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ORIGINAL ARTICLE

A CARD9 single-nucleotide polymorphism rs4077515 is associated with reduced susceptibility to and severity of primary immune thrombocytopenia

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Abstract

Primary immune thrombocytopenia (ITP) is an acquired autoimmune disease characterized by a low platelet count and consequent increased risk of bleeding. The etiology underlying this condition remains poorly understood. The aim of this study is to evaluate the association of a single nucleotide polymorphism (SNP) rs4077515 in the caspase recruitment domain-containing protein 9 (CARD9) gene with the pathogenesis and therapy of ITP. Two hundred ninety-four patients with ITP and 324 age-matched healthy participants were recruited in this case-control study. Genotyping of CARD9 rs4077515 polymorphism was performed by Sanger sequencing. Our results revealed that a polymorphism rs4077515 in CARD9 gene is associated with decreased risk of susceptibility to and severity of ITP (susceptibility: codominant, AA vs. GG, OR = 0.175, 95% CI = 0.054-0.776, $p = 0.001$; recessive, GG + AG vs. AA, OR = 6.183, 95% CI = 2.287–16.715, $p < 0.001$; severity: allele, A vs. G, OR = 0.685, 95% CI = 0.476–0.985, $p = 0.041$; codominant, AG vs. GG, OR = 0.571, 95% CI = 0.350–0.931, $p = 0.025$; dominant, AA + AG vs. GG, OR = 0.558, 95% CI = 0.343–0.907, $p = 0.019$). The existence of the allele A, the mutant AA genotype and the heterozygous AG genotype of CARD9 rs4077515, plays a protective role in ITP. However, CARD9 rs4077515 polymorphism had no effect on corticosteroid sensitivity or refractoriness of ITP.

Keywords Primary immune thrombocytopenia · CARD9 · Single-nucleotide polymorphism · Susceptibility · Severity

Abbreviations

CARD9	caspase recruitment domain-containing protein 9
ITP	immune thrombocytopenia
SNP	single nucleotide polymorphism
Th cell	T helper cell
IBD	inflammatory bowel disease
ASH	American Society of Hematology
IWG	International Working Group
PCR	polymerase chain reaction
ABI	applied biosystems
χ^2	chi-squared
ORs	odds ratios
95% CIs	95% confidence intervals
HWE	Hardy–Weinberg equilibrium
IL	interleukin
IFN	interferon
TNF	tumor necrosis factor
WTCCC	Wellcome Trust Case Control Consortium

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Introduction

Primary immune thrombocytopenia (ITP), formerly known as idiopathic or immune thrombocytopenic purpura, is a clinically common acquired autoimmune disorder characterized by a transient or persistent decrease in platelet count [1]. As the most common hemorrhagic disease clinically, it accounts for nearly

30% of all hemorrhagic events. Therefore, it is important to understand the pathophysiology of primary ITP for seeking more effective clinical interventions.

Pathogenesis of ITP includes accelerated peripheral platelet destruction by the activated reticuloendothelial system and impaired platelet production in the bone marrow, which is related to defects in immune tolerance [2]. Impaired self-tolerance leads to abnormal humoral and cellular responses [3–5]. Derangement of cellular immunity is considered important in ITP pathophysiology [6]. Dysregulation of T cell subgroup proportion (elevated T helper (Th) 1/Th2 ratio) suggests that ITP is a Th1-dominated disease [7]. Previous studies suggested that Th1 polarization and inflammatory cytokine abnormalities due to gene polymorphisms play a role in the clinical features of ITP as well as in the response to treatment [8–11].

Caspase recruitment domain family member 9 (CARD9) is an inflammation-related molecule, which can trigger inflammatory cytokine cascade [12]. It mediates the production of pro-inflammatory cytokines, which drive Th1 and Th17 responses [13, 14]. Single nucleotide polymorphisms (SNPs), as the most common form of genetic variants, affect the human genome and mediate individual susceptibility to some diseases. Recently published studies of genome-wide association have demonstrated that the SNP rs4077515 in CARD9 in the human genome is strongly linked to the development of several human autoimmune inflammatory diseases, including ankylosing spondylitis, rheumatoid arthritis, IgA nephropathy, Crohn's disease, ulcerative colitis, and inflammatory bowel disease (IBD) [15–21]. Nevertheless, this polymorphism does not affect the susceptibility to candidemia or recurrent vulvovaginal candidiasis [22, 23]. However, to our knowledge, there are no studies attempting to clarify the link between CARD9 rs4077515 polymorphism and ITP. With regard to participation of dysregulated Th subsets in initiation and progression of ITP [12], as well as the important role of CARD9 in inflammatory cytokine production and Th polarization, we hypothesize that CARD9 polymorphism may be involved in the pathogenesis of ITP.

Taken together, established studies have investigated the associations between CARD9 rs4077515 polymorphism and several common autoimmune inflammatory diseases. And the results showed that the polymorphism played a protective, risky, or meaningless role in different diseases. ITP is also a typical autoimmune inflammatory disease. Herein our study was designed to explore the association between CARD9 rs4077515 polymorphism and primary ITP in the Chinese Han population.

Materials and methods

Study participants

The inclusion criteria applied were patients diagnosed with primary ITP as defined by standard criteria of the ITP International Working Group (IWG). The clinical criteria for the diagnosis of ITP were (1) that isolated thrombocytopenia with a peripheral platelet count less than 100×10^9/L, (2) normal or increased marrow megakaryocytes, and (3) no other causes or disorders associated with thrombocytopenia [1].

The exclusion criteria were as follows: patients with secondary ITP caused by other autoimmune diseases or underlying immune dysregulation (e.g., systemic lupus erythematosus-associated ITP); seropositive detection of human immunodeficiency virus (HIV) or hepatitis C virus (HCV); a pregnant or lactating status; active infection; a history of hypertension, diabetes, or cardiovascular diseases; significantly impaired liver and kidney function; patients with other congenital or acquired hemorrhagic diseases (e.g., severe anemia); and patients received drugs which could cause bleeding (e.g., aspirin).

Based on the criteria, we consecutively recruited 294 patients with ITP from January 2007 to April 2016 at the Department of Hematology, Qilu Hospital.

By contrast, a total of 324 age-matched healthy participants were enrolled in our study. They were randomly selected from healthy volunteers with no HIV or HCV infection, no symptoms of thrombocytopenia, and no other autoimmune diseases. Since ethnicity can influence gene polymorphisms, patients and controls in this study were all Han Chinese and there was no genetic association among them.

Previous studies usually focused on the association between SNPs and susceptibility to ITP. We would like to explore the link between the SNP and more complex clinical data. Based on the disease course and the response to treatment, patients were further stratified in terms of severity, corticosteroid sensitivity, and refractoriness [1]. Severe ITP is defined by the bleeding events sufficient to mandate treatment or by the new onset bleeding symptoms requiring additional therapeutic intervention according to the criteria of the ITP IWG. A platelet count of less than 10×10^9/L is regarded as the threshold for severe ITP. All the rest remaining are put in the non-severe category. The corticosteroid regimen is the first-line treatment strategy for newly diagnosed ITP patients, which includes high-dose dexamethasone at a dose of 40 mg/day for 4 days or prednisone at a dose of 1 mg/kg/day orally for 4 weeks. The standard definition for corticosteroid sensitivity is platelet counts over 30×10^9/L with at least a twofold increase from the baseline count in the absence of bleeding after receiving corticosteroid management. If patients required additional interventions, they were considered corticosteroid-resistant. Refractory ITP is defined as a failure to achieve at least a response or relapse after splenectomy. Meanwhile, the patients should either exhibit severe ITP or have a tendency of severe bleeding that requires therapy [1].

DNA preparation and genotyping

About 5 mL of peripheral venous blood from all participants was collected with vacuum anticoagulant tubes and stored at − 80 °C. Following the instruction, genomic DNA was extracted and purified from whole blood using a commercially available genomic DNA extraction kit (Cat. #DP304-03, TianGen, Beijing, China) according to the standard protocol. The CARD9 gene polymorphism was amplified by polymerase chain reaction (PCR) from extracted DNA. The forward primer (5′-GCGTCTGAGAAGGAGTGGGA-3′) and the reverse primer (5′-CTGACCCACTTTCCGTTTGC-3′) were used to generate a 285-bp PCR product. In brief, the PCR amplification consisted of an initial denaturation step at 95 °C for 5 min, followed by 40 cycles of 95 °C for 30 s, 58 °C for 30 s, and 72 °C for 1 min, and a final extension at 72 °C for 5 min. The amplified PCR products of the CARD9 gene were purified using Axygen AxyPrep DNA Gel Extraction Kit (AP-GX-250, Corning Life Sciences, NY, USA). Genotyping was performed by Sanger sequencing (Invitrogen, Shanghai, China), which was based on the Applied Biosystems (ABI) Prism BigDye Terminator v3.1 Cycle Sequencing Kit and was run on an ABI 3730XL Genetic Analyzer.

Statistical analysis

The calculator available at the Helmholtz Center Munich website was used to calculate the p value of the Hardy–Weinberg equilibrium. The data of genotypic frequencies under codominant, dominant, and recessive models and allelic frequencies were obtained. Then, the chi-squared (χ^2) test or Fisher's exact test were used to analyze the association between the SNP and ITP susceptibility, severity, corticosteroid sensitivity, and refractoriness. Odds ratios (ORs) were calculated and the 95% confidence intervals (95% CIs) were estimated using univariate binary logistic regression analyses. Statistically significant values were set at a two-tailed $p < 0.05$. All statistical analyses were performed using computer programs Microsoft Excel (Microsoft Corporation, NY, USA) and SPSS 22.0 (Statistical package for the social science) statistical software (SPSS, Inc., Chicago, IL, USA).

Results

Study population

Descriptive statistics of the main demographic and clinical characteristics of ITP patients and healthy controls are summarized in Table 1. No significant deviations from the Hardy–Weinberg equilibrium were observed in the control group ($p = 0.156$, Table 2). There was no statistical difference in age composition between ITP patients and healthy controls ($t = -0.225$, $p = 0.822$), while a significant difference was found in the gender distribution between the two groups ($\chi^2 = 47.325$, $p < 0.001$). A total of 249 patients accepted corticosteroid therapy among all ITP patients in the current study.

Table 1 Demographic and clinical characteristics

	ITP patients	Controls
No.	294	324
Age, mean ± SD	41.18 ± 17.44	40.91 ± 11.92
Gender (M/F)	111/183	212/112
ITP severity, *n* (%)		
Severe ITP	180	NA
Non-severe ITP	114	NA
ITP refractoriness, *n* (%)		
Refractory ITP	24	NA
Non-refractory ITP	270	NA
Treatment, *n* (%)		
Corticosteroid-sensitive	133	NA
Corticosteroid-resistant	116	NA
No use of corticosteroid	45	NA

M, male; *F*, female; *NA*, not applicable; *ITP*, immune thrombocytopenia

Association of CARD9 rs4077515 polymorphism with ITP susceptibility

Firstly, genotypic frequencies under three genetic models and allelic frequencies of CARD9 rs4077515 polymorphism were evaluated between all ITP patients and healthy controls. The statistical data are detailed in Table 2. After preliminary screening, a significantly different distribution of genotypes under the codominant and recessive models indicated an association between CARD9 rs4077515 polymorphism and ITP susceptibility ($p = 0.001$ and $p < 0.001$, respectively, Table 2). However, there was no evidence for the significant difference in the genotypic distribution under the dominant model or in the allelic frequencies between ITP patients and controls ($p = 0.653$ and $p = 0.387$, respectively, Table 2).

Univariate binary logistic regression analysis further revealed that as for CARD9 rs4077515, the AA rather than GG genotype was significantly associated with a decreased risk of susceptibility to ITP under the codominant model (OR = 0.175, 95% CI = 0.054-0.776, $p = 0.001$, Table 2). This polymorphism exhibited a protective effect. In accordance with the above finding, ITP patients carrying the GG/AG genotypes of CARD9 rs4077515 showed a 6.183-fold increase in susceptibility to ITP compared with patients carrying the minor genotype AA under the recessive model after adjusting for age and gender by univariate binary logistic regression (GG + AG vs. AA: OR = 6.183, 95% CI = 2.287–16.715, $p < 0.001$, Table 2). Thus, the GG/AG genotypes of CARD9 rs4077515 greatly increased ITP susceptibility.

Table 2 Association between CARD9 rs4077515 and ITP susceptibility

Gene polymorphism	Model/allele		ITP patients (N = 294)		Controls (N = 324)		HWE-p	p value	OR (95% CI)	Adjusted p value
			Count	%	Count	%				
CARD9 rs4077515	Codominant	GG (wild type)	129	43.9	148	45.7	0.156			Reference
		AA (mutant)	5	1.7	26	8		*0.001*	0.175 (0.054–0.776)	*0.001*
		AG (hetero)	160	54.4	150	46.3			1.152 (0.820–1.619)	0.414
	Dominant	GG	129	43.9	148	45.7		0.653		
		AA/AG	165	56.1	176	54.3			NA	NA
	Recessive	AA	5	1.7	26	8		*< 0.001*		Reference
		GG/AG	289	98.3	298	92			6.183 (2.287–16.715)	*< 0.001*
	Allele	G	418	71.1	446	68.8		0.387		
		A	170	28.9	202	31.2			NA	NA

CI, confidence interval; *OR*, odds ratio; *ITP*, immune thrombocytopenia; *HWE-p*, p value of Hardy–Weinberg equilibrium. p value calculated with the chi-squared test; adjusted p value calculated with univariate logistic regression. Values in italics are statistically significant ($p < 0.05$)

Association of CARD9 rs4077515 polymorphism with ITP severity

We divided all patients into severe and non-severe groups to determine the association between CARD9 rs4077515 polymorphism and disease severity. In these analyses, CARD9 rs4077515 polymorphism was shown to affect ITP severity. There was a different distribution in alleles and genotypes under codominant and dominant models between severe and non-severe ITP patients according to the chi-squared analyses or Fisher's exact tests (p = 0.039, p = 0.031, and p = 0.016, respectively, Table 3). After adjusting for age and gender by univariate binary logistic regression, ITP patients carrying the AG and AA/AG genotypes showed a decreased risk of developing severe ITP compared with patients carrying the major genotype GG under the codominant and dominant models (OR = 0.571, 95% CI = 0.350–0.931, p = 0.025 and OR = 0.558, 95% CI = 0.343–0.907, p = 0.019, respectively, Table 3). In addition, the A allele of CARD9 rs4077515 polymorphism may protect patients from severe ITP compared with the G allele (OR = 0.685, 95% CI = 0.476–0.985, p = 0.041, Table 3).

Association of CARD9 rs4077515 polymorphism with sensitivity to corticosteroid therapy

We next explored the association between CARD9 rs4077515 polymorphism and response to treatment of ITP, specifically in terms of sensitivity to corticosteroid therapy and refractoriness. The patients who received corticosteroid therapy were stratified into the corticosteroid-sensitive group (n = 133) and the corticosteroid-resistant group (n = 116). Neither allelic frequencies nor genotypic frequencies of CARD9 rs4077515 polymorphism differed significantly between the two groups (Table 4).

Table 3 Association between CARD9 rs4077515 and ITP severity

Gene polymorphism	Model/allele		Severe ITP patients (N = 180)		Non-severe ITP patients (N = 114)		p value	OR (95% CI)	Adjusted p value
CARD9 rs4077515			Count	%	Count	%			
	Codominant	GG (wild type)	89	49.4	40	35.1	*0.031*		Reference
		AA (mutant)	2	1.2	3	2.6		0.276 (0.044–1.747)	0.172
		AG (hetero)	89	49.4	71	62.3		0.571 (0.350–0.931)	*0.025*
	Dominant	GG	89	49.4	40	35.1	*0.016*		Reference
		AA/AG	91	50.6	74	64.9		0.558 (0.343–0.907)	*0.019*
	Recessive	AA	2	1.2	3	2.6	0.379		
		GG/AG	178	98.8	111	97.4		NA	NA
	Allele	G	267	74.2	151	66.2	*0.039*		Reference

CI, confidence interval; *OR*, odds ratio; *ITP*, immune thrombocytopenia; *HWE-p*, p value of Hardy–Weinberg equilibrium. p value calculated with the chi-squared test; adjusted p value calculated with univariate logistic regression. Values in italics are statistically significant ($p < 0.05$)

Table 4 Association between CARD9 rs4077515 and corticosteroid sensitivity

Gene polymorphism	Model/Allele		Corticosteroid-sensitive ITP patients (N = 133)		Corticosteroid-resistant ITP patients (N = 116)		p value
			Count	%	Count	%	
CARD9 rs4077515	Codominant	GG (wild type)	50	37.6	47	40.5	0.898
		AA (mutant)	3	2.3	2	1.7	
		AG (hetero)	80	60.1	67	57.8	
	Dominant	GG	50	37.6	47	40.5	
		AA/AG	83	62.4	69	59.5	1
	Recessive	AA	3	2.3	2	1.7	
		GG/AG	130	97.7	114	98.3	
	Allele	G	180	67.7	161	69.4	0.679
		A	86	32.3	71	30.6	

CI, confidence interval; *OR*, odds ratio; *ITP*, immune thrombocytopenia; *HWE-p*, p value of Hardy–Weinberg equilibrium. p value calculated with the chi-squared test; adjusted p value calculated with univariate logistic regression. Values in italics are statistically significant ($p < 0.05$)

Association of CARD9 rs4077515 polymorphism with ITP refractoriness

The enrolled patients were further subdivided into two groups: those refractory to splenectomy treatment and the rest remaining who responded to medical treatment or to splenectomy to study the influence of CARD9 rs4077515 polymorphism on the refractoriness of ITP. We compared the distribution of genotypes and alleles between the two groups. No significant differences were found (Table 5).

Discussion

In this study, we assessed the associations between CARD9 rs4077515 polymorphism and the incidence and treatment of ITP in the Chinese Han population. Interestingly, we found that CARD9 rs4077515 polymorphism was significantly associated with susceptibility to ITP under the codominant and recessive models. Individuals carrying the AA genotype rather than those with GG genotype had a significant decrease in the ITP susceptibility under the codominant model, while the GG/AG genotypes of CARD9 rs4077515 showed a 6.183-fold increase in the risk of developing ITP compared with the minor AA genotype under the recessive model. The results indicated that the inheritance of minor genotype AA should be considered as a significant protective factor for susceptibility in ITP patients. Strong associations between CARD9 rs4077515 polymorphism and severity of ITP were also observed under allelic and genotypic frequencies in codominant and dominant models, suggesting that this polymorphism may affect the severity of ITP. We observed that the A allele and the AG and pooled AA/AG genotypes of CARD9 rs4077515 can protect patients from severe ITP. In other words, the existence of the A allele may protect patients from severe ITP. However, there was no different distribution of genotypes or alleles between the corticosteroid-sensitive and corticosteroid-resistant groups. We also did not find a significant difference between

Table 5 Association between CARD9 rs4077515 and ITP refractoriness

Gene polymorphism	Model/Allele		Refractory ITP patients (N = 24)		Non-refractory ITP patients (N = 270)		p value
			Count	%	Count	%	
CARD9 rs4077515	Codominant	GG (wild type)	10	41.7	119	44.1	0.081
		AA (mutant)	2	8.3	3	1.1	
	Dominant	AG (hetero)	12	50	148	54.8	
		GG	10	41.7	119	44.1	0.82
		AA/AG	14	58.3	151	55.9	
	Recessive	AA	2	8.3	3	1.1	0.055
		GG/AG	22	91.7	267	98.9	
	Allele	G	32	66.7	386	71.5	0.481
		A	16	33.3	154	28.5	

CI, confidence interval; *OR*, odds ratio; *ITP*, immune thrombocytopenia; *HWE-p*, p value of Hardy–Weinberg equilibrium. p value calculated with the chi-squared test; adjusted p value calculated with univariate logistic regression. Bold highlights statistical significance ($p < 0.05$)

the refractory ITP patients and the controls. Taken together, our studies have demonstrated that CARD9 rs4077515 polymorphism is associated with reduced susceptibility to and severity of ITP and may serve as a predictive factor to monitor the progression of ITP.

Recent studies have shown that CARD9 SNPs are associated with several autoimmune diseases [16, 24–27]. Of all the SNPs in the CARD9 gene, rs4077515 is one of the mostly studied. CARD9 rs4077515 in the human genome results from the substitution from the wild-guanine (G) to adenine (A) nucleotide, which encodes substitution of asparagine for serine at position 12 (S12N) in the protein CARD9 ($CARD9^{S12N}$). It has been identified as a genome-wide significant signal for IgA nephropathy, ankylosing spondylitis, Crohn's disease, and ulcerative colitis by the Wellcome Trust Case Control Consortium (WTCCC) [17, 18, 26, 28, 29]. The CARD9 rs4077515-A allele confers an increased risk of these diseases. This is probably because this substitution is associated with higher expression of CARD9 in monocytes, lymphoblastoid cell lines, and peripheral blood cells, which leads to a hyper-reactive immune state [24, 25, 30–32]. The CARD9 rs4077515 allele C and the genotype CC were found to be significantly protective against ankylosing spondylitis in HLA-B27-negative Iranian patients [33].

Previous studies demonstrated that ITP shared common signaling pathways, especially inflammation-related, with other immune-mediated diseases. CARD9 is an inflammation-related molecule, mediating signals through transcription factor NF-κB subunit p65 and the kinase ERK (extracellular signal-regulated protein kinase) to initiate production of various cytokines, including TNF, IL-6, IL-12, IL-17, and IL-23 [34]. In addition, CARD9 can stimulate naïve T cells to become IFN-γ-producing Th1 cells and/or IL-17 producing Th17 cells [34]. Substitution of nucleotides in CARD9 gene may change Th1/Th2 ratio and alter the secretion of inflammatory cytokines [8, 35]. A recent study indicated that the CARD9 rs4077515 polymorphism facilitates the induction of type 2 immune responses after engagement of C-type lectin receptors (CLRs) [36]. To determine the functional importance of the CARD9 rs4077515 polymorphism, researchers generated mice homozygous for the S12N-encoding mutation knocked into Card9 ($CARD9^{S12N}$ knock-in (KI) mice). After infection with *Aspergillus fumigatus* (Af), they found impaired production of some Th1 cell cytokines, including IL-12p40 and IFN-γ, as well as increased production of the canonical type 2 cytokines IL-4 and IL-5 in the lungs of $CARD9^{S12N}$ KI mice than in those of wild-type mice, indicating an important role of the CARD9 rs4077515 polymorphism in directing Th2 cell polarization.

As we know, the aberrant cellular immunity plays an important role in the pathogenesis of ITP, which is mainly manifested in $CD4^+$ T cell subtype drift. Naïve $CD4^+$ T cells differentiate into several different Th cells, including Th1, Th2, Th17, and regulatory T (Treg) cells. Th cell functions are determined by their cytokine secretion patterns. Th1 and Th2 cells can produce cytokines involved in the development and suppression of inflammation respectively [37]. The equilibrium between Th1 and Th2 cells is essential for the regulation and normal function of the immune system [38]. Disturbed Th1/Th2 ratio plays an important role in the pathogenesis of ITP. Experimental data consistently support the concept that adult ITP is the manifestation of a type-1 polarized immune process [7]. Th1/Th2 ([IL-2 + IFN-γ]/[IL-4 + IL-5]) cytokine ratio, reflecting the Th deviation of the pathogenic disease-specific T cells, is significantly increased in patients with ITP. The increased Th1 cells in ITP patients result in overproduction of several pro-inflammatory cytokines, including IL-1α, IL-2, IL-6, IL-16, IL-17, IL-23, IFN-γ, and tumor necrosis factor (TNF), eventually leading to tissue inflammation in genetically predisposed individuals [34, 39–48]. In addition, Th17 cells also exhibit a critical role in the pathogenesis of ITP. It is probably through increasing the secretion of IL-17, which may further aggravate the enormous imbalance between Th1 and Th2 cells and lead to increased autoimmunity [2]. In recent years, numerous studies have shown that the high Th1/Th2 ratio is reversed by the standard therapy for ITP, including administration of intravenous immunoglobulin G (IVIg), high-dose dexamethasone (HD-DXM), and splenectomy. Therefore, correcting Th1/Th2 disorder has an important impact on the treatment and prognosis of ITP, which provides a theoretical basis for us to carry out the immune intervention in ITP patients. Considering the role of the CARD9 rs4077515 polymorphism in Th2 polarization as mentioned above, we hypothesize that this polymorphism could correct the disequilibrium between Th1 and Th2 cells and restore the immune tolerance status in ITP patients, and then affect the susceptibility to and severity of ITP.

Some limitations of our study should be addressed. Firstly, the limited samples, which were only from the Chinese Han population, may not result in enough statistical power to reveal the correlation between CARD9 rs4077515 polymorphism and the incidence of ITP. Therefore, further multicentric studies with larger patient populations from multiple ethnicities should be conducted to verify these results in the general population. Secondly, our study is a hospital-based case-control design, in which the patients' symptoms were relatively severe among all ITP patients. There may be a potential selection bias of subjects. Thirdly, although our study has suggested CARD9 rs4077515 polymorphism was significantly associated with the development of ITP, the underlying molecular mechanisms are not clear so far and required further research. Last but not least, not all CARD9

polymorphisms were incorporated into our study. Several polymorphisms in the CARD9 gene were reported to be associated with the susceptibility to autoimmune diseases in previous researches. For example, strong associations were observed between rs59902911, a synonymous SNP within the CARD9 gene, and joint damage in European Americans with rheumatoid arthritis ($p < 1 \times 10^{-4}$) [16]. In addition, Hong et al. demonstrated that a novel rare nonsynonymous polymorphism, rs200735402, in the CARD9 gene had a significant protective effect against Crohn's disease (OR = 0.09, $p = 5.28 \times 10^{-5}$) [49]. The candidate gene approach did not cover all susceptible loci, which may also lead to selection bias.

In conclusion, our investigation of the association between CARD9 rs4077515 polymorphism and ITP provides interesting results. The obtained data indicated that this polymorphism, associated with decreased risk of susceptibility to and severity of ITP, may be a genetic protective factor associated with the development of ITP in the Chinese Han population. To our knowledge, this is the first study attempting to clarify the association between CARD9 rs4077515 polymorphism and ITP. However, further studies on larger samples are needed to verify these findings and explore the role of CARD9 rs4077515 polymorphism in the etiology of ITP.

Authors' contribution ZS was the main researcher for this study and contributed to writing this manuscript. MH, JP, and SL were involved in the collecting of blood samples and clinical data. JL and Y-JW did the technical work. QF planned the study and revised the manuscript. All authors agree that this manuscript can be published in the Journal of Annals of hematology.

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Availability of data and material All data and material are available in this manuscript.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval All procedures conducted in this study were in accordance with the ethical standards of our institutional research committee and with the 1964 World Medical Association Declaration of Helsinki and its later amendments or comparable ethical standards. The Medical Ethics Committee of Qilu Hospital, Shandong University reviewed and approved this study.

Informed consent Informed consent was obtained from all individual participants included in the study.

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