activity Index		5.4 ±4.4	6.4 ±4.6	* P = 0.032
Percentage of the histological lesion (%)				
Cellular crescent		36 (28.8)	51 (40.0)	NS
Karyorrhexis		16 (12.8)	22 (15.7)	NS
Glomerular thrombi		13 (10.4)	38 (27.1)	¥ P = 0.001
Glomerular capillary wall necrosis		15 (12.0)	17 (12.1)	NS
Glomerular sclerosis		43 (34.4)	38 (27.1)	NS
Tubular Injury		52 (41.6)	57 (40.7)	NS
Interstitial vasculitis		27 (21.6)	44 (31.4)	NS
Counts of infiltrating/proliferating cells		n = 39	n = 42	
CD68	Maximal per glomerulus	21.5 ± 22.9	27.6 ± 22.4	NS
	Mean per glomerulus	10.0 ± 11.4	14.3 ± 12.9	NS
	Mean in interstitum	342.7 ± 207.9	424.1 ± 260.8	NS
PCNA	Maximal per glomerulus	3.6 ± 4.5	4.8 ± 5.9	NS
	Mean per glomerulus	1.0 ± 1.6	1.6 ± 2.3	NS
	Mean in interstitum	9.1 ± 9.2	15.0 ± 16.7	NS

* Mann-Whitney U test.
¥ Pearson's Chi-square test: OR 3.210, 95% CI 1.619 – 6.364 (AC/CC compared with AA).
NS represents no significance.
PCNA represents proliferating cellular nuclear antigen.

been few studies concentrating on the association between IL-10 and LN. In this study, we investigated the distribution of IL-10 –592 A/C polymorphism in patients with biopsy-proven LN, and for the first time we have found evidence of a genetically determined subgroup of LN patients predisposed to proliferative glomerulonephritis.

The promoter of the IL-10 gene has been shown to be highly polymorphic. Studies to find associations between IL-10 promoter polymorphisms and SLE have yielded varying results among different populations. Two CA-repeat microsatellites, IL-10.R at –4 kb and IL-10.G at –1.1 kb, and eight SNP haplotypes in the distal promoter region have been shown to be associated with SLE incidence in some populations but not in others (27-31). For the most frequently studied –1082/–819/–592 SNPs, no association of the identified haplotypes with SLE incidence has been found in the Chinese, Dutch or British populations (17,32-35). However, the GCC haplotype was associated with anti-Ro autoantibodies in British Caucasians, while the ATA haplotype was associated with renal disease in the Chinese (17) but not Dutch (34) or British (35) populations, and with neuropsychiatric SLE in Dutch Caucasians (34) but not Chinese patients (17). Associations with disease outcome have been implicated especially with –592 A. The –592 A allele was associated with increased incidence of sepsis and mortality in critically ill patients (20) and with renal involvement in SLE patients (17). In hematopoietic-cell transplant recipients, the –592 AA genotype was associated with a decreased risk of grade III or IV acute graft-versus-host disease and death in remission (36). These findings suggested the influence of the IL-10 –592 A/C polymorphism in disease progression and prognosis.

In this study, we have found in the Chinese population that –592 A was the prevalent allele (64.5%) compared with –592 C, which was consistent with its distribution pattern in Hong Kong Chinese (67%) (17) but conspicuously different from British Caucasians (21%) (10). These results revealed significant racial variation in the distribution of the –592 A/C polymorphism of the IL-10 gene. In comparison with the significant difference between SLE patients with and without renal involvement in the Hong Kong Chinese population (17), there was no statistical difference in the distribution of the IL-10 –592 genotypes between LN patients and healthy controls, suggesting that the –592 polymorphism in the IL-10 gene may not be associated with LN susceptibility in the Chinese population.

The association of IL-10 with glomerular hypercellularity has been reported by several studies. IL-10 can induce significant mesangial cell proliferation *in vitro* and *in vivo* (37), and inhibition of IL-10 by the immunomodulator AS101 reduced mesangial cell proliferation in experimental mesangioproliferative glomerulonephritis (38). *In situ* hybridization and immunohistochemistry revealed that IL-10 mRNA and protein expression were significantly upregulated in the glomeruli with marked proliferative responses and in acute phases of microscopic polyangiitis

(39). In this study, we compared the percentage of PCNA positive cells in the glomeruli as an index of proliferation, but found no difference between the patients with different genotypes. Intriguingly, we observed in patients with distinctive pathological classes that those with LN-IV, characterized by significant glomerular hypercellularity, had a higher frequency of C-allele-carrying genotypes than those with either LN-II or LN-Va. This result revealed that the IL-10 gene polymorphism may play a role in diverse renal pathological changes in LN patients and that those carrying a high-IL-10-producing allele were more likely to have diffuse proliferative lesions in the kidney. We also found that the C allele was associated with both higher systemic activity (higher DAI and frequency of positive anti-dsDNA) and more active renal disease (more urinary protein and erythrocytes and higher AI and frequency of glomerular thrombi), consistent with the higher frequency of –592 C allele in patients with LN-IV, which is characterized by more active glomerular lesions and systemic disease than other pathological types of LN.

SLE is a typical immune-complex-mediated disease, but kidney involvement may be marked by different degrees of immune cell activation and resident cell proliferation. Patients with LN-IV usually have significant diffuse glomerular immune complex deposition as well as conspicuous inflammatory cell infiltration and hypercellularity. In patients with LN-V (Va in particular, since Vb is not a pure membranous lesion and has some characteristics of proliferation), cell infiltration and proliferation are insignificant and immune complexes are always localized to the subepithelial region. Patients with LN-IV have better responses to immunosuppressive agents than those with LN-V. These characteristics suggest that there may be different immunological mechanisms underlying the pathogenesis of LN class IV and class V. Our finding that patients with LN-IV had a higher frequency of AC/CC genotypes than those with LN-II and LN-Va suggested that genetic factors may contribute to the glomerular lesions in patients with LN.

The IL-10 –592 A/C polymorphism has been shown to influence IL-10 secretion in studies *in vitro* and the A allele was associated with lower IL-10 release in lipopolysaccharide-stimulated peripheral blood mononuclear cells (PBMCs) from healthy controls (20). We investigated the influence of the IL-10 –592 A/C polymorphism on the serum IL-10 level of LN patients where no related data had been reported, but failed to see a genetic regulation of IL-10 production among the patients. It was possibly due to the complexity of conditions *in vivo* that could regulate IL-10 gene expression. Compared with the supernatants from cultured PBMCs, serum IL-10 reflects its production from various sources, including PBMCs, tissue cells such as macrophages and certain epithelial cells, and viruses such as EBV, and therefore it may be affected by many factors such as infections and tissue injuries (40). In this study, the patients were either in an active disease status prior to therapy at the time of serum sample collection, or had failed steroid treatments, but there existed differences in the degree of disease activity, target organ injuries, number and type of IL-10-producing cells, and in the dosage of immunosuppressive agents used that might perturb the constitutive IL-10 expression. Although not associated with serum IL-10, the –592 A/C polymorphism has been shown to influence DAI and serum anti-dsDNA antibodies of LN patients, indicating the intrinsic association of the IL-10 gene polymorphism with the immunological dysregulation and disease activity of LN.

It still remains to be determined how the genetic polymorphism not associated with serum IL-10 level may have impact on renal lesions of LN. Our study showed that the –592 C allele was associated with a higher frequency of positive anti-dsDNA antibodies, which have been considered to be responsible for the initiation of lupus nephritis (41). In fact, the pattern of immune complex deposition of each pathological class of LN appears to be different. It is possible that the IL-10 gene polymorphism may influence the renal lesions through anti-dsDNA antibodies but the mechanism is still to be identified. Secondly, the IL-10 gene polymorphism might possibly impact on the local IL-10 level in the glomeruli, other than the serum IL-10 level, that could be responsible for the renal lesions in LN and differs between LN-IV and other pathological classes. It has been shown that IL-10 could act as a monocyte chemotactic factor in the presence of immune complex since it may enhance the expression of Fcγ receptor I and III on monocytes, which is correlated with enhanced antibody-dependent cell-mediated cytotoxicity (ADCC). The degree of immune cell infiltrating and glomerular hypercellularity, therefore, can be influenced by renal IL-10 levels. It is also possible that the IL-10 gene polymorphism may act indirectly through linkages with some other genes that play a role in the pathological lesions in the lupus glomeruli. Further studies concerning these aspects are to be carried out to identify the role of the IL-10 gene polymorphism in renal pathology of LN. Furthermore, we cannot neglect the fact that the pathological type in a LN patient may not always be invariable and we need to follow up these patients to collect more evidence, especially the pathological diagnosis from repeated renal biopsies.

In conclusion, our results reveal that the IL-10 gene –592 A/C polymorphism, though not associated with susceptibility to LN or the difference of the patients' serum IL-10 levels, may play a role in the clinical and pathological diversity of LN. The –592 C allele of the IL-10 gene is associated with higher susceptibility of class IV LN, and this genetic susceptibility might possibly contribute to the response of IL-10-mediated immune reaction and tissue damage in patients with LN.

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