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RESEARCH ARTICLE

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Association of interleukin-18 gene polymorphism and its protein expression with the lower extremity deep venous thrombosis in the chinese han population: A case-control study

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Objective: We aim to explain the correlation among IL-18 gene polymorphism, its protein expression and LEDVT in the Chinese Han population.

Methods: A total of 138 LEDVT patients and 150 healthy people volunteered as LEDVT and control groups. All the data, including the gender, age, BMI, levels of TG, LDL/HDL, TC, GLU, APTT, BUN, Cr, ALT, AST, ApoA1, ApoB, and Fg was detected. IL-18 level, IL-18 −137G/C and −607C/A polymorphism, and risk factors of LEDVT were detected using ELISA, PCR-RFLP and multivariate logistic regression analysis, respectively.

Results: Increased BMI, GLU, Fg, BUN, ApoB and IL-18 and decreased APTT were found in the LEDVT group. The GC + CC genotype and C allele in −137G/C polymorphism was elevated in the control group when compared to that in the LEDVT group. The IL-18 level was elevated in the case group when compared to the control group with respect to the same genotype in −607C/A and −137G/C polymorphisms, and in the LEDVT group, IL-18 level was higher in the GG genotype than that in the GC + CC genotype of −137G/C polymorphism. BUN, GG genotype and IL-18 level were independent risk factors, but APTT was a protective factor of LEDVT.

Conclusion: On the basis of our results, we concluded that the GG genotype of −137G/C polymorphism and IL-18 level are independent risk factors of LEDVT, and IL-18 gene polymorphism affects the level of IL-18 in LEDVT patients.

KEYWORDS

−137G/C, −607C/A, interleukin-18, lower extremity deep venous thrombosis, polymorphism

1 | INTRODUCTION

Venous thromboembolism (VT), including pulmonary embolism (PE) and deep vein thrombosis (DVT), is a widely-recognized disorder with a high incidence rate of ~131.5/100 000 people every year.[1] DVT and PE are the two different stages of VT, which are associated with common risk factors caused by environmental, behavioral, and genetic interactions.[2,3] DVT is the fundamental reason for cardiovascular-related mortality and its treatment such as anticoagulation therapy and mechanical leg compression only has a function of palliation.[4] Systemic or oral anticoagulation therapy remains ineffective in eliminating thrombus burden and it cannot prevent the post-thrombotic syndrome as well.[5] Lower extremity deep vein thrombosis (LEDVT), is predominantly a disease of older age which is a now more prevalent in clinical studies and it typically starts below the knee, and yet may extend proximally, leading to PE.[6] It is a prevailing disease with an incidence rate of 1/1000 adults annually.[7] Acute DVT may result in PE, thrombus progression, phlegmasia cerulea dolens, venous gangrene, phlegmasia alba dolens, and even death.[8] The standard treatment for DVT is oral anticoagulation, aiming to restrict the thrombus

propagation and reduce PE risk.[9] As a prevalent disorder, various genetic factors have been indicated to affect the susceptibility toward thrombosis as well as DVT incidence.[10]

Interleukin-18 (IL-18), a member of the IL-1 superfamily, can enhance both acquired and innate immune responses, which are highly expressed in synovial fluids, sera, and synovial tissues of patients having rheumatoid arthritis.[11] IL-18 is also a pro-inflammatory cytokine which plays an important role in the Th1 response, and is capable of inducing interferon (IFN)-γ production in natural killer cells and T cells.[12] Moreover, gene polymorphism of IL-18 plays a significant role in the hepatitis C virus infection among Americans, Europeans, Indians and also in the Chinese Han population.[13] Due to its important role in the immune response, pathophysiology and pathogenesis of various infectious diseases, IL-18 gene polymorphism is known to affect expression levels and also the outcome of the infection.[14] IL-18 promoter polymorphisms were involved in different inflammatory diseases, among which three single nucleotide polymorphisms at positions of −607C/A, −656G/T and −137G/C among promoters of the IL-18 gene were found.[15] Moreover, a study also explained the possible relationship between two different promoter polymorphisms, −607C/A (rs1946518) and −137G/C (rs187238) in IL-18 gene and prognosis and occurrence of prostate cancer in the Han Chinese population.[16] IL-18, an immune response modulator, plays an important role in the pathogenesis of various inflammatory-related disorders.[17] The serum IL-18 level is elevated in the DVT, which might damage the venous endothelial cells, leading to venous thrombosis.[18] Thus, our study aims to explain the association of IL-18 gene polymorphism and its protein expression with LEDVT in the Han population.

2 | MATERIALS AND METHODS

2.1 | Ethics statement

All experiments in this study were approved by the Ethics Committee of Huzhou Central Hospital and all patients signed the informed consent.

2.2 | Study subjects

Between October 2014 and December 2015, a total of 138 LEDVT patients of the Chinese Han population treated in the Vasculocardiology Deparment of Huzhou Central Hospital volunteered as the LEDVT group in our study, among those 73 were male and 65 were female with a mean age of (58.19 ± 12.71) and a mean weight of (69.3 ± 9.8) kg. The included criteria were as follows: according to the LEDVT diagnosis criteria revised in 1995 by the Committee Specialized in Peripheral Vascular Disease of the Society of Integrated Traditional Chinese and Western Medicine[19]; (i) acute stage of LEDVT: patients have an abrupt onset of LEDVT (often within 7 days), accompanied by severe distending pain in affected limb, tenderness in the shank and femoral triangle, maroon skin color, increased body temperature, extensive swelling, dilatation of the superficial vein of the affected limb and a "positive Homans" sign; (ii) In chronic stage of LEDVT, patients have an onset period lasting over 1 month, accompanied by obstruction to the venous return, vein blood reverse flow in the late stage, varicose veins and vein engorgement, limb swelling and pain, hollowness and alogotrophy after activity. All the patients were detected with DVT using lower limb deep vein angiography and were later diagnosed with LEDVT. The excluded criteria were as follows: patients had acute arterial embolism, erysipelas, acute lymphangitis, primary pelvic tumor, leg fibrositis, and leg injury hematoma. Later, in the corresponding period another 150 healthy people (Han population) with no history of LEDVT underwent physical examination in Huzhou Central Hospital and were enrolled as control group, among which 79 were male and 71 were female with a mean age of (55.92 ± 13.22) years old and a mean weight of (68.4 ± 10.6) kg.

2.3 | Clinical data collection

All the data, including the gender, age, body mass index (BMI), triglycerides (TG), low density lipoprotein (LDL), high density lipoprotein (HDL), total cholesterol (TC), fasting blood-glucose (GLU), partial thromboplastin time (APTT), blood urea nitrogen (BUN), Cr, alanine transaminase (ALT), aspartate transaminase (AST), apolipoprotein A1 (ApoA1), apolipoprotein B (ApoB), and fibrinogen (Fg) was detected in both the groups.

2.4 | Evaluating the Serum IL-18 level

A total of 2 mL of peripheral venous blood was collected from all the subjects on an empty stomach, later it was put in an acid-citrate dextrose (ACD) anticoagulant tube (Shanghai Qian Ling Chemical Co., Ltd., Shanghai, China), for centrifugation (3000 r/min) for 5 minutes, along with the serum preserved at a temperature of −20°C in the refrigerator for further use. An enzyme-linked immunosorbent kit (ELISA, R&D System, Minneapolis, Minn., USA) was used to determine the serum IL-18 level.

2.5 | Polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP)

The PCR-RFLP was used to detect the polymorphism of IL-18 gene promoters −137G/C and −607C/A. The modified salting out method was used to extract the leukocyte DNA and based on the Primer 5.0 software (Shanghai Sangon Biological Engineering Technology & Services Co., Ltd, Shanghai, China), the primer sequences were designed for the target gene. After annealing, temperature and cycle times were continuously changed and later tested by the polyacrylamide gel electrophoresis (PAGE). The final primer sequences were obtained as follows: −607C/A: forward primer, 5′-TTGTAACATTGTAGGAATTACC-3′; reverse primer, 5′-ATGTAATATCACTATTTTCATGAGA-3′; −137G/C: forward primer, 5′-ATGCTTCTAATGGACTAAGGA-3′; reverse primer, 5′-GTAATATCACTATTTTCATGAATT-3′. The PCR reaction conditions of −607C/A

were as follows: predenaturation at 95°C for 5 minutes, denaturation at 94°C for 30 seconds, annealing at 64°C for 30 seconds, extension at 72°C for 30 seconds, and final extension at 72°C for 7 minutes. The PCR reaction conditions of −137G/C were as below: predenaturation at 95°C for 5 minutes, denaturation at 94°C for 30 seconds, annealing at 50°C for 1 minute, extension at 72°C for 5 seconds, and final extension at 72°C for 7 minutes. Each PCR had 35 cycles. Enzyme digestion and electrophoresis detection were as follows: After enzyme digestion, the PCR amplification products were obtained and digested by the MseI restriction enzyme (Shanghai Yubo Biotech Co., Ltd, Shanghai, China) and EcoRI restriction enzyme (Shanghai Yubo Biotech Co., Ltd, Shanghai, China), followed by a gel electrophoresis by 4% agarose (Genetech, Vacaville, CA, USA) to detect the genotype and the mutant sites.

2.6 | Statistical analysis

The SPSS 21.0 software (IBM Corp., Armonk, NY, USA) was used for the data analysis. The measured data was presented as mean ± standard deviation and the *t*-test was used to compare the data between the two groups. The data calculated was also demonstrated in percentages and ratios and the chi-square test was adapted for group comparisons. The Hardy-Weinberg equilibrium (HWE) test was performed to detect the group representation, genotype and allele frequency using the chi-square test and multivariate analysis of variance using binary logistic regression analysis. $P < .05$ meant there was a statistically significant difference.

3 | RESULTS

3.1 | Clinical data of the subjects in the LEDVT and control groups

According to the comparison the LEDVT group had increased levels of BMI, GLU, Fg, BUN, and ApoB but decreased APTT when compared to that in the control group (all $P < .05$). There were no significant difference in the gender, age, TG, LDL, HDL, CHOL, Cr, ALT, AST, and $ApoA_1$ between the two groups ($P > .05$) (Table 1).

3.2 | Identification of PCR restriction enzyme electrophoresis

As shown in the Figure 1, the PCR restriction enzyme electrophoresis images showed that IL-18 −607C/A (PCR product length: 196 bp) polymorphism had CC, AA and CA genotypes, but the IL-18 −137G/C (PCR product length: 261 bp) polymorphism had GG, CC and GC genotypes after the restriction enzyme digestion.

TABLE 1 The clinical data between the LEDVT and control groups

	LEDVT (n = 138)	Control (n = 150)	*P*
Gender (male/female)	74/64	79/71	.906
Mean age	58.19 ± 12.71	55.92 ± 13.22	.139
BMI (kg/m2)	24.58 ± 2.16	23.00 ± 2.49	<.0001
Affected limb			
Left LEDVT	75	–	
Right LEDVT	29	–	
Left- and right LEDVT	34	–	
TG (mmol/L)	1.69 ± 0.79	1.64 ± 0.73	.577
LDL (mmol/L)	3.13 ± 0.86	3.04 ± 0.72	.335
HDL (mmol/L)	1.10 ± 0.24	1.14 ± 0.34	.253
TC (mmol/L)	4.72 ± 1.00	4.74 ± 0.79	.850
GLU (mmol/L)	5.25 ± 0.88	4.98 ± 0.71	.004
APTT (min)	34.18 ± 5.54	37.33 ± 7.44	<.0001
BUN (mmol/L)	5.84 ± 1.69	5.37 ± 1.38	.010
Cr (mmol/L)	80.40 ± 18.62	78.33 ± 16.29	.315
ALT (IU/L)	32.06 ± 12.08	29.85 ± 9.56	.085
AST (IU/L)	31.98 ± 10.50	29.78 ± 9.31	.061
ApoA1 (g/L)	1.30 ± 0.38	1.33 ± 0.40	.515
ApoB (g/L)	0.99 ± 0.49	0.80 ± 0.42	.001
Fg (g/L)	3.83 ± 1.11	3.25 ± 0.78	<.0001

ALT, alanine transaminase; ApoA1, apolipoprotein A1; ApoB, apolipoprotein B; APTT, partial thromboplastin time; AST, aspartate transaminase; BMI, body mass index; BUN, blood urea nitrogen; Cr, creatinine; Fg, fibrinogen; GLU, fasting blood-glucose; HDL, high density lipoprotein; LDL, low density lipoprotein; LEDVT, lower extremity deep venous thrombosis; TC, total cholesterol; TG, riglycerides.

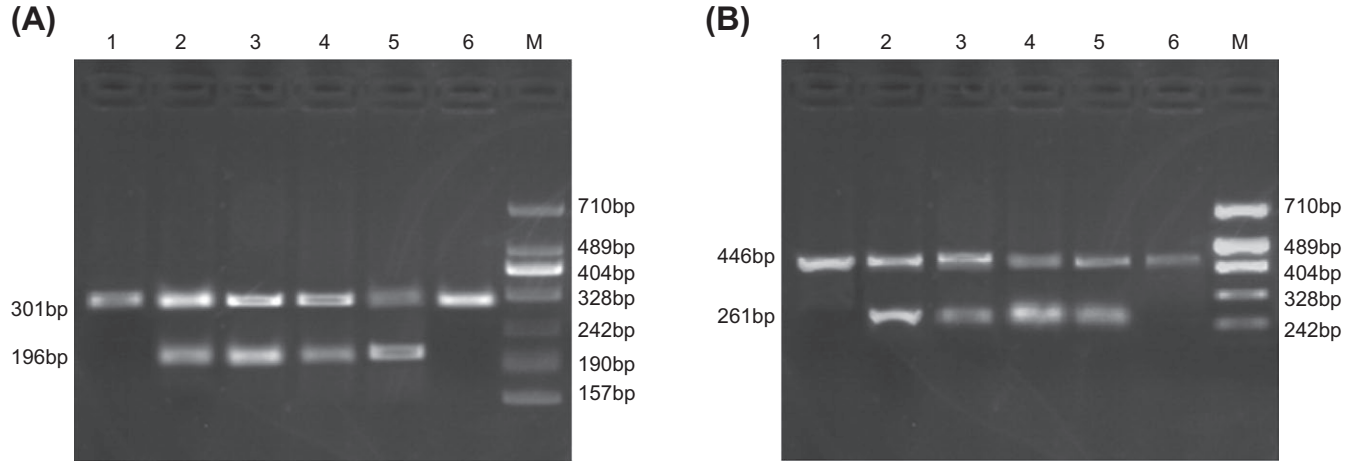

FIGURE 1 Electrophoresis Photo images for the polymorphisms of IL-18 gene promoters −607C/A and −137G/C. A, Electrophoresis images for the IL-18 −607C/A polymorphism; Lane 1 and 2, CC genotype; Lane 3 and 4, CA genotype; Lane 5 and 6, AA genotype; B, Electrophoresis images for the IL-18 −137G/C polymorphism; Lane 1 and 2, GG genotype; Lane 3 and 4, GC genotype; Lane 5 and 6, CC genotype; IL-18, interleukin-18

3.3 | Frequency distribution of IL-18 genotypes and its alleles in the LEDVT and control groups

The HWE demonstrated that both IL-18 −137G/C polymorphism and −607C/A polymorphism were in accordance with the HWE, which verified that all the selected data were of group representation.

The GC + CC genotype and C allele in −137G/C polymorphism significantly increased in the control group compared to those in the LEDVT group (all $P < .05$), suggesting that GC + CC genotype and C allele in −137G/C polymorphism were the protective gene for the LEDVT (OR = 0.826, 95% CI = 0.586-0.996, $P = .008$; OR = .743, 95% CI = 0.047-0.989, $P = .011$). There was no significant difference in the genotype and allele frequency of −607C/A polymorphism between the LEDVT and the control group ($P > .05$) (Table 2).

3.4 | Changes of serum IL-18 level in different genotypes of IL-18 −137G/C polymorphism and −607C/A polymorphism

As shown in Figure 2, the IL-18 levels in the LEDVT and control groups were (268.74 ± 37.91) pg/mL and (165.28 ± 28.15) pg/mL, respectively, indicating that the serum IL-18 level increased significantly in the LEDVT group compared to the control group ($P < .05$). IL-18 level was high in the LEDVT group when compared to the control group with respect to the same genotype in −607C/A and −137G/C polymorphisms ($P < .05$), and within the LEDVT group, IL-18 level was higher in the GG genotype than that in the GC + CC genotype of −137G/C polymorphism ($P < .05$), while no such significant difference was found in the control group ($P > .05$). Hence, this experiment was not able to establish any association between genotypes of −607C/A polymorphism and serum IL-18 level in the LEDVT and control groups ($P > .05$) (Table 3).

3.5 | Multivariate logistic regression analysis for the risk factor of LEDVT

The different genotypes of −137G/C polymorphism and levels of IL-18, BMI, GLU, APTT, BUN, ApoB and Fg, were considered as the potential risk factors in multivariate logistic regression analysis of our study. The input method was used for the analysis and BUN, IL-18-GG polymorphism and IL-18 level were independent risk factors, but APTT was a protective factor of LEDVT (all $P < .05$); whereas the BMI, GLU, ApoB, and Fg made no significant difference ($P > .05$) (Table 4).

4 | DISCUSSION

LEDVT, along with its complications still has a high incidence among the hospitalized patients.[20] It is a multifactorial disease with acquired and genetic risk factors playing a significant role in its pathogenesis.[21-24] Some genetic factors were mentioned in the etiology of LEDVT.[25,26] Thus, in this study we aim to demonstrate the association of IL-18-GG polymorphism and IL-18 level with LEDVT. In the end, our study provided evidence that GG genotype of −137G/C polymorphism and IL-18 level were independent risk factors of LEDVT and IL-18 gene polymorphism can influence the serum IL-18 level in LEDVT patients.

Initially, the LEDVT group had increased levels of BMI, GLU, Fg, BUN, ApoB and IL-18 but decreased APTT when compared to the control group. BMI was demonstrated as a biomarker for improvement in proximal DVT disease.[27] GLU and FBG levels were also a main criterion used to diagnose the glucose intolerance.[28] Fg, being a multifunctional plasma protein, plays a crucial role in several biological processes.[29] A study examined the effect of Fg on intracranial hemorrhage and concluded that Fg concentrate was a suitable therapy for enhancing plasma Fg for the treatment of intracranial hemorrhage along with hematoma expansion.[30] The BUN, which

TABLE 2 Frequency distribution of IL-18 genotype and allele between the LEDVT and control groups

	LEDVT (n, %)	Control (n, %)	OR	95% CI	χ^2	*P*
−607C/A						
CC	41 (29.71)	40 (26.67)	1			
CA	69 (50.00)	72 (48.00)	1.070	0.619-1.848	0.058	.809
AA	28 (20.29)	38 (25.33)	1.391	0.723 - 2.676	0.980	.322
CA + AA	97 (70.29)	110 (73.33)	1.162	0.695-1.944	0.329	.566
C	151 (54.71)	152 (50.67)	1			
A	125 (45.29)	148 (49.33)	1.176	0.848-1.632	0.943	.332
−137G/C						
GG	120 (86.96)	108 (72.00)	1			
GC	16 (11.59)	33 (22.00)	0.436	0.228-0.837	6.441	.011
CC	2 (1.45)	9 (6.00)	0.200	0.042-0.947	4.984	.026
GC + CC	18 (13.04)	42 (28.00)	0.386	0.210-0.710	9.749	.002
G	256 (92.75)	249 (83.00)	1			
C	20 (7.25)	51 (17.00)	0.381	0.221-0.658	12.650	<.001

LEDVT, lower extremity deep venous thrombosis; CI, combination index; OR, odd ratio; IL-18, interleukin-18.

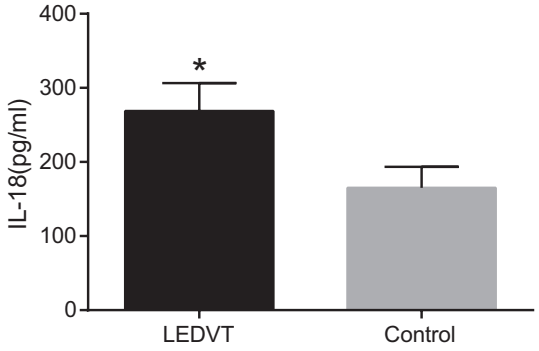

FIGURE 2 Serum IL-18 levels between the LEDVT and control groups. *P < .05, compared with the control group; LEDVT, lower extremity deep venous thrombosis; IL-18, interleukin-18

is a broadly available, easily determinable and inexpensive marker, could be adapted to confirm patients who are at the risk of cardiovascular endpoints, and the elevated BUN was inconsistency with increased mortality.[31,32] The apoB to apoA1, an indicator to balance the atheroprotective and atherogenic cholesterol transport,[33] was found to be independently related to cardiovascular diseases.[34] IL-18-stimulated macrophages triggered endothelial cell apoptosis, indicating that, in vivo, excess IL-18 suppressed tumor blood vessel formation.[35] The APTT is widely used to monitor therapeutic anticoagulation with standard heparin.[36] Together, BUN, and IL-18 level were independent risk factors, but APTT was a protective factor of LEDVT, which was also further proved by the multivariate logistic regression analysis.

The IL-18 level elevated remarkably in the LEDVT group when compared to the control group with respect to the same genotype in −607C/A and −137G/C, and in the LEDVT group IL-18 level was higher in the GG genotype of −137G/C than that in the GC + CC genotype. A study also revealed that IL-18 gene −137G/C polymorphism as well as −137C/-607A haplotype is related to the colorectal cancer.[37] Another study indicated that IL-18 −137G/C and −607A/C polymorphisms could control the IL-18 protein levels and

TABLE 3 Changes of serum IL-18 level in different genotypes of IL-18 −137G/C polymorphism and −607C/A polymorphism between the LEDVT and control groups

	LEDVT		Control	
Genotype	Case (n)	IL-18 level (pg/mL)	Case (n)	IL-18 level (pg/mL)
−607C/A				
CC	41	278.34 ± 38.97#	40	173.85 ± 38.93
CA + AA	97	271.25 ± 43.62#	110	159.25 ± 43.62
−137G/C				
GG	120	258.65 ± 32.30#*	108	154.65 ± 32.30
GC + CC	18	239.34 ± 29.13#	42	151.34 ± 29.13

#P < .05, compared with the same genotype between the LEDVT group and the control group.
*P < .05, GG genotype compared with the GC + CC genotype; LEDVT, lower extremity deep venous thrombosis; IL-18, interleukin-18.

TABLE 4 Multivariate logistic regression analysis for the risk factor of LEDVT

Factor	B	SE	Wals	Sig.	Exp (B)	95% CI
137G/C	−2.66	1.21	4.82	0.028	0.07	0.01-0.75
BMI	0.31	0.17	3.17	0.075	1.36	0.97-1.90
GLU	−0.35	0.51	0.47	0.492	0.71	0.26-1.91
APTT	−0.12	0.05	5.31	0.021	0.88	0.8-0.98
BUN	0.53	0.25	4.64	0.031	1.70	1.05-2.77
ApoB	0.49	0.80	0.37	0.543	1.63	0.34-7.76
Fg	0.45	0.36	1.54	0.214	1.57	0.77-3.19
IL-18	0.1	0.02	35.24	<0.001	1.11	1.07-1.14

LEDVT, lower extremity deep venous thrombosis; BMI, body mass index; GLU, fasting blood-glucose; APTT, partial thromboplastin time; BUN, blood urea nitrogen; ApoB, apolipoprotein B; Fg, fibrinogen; CI, combination index; IL-18, interleukin-18.

also influence an individual's sensitivity to oral cancer.[38] Vairaktaris et al. and Asefi et al. have detected the impact of IL-18 −607A/C and −137G/C polymorphisms on clinical parameters and the occurrence of oral cancer.[39,40] Also, Saenz-Lopez et al[41] demonstrated that IL-18 −137 GG genotype was remarkably associated with a high tumor grade, size, and stage in renal cell carcinoma patients. And it was further proved in a study that GG genotype of the −137G/C polymorphism was 2.165-times more risky than the GC genotype for the prostate cancer progression and lowered the rate of progression-free survival.[16] Importantly, IL-18-607A/C and also −137G/C promoter polymorphisms were associated with susceptibility to penicillin allergy and in particular, the −137G/C position plays an important role in IL-18 expression.[42] Serum IL-18 level increased in the DVT, which potentially impaired venous endothelial cells, leading to venous thrombosis, hence IL-18 could be a new potential target for the prevention of DVT.[18] In addition, interleukin polymorphisms have been reported to be involved in similar diseases along with LEDVT: IL-6 and its promoter polymorphism at −572G/C are associated with the risk of venous thromboembolisms (VTE) and IL-10 −1082A/G polymorphism is correlated with the risk of DVT.[43,44] IL-22 and IL-17, which are secreted from Th17 cells, have been considered as the new diagnostic markers of DVT, and they both might provide the novel molecular target for accelerating thrombus resolution in patients with DVT.[45] All the data mentioned above was consistent with our findings, and we reached to the conclusion that LEDVT patients with GG genotype of the IL-18 −137G/C gene had elevated IL-18 level.

In conclusion, our study demonstrated that the GG genotype of IL-18 −137G/C polymorphism and IL-18 level are independent risk factors of LEDVT, and IL-18 gene polymorphism can influence the serum IL-18 level in LEDVT patients. However, due to the limited data and experimental conditions, more improvements in the study could be done in the future.

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COMPETING INTERESTS

The authors have declared that no competing interests exist.

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