Absence of Association of *Interleukin-18* Gene Polymorphisms with Primary Immune Thrombocytopenia in a Chinese Han Population

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Interleukin-18 (IL-18) is an inflammatory cytokine that plays an important role in autoimmune disease by inducing interferon- γ secretion. Considering the abnormal serum concentration of IL-18 in primary immune thrombocytopenia (ITP) patients and the regulated effect of *IL-18* gene polymorphisms on its production, the aim of this study was to investigate a possible association between the *IL-18* promoter polymorphisms (–137 G/C and –607 C/A sites) and genetic susceptibility to ITP in a Chinese Han population. A total of 181 ITP patients and 163 healthy controls were included in this study; *IL-18* gene promoter polymorphisms were analyzed by a polymerase chain reaction with sequence-specific primers. No significant differences in genotype (–607: $\chi^2=0.307$, $p=0.858$; –137: $\chi^2=0.378$, $p=0.828$) and allele frequencies (–607: $\chi^2=0.004$, $p=0.949$; –137: $\chi^2=0.307$, $p=0.858$) were found between total ITP patients and normal controls. We further analyzed the association of *IL-18* polymorphisms with clinical parameters of ITP patients, including first onset age and clinical therapy response to glucocorticoids, and no difference was revealed. In conclusion, *IL-18* promoter polymorphisms may not be associated with genetic susceptibility to ITP in a Chinese Han population.

Introduction

PRIMARY IMMUNE THROMBOCYTOPENIA (ITP) is an acquired autoimmune disease characterized by decreased platelet count due to both increased platelet destruction and insufficient platelet production (Stasi *et al.*, 2008; Wang *et al.*, 2011). The etiology of ITP is still unclear; besides autoreactive B lymphocytes secreting antiplatelet antibodies (Stasi *et al.*, 2008; Semple *et al.*, 2010), dysfunctional cellular immunities are also involved in ITP (Zhou *et al.*, 2005). Among them, the T-cell subsets skewed to T1 polarization play a critical role in ITP pathology, and the Th1:Th2 ratio is even closely related to the etiology and disease status of ITP (Semple *et al.*, 1996; Ogawara *et al.*, 2003; Wang *et al.*, 2005).

Interleukin-18 (IL-18), a member of the IL-1 family, is produced by monocytes, macrophages, and dendritic cells. It has an important regulation effect both on innate and adaptive immune responses against intracellular pathogen and viruses through binding to the IL-18 receptor (Srivastava *et al.*, 2010). IL-18 promotes Th1 responses through stimulating interferon (IFN)- γ production by T lymphocytes and natural killer cells in synergy with IL-12 (Nakanishi *et al.*, 2010). However, IL-18 also can induce a type 2 response in the absence of IL-12 by inducing the production of

IgE and Th2 cytokines such as IL-4 and IL-13 (Hoshino *et al.*, 2001; Nakanishi *et al.*, 2001; Gracie *et al.*, 2003). Because of the important role of the Th1/Th2 balance in immunological homeostasis, the expression of IL-18 has been correlated with several autoimmune and inflammatory disorders. In ITP patients, the serum concentration of IL-18 is upregulated. The elevated levels of IL-18 might enhance Th1 immune responses, including excessive IFN- γ and TNF- α production, finally leading to exacerbation of ITP (Shan *et al.*, 2009a). In addition, high-dose dexamethasone administration could downregulate *IL-18* expression in ITP patients (Shan *et al.*, 2009b), validating the important role of IL-18 in ITP pathogenesis.

Three single-nucleotide polymorphisms (SNPs) in the promoter of the *IL-18* gene at positions –656 G/T (rs1946519), –607 C/A (rs1946518), and –137 G/C (rs187238) relative to the transcriptional start site have been identified. Among them, the dysfunctions of –607 C/A and –137 G/C sites have been identified clearly. It was reported that the –607 C and –137 G alleles could induce higher *IL-18* expression at transcription levels through favoring the binding of cAMP-responsive element protein and histone 4 transcription factor-1, respectively (Giedraitis *et al.*, 2001; Smith and Humphries, 2009). Recently, these promoter SNPs have been found associated with genetic susceptibility to many autoimmune diseases, such

TABLE 1. THE CHARACTERISTICS OF IMMUNE THROMBOCYTOPENIA PATIENTS AND NORMAL CONTROLS ENROLLED IN THIS EXPERIMENT

Characteristics	Total ITP	Normal controls
Number of patients	181	163
Gender (male:female)	58:123	57:106
Age at diagnosis median (range) (year)	29 (1–81)	
Child-onset cITP (age at diagnosis ≤ 18)	48	
Adult-onset cITP (age at diagnosis > 18)	133	
Disease course median (range) (month)	12 (0.2–360)	
Number of patients treated with GCs	139	
Clinical responses to GCs (<i>n</i>)		
Complete response	72	
Response	24	
No response	43	

GCs, glucocorticoids; ITP, immune thrombocytopenia.

as systemic lupus erythematosus, type 1 diabetes, and rheumatoid arthritis (Dong *et al.*, 2007; Sánchez *et al.*, 2009; Wen *et al.*, 2014).

Taking into consideration the function of –607 C/A and –137 G/C sites polymorphism on *IL-18* expression, and elevated *IL-18* serum concentration in ITP patients, we supposed that *IL-18* promoter polymorphisms (–607 C/A and –137 G/C sites) may have important roles in the pathogenesis of ITP by affecting *IL-18* expression at transcription levels. Thus, we investigated the associations of the –137 C/G and –607 A/C polymorphisms in the promoter region of *IL-18* and risk for ITP in a Chinese Han population.

Materials and Methods

Patients

A total of 181 ITP patients and 163 ethnic background and sex-matched healthy controls were included in this study. Informed consent was obtained from the patients' legal guardians or the patients themselves, and this study was approved by the hospital-based ethics committee. The diagnosis of ITP met the diagnostic criteria as previously described

(Rodeghiero *et al.*, 2009). According to the age of first onset, the cases were further stratified into childhood ITP (onset age at 0–18 years) and adult ITP (onset age more than 18 years). In addition, for most enrolled patients ($n=139$) treated with glucocorticoids (GCs), we also analyzed the response to GCs according to the response criteria of the ITP International Working Group (Rodeghiero *et al.*, 2009). A complete response is defined as any platelet count of at least $100 \times 10^9/L$, and a response was defined as any platelet count between 30 and $100 \times 10^9/L$ or at least doubling of the baseline count. No response was defined as any platelet count lower than $30 \times 10^9/L$ or less than doubling of the baseline count. All patients were followed up in the outpatient department for at least 12 months from diagnosis. The characteristics of the subjects are shown in Table 1.

Extraction of genomic DNA

Peripheral blood samples were collected in vacuum tubes containing 5% EDTA. Genomic DNA was isolated from peripheral blood using the Wizard genomic DNA purification kit (Promega, Madison, WI) according to the instructions.

Genotyping

Genotyping of –607 C/A and –137 G/C sites was performed by using polymerase chain reaction (PCR) with sequence-specific primers and confirmed by DNA sequencing. The primer sequences are listed in Table 2. PCRs were performed with mixtures consisting of 50 ng of genomic DNA, 10 pM of each primer, and Premix Taq (Takara, Dalian, China) in a total volume of 25 μ L. For PCR amplification, an initial denaturation at 94°C for 5 min was followed by 35 cycles at 94°C for 30 s, 60°C for 30 s, 72°C for 30 s, and a final extension at 72°C for 10 min. Control primers were used to amplify fragments covering the polymorphic sites as a positive amplification control. PCR products were separated on a 2% agarose gel and then visualized by ethidium bromide staining under UV light.

Statistic analysis

Statistical analysis was performed using SPSS 16.0, and genotype and allele frequencies were analyzed using the χ^2 test or Fisher's exact test in case of expected frequencies < 5 . In addition, linkage disequilibrium between polymorphisms and haplotype analysis were assessed using the SHEsis software (Shi and He, 2005). $p < 0.05$ was considered as statistically significant.

TABLE 2. PRIMER SEQUENCES FOR POLYMERASE CHAIN REACTION

SNP	Primer sequence (5' $\rightarrow$ 3')	Fragment size (bp)
–607	Forward 1: 5' GTT GCA GAA AGT GTA AAA ATT ATT AC 3'	196
	Forward 2: 5' GTT GCA GAA AGT GTA AAA ATT ATT AA 3'	196
	Control forward: 5' CTT TGC TAT CAT TCC AGG AA 3'	301
	Reverse: 5' TAA CCT CAT TCA GGA CTT CC 3'	
–137	Forward 1: 5' CCC CAA CTT TTA CGG AAG AAA AG 3'	261
	Forward 2: 5' CCC CAA CTT TTA CGG AAG AAA AC 3'	
	Control forward: 5' CCA ATA GGA CTG ATT ATT CCG CA 3'	261
	Reverse: 5' AGG AGG GCA AAA TGC ACT GG 3'	449

Bold letters indicate the sequence-specific base.
SNP, single-nucleotide polymorphism.

TABLE 3. DISTRIBUTION OF THE GENOTYPE AND ALLELE FREQUENCIES OF *IL-18* –607 C/A AND –137 G/C SITES IN ALL ITP PATIENTS AND CONTROLS

	<i>tITP</i> n = 181 (%)	<i>Childhood</i> <i>ITP</i> n = 48 (%)	<i>Adult ITP</i> n = 133 (%)	<i>Control</i> n = 163 (%)	p-Value	OR (95% CI)
–607						
Genotype						
CC	51 (28.2)	16 (33.3)	35 (26.4)	44 (27.0)		Ref [(CA + AA)/CC]
CA	97 (53.6)	25 (52.1)	72 (54.1)	92 (56.4)	0.858 ^a	1.061 (0.661–1.704) ^a
AA	33 (18.2)	7 (14.6)	26 (19.5)	27 (16.6)	0.690 ^b	1.352 (0.676–2.703) ^b
CA + AA	130 (71.8)	32 (66.7)	98 (73.6)	119 (73.0)	0.799 ^c	0.966 (0.575–1.622) ^c
Allele						
C	199 (55.0)	57 (59.4)	142 (53.4)	180 (55.2)		Ref
A	163 (45.0)	39 (40.6)	124 (46.6)	146 (44.8)	0.949 ^a	0.990 (0.733–1.338) ^a
					0.470 ^b	0.844 (0.531–1.339) ^b
					0.656 ^c	1.077 (0.778–1.490) ^c
–137						
Genotype						
GG	148 (81.8)	43 (89.6)	105 (78.9)	129 (79.1)		Ref [(GC + CC)/GG]
GC	32 (17.6)	5 (10.4)	27 (20.3)	33 (20.2)	0.828 ^a	1.182 (0.693–2.016) ^a
CC	1 (0.6)	0	1 (0.8)	1 (0.7)	0.249 ^b	2.267 (0.834–6.163) ^b
GC + CC	33 (18.2)	5 (10.4)	28 (21.1)	34 (20.9)	0.989 ^c	0.988 (0.563–1.735) ^c
Allele						
G	328 (90.6)	91 (94.8)	237 (89.1)	291 (89.3)		Ref
C	34 (9.4)	5 (5.2)	29 (10.9)	35 (10.7)	0.558 ^a	0.862 (0.524–1.418) ^a
					0.104 ^b	2.189 (0.833–5.752) ^b
					0.948 ^c	0.983 (0.584–1.655) ^c

^aTotal ITP group compared with controls.

^bChildhood ITP group compared with controls.

^cAdult ITP group compared with controls.

CI, confidence interval; *IL-18*, interleukin-18; OR, odds ratio.

TABLE 4. STRATIFICATION ANALYSES OF *IL-18* –607 C/A AND –137 G/C POLYMORPHISMS WITH CLINICAL RESPONSES TO GCs IN ITP PATIENTS

	<i>Total ITP (%)</i>				<i>Controls</i> n = 163 (%)	p	OR (95% CI)
	<i>Complete</i> <i>response</i> , n = 72	<i>Response</i> , n = 24	<i>Complete response</i> + <i>response</i> , n = 96	<i>No response</i> , n = 43			
–607							
Genotype							
CC	20 (27.8)	8 (33.3)	28 (29.2)	11 (25.5)	44 (27.0)		Ref [(CA + AA)/CC]
CA	41 (56.9)	11 (45.8)	52 (54.1)	26 (60.5)	92 (56.4)	0.968 ^a	1.040 (0.559–1.935) ^a
AA	11 (15.3)	5 (20.9)	16 (16.7)	6 (14.0)	27 (16.6)	0.621 ^b	1.352 (0.541–3.381) ^b
CA + AA	52 (72.2)	16 (61.7)	68 (70.8)	32 (74.5)	119 (73.0)	0.923 ^c	1.114 (0.636–1.949) ^c
						0.875 ^d	0.930 (0.432–2.003) ^d
Allele							
C	81 (56.2)	27 (56.2)	108 (56.2)	48 (55.8)	180 (55.2)		Ref
A	63 (43.8)	21 (43.8)	84 (43.8)	38 (44.2)	146 (44.8)	0.835 ^a	1.043 (0.703–1.548) ^a
						0.893 ^b	1.043 (0.566–1.921) ^b
						0.819 ^c	1.043 (0.728–1.493) ^c
						0.921 ^d	1.024 (0.635–1.652) ^d
–137							
Genotype							
GG	59 (81.9)	21 (87.5)	80 (83.3)	38 (88.4)	129 (79.1)		Ref [(CC + GC)/GG]
GC	13 (18.1)	3 (12.5)	16 (16.7)	5 (11.6)	33 (20.2)	0.736 ^a	1.196 (0.588–2.432) ^a
CC	0	0	0	0	1 (0.7)	0.612 ^b	1.845 (0.519–6.552) ^a
GC + CC	13 (18.1)	3 (12.5)	16 (16.7)	5 (11.6)	34 (20.9)	0.569 ^c	1.318 (0.684–2.541) ^c
						0.369 ^d	2.003 (0.732–5.478) ^d
Allele							
G	131 (91.0)	45 (93.8)	176 (91.7)	81 (94.2)	291 (89.3)		Ref
C	13 (9.0)	3 (6.2)	16 (8.3)	5 (5.8)	35 (10.7)	0.573 ^a	0.825 (0.423–1.611) ^a
						0.337 ^b	0.554 (0.163–1.878) ^b
						0.375 ^c	0.755 (0.406–1.406) ^c
						0.170 ^d	0.513 (0.195–1.352) ^d

^aComplete response ITP group compared with controls.

^bResponse ITP group compared with controls.

^cComplete response + response ITP group compared with controls.

^dNo response ITP group compared with controls.

Results

A total of 181 ITP patients and 163 normal controls were genotyped for the *IL-18* promoter region –607 C/A and –137 G/C polymorphisms by PCR with sequence-specific primers. Also, the genotyping result was confirmed by DNA sequencing analysis. Genotypic frequencies in controls and patients were in the Hardy–Weinberg equilibrium (both $p > 0.05$). Moreover, –607 C/A and –137 G/C sites were not in linkage disequilibrium with each other ($D' = 0.668$, $r^2 = 0.0061$).

We evaluated *IL-18* –607 C/A and –137 G/C genotypes and allele distributions between total ITP patients and normal controls (Table 3). There was no significant difference in genotype and allele frequencies between them. Based on the onset age of ITP, the patients were subdivided into childhood ITP and adult ITP. No statistical differences were detected in genotype and allelic frequencies between each of the two groups and the controls (Table 3).

As a first-line therapy strategy, most of the enrolled patients ($n = 139$) received the GC treatment, thus, we also analyzed the association between *IL-18* polymorphisms and the GC treatment. First, we classified the patients who received GCs into the complete response, response, and no response group according to the clinical responses to GCs.

Then, we compared the distribution of –607 C/A and –137 G/C genotype and allele frequencies between each of the four groups and the controls, respectively. No statistical differences were found (Table 4). We also evaluated the impact of these SNPs on the response to GCs for the childhood group and the adulthood group separately, but we still did not find any differences between the each of the groups and the controls (Supplementary Tables S1 and S2; Supplementary Data are available online at www.liebertpub.com/dna).

We also compared the differences in disease course, gender, first onset age, and responses to GCs among ITP patients harboring the different genotypes or alleles. As shown in Table 5, no differences in any clinical parameter were found among these patients harboring the different genotypes or alleles.

In addition, we compared haplotype frequencies between ITP patients and controls (Table 6). This showed that none of the four possible haplotypes was significantly associated with genetic susceptibility to ITP.

Discussion

IL-18, a member of the *IL-1* family, promotes Th1 responses through stimulating IFN- γ production by T lymphocytes and

TABLE 5. THE ASSOCIATION ANALYSIS BETWEEN THE GENETIC POLYMORPHISMS AND THE CLINICAL PARAMETERS IN ITP PATIENTS

–607	Genotype (%)			χ^2	p	Allele (%)		χ^2	p
	CC	CA	AA			C	A		
Disease course									
New diagnosed	8 (38.1)	8 (38.1)	5 (23.8)	3.116	0.439	24 (57.1)	18 (42.9)	0.829	0.661
Persistent	7 (21.2)	19 (57.6)	7 (21.2)			33 (50)	33 (50)		
Chronic	36 (28.3)	70 (55.1)	21 (16.5)			142 (55.9)	112 (44.1)		
Gender									
Male	17 (29.3)	29 (50)	12 (20.7)	0.527	0.768	63 (54.3)	53 (45.7)	0.030	0.862
Female	34 (27.6)	68 (55.3)	21 (17.1)			136 (55.3)	110 (44.7)		
First onset age									
Adult ITP	35 (26.4)	72 (54.1)	26 (19.5)	1.121	0.571	142 (53.4)	124 (46.6)	1.023	0.312
Childhood ITP	16 (33.3)	25 (52.1)	7 (14.6)			57 (59.4)	39 (40.6)		
Response to GCs									
Complete response	20 (27.8)	41 (56.9)	11 (15.3)	1.429	0.856	81 (56.2)	63 (43.8)	0.005	0.998
Response	8 (33.3)	11 (45.8)	5 (20.9)			27 (56.2)	21 (43.8)		
No response	11 (25.5)	26 (60.5)	6 (14)			48 (55.8)	38 (44.2)		
–137	Genotype (%)			χ^2	p	Allele (%)		χ^2	p
	CC	GC	GG			C	G		
Disease course									
New diagnosed	1 (4.8)	6 (28.6)	14 (66.7)	9.910	0.061	8 (19)	34 (81)	5.234	0.073
Persistent	0	5 (15.2)	28 (84.8)			5 (7.6)	61 (92.4)		
Chronic	0	21 (16.5)	106 (83.5)			21 (8.3)	233 (91.7)		
Gender									
Male	0	7 (12.1)	51 (87.9)	2.388	0.314	7 (6)	109 (94)	2.262	0.133
Female	1 (0.8)	25 (20.3)	97 (78.9)			27 (11)	219 (89)		
First onset age									
Adult ITP	1 (0.7)	27 (20.3)	105 (78.9)	2.798	0.247	29 (10.9)	237 (89.1)	2.687	0.101
Childhood ITP	0	5 (11.6)	43 (89.6)			5 (5.2)	91 (94.8)		
Response to GCs									
Complete response	0	13 (18.1)	59 (81.9)	1.021	0.664	13 (9)	131 (91)	0.938	0.686
Response	0	3 (12.5)	21 (87.5)			3 (6.2)	45 (93.8)		
No response	0	5 (11.6)	38 (88.4)			5 (5.8)	81 (94.2)		

TABLE 6. HAPLOTYPE FREQUENCIES OF *IL-18* –607 C/A AND –137 G/C POLYMORPHISMS IN ITP PATIENTS AND NORMAL CONTROLS

Haplotype	Case (freq %)	Control (freq %)	χ^2	p	OR (95% CI)
–607 C/–137 C	2.7	1	–	–	–
–607 C/–137 G	52.3	54.2	0.073	0.787	0.959 (0.708–1.299)
–607 A/–137 C	6.7	9.7	1.930	0.165	0.677 (0.390–1.177)
–607 A/–137 G	38.3	35.1	1.140	0.286	1.186 (0.867–1.621)

natural killer cells through the IL-18 receptor (Nakanishi *et al.*, 2010). As a proinflammatory cytokine, it was upregulated in some autoimmune diseases and often in correlation with disease activity (Migliorini *et al.*, 2010). It is reported that the serum IL-18 concentrations in ITP patients were higher compared with normal controls, which confirmed that IL-18 were involved in ITP pathogenesis (Shan *et al.*, 2009b). For the –607 C/A and –137 G/C polymorphisms to cause transcription factor binding and regulate gene expression (Giedraitis *et al.*, 2001), we speculated that there was a correlation between *IL-18* promoter polymorphisms and the clinical implications of ITP. However, no differences were found in the genotype and allele distributions of these two SNPs between the patients with ITP and the healthy controls in this study. We further stratified the patients by first onset age, but also did not find any association between *IL-18* polymorphisms and the risk of ITP.

GCs, as a first-line therapy strategy, were widely used in ITP treatment. The research about the mechanism of GC treatment in ITP patients demonstrated that high-dose dexamethasone administration could downregulate IL-18 expression in ITP patients (Shan *et al.*, 2009b), suggesting that GCs work in ITP patients partly by downregulation of IL-18. Thus, in this study, we also analyzed the association between *IL-18* polymorphisms and the clinical responses to GC treatment, but no significant association was found.

Besides these, to well understand the contribution of *IL-18* promoter polymorphism to ITP pathogenesis, we analyzed the differences in clinical parameters among the ITP patients harboring different genotypes of alleles; meanwhile, we also compared haplotype frequencies between ITP patients and controls. Nevertheless, no positive results were found.

Overall, our study determined whether the promoter polymorphisms of *IL-18* gene were associated with ITP, and all these negative results remind us that gene polymorphisms neither for *IL-18* –607 C/A site nor for –137 G/C site were associated with susceptibility to ITP in a Chinese Han population. Additional prospective studies with large samples and different ethnic populations are needed to evaluate the role of *IL-18* gene polymorphism in ITP patients in the future.

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Disclosure Statement

No competing financial interests exist.

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