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Original Article

Genetic association between *IL-10* gene polymorphisms and diabetic nephropathy susceptibility

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Abstract: Objective: The aim of this study was to explore the association between Interleukin-10 (IL-10) gene rs1518111 and rs3021094 single nucleotide polymorphisms (SNPs) and diabetic nephropathy (DN) susceptibility. Methods: A cohort of 265 Chinese Han population were enrolled in this case-control study. IL-10 gene rs1518111 and rs3021094 SNP were genotyped by polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) approach. Chi-square test was employed to analyze the differences of genotype and allele frequencies of the two polymorphisms between case and control groups. Odds ratios (ORs) and 95% confidence intervals (CIs) were applied to explain relative susceptibility of DN. Results: AA genotype and A allele frequencies of IL-10 SNP rs1518111 were significantly increased in cases compared with controls ($P < 0.05$), suggesting significant association with susceptibility of DN (OR = 3.091, 95% CI = 1.037-9.209; OR = 1.615, 95% CI = 1.083-2.406). Nevertheless, rs3021094 is weakly associated with DN risk ($P > 0.05$). Strong linkage disequilibrium (LD) level (D' = 1.0) was observed for IL-10 SNPs rs1518111 and rs3021094, and haplotype (G-A) frequencies in cases were significantly differ from controls ($P < 0.05$), revealing haplotype G-A could decrease the risk of DN occurrence (OR = 0.602, 95% CI = 0.397-0.913). Conclusion: IL-10 gene rs1518111 SNP might be associated with ND risk while rs1518111 were not in a Chinese Han population. A allele of rs1518111 was suggested to be a risk factor for DN. Haplotype G-A for rs1518111 and rs3021094 could be protective against DN susceptibility.

Keywords: *IL-10*, polymorphism, DN, PCR-RFLP

Introduction

Diabetic nephropathy (DN) is the most common chronic microvascular complications of diabetes. With higher mortality in diabetic patients, DN has been the major cause of end-stage renal disease (ESRD) [1]. The clinical manifestation of DN patients is albuminuria, progressive renal damage, hypertension and oedemathen, and ultimately lead to kidney failure which is one of the leading causes of death in patients with diabetes. DN is growing rapidly around the world, and has become the leading cause of ESRD in Japan [2]. Therefore, early diagnosis and treatment has an important significance for illness recovery of patients with DN. Various risk factors for DN have been reported, such as hypertension, high glomerular filtration rate, lipid abnormalities, and race [3]. Seaquist ER et al. have provided an evidence for genetic susceptibility to DN [4]. Besides, epidemiological studies have indicated that a genetic susceptibility might be involved in the etiology of DN [5].

Interleukin-10 (IL-10) is a cytokine that down-regulates pro-inflammatory responses and plays an important role in the regulation of the immune system. IL-10 has now measured up to the criteria for an anti-inflammatory and an immunosuppressive cytokine which acts as an inhibitor of many cytokines such as IL-6, IL-1α, IL-1β and TNF-α in activated macrophage and IFNg by T cells [6]. *IL-10*, the encoding gene of IL-10 cytokine, is located on human chromosome 1q31-1q32 containing 5 exons and 4 introns [7]. Many genetic variants of IL-10 gene have been reported [8-10]. Previous researches have indicated that human diseases might be affected by *IL-10* polymorphism. Chambrone L et al. have speculated AA genotype of the -1082 IL-10 gene polymorphism seem to be an increased risk factor for developing chronic

Table 1. Primer sequences of *IL-10* gene rs1518111 and rs3021094 polymorphisms

SNP	Primer sequences		Size (bp)
rs1518111	upstream	5'-CTGGAGGACTTTAAGGTGA-3'	294
	downstream	5'-AGCTTGGTTCTAGCGATC-3'	
rs3021094	upstream	5'-GCTCCGCAGAAAGAAGAC-3'	291
	downstream	5'-TTGAGGTTTGGGGAAATA-3'	

periodontitis (CP) [11]. Qi M et al. have suggested that the *IL-10* gene -592C>A polymorphism might be associated with gastric cancer risk in Asians [12].

IL-10 is a major anti-inflammatory cytokine and its activity might play a crucial role in clinical outcome of DN. Myśliwska et al. have found a higher level of circulating IL-10 in diabetes mellitus (DM) patients with DN compared to patients without DN [13]. Wong CK et al. have found that the level of IL-10 is correlated with the extent of renal damage in DN [14]. Overall, all results indicate an involvement of IL-10 in the pathogenesis of DN.

Various researches have studied the correlation of *IL-10* gene polymorphism with DN risk. Babel et al. have speculated *IL10*-1082A/G polymorphism might be associated with DN susceptibility [15]. A meta-analysis has also confirmed that *IL10*-1082A/G polymorphism might increase the risk of DN [16]. In this present study, we aimed at two another SNPs (rs1518111 and rs3021094) of *IL-10* gene, and explored their correlation with DN susceptibility in Chinese Han population.

Materials and methods

Objects of study

DN patients and healthy controls were enrolled in this case-control study. This research was consented and approved by Ethics committee of Shouguang People's Hospital. Sample collection is conformed to ethics criteria of national human genome research. Written informed consent was obtained from all participants before enrollment. All participants were Chinese Han population.

135 DN patients including 79 females and 56 males (aged 35-64 years old) were selected as cases. DN patients were those admitted to Shouguang People's Hospital from June 2013 to May 2014 and were diagnosed based on the 2003 American Diabetes Association diagnostic criteria for diabetes. 130 age and gender matched healthy volunteers including 73 females and 57 males (aged 33-65 years old) were recruited from healthy check-up center as controls. Controls were according to the cases in age and gender.

Sample collection and DNA extraction

2 ml peripheral venous blood were collected from every individual, anticoagulated by 0.5% EDTA (pH 8.0), separated to serum and hemocyte. Genomic DNA was extracted by TaKaRa Genome DNA Extraction Kit (Dalian Biological Engineering CO., LTD, China) and stored at -20°C.

PCR amplifications and genetic typing assay

The genotypes of *IL-10* gene rs1518111 and rs3021094 polymorphisms were examined by polymerase chain reaction restriction fragment length polymorphism (PCR-RFLP). Primer sequences for *IL-10* gene SNPs rs1518111 and rs3021094 were designed by Primer Premier 5.0, and synthesized by Sangon Biotech (Shanghai, China) (**Table 1**). PCR amplification was performed in a total volume of 25 μl containing 5 μl 10 × Buffer, 2 μl template DNA, 1 μl upstream primer, 1 μl downstream primer, 0.5 μl Taq DNA polymerase, 2 μl dNTP, and 13.5 μl deionized sterile water. PCR procedures were as the following: 95°C initial denaturation for 5 min; followed by 38 cycles of denaturing at 95°C for 30 s, annealing at different temperatures (51°C for rs1518111 and 49°C for rs3021094) for 30 s and extension for 30 s at 72°C; finally extension at 72°C for 5 min. PCR products were examined by agarose gel electrophoresis.

Then the amplified PCR products of *IL-10* SNPs rs1518111 and rs3021094 were digested with Csp6I and BglI, respectively. The digestions of PCR production were performed in accordance with Manufacturer's instructions. Finally, digested DNA products were then analyzed by 2% agarose gel electrophoresis and visualized by UV light.

Statistical analysis

The data analysis was performed by using PASW statistics 18.0 statistical software. Hardy-

Table 2. Genotype and allele distributions of *IL-10* gene rs1518111 and rs3021094 polymorphisms in case and control groups

Genotype/ Allele	Case *N* = 135 (%)	Control *n* = 130 (%)	χ^2	*P*	OR (95% CI)
rs1518111					
GG	5 (3.70)	12 (9.23)	-	-	1
GA	45 (33.34)	52 (40.00)	1.694	0.289	2.077 (0.680-6.346)
AA	85 (62.96)	66 (50.77)	4.439	0.042	3.091 (1.037-9.209)
G	55 (20.37)	76 (29.23)	-	-	1
A	215 (79.63)	184 (70.77)	5.588	0.021	1.615 (1.083-2.406)
rs3021094					
CC	45 (33.33)	33 (25.38)	-	-	1
CA	71 (52.59)	68 (52.31)	0.878	0.396	0.766 (0.438-1.339)
AA	19 (14.08)	29 (22.31)	3.899	0.066	0.480 (0.231-0.999)
C	161 (59.63)	134 (51.54)	-	-	1
A	109 (40.37)	126 (48.46)	3.514	0.067	0.720 (0.510-1.016)

Weinberg equilibrium (HWE) was assessed to test the representativeness of case and control groups. Genotype and allele frequencies of *IL-10* SNPs rs1518111 and rs3021094 were estimated by direct counting. Differences of the polymorphisms distributions between case and control groups were compared via Chi-square test. Association between *IL-10* gene polymorphisms and DN susceptibility was evaluated by odds ratios (ORs) and 95% confidence intervals (CIs). Linkage disequilibrium (LD) level was analyzed by haploview software. The differences had statistical significance when $P < 0.05$.

Results

HWE test

In our present cohort, 265 subjects including 135 cases and 130 controls were enrolled in this study. Genotype and allele distributions of *IL-10* gene rs1518111 and rs3021094 polymorphisms were revealed in **Table 2**. Genotypes of the two polymorphisms all conformed to HWE, which revealed the representativeness of participants.

Distributions of IL-10 gene polymorphisms

Genotype and allele distributions of *IL-10* gene rs1518111 and rs3021094 polymorphisms were shown in **Table 2**. As shown, GG, GA and AA genotype frequencies of rs1518111 were 3.70%, 33.34%, 62.96% in cases and 9.23%, 40.00%, 50.77% in controls, respectively. There were statistically differences of AA genotype between case group and control group ($P = 0.042$, OR = 3.091, 95% CI = 1.037-9.209). Meanwhile, G and A allele frequencies were respectively 20.37%, 79.63% in cases and 29.23%, 70.77% in healthy controls. Obviously, A allele frequencies were significantly increased in case group compared with control group ($P = 0.021$, OR = 1.615, 95% CI = 1.083-2.406). All results demonstrated that *IL-10* gene rs1518111 had an obvious association with DN susceptibility and A allele was probably a risk factor for DN.

The same as shown in **Table 2**, the frequencies of CC, CA and AA genotype of rs3021094 were respectively 33.33%, 52.59%, 14.08% in DN patients and 25.38%, 52.31%, 22.31% in healthy individuals; C and A allele frequencies were 59.63%, 40.37% in cases and 51.54%, 48.46% in controls. A allele carriers decreased in cases compared with controls, but the differences had no statistically significance ($P > 0.05$). The results showed that rs3021094 might have no obvious association with DN susceptibility.

Haplotype analysis of IL-10 SNPs and DN

Strong LD level (D' = 1.0) was observed for *IL-10* SNPs rs1518111 and rs3021094 by haploview software. Four haplotypes were detected including A-C, G-A, and A-A, and the detailed information was displayed in **Table 3**. The haplotype (G-A) frequency was lower in DN patients than in controls, and the differences were statistically significant ($P = 0.021$). It indicated that haplotype G-A formed by rs1518111 and rs3021094 was correlated with the risk of DN occurrence (OR = 0.602, 95% CI = 0.397-0.913).

Discussion

Chronic kidney disease (CKD) has affected millions of people from all over the world, and has been identified as a worldwide public health

Table 3. LD and Haplotype analyses of *IL-10* gene rs1518111 and rs3021094 polymorphisms in case and control groups

Haplotype	Case $2n$ = 270 (%)	Control $2n$ = 260 (%)	X^2	P	OR (95% CI)
AC	161 (59.63)	134 (51.54)	-	-	1
GA	55 (20.37)	76 (29.23)	5.754	0.021	0.602 (0.397-0.913)
AA	54 (20.00)	50 (19.23)	0.218	0.649	0.899 (0.574-1.407)

problem. DN is a common and serious complication of diabetes and has became the leading cause of ESRD [17]. In recent years, the prevalence of diabetes has increased rapidly worldwide. The total number of individuals with diabetes is projected to reach 221 million with diabetes is projected to reach 221 million and the main region with greatest increasing rate are Asia and Africa [18]. Furthermore, about 20-40% of diabetic patients develop nephropathy and progressed to ESRD finally in developed countries [19]. The global prevalence of DN has attracted more attention of scholars. In recent years, a deeper understanding of the genetic and molecular basis of the development and progression of DN has presented. To our knowledge, DN has been considered as a nonimmune disease, but recent researches have revealed a great role of immune-mediated inflammatory processes in the pathophysiology of DN [20]. Recent findings have also found that genetic variations play a crucial role in the development of DN such as *TRIB3*, *eNOS*, *PARP-1* and *APOE* [21-23].

IL-10 is first observed by Mosmann TR et al. in 1989 and named as cytokine synthesis inhibitory factor (CSIF) originally which inhibits cytokine synthesis by Th1 cells [24]. IL-10 is confirmed to the criteria for an anti-inflammatory and immunosuppressive cytokine [13]. Wong CK et al. have explored a significant positive correlation of plasma concentrations of IL-10 with urine albumin/creatinine ratio in DN patients [14]. Besides, the level of IL-10 has been reported to be correlated with the severity of nephropathy in DN patients by Wong CK et al. in 2007 [14]. To investigate the effect of *IL-10* gene polymorphism on DN risk, several studies have been performed. To our known of the latest knowledge, associations between three SNPs of *IL-10* gene and DN risk have been studied. For example, Babel N et al. have indicated that *IL-10* -1082A/G (rs1800896) polymorphism might be a risk factor for DN susceptibility in 2006 [15]. A meta-analysis performed by Peng X et al. in China have confirmed the contribution of *IL-10*-1082A/G polymorphism to the risk for DN [16]. Two studies performed in Tunisian population have also indicated the protective effect of T allele of rs1800871 for DN susceptibility while weakly association of rs1800872 with DN has been found [25, 26]. However, a study was done in Taiwanese T2DM patients with opposite results of *IL-10*-(-592) promoter polymorphisms might influence cytokine expressions involved in the progression of DN [27].

In the present study, we aimed at two another SNPs (rs1518111 and rs3021094) of *IL-10* gene. There were statistically significant differences in the genotype and allele distributions of *IL-10* gene rs1518111 between DN patients and healthy controls. As showed in **Table 2**, AA genotype and A allele frequencies were significantly increased in cases compared with controls, which suggested A allele might be a risk factor for DN risk. In the previous study, rs1518111 has been reported to be associated with Behçet's disease (BD) and showed strong linkage disequilibrium with allele rs1800871 [28]. And the strong LD level between allele rs1518111 and rs1800871 might be the reason for the present results while association between rs1800871 and DN risk has been reported [25]. Unfortunately, weakly association was found between rs3021094 and DN susceptibility. However, Wu Z et al. have observed significant association between rs3021094 and BD risk in Chinese Han population [28]. Besides, strong LD level was observed for rs1518111 and rs3021094, and three haplotypes were detected in which haplotype G-A was associated with DN risk.

In conclusion, *IL-10* SNP rs1518111 was suggested to be correlated with DN risk in a Chinese Han population in our present study while rs3021094 was not. The results further confirmed that *IL-10* genetic polymorphism affected the cytokine synthesis which participant in the progression of DN. Our study was performed only in a Chinese Han Population, the results remain to be confirmed in different

races. Although rs3021094 seemed to have no association with DN risk in our cohort, a larger or different population should be involved to verify our result. Besides, deeper investigations with multivariate risk assessments should be warranted, and interactions of genetic and environmental factors need to be taken into account.

Disclosure of conflict of interest

None.

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