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GENETICS

The association of APOH and NCF1 polymorphisms on susceptibility to recurrent pregnancy loss in women with antiphospholipid syndrome

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Abstract

Background Recurrent pregnancy loss (RPL) is the main manifestation of pathological pregnancy in antiphospholipid syndrome (APS) women. The immune state plays a significant role in the occurrence/development of APS and RPL susceptibility, but there is little research on genetic factors.

Method Previous studies have described the important role of *APOH* and *NCF1* in APS and pregnancy. To explore the association of *APOH* and *NCF1* gene variants with RPL susceptibility in APS patients, we collected and analyzed 871 controls, 182 APS and RPL, and 231 RPL patients. Four single nucleotide polymorphisms (SNPs) (rs1801690, rs52797880, and rs8178847 of *APOH* and rs201802880 of *NCF1*) were selected and genotyped.

Results We found rs1801690 ($p = 0.001$, $p = 0.003$), rs52797880 ($p = 8.73e\text{-}04$, $p = 0.001$), and rs8178847 ($p = 0.001$, $p = 0.001$) of *APOH* and rs201802880 ($p = 3.77e\text{-}26$, $p = 1.31e\text{-}26$) of *NCF1* showed significant differences between APS and RPL patients and controls in allelic and genotype frequencies respectively. Moreover, rs1801690, rs52797880, and rs8178847 showed strong linkage disequilibrium. Especially, our results revealed a complete linkage disequilibrium ($D' = 1$) between rs52797880 and rs8178847. Furthermore, higher serum TP (total protein) level was described in *APOH* rs1801690 CG/GG ($p = 0.007$), rs52797880 AG/GG ($p = 0.033$), and rs8178847 CT/TT ($p = 0.033$), while the higher frequency of positive serum ACA-IgM was found in *NCF1* rs201802880 GA ($p = 0.017$) in APS and RPL patients.

Conclusion Rs1801690, rs52797880, and rs8178847 of *APOH* and rs201802880 of *NCF1* were associated with RPL susceptibility in APS patients.

Keywords Antiphospholipid syndrome (APS) · Recurrent pregnancy loss (RPL) · Apolipoprotein H (APOH) · The neutrophil cytosol factor 1 (NCF1) · Single nucleotide polymorphisms (SNP)

Introduction

Antiphospholipid syndrome (APS) is a prothrombotic, autoimmune, multisystem disorder characterized by a peculiar combination of thrombocytopenia, venous and/or

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arterial thrombosis, recurrent fetal loss, and/or pregnancy complications in patients with persistently positive antiphospholipid antibodies (APA), namely anti-cardiolipin (ACL), lupus anticoagulant (LA), and anti-beta-2 glycoprotein I (anti-β2GPI) antibodies [1]. The main manifestations of pathological pregnancy in APS women were recurrent pregnancy loss (RPL). RPL was defined as at least two consecutive spontaneous miscarriages occurring before 20 weeks of gestation [2] and was observed in the majority (~ 54%) of APS patients [3]. APA are a heterogeneous group of antibodies directed against phospholipids or phospholipid-binding proteins which are situated in the endothelial and trophoblast cell membrane, platelets, and other cells involved in the coagulation cascade. APA can produce inflammation-related factors and activate complement and cascade reaction, which cause thrombosis and trophoblast cell destruction, and even ultimately lead to abnormal pregnancy and intrauterine fetal demise [4]. Without specific treatment, the rate of fetal loss in pregnant women with positive APA can be as high as 90% [5]. Over the past two decades, the prognosis of pregnancies in APS women has highly improved. Nevertheless, about 20–30% of APS women remain unable to give birth to a healthy newborn despite conventional treatment [6].

Genetic factors have been found to act a part in the etiology and course progress of APS [7] and RPL [8]. Previous studies have revealed that in primary APS patients, *STAT4* SNPs (rs3024866, rs3821236, and rs7574865) and *BLK* SNP (rs2736340) displayed a significant genetic association, while *IRF5* SNPs (rs10954213 and rs2070197) displayed a weak association [9, 10]. Sugiura-Ogasawara et al. described that SNP (rs2288493) located on the 3′-UTR of *TSHR* exhibited an experiment-wide significant APS association, while SNP (rs79154414) located around the *C1D* indicated a genome-wide significant APS association [11]. Additionally, Ochoa et al. found a TAC risk haplotype containing one SNP (rs3184504) in the *SH2B3* gene and two SNPs (rs10774625 and rs653178) in the *ATXN2* gene showed the strongest association with thrombotic APS [12]. In recent years, genetic susceptibility related to APS has been extensively studied. However, there is a relative lack of research on genetic factors related to the susceptibility of APS patients to RPL.

APOH (apolipoprotein H, also denoted β2GPI) is a component of circulating plasma lipoproteins. It has been implicated in a diversity of physiologic pathways including hemostasis, coagulation, lipoprotein metabolism, and the production of APA. Prieto et al. revealed *β2GPI* gene polymorphism located at position 247 valine or leucine in a population of Brazilian and Mexican patients with APS and found significantly higher frequencies of the V allele and VV genotype expression [13]. APOH locus had also been shown to be associated with the presence of APA [14]. Another study in GWAS described that *APOH* had a significant correlation with the inflammatory process which is an increased risk of autoimmune disorders such as APS through modulating thrombotic response [15]. It is worth noting that *APOH* also plays a critical role in pregnancy. Kolialexi et al. identified a decrease of APOH in early-onset preeclampsia [16], while Provost et al. described an increase of APOH in late gestation [17]. Moreover, *APOH* was also found damaged in gestational diabetes and was related to oxidative stress and inflammation [18]. Oxidative stress and inflammation affect placental dysfunction by affecting placental angiogenesis and immune response and ultimately lead to miscarriage. The above studies suggest that *APOH* not only plays a regulatory role in APS but also may associate with the pathological process of RPL [13–18].

NCF1 (the neutrophil cytosol factor 1, also known as $p47^{PHOX}$) is a cytosolic subunit of the phagocytic NADPH oxidase isoform 2 (NOX2) complex that converts oxygen into superoxide anion. Previous studies have revealed a significant association between *NCF1* gene polymorphism and autoimmune diseases, including APS. Zhao et al. and Olsson et al. described that naturally occurring polymorphisms in the *NCF1* genes are associated with reactive oxygen species (ROS) production in autoimmune diseases [19, 20]. In mice, the *NCF1* mutation was described to be associated with autoimmune encephalomyelitis [21], arthritis, and a lupus-like phenotype with feathers of glomerulonephritis [22]. The missense variant rs201802880 in *NCF1* leads to reduced NADPH oxidase function and was believed to be associated with susceptibility to systemic lupus erythematosus (SLE) [19, 23]. Linge et al. discovered a specific influence of the ROS-deficient *NCF1-339* genotype promoting susceptibility to develop APA and possibly secondary APS [24]. Notably, c.579G > A mutation in *NCF1* has been described extensive significance for pre-pregnancy screening [25], which means the important role of *NCF1* gene polymorphism in normal pregnancy. However, no studies have examined the effect of *NCF1* gene polymorphism on susceptibility to RPL in APS patients.

Given the involvement of *APOH* and *NCF1* in the molecular mechanism of APS and the paucity of studies concentrating on the association between *APOH/NCF1* variant and susceptibility to RPL, an association study was carried out to explore whether the three *APOH* SNPs (rs1801690, rs52797880, and rs8178847) and one *NCF1* SNP (rs201802880) are associated with susceptibility to RPL in APS patients.

Materials and methods

Study subjects

In this study, 182 unrelated APS female subjects with idiopathic RPL (APS and RPL) (23 to 44 years old) and 231 idiopathic

RPL subjects (25 to 45 years old) were recruited from Shanghai First Maternity and Infant Hospital affiliated with Tongji University School of Medicine from June to October 2019. A total of 871 healthy females as controls (22 to 43 years old) were recruited from Shanghai East Hospital affiliated with Tongji University. All the groups were age-matched. APS was diagnosed in accordance with the 2006 Sydney revised Sapporo guidelines [1]. In brief, subjects with a combination of at least one positive clinical criterion (vascular thrombosis or pregnancy morbidity) and one positive laboratory criterion (LA, IgG/IgM anti-β2GP1 or IgG/IgM ACL antibodies at medium titer detected by ELISA on two different situations separated by 12 weeks) were diagnosed with APS. The subjects with idiopathic RPL we recruited had gone through at least two unexplained successive spontaneous abortions before 20 weeks of pregnancy in accordance with the definition set by the American Society for Reproductive Medicine (Practice Committee of the American Society for Reproductive Medicine 2013) [2]. None of the enrolled idiopathic RPL patients had an acceptable explanation for their miscarriages. Healthy females as controls had no history of miscarriage and immunological disorders. All subjects gave signed informed consent.

Gene and SNP screening

We reviewed the published literature and found that eight genes were associated with APS and RPL. Then, we searched for common SNPs on the exons and untranslated region (UTR) of these genes in the University of California Santa Cruz (UCSC) database and analyzed a total of 65 SNPs together with the SNPs reported in the literature.

Statistical analysis

The Student's *t*-test was used to evaluate whether demographic and relevant clinical characteristics were balanced in all participants. A chi-square test was performed to compare the allele and genotype frequencies between the two groups. To analyze the association between clinical laboratory indicators and polymorphism of studied genes, we used the Student's *t*-test to assess differences in normally distributed data and the chi-square test to evaluate differences in categorical variables data. A web-based platform (http://shesisplus.bio-x.cn/SHEsis.html) was used for statistical pairwise linkage disequilibrium (LD) and haplotype analysis [26]. Haplotypes with frequency <0.03 are ignored. Chi-square or Fisher's exact tests were performed to compare categorical variables. *p*-values ≤ 0.05 (two-sided) were considered statistically significant.

Results

Demographic and clinical characteristics of study subjects

Table 1 lists the demographic and clinical characteristics of APS and RPL patients and controls. No significant difference was found between the two groups in terms of age ($t = -1.039$, $p = 0.299$) and BMI ($t = -0.848$, $p = 0.398$). Compared with the control group, the APS and RPL group showed a higher number of recurrent pregnancy losses ($p < 0.001$).

Basic information on positive SNPs

We observed three positive SNPs of *APOH* (rs1801690, rs52797880, and rs8178847) and one positive SNP of *NCF1* (rs201802880) between APS and RPL patients and controls. SNP rs1801690, rs52797880, and rs8178847 of *APOH* are located on chromosome 17 (64208285, 64216815, 64216854), whereas SNP rs201802880 of *NCF1* is located on Chr17:74193642. The basic information on the positive SNPs including respective Human Genome Variation Society (HGVS) names is shown in Table 2.

Allelic and genotypic distributions

The allelic and genotypic distributions are displayed in Table 3. Among APS and RPL patients, RPL patients and controls, allele and genotype frequencies of rs1801690 ($p = 0.006$, $p = 0.008$), rs52797880 ($p = 0.004$, $p = 0.003$), and rs8178847 ($p = 0.006$, $p = 0.004$) of *APOH* and rs201802880 ($p = 1.74e\text{-}40$, $p = 3.97e\text{-}42$) of *NCF1* all exhibit significant differences.

Rs1801690 ($p = 0.001$), rs52797880 ($p = 8.73e\text{-}04$), and rs8178847 ($p = 0.001$) of *APOH* and rs201802880 ($p = 3.77e\text{-}26$) of *NCF1* showed significant difference between

Table 1 Demographic characteristics of study subjects

Variables	Control group ($n = 871$)	APS and RPL group ($n = 182$)	Statistics, *t*	*p*-values
Ages (years)	30.18 ± 4.05	30.61 ± 5.19	−1.039	0.299
BMI (kg/m^2)	20.67 ± 2.18	21.14 ± 2.71	−0.848	0.398
No. of recurrent pregnancy loss	0	2.66 ± 1.03		

Data are presented as mean ± SD

Table 2 Basic information on the SNPs

SNP	rs1801690	rs52797880	rs8178847	rs201802880
Gene	APOH	APOH	APOH	NCF1
Position	Chr17:64208285	Chr17:64216854	Chr17:64216815	Chr7:74193642
HGVS name	c.1004G>C(p.Trp335Ser)	c.422T>C (p.Ile141Thr)	c.461G>A (p.Arg154His)	c.269G>A (p.Arg90His)

Table 3 Genetic association of SNPs of *APOH* and *NCF1*

Gene	SNP ID	Allele frequency		*p*-values	Genotype frequency			*p*-values
APOH	rs1801690	C	G	0.006[a]	CC	CG	GG	0.008[a]
	APS and RPL	322 (0.884)	42 (0.115)		145 (0.796)	32 (0.175)	5 (0.027)	
	RPL	430 (0.930)	32 (0.069)	0.021[*b]	199 (0.861)	32 (0.138)	0 (0)	0.016[*b]
	Control	1625 (0.932)	117 (0.067)	0.001[**c]	758 (0.87)	109 (0.125)	4 (0.004)	0.003[**c]
	rs52797880	A	G	0.004[a]	AA	AG	GG	0.003[a]
	APS and RPL	323 (0.887)	41 (0.112)		147 (0.807)	29 (0.159)	6 (0.032)	
	RPL	429 (0.928)	33 (0.071)	0.039[*b]	198 (0.857)	33 (0.142)	0 (0)	0.014[*b]
	Control	1632 (0.936)	110 (0.063)	8.73e-04[***c]	765 (0.878)	102 (0.117)	4 (0.004)	0.001[**c]
	rs8178847	C	T	0.006[a]	CC	CT	TT	0.004[a]
	APS and RPL	323 (0.887)	41 (0.112)		147 (0.807)	29 (0.159)	6 (0.032)	
	RPL	429 (0.928)	33 (0.071)	0.039[*b]	198 (0.857)	33 (0.142)	0 (0)	0.014[*b]
	Control	1629 (0.935)	113 (0.064)	0.001[**c]	762 (0.874)	105 (0.12)	4 (0.004)	0.001[**c]
NCF1	rs201802880	G	A	1.74e-40[a]	GG	GA		3.97e-42[a]
	APS and RPL	333 (0.914)	31 (0.085)		151 (0.829)	31 (0.17)		
	RPL	402 (0.870)	60 (0.129)	0.041[*b]	171 (0.740)	60 (0.259)		0.031[*b]
	Control	1735 (0.995)	7 (0.004)	3.77e-26[***c]	864 (0.991)	7 (0.008)		1.31e-26[***c]

[a]Statistical analysis among three groups

[b]Statistical analysis between APS and RPL patients and RPL patients

[c]Statistical analysis between APS and RPL patients and controls

[*]$p < 0.05$, [**]$p < 0.01$, [***]$p < 0.001$. APS and RPL patients ($n = 182$), RPL patients ($n = 231$), controls ($n = 871$)

APS and RPL patients and controls in allelic frequencies. The *APOH* allele frequencies of rs1801690 G, rs52797880 G, and rs8178847 T were respectively 4.8%, 4.9%, and 4.8% higher in the APS and RPL patients than controls, while the *NCF1* allele frequency of rs201802880 A was 8.1% higher in the APS and RPL patients. Similarly, the genotype frequency of rs1801690 ($p = 0.003$), rs52797880 ($p = 0.001$), and rs8178847 ($p = 0.001$) of *APOH* and rs201802880 ($p =$ 1.31e-26) of *NCF1* between APS and RPL patients and controls also exhibited significant differences. Especially, the genotype frequency of *NCF1* rs201802880 GA was 16.2% higher in the APS and RPL patients than in controls.

To further reveal the role of *APOH* and *NCF1* polymorphisms, we also compared the allelic and genotypic distributions between APS and RPL patients and RPL patients (Table 3). The results of allelic distributions showed that rs1801690 ($p = 0.021$), rs52797880 ($p = 0.039$), and rs8178847 ($p = 0.039$) of *APOH* and rs201802880 ($p =$ 0.041) of *NCF1* showed significant differences between APS and RPL patients and RPL patients. The genotype frequency of rs1801690 ($p = 0.016$), rs52797880 ($p = 0.014$), and rs8178847 ($p = 0.014$) of *APOH* and rs201802880 ($p =$ 0.031) of *NCF1* also exhibited significant differences. Allelic and genotypic distributions were also compared between RPL patients and controls (Supplementary Table 1). The results showed that three SNPs of *APOH* described no significant differences both in allele and genotype frequencies, while rs201802880 of *NCF1* showed significant differences in allele ($p = 1.65e\text{-}34$) and genotype ($p = 5.52e\text{-}36$) distribution.

Haplotype analysis within the block

Pairwise LD estimates defined by *D*' exhibited strong LD among rs1801690, rs52797880, and rs8178847 in the *APOH* gene (Fig. 1). Especially, LD analyses revealed that the *D*' was 1 between rs52797880 and rs8178847, indicating a complete linkage disequilibrium between the two SNPs.

Significant differences were described in the global frequencies of haplotypes with different combinations of

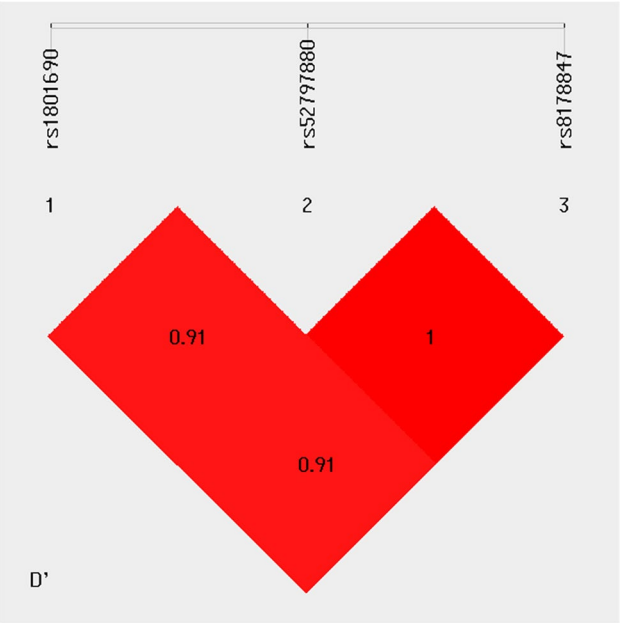


Fig. 1 LD structure of the three *APOH* gene polymorphisms with Haploview analysis. Red squares indicate significant statistical LD between the pair of polymorphisms (*D*' > 80)

three polymorphisms (rs1801690-rs52797880-rs8178847) between the cases and controls ($p = 0.002$). The individual haplotypes CAC ($p = 0.001$) and GGT ($p = 0.001$) displayed a significant difference between APS and RPL patients and controls (Table 4).

Association between polymorphism and clinical laboratory indicators

We further explored the association between *APOH/NCF1* SNPs and relative clinical laboratory indicators (Table 5). In APS and RPL patients, higher serum TP level was described in the mutant genotype of *APOH* rs1801690 CG/GG ($p = 0.007$) (Fig. 2A), rs52797880 AG/GG ($p = 0.033$) (Fig. 2B), and rs8178847 CT/TT ($p = 0.033$) (Fig. 2C), while the higher frequency of positive serum ACA-IgM level was found in the mutant genotype of *NCF1* rs201802880 GA ($p = 0.017$) (Fig. 2D). Because of the complete linkage

Table 4 Haplotype analysis within the block

Haplotype	Case frequency	Control frequency	*p*-values	Global *p*
rs1801690-rs52797880-rs8178847				0.002**
CAC	319 (0.876)	1616 (0.927)	0.001***	
GGT	38 (0.104)	101 (0.057)	0.001***	

**$p < 0.01$, ***$p < 0.001$

APS and RPL patients ($n = 182$), controls ($n = 871$)

Table 5 Association between polymorphism and clinical laboratory indicators

Variable	*p*-values			
	APOH			NCF1
	rs1801690	rs52797880	rs8178847	rs201802880
ACA-IgM[a]	0.250	0.430	0.430	0.017*
ACA-IgG[a]	0.827	0.583	0.583	0.666
β2GP1-IgM[a]	0.539	0.573	0.573	0.709
β2GP1-IgG[a]	0.222	0.834	0.834	1.000
TC[b]	0.635	0.621	0.621	0.339
TG[b]	0.826	0.885	0.885	0.945
TP[b]	0.007**	0.033*	0.033*	0.751
ApoA1[b]	0.144	0.229	0.229	0.128
ApoB[b]	0.782	0.920	0.920	0.931

[a]Student's *t*-test, [b]chi-square test

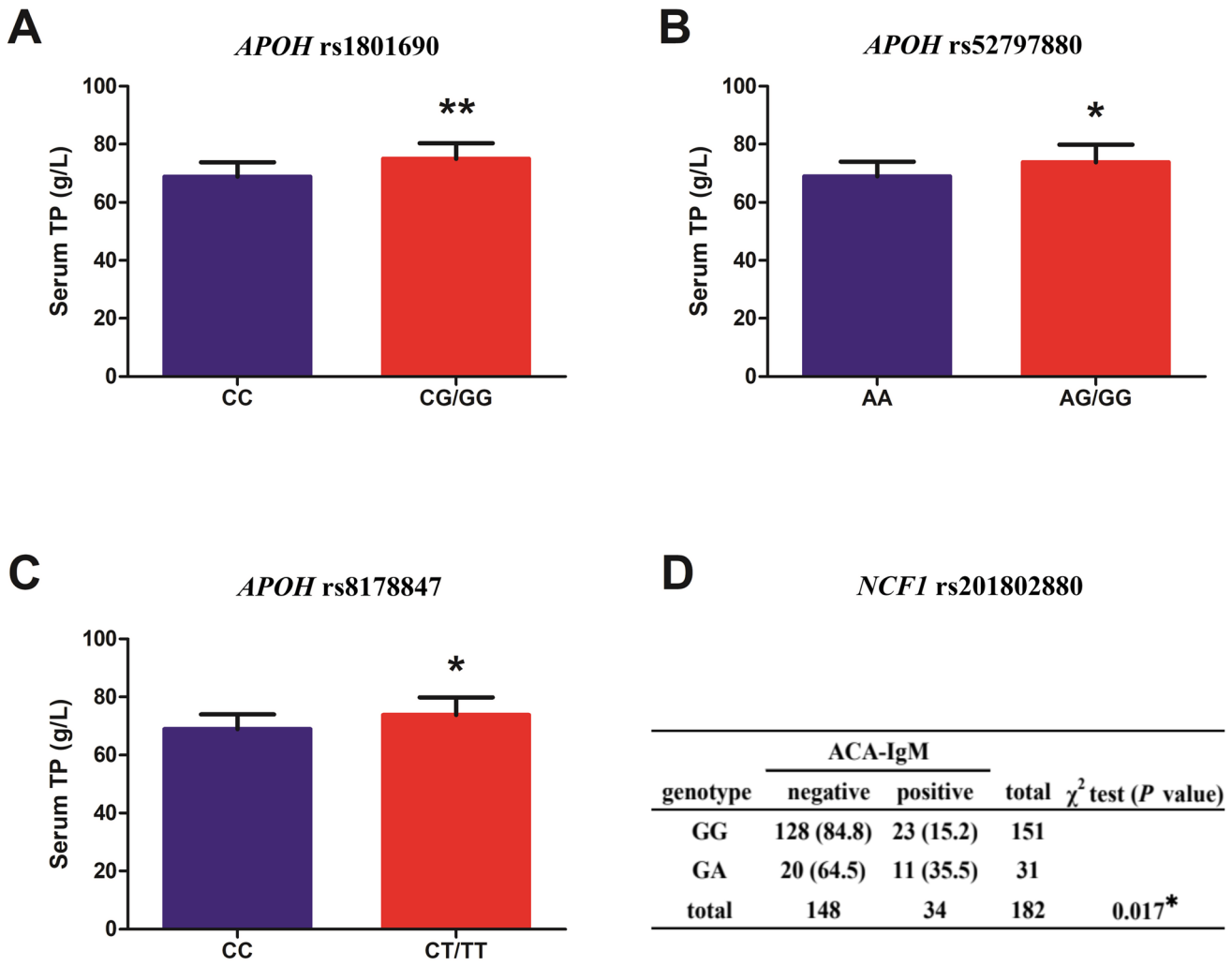
*$p < 0.05$, **$p < 0.01$

ACA-IgM: anti-cardiolipin antibody IgM, *ACA-IgG:* anti-cardiolipin antibody IgG, *β2GP1-IgM:* anti-β2-glycoprotein1-IgM, *β2GP1-IgG:* anti-β2-glycoprotein1-IgG, *TC:* total cholesterol, *TG:* triglyceride, *TP:* total protein, *ApoA1:* apolipoprotein A1, *ApoB:* apolipoprotein B

disequilibrium between rs8178847 and rs52797880 in the *APOH* gene, we noticed that the association statistic differences between the two SNPs and clinical characteristics were exactly similar.

Discussion

To explore the association between *APOH/NCF1* SNPs and APS and RPL, a study with 1284 subjects (871 controls, 182 APS and RPL patients, and 231 RPL patients) was performed. Rs1801690, rs52797880, and rs8178847 of *APOH* and rs201802880 of *NCF1* were strongly associated with APS and RPL. The allele frequencies of *APOH* rs1801690 G, rs52797880 G, and rs8178847 T in APS and RPL patients were approximately 5% higher than in controls, while the *NCF1* allele frequency of rs201802880 A was 8.1% higher in APS and RPL patients. Additionally, the genotype frequency of *NCF1* rs201802880 GA was 16.2% higher in APS and RPL patients. According to LD analysis, rs1801690, rs52797880, and rs8178847 of *APOH* were in a strong LD block. Especially, rs52797880 and rs8178847 showed a complete linkage disequilibrium. What is more, the allele and genotype frequencies of the three *APOH* SNPs and one *NCF1* SNP also described significant differences between APS and RPL patients and RPL patients. This was without precedent that rs1801690, rs52797880, and rs8178847 of *APOH* have been revealed to be haplotype blocks and disclosed to be associated with APS and RPL. Haplotype CAC was a genetic protective factor for APS and RPL patients and



genotype	ACA-IgM negative	ACA-IgM positive	total	χ^2 test (P value)
GG	128 (84.8)	23 (15.2)	151	
GA	20 (64.5)	11 (35.5)	31	
total	148	34	182	0.017*

Fig. 2 Clinical laboratory indicator difference in different *APOH* and *NCF1*genotypes. **A**, **B**, **C** Comparison of serum TP levels between individuals with different genotypes of *APOH* rs1801690 (**A**), rs52797880 (**B**), and rs8178847 (**C**). *APOH* rs1801690 CC (**A**), rs52797880 AA (**B**), and rs8178847 CC (**C**) were wild genotypes. **D** Comparison of serum ACA-IgM levels between individuals with wild (GA) and mutant (GG) genotypes of *NCF1* rs201802880. The Student's *t*-test was used in (**A**), (**B**), and (**C**). The chi-square test was used in (**D**). $^{*}p < 0.05$, $^{**}p < 0.01$

haplotype GGT was positively related to individuals with APS and RPL. Furthermore, higher serum TP level was described in the mutant genotype of *APOH* rs1801690 CG/GG, rs52797880 AG/GG, and rs8178847 CT/TT in APS and RPL patients, while a higher frequency of positive serum ACA-IgM level was found in the mutant genotype of *NCF1* rs201802880 GA.

APOH is the main target of APA found in patients with APS and other autoimmune diseases [27, 28, 29]. APOH can directly enter human umbilical vein endothelial cells (HUVECs) and act a role through the phospho-extracellular signal-regulated kinase pathway and ultimately damage vascular development [30]. In our study, we explored three *APOH* SNPs (rs1b801690, rs52797880, and rs8178847) of which the rs1801690 has been reported as G1025C (Try316Ser) [31] while the other two SNPs (rs52797880 and rs8178847) have not been reported in Chinese population. Our results not only confirm the important role of APOH in previous studies but also further discover its genetic vital impact on the susceptibility to RPL in APS patients.

APOH-related faulted lipid metabolism also accounts for abnormal fetal growth and development [18]. In a Chinese sample study, rs1801690 and three other *APOH* SNPs in high LD were associated with increased risk of APOH levels and venous thrombosis [31]. Different studies have demonstrated the association of *APOH* SNPs rs1801690, rs52797880, and rs8178847 respectively with serum lipids [32], body mass index [33], and obesity [34]. Another study found that the missense *APOH* SNP rs1801690 interpreted up to 14% of the detected plasma *APOH* variation in Caucasians [28, 35]. However, our study found no significant difference between

APOH SNPs and circulating lipids, which may suggest that APOH-related faulted lipid metabolism was not regulated by SNPs in APS and RPL patients.

Studies concerning the association between *APOH* SNPs and APA have been inconsistent due to the conflicting reports [36, 37]. Liu revealed that the β2GPI-IgM accounted for the largest proportion of antibodies in APS-related RPL patients [38]. Another study described six *APOH* SNPs associated with anti-β2GPI and the most significant SNP was rs1801690 [39] which is located in the 5th domain of β2GPI affecting the phospholipid-binding site [40]. In our study, we did not find a significant difference between the three *APOH* SNPs and APA in APS and RPL patients. But we revealed that both the allele and genotype frequencies were highest in APS and RPL patients, followed by RPL patients, and there were no significant differences between RPL patients and controls. These results suggest that *APOH* SNPs may contribute more to APS and do not play a role directly by affecting APA in APS and RPL patients. Meanwhile, our study described higher serum TP level in the mutant genotype of *APOH* rs1801690 CG/GG, rs52797880 AG/GG, and rs8178847 CT/TT, perhaps indicating that *APOH* SNPs act a role through some serum proteins. Besides the strong association between *APOH* SNP rs1801690 and the susceptibility to RPL in APS, we also further described that *APOH* allele frequencies of rs1801690 G in APS and RPL patients were 4.8% higher than controls and 4.6% higher than RPL patients, which have not been revealed before and may be considered further as a new therapeutic target through more in-depth research.

In early pregnancy, functionally active NADPH oxidase exists in the stem villous arteries and cytotrophoblast of the human placenta, which can produce more superoxide than in the full-term placenta [41]. Studies have shown that increased placental NADPH oxidase activity can lead to augmentation of effective antioxidant defenses [42, 43], suggesting that impaired antioxidant capacity may contribute to early pregnancy loss [44, 45]. Previous studies have exhibited that the polymorphism of *NCF1* is a major factor associated with autoimmune diseases, most likely through the regulation of peroxide [46]. *NCF1* gene which encodes the $p47^{phox}$/NCF1 protein of the NOX2 complex is critical for ROS induction. ROS was believed to be a major cause of chronic inflammation in autoimmune diseases such as APS, SLE, and rheumatoid arthritis (RA).

A previous study explored the function of three SNPs (*NCF1-339*, *NCF1-365*, and *NCF1-566*) in *NCF1* and revealed that the minor allele (T) of the *NCF1-339* SNP in exon four reduced ROS production in transfected cell constructs [47]. Olsson et al. described that the SLE-associated T allele of rs201802880 in *NCF1* reduced the effects of NADPH oxidase, resulting in reduced ROS production [23]. Additionally, Yokoyama et al. observed a remarkable enrichment of *NCF1* rs201802880 A allele in SLE patients with younger age of onset [48]. In our study, we found the *NCF1* allele frequency of rs201802880 A was the highest in RPL patients, followed by APS and RPL patients, and the lowest in controls, which may suggest a more contribution of *NCF1* SNP to RPL in APS and RPL patients. Our results are consistent with some previous studies relating to autoimmune diseases and some contradictory views may exist because of the RPL history in the APS and RPL subjects.

Independent reports have associated the non-synonymous SNP rs201802880 with Swedish, American, and Asian autoimmune disease cases [19, 23]. A previous study revealed that SNP rs201802880 in the *NCF1* gene was associated with the impaired production of neutrophil extracellular traps (NETs) and ROS and connected with the presence of APA and APS [24]. Especially, another study found lower ACA-IgM and higher ACA-IgG in pregnant women compared with non-pregnancy women [49]. Moreover, reduced ACA-IgG was described as a protective effect in the autoimmune disease, while ACA-IgM indicates a poor prognosis [50]. We found that the above studies tended to show us that ACA-IgG may be a protective antibody while ACA-IgM may be a detrimental antibody. Consistently, our study described a higher frequency of positive serum ACA-IgM levels in the mutant genotype of *NCF1* rs201802880 GA in APS and RPL patients, which implies that *NCF1* rs201802880 GA probably plays a pathogenic role directly or indirectly through serum ACA-IgM.

Despite genetic susceptibility related to APS having been examined in a lot of studies, identifying genetic risk factors for APS is still difficult due to the heterogeneity of APS-related antigen specificity and the pathogenesis of its clinical manifestations. Race is also a factor as some of these genetic associations have only been found in certain populations. Notably, little research has been conducted on the genetic factors involved in the susceptibility of APS women to RPL. Therefore, although the association between different SNPs in key genes and APS has been demonstrated by previous studies, the pathogenesis of this complex multisystem disorder, especially the susceptibility to RPL, still needs a large number of experimental investigations. Thus, due to the relative lack and inconsistency of relevant research, we need to perform further studies with more comprehensive SNPs and larger sample sizes as well as functional studies such as the detection of protein function.

Our study still exists some limitations such as the relatively small case sample size, the study performation in only one country, not very strict control group inclusion criteria (for example the lack of APL determination and the only one full-term pregnancy), and lack of further experimental validation (for example the lack of APS patients with other obstetrical complications to confirm the RPL specific association) because of the difficulty of collecting samples. Further studies with bigger sample size and more strict sample

inclusion criteria are needed to elucidate the mechanisms by which miRNAs control *APOH* and *NCF1* transcription and to validate it in vitro and in vivo.

Taken together, we have described that *APOH* and *NCF1* were associated with APS and RPL in our study population. We found that rs1801690, rs52797880, and rs8178847 of *APOH* and rs201802880 of *NCF1* were strongly associated with APS and RPL. Moreover, rs52797880 and rs8178847 of *APOH* showed a complete linkage disequilibrium. Furthermore, we described higher serum TP in three mutant genotypes of *APOH* and higher frequency of positive serum ACA-IgM in one mutant genotype of *NCF1* in APS and RPL patients. These findings lay the groundwork for personalized diagnosis and treatment of APS and RPL patients. APS patients with reproductive requirements and a history of RPL during childbearing age can contemplate an early screening for SNP mutation sites in *APOH* and *NCF1* genes to estimate the hazard of miscarriage and recurrent miscarriage. Patients with divergent SNP loci may have divergent therapeutic effects on drugs. Subsequent research can further explore drug screening for SNP mutation sites in *APOH* and *NCF1* genes, in order to achieve early intervention and tailored treatment for APS patients with RPL susceptibility. Furthermore, by integrating SNP research and pharmacogenomics, it will be feasible to design drugs in accordance with the specific genotype of APS patients identified as susceptible to RPL by pre-screening differential SNP mutation sites.

In a word, this study provides new evidence for the association analysis between gene polymorphisms and the susceptibility to RPL in APS patients, as well as the molecular pathogenesis underlying APS and RPL, which will provide new ideas for the diagnosis and treatment of APS and RPL in the future.

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Author contribution All authors contributed to the study conception and design. Shihua Bao plays a guiding role in the conceptualization and the final review, Xujing Deng takes charge of data collection and writing of the paper, Jian Mu is responsible for methodology and revision of the paper, Qing Sang was chiefly accountable for data analysis, and Ruixiu Zhang is responsible for revising the paper. All authors read and approved the final manuscript.

Data availability The datasets used and/or analyzed during the current study are available from the corresponding author upon reasonable request.

Declarations

Ethics approval This study was conducted in accordance with the Declaration of Helsinki and approved by the Ethics Committee of Shanghai First Maternity and Infant Hospital.

Informed consent Written informed consent has been obtained from the patients to publish this paper.

Conflict of interest The authors declare no competing interests.

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