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Genetic analysis of *ANXA5* haplotype and its effect on recurrent pregnancy loss

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Abstract. Recurrent pregnancy loss (RPL) is often associated with dysregulated Annexin A5 (*ANXA5*) expression. Moreover, the variants of Anxa5, a protein that is enriched in the placenta to prevent coagulation, have been reported to affect RPL risks. The haplotypes M1 [including single nucleotide polymorphisms (SNPs) 1A/C and 27T/C] and M2 (including SNPs 19G/A, 1A/C, 27T/C and 76G/A) of *ANXA5* were also reported to affect RPL risks. The present study aimed to investigate the association between the haplotype located in the promoter region of *ANXA5* and the risk of RPL. Patients with RPL (n=235) or intrauterine fetus death (IUFD; n=154), as well as healthy control subjects (n=375) were enrolled in the current research. Their haplotypes of *ANXA5* were determined using genotyping, and the association between *ANXA5* haplotypes and the risk of RPL was accordingly analyzed. A luciferase assay was conducted to investigate the haplotype responsible for *ANXA5* activity. Reverse transcription-quantitative PCR, western blot analysis, immunohistochemistry and ELISA were performed to assess the expression level and activity of *ANXA5* in patients with RPL. Consequently, the majority (n=214) of patients with RPL had a history of early RPL, whereas 31 patients with RPL had a history of both early and late RPL episodes. A significant difference was found between cases and controls in terms of gravidity and parity, whereas no significant differences were found in terms of age. The percentage of patients with RPL carrying the M2 haplotype of *ANXA5* was significantly higher compared with that in control subjects, indicating that the M2 haplotype of *ANXA5* was an independent risk of RPL as it influenced the transcription efficiency of *ANXA5* promoter. In patients with RPL, ANXA5 activity was suppressed and the mRNA and protein expression levels of Anxa5 were decreased. Thus, the *ANXA5* M2 haplotype may be an independent risk factor of RPL by affecting Anxa5 activity.

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Key words: Annexin A5, recurrent pregnancy loss, single nucleotide polymorphism, haplotype, coagulation

Introduction

Recurrent pregnancy loss (RPL) accounts for 1/3 of all cases of miscarriages and is characterized by ≥2 consecutive miscarriages occurring before week 20 of gestation (1,2). RPL has been attributed to multiple risk factors, such as anti-phospholipid antibody syndrome, infections and endocrine, anatomic and genetic disorders. However, after comprehensive assessments, the pathological trigger of >1/2 of RPL cases remains unknown, although environmental factors and aged ovaries were suspected to serve a role (3). In addition, immune disorders, such as endometritis and anti-phospholipid antibody syndrome, are also implicated in the development of RPL (3-5).

Anxa5 protein is enriched in the placenta to prevent coagulation (6). In the presence of calcium, *ANXA5* can bind to phosphatidylserine located on upper surface of syncytiotrophoblasts in the placenta to prevent clotting (6-8). Moreover, *ANXA5* plays a critical role in enhancing epithelial repair to maintain placental integrity (9). It has been reported that the activity of *ANXA5* in placenta tissue samples harvested from patients with RPL was notably reduced, along with inhibited *ANXA5* expression. The aforementioned results suggested that a decreased protein expression level of Anxa5 increased RPL risk.

Recently, four single nucleotide polymorphisms (SNPs), including rs112782763, rs28717001, rs28651243 and rs113588187, were discovered in the *ANXA5* promoter via functional and genetic assessments. The group of four minor alleles corresponding to these four SNPs, rs112782763 (c.-467 GNA), rs28717001 (c.-448 ANC), rs28651243 (c.-422 TNC) and rs113588187 (c.-373 GNA), was named haplotype M2 (a combination of SNPs of 19G/A, 1A/C, 27T/C and 76G/A) and it can be inherited (10). On the other hand, a group of two alleles, c.-448 ANC and c.-422 TNC, was named haplotype M1 (a combination of SNPs of 1A/C,27T/C). *ANXA5* expression

is notably affected by the presence of the M2 haplotype. In addition, the activity of the *ANXA5* promoter carrying the M1 alleles is higher than the activity of the *ANXA5* promoter carrying the M2 alleles (10,11). Another allele termed 'N' in the *ANXA5* promoter shows certain advantages over M1 and M2 alleles by expressing a higher level of anti-coagulation factors in the villi of the placenta to decrease the susceptibility to pre-eclampsia (10).

It has been reported that the *ANXA5* variants can affect RPL risks (12). In addition, in a study of 70 RPL cases, the promoter and coding sequences of *ANXA5* showed various mutations, including the loss of prothrombin G20210A mutation (10). Moreover, haplotypes M2 and M1 were shown to affect RPL risks. The present study enrolled subjects with or without RPL to study the effect of the haplotype located in the promoter region of *ANXA5*, as well as enrolled another group of patients with intrauterine fetus death (IUFD) to compared them with RPL cases and controls.

Materials and methods

Human sample collection. All patients were recruited during their visits to the Outpatient Department of Qilu Hospital (Jinan, China) from December 2014 to November 2019. Based on their diagnosis, the present study enrolled 235 patients showing symptoms of RPL into the RPL group, 154 patients diagnosed with IUFD into the IUFD group and 375 healthy control subjects free of RPL and IUFD were recruited into the control group. The inclusion criteria were based on the diagnosis of each patient with RPL or IUFD without any other underlying health conditions. Patients who were diagnosed with endometriosis, endometrial carcinoma, renal failure, cirrhosis, cardiac failure or had an abortion with known reason, including abnormal chromosomes, abnormal uterus structure or antiphospholipid syndrome, were excluded from the present study. For the patients in the control group, they were age-matched with the RPL cases and they were free of any obstetrics/gynecology medical condition.

Peripheral blood samples and placenta tissue samples were collected from each subject in the three groups. The clinical features of the subjects in the RPL, IUFD and control groups were analyzed, summarized and compared in Table I; the differences among the three groups in terms of gravidity and parity were examined using the Kruskal Wallis test.

The Human Research Ethics Committees of The Third Affiliated Hospital of Wenzhou Medical University approved this research (approval no. 153368XX06) and all methods were performed in accordance with the last vision of the Declaration of Helsinki. Written informed consent was obtained from all participants before the initiation of this study.

Cell culture. Hs 815.Pl cells and HUVECs were originally purchased from the American Type Culture Collection. These two cell lines, a placenta osteosarcoma cell line (H 815.PI cells) and an umbilical vein endothelial cell line (HUVECs), were utilized since they were both appropriate models to study RPL (13). These cells were conventionally cultured in MEM (Invitrogen; Thermo Fisher Scientific, Inc.) supplemented with 10% (v/v) FCS (Sigma-Aldrich; Merck KGaA), 0.3 mg/ml glutamine, 100 μg/ml streptomycin, 100 U/ml penicillin and 1% (v/v) sodium pyruvate, according to the instructions provided by the supplier of the cells. Both cell types were cultured under saturated humidity in a 37˚C tissue culture incubator supplied with 5% of CO_2. Prior to the start of cell culture experiments, all cells were tested for the presence of contaminations and only cells that passed the tests were selected for cell culture. During the cell experiments, the cells were randomly allocated into various groups for transfection, which are detailed in the luciferase assay section.

Genotyping using a Taqman assay. To determine the genotypes of haplotypes located in the promoter region of *ANXA5*, a Taqman genotyping assay kit (Applied Biosystems; Thermo Fisher Scientific, Inc.) was used according to the manufacturer's instructions to examined the genotypes of the four SNPs located in the promoter region of *ANXA5*, including rs112782763 SNP (c.-467), rs28717001 SNP (c.-448), rs28651243 SNP (c.-422) and rs113588187 SNP (c.-373).

RNA isolation and reverse transcription-quantitative (RT-q) PCR. The RNA of cultured cells and collected blood and tissue samples was extracted using a RNeasy FFPE Assay Kit (Qiagen, Inc.) based on the manufacturer's instructions. Total RNA was reverse-transcribed into cDNA using the PrimeScript® RT Reagent Kit (Takara Biotechnology Co., Ltd.) according to the manufacturer's protocol. Subsequently, the relative expression of *ANXA5* was determined using an Assay On Demand assay kit (Applied Biosystems; Thermo Fisher Scientific, Inc.) and Power SYBR Green PCR Master Mix (Takara Bio, Inc.) on a PRISM 7300 real time PCR machine (Applied Biosystems; Thermo Fisher Scientific, Inc.) following the RT-qPCR conditions as below: 95˚C for 10 min; followed by 40 cycles of 95˚C for 15 sec and 60˚C for 60 sec. The analysis of relative *ANXA5* (forward primer, 5'-GTGGCTCTG ATGAAACCCTCTC-3' and reverse primer, 5'-GGCTCTCAG TTCTTCAGGTGTC-3') expression was conducting using the software equipped in the PRISM 7300 real time PCR machine and the $2^{-\Delta\Delta Cq}$ (14) method. GAPDH (forward primer, 5'-CAG CCTCAAGATCATCAGCA-3' and reverse primer, 5'-GGC ATGGACTGTGGTCATGAG-3') was used as the reference gene to normalize the expression of *ANXA5*.

Luciferase assay. To determine the effects of various haplotypes on the transcription efficiencies of the *ANXA5* promoter, a dual-luciferase reporter system based on the pcDNA 3.1 basic luciferase reporter vectors (Promega Corporation) were constructed to contain the M1 and M2 haplotypes of the *ANXA5* promoter (ranging from -300 bp to +300 bp). *Renilla* luciferase reporter gene was used. Then, Hs 815.Pl cells and HUVECs were seeded into 24-well tissue culture plates at a density of $5x10^5$ cells/well and transfected with 300 ng of each of the different vectors containing the M1 or M2 haplotype of the *ANXA5* promoter for 24 h at 4˚C. At 48 h after the initialization of the transfection, the luciferase activity of different groups of cells was measured using a Bright-Glo™ Luciferase Assay System (Promega Corporation) to compare the transcription efficiencies of the *ANXA5* promoter harboring various haplotypes with the luciferase activity of *Renilla* as the normalization reference. All transfection operations were carried out using the Fugene HD transfection reagent

Table I. Characteristics of the study participants of RPL (n=235), IUFD (n=154) and control (n=375) groups.

Characteristic	RPL	IUFD	Control	P-value
Age, years	33 (24-47)	31 (21-46)	32 (26-46)	0.0838
Gravidity	5 (3-11)	4 (2-11)	1 (1-3)	P<0.0001
Parity	0 (0-3)	0 (0-2)	1 (0-3)	P<0.0001
No. of fetal losses	5 (1-8)	6 (3-13)	-	0.0778
Weeks of early fetal losses	8 (5-17)	9 (5-17)	-	0.2501
Weeks of late fetal losses	18 (10-22)	16 (10-19)	-	0.0835

Data are presented as the median (range). RPL, Recurrent pregnancy loss; IUFD, intrauterine fetus death.

Table II. Genotype frequencies of haplotypes in the ANXA5 promoter in RPL, IUFD and control groups.

Index	RPL (n=235)	IUFD (n=154)	Control (n=375)	RPL vs. Control P-value	IUFD vs. Control P-value
Genotypes				N/A	N/A
N/N	148 (63.0)	125 (81.2)	298 (79.5)	N/A	N/A
N/M1	14 (6.0)	7 (4.5)	17 (4.5)	N/A	N/A
M1/M1	0	0	0	N/A	N/A
N/M2, M1/M2[a]	71 (30.2)	22 (14.3)	60 (16.0)	P<0.0001	P>0.05
M2/M2	2 (0.8)	0	0	N/A	N/A
Total	235	154	375	N/A	N/A

The data are presented as n (%). [a]M1/M2 was observed in one patient with RPL, one patient with IUFD and two controls. N/N, wild-type of all four variants implicated in the haplotypes of Annexin A5 promoter; N/M1, heterozygotes of only two variants 1A/C and 27T/C; M1/M1, homozygotes of only two variants 1A/C and 27T/C; M1/M2, heterozygotes of variants-19G/A, 76G/A and homozygotes of 1A/C,27T/C; N/M2, heterozygotes of variants-19G/A, 1A/C, 27T/C and 76G/A; M2/M2, homozygotes of variants-19G/A, 1A/C, 27T/C and 76G/A. RPL, recurrent pregnancy loss; IUFD, intrauterine fetus death; N/A, not applicable. The P-value was only presented for the combination of N/M2 and M1/M2 genotypes, which had an adequate sample size for the generation of meaningful statistical power. In the functional analysis, the comparison was performed between all other genotypes and the combination of N/M2 and M1/M2 genotypes in the functional analysis.

(Promega Corporation). The luciferase signal was read using a microplate reader (BMG; Molecular Devices LLC).

Western blot analysis. The protein content of cultured cells and collected blood and tissue samples was extracted using RIPA buffer (cat. no. R0278; Sigma-Aldrich; Merck KGaA). Subsequently, the protein concentration was determined with a BCA assay kit. Then, the protein content in each sample lysate was resolved using 10% SDS-PAGE. Subsequently, the resolved protein in each sample lysate was transferred onto a PVDF membrane, which was initially blocked with 7% non-fat milk for 1 h at room temperature. Then, the membranes were incubated consecutively with anti-Anxa5 (1:1,000; cat. no. ab108321; Abcam) primary antibodies for 8 h at room temperature and then an appropriate HRP-conjugated secondary antibody 1:1,000; cat. no. 56970; Cell Signaling Technology, Inc.) for 1 h at room temperature. After development using a Pierce™ ECL Western Blotting Substrate (cat. no. 32109; Pierce; Thermo Fisher Scientific, Inc.), the relative expression of Anxa5 protein was calculated with the expression of β-actin (1:1,000; cat. no. ab8226; Abcam) being used as the internal control.

Immunohistochemistry analysis. The tissue samples collected from the RPL, IUFD and control groups were fixed for 15 min at room temperature in PBS containing 10% neutral formalin and sliced into 5-μm thick tissue slides to prepare paraffin-embedded specimens. Samples were blocked in 10% goat serum (Thermo Fisher Scientific, Inc.) for 1 h at room temperature, permeabilized for 1 h at room temperature with 0.1% Triton X-100 (Sigma-Aldrich; Merck KGaA) and immunostained for 24 h at 4˚C with anti-Anxa5 monoclonal primary antibodies (1:100; cat. no. ab108321; Abcam). After washing with PBS, the slides were stained with the appropriate biotin-conjugated secondary antibodies (1:1,000; cat. no. 39681; Cell Signaling Technology. Inc.) for 2 h at room temperature, treated with DAB, counterstained with hematoxylin for 30 min at room temperature and observed under a fluorescence microscope (magnification, x200).

Detection of Anxa5 activity using ELISA. The activity of Anxa5 in the placenta tissue samples collected from the RPL (n=28), IUFD (n=26) and control (n=32) groups was determined using the Human ANXA5/Annexin V (Sandwich ELISA) ELISA Kit

Table III. Logistics regression analysis of the association between Annexin A5 haplotype and RPL/IUFD.

Group	OR; 95% CI	P-value
RFL	3.1; (1.1-9.5)	0.047
IUFD	1.31; (0.7-2.3)	0.250

Potential confounding variables: Age, gravidity and parity. OR, odds ratio; RPL, recurrent pregnancy loss; IUFD, intrauterine fetus death.

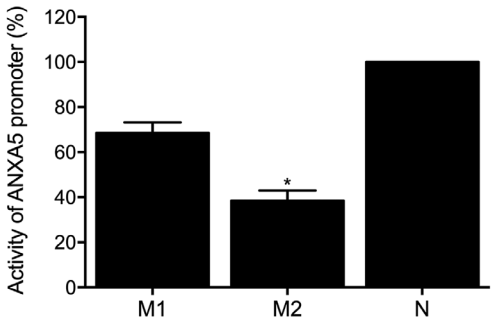

Figure 1. Effect of variants in the *ANXA5* promoter on its activity, as determined using luciferase reporter gene assays. *P<0.05 vs. N group; one-way ANOVA and Newman-Keuls test. N denotes the wild-type promoter sequence, whose activity was set to 100%. M1 contains nucleotide changes 1A→C and 27T→C; M2 contains all four substitutions, -19G→A, 1A→C, 27T→C and 76G→A. *ANXA5*, Annexin A5.

(cat. no. LS-F21936; LifeSpan Biosciences, Inc.) according to the manufacturer's instructions.

Statistical analysis. The clinical features of all recruited patients were analyzed using a Kruskal Wallis test followed by Dunn's post hoc. The associations between *ANXA5* haplotypes and the risk of RPL were determined using Fisher's exact test and logistic regression. The other data obtained during this research were analyzed using one-way ANOVA with Newman-Keuls test as the post hoc test. Data are presented as the mean ± SD and the average of each value was derived from three repeated experiments. All data analysis was performed using SAS 9.0 software (SAS Institute, Inc.) in conjunction with Microsoft Excel 2013 (Microsoft Corporation).

To clarify the relationship between M2 and RPL, a logistic regression model was built based on the corrected age, gravidity and parity. The establishment and assessment of the logistic regression model were carried out using SPSS 22.0 (IBM Corp.). The intensity of immunochemistry staining was evaluated using a Cox regression model with multiple variables. The sample size was evaluated using Raosoft Sample Size Calculator (http://www.raosoft.com/samplesize.html; version 2004; Raosoft, Inc.). Each experiment in this study was repeated for three times. P<0.05 was considered to indicate a statistically significant difference.

Results

Clinical features of RPL, IUFD and control cases. Peripheral blood was collected from RPL and IUFD cases, as well as from healthy control subjects. Their clinical features were analyzed and are listed in Table I. It was found that the majority (n=214) of women with RPL showed a history of early RPL, whereas 31 (13.1%) RPL cases had a history of both early and late episodes of RPL. In the IUFD group, 95/154 (61.7%) subjects experienced IUFD. Moreover, a significant difference was found between case and control groups in terms of gravidity (Kruskal Wallis test, P<0.001) and parity (Kruskal Wallis

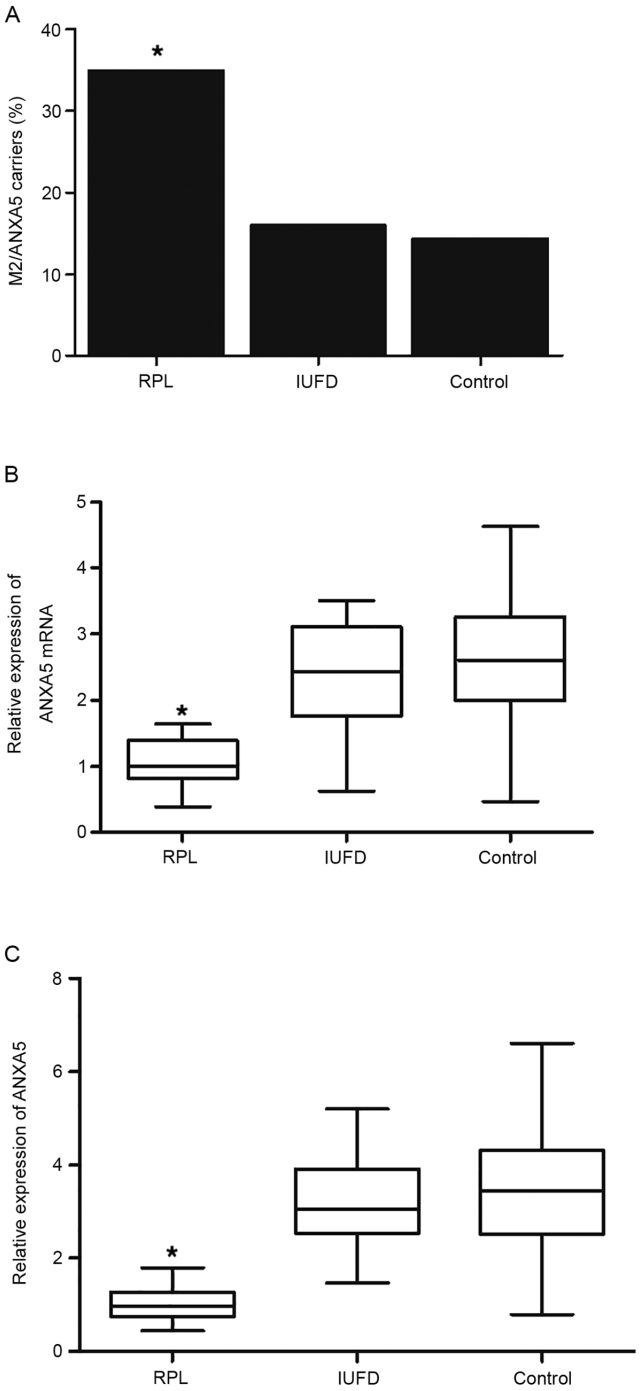

Figure 2. *ANXA5* mRNA and Anxa5 protein expression levels in placenta tissues of RPL, IUFD and control groups. (A) M2 carriage ratio of RPL, IUFD and control groups. (B) mRNA expression level of *ANXA5* in RPL, IUFD and control groups. (C) Protein expression level of Anxa5 in RPL, IUFD and control groups. *P<0.05 vs. control group, one-way ANOVA and Newman-Keuls test. *ANXA5*, Annexin A5; RPL, recurrent pregnancy loss; IUFD, intrauterine fetus death.

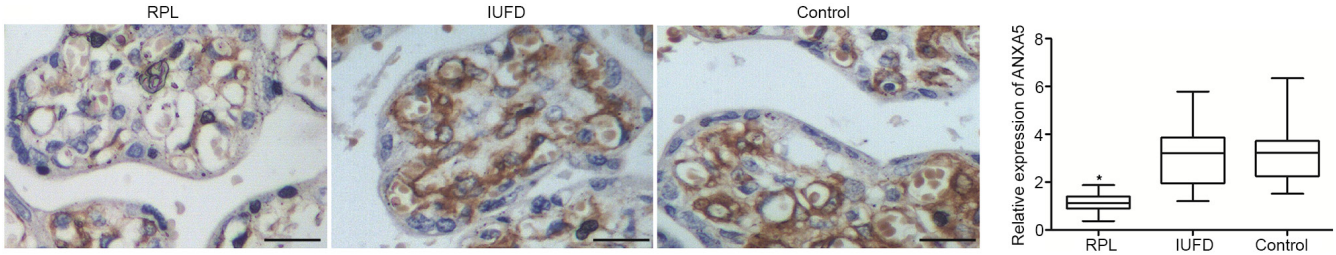

Figure 3. Protein expression level of Anxa5 in placenta tissues of RPL, IUFD and control groups, as measured via immunohistochemistry. Scale bar, 50 μm. *P<0.05 vs. control group, one-way ANOVA and Newman-Keuls test. Anxa5, Annexin A5; RPL, recurrent pregnancy loss; IUFD, intrauterine fetus death.

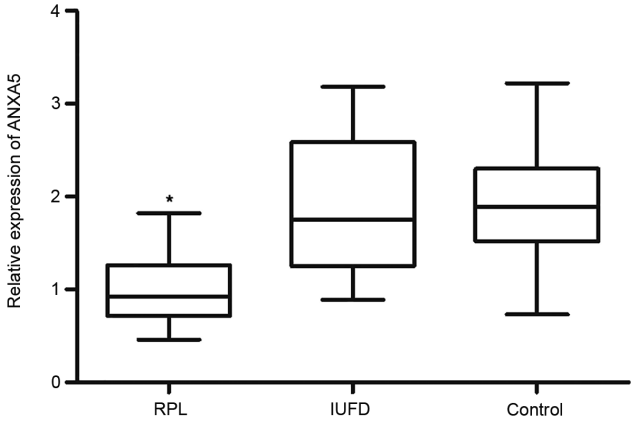

Figure 4. Anxa5 activity in peripheral blood of RPL, IUFD and control groups, as measured using ELISA. *P<0.05 vs. control group, one-way ANOVA and Newman-Keuls test. Anxa5, Annexin A5; RPL, recurrent pregnancy loss; IUFD, intrauterine fetus death.

test, P<0.05), whereas no significant differences were noted (P>0.05) in terms of age.

Association between the ANXA5 M2 haplotype and RPL. The genotype frequencies of haplotype M2 located in the promoter of *ANXA5* was further analyzed. The results are summarized in Table II and demonstrated that the rate of M2 haplotype in the control group (60 cases, 16%) was lower compared with that in the RPL group [71 cases, 30.2%; Fisher's exact test, P<0.0001; odds ratio (OR), 0.41; 95% CI, 0.28-0.62, RPL vs. control]. The incidence of the M2 haplotype in patients with IUFD was 22/154 (14%; Fisher's exact test, P>0.05, IUFD vs. controls) and showed no difference with the control group.

To clarify the relationship between M2 and RPL, a logistic regression model was established based on the corrected age, gravidity and parity. The results demonstrated that the M2 haplotype was significantly and independently associated with the risk of RPL (P=0.047; OR, 3.1; 95% CI, 1.1-9.5; Table III).

Taken together, it was suggested that the M2 haplotype was involved in the risk of RPL as an independent risk factor.

Effects of the M2 haplotype of ANXA5 on the ANXA5 promoter activity. To determine which haplotype is critical for *ANXA5* activity, luciferase assays were performed to detect the effects of different *ANXA5* haplotypes on the transcription efficiency of *ANXA5* promoter. The results (Fig. 1) showed a reduction of *ANXA5* promoter activity in the presence of all four minor alleles of the SNPs, i.e., the M2 haplotype. Furthermore, the M1 haplotype induced no significant change compared with the control group.

Placenta tissues were collected from RPL (n=28), IUFD (n=26) and control (n=32) groups with one sample collected from each patient to examine the effect of the M2 haplotype on the risk of RPL. As shown in Fig. 2A, 10/28 (35.7%) of patients with RPL carried the M2 haplotype and the rate was significantly higher compared with that in the control (5/32, 15.6%) and IUFD (4/26, 15.4%) groups. The results of statistical analysis with samples from RPL, IUFD and control groups also showed a similar trend (Table II).

Subsequently, the ANXA5 mRNA and Anxa5 protein expression levels in the placenta tissue samples were determined via RT-qPCR and western blotting. As shown in Fig. 2B, *ANXA5* mRNA expression was significantly decreased in patients with RPL compared with that in the IUFD and control groups. Moreover, western blot analysis of Anxa5 protein expression (representative blots shown in Fig. S1) demonstrated significantly suppressed Anxa5 expression in patients with RPL compared with the IUFD and control groups (Fig. 2C).

The expression level of Anxa5 protein in placental tissues was further examined via immunohistochemistry. As shown in Fig. 3, the protein expression level of Anxa5 was decreased in the RPL group with compared with the control and IUFD groups.

Next, peripheral blood samples were collected from the three groups to assess their Anxa5 activity using ELISA. As shown in Fig. 4, the activity of Anxa5 was decreased in the RPL group compared with that in the control and IUFD groups.

Taken together, these results suggested that the *ANXA5* M2 haplotype was essential for Anxa5 activity, which was decreased in patients with RPL.

Discussion

The Anxa5 protein is highly expressed in the placenta and can promote its anti-coagulant activity (15). In addition, a haplotype termed M2 located in the promoter of *ANXA5* was shown to increase the risk of RPL by reducing the expression of *ANXA5* (10). In fact, Anxa5 proteins were shown to generate a protective layer on the apical cell membrane of syncytiotrophoblasts located in the villi of the placenta to maintain a balance of hemostasis during pregnancy (16). The plasma expression of *ANXA5* in healthy pregnant women is typically <5 ng/ml (17). However, a case controlled comparative research on the plasma levels of ANXA5

in both healthy pregnant women and patients with RPL showed a significantly decreased level of circulating Anxa5 in RPL participants, especially in those patients who have experienced ≥3 episodes of RPL (18). It was also reported that a combination of SNPs located in *ANXA5* promoter, i.e., the M2 haplotype formed by four minor alleles of SNPs [SNP4: (-)373 G>A, SNP3: (-)422 T>C, SNP2: (-)448 A>C and SNP1: (-)467 G>A] located in chromosome 4q27, can promote the onset and development of RPL, as well as other complications including pre-eclampsia and thrombophilia or thromboembolism related to pregnancy, presumably by reducing the expression of Anxa5 and by increasing the coagulation in the placenta (10,19-23). It was revealed that the transcription efficiency of the promoter of *ANXA5* harboring the M2 haplotype was reduced by ~60% when compared with the transcription efficiency of the promoter of *ANXA5* harboring the N haplotype, i.e., the wild-type haplotype of the promoter of *ANXA5* (10). Moreover, both M2 and M1 haplotypes can be frequently observed in the promoter of *ANXA5* expressed in the healthy population (10). It was also demonstrated that the promoter of *ANXA5* harboring the M2 and M1 haplotypes showed similar transcriptional efficiencies, in spite of the fact that the M2 haplotype in the promoter of *ANXA5* involves four SNPS, i.e., rs112782763 (c.-467 GNA), rs28717001 (c.-448 ANC), rs28651243 (c.-422 TNC) and rs113588187 (c.-373 GNA), while the M1 haplotype in the promoter of *ANXA5* only involves two SNPS, i.e., c.-448 ANC and c.-422 TNC (10). The present study collected peripheral blood samples from RPL, IUFD and control groups to show that the incidence of M2 haplotype was notably higher (35%) in patients with RPL. Next, a logistic regression model was built based on corrected age, gravidity and parity to show that the M2 haplotype was significantly and independently associated with the risk of RPL.

Although it was previously reported that several women with a history of RPL that could not be explained by pathological diagnosis showed decreased Anxa5 expression, the incidence of RPL induced by abnormal Anxa5 expression remains unknown (18). The transcription efficiency of the promoter of *ANXA5* appears to be correlated to the expression of IgG antibodies against domain I (24). At present, accumulating evidence obtained from thrombo-elastography experiments and coagulation assays has shown an increased level of coagulation activity in patients with RPL (25,26). In women with RPL, the prevalence of M2 haplotype is notably increased compared with that observed in healthy controls (10). In addition, M2 haplotype was detected in ~30% of RPL cases in a Japanese population, while the incidence of M2 haplotype in healthy controls of the Japanese population was <10% (27). Moreover, it was revealed that the M2 haplotype could be found in ~20% of residents in Europe, while the M2 haplotype was found in >30% of European women suffering from RPL (21,28). In addition, there is a close relationship between the M2 haplotype and the occurrence of pre-eclampsia and gestational disorders, while the susceptibility to FGR is increased by M2 haplotype (29,30). It has also been shown that the activity of *ANXA5* promoter in placental tissue samples harvested from patients with RPL was markedly reduced to decrease Anxa5 expression, suggesting that the M2 haplotype increased RPL risk by reducing the expression of Anxa5 (31). In the present study, the luciferase assays demonstrated that the M2 haplotype was responsible for the transcription efficiency of the *ANXA5* promoter. In addition, the current results indicated that the rate of the M2 haplotype was higher in patients with RPL. It was also identified that the mRNA and protein expression levels of ANXA5 in the placenta tissues were decreased in patients with RPL, and that the activity of ANXA5 was decreased in this group.

The present study investigated the effect of the ANXA5 haplotype on RPL, thereby providing a possible approach and candidate biomarker for the prediction, diagnosis and prognosis of RPL in future clinical applications. However, due to the limitation of available cases in Qilu Hospital, the sample size was relatively limited for the correlation analysis between haplotype and RPL risk. The outcome of the statistical analysis may be different or influenced by the different sample size. Moreover, the cases recruited in the current study were all patients who attended Qilu Hospital, which raised a potential selection bias since the recruited cases may be suffering from other health complications. In addition, an appropriate animal study to validate the current finding is also necessary. Therefore, in future studies, to validate the aforementioned finding, a larger sample size from the general population, as well as animal studies, are necessary to eliminate the potential selection bias generated during the process of patient recruitment.

In conclusion, the present study demonstrated that the M1/M2 haplotypes in *ANXA5* were associated with an increased risk of RPL. The results also indicated that *ANXA5* expression was decreased in the carriers of the M2 haplotype. Therefore, the presence of the M2 haplotype in *ANXA5* may be used as a novel biomarker to predict the prognosis of RPL, thus providing a potential approach for the prediction of RPL risk in future clinical applications.

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Availability of data and materials

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

Authors' contributions

ZC supervised the study and XD designed the study. XZ, YC and FC collected the literature. ZC, XZ, YC and LC conducted the experiments. FC and XD collected and analyzed the data. ZC and XZ drafted the manuscript and XD revised the manuscript. ZC and XD confirmed the authenticity of all the raw data. All authors have approved the final version for publication.

Ethics approval and consent to participate

The Human Research Ethics Committees of The Third Affiliated Hospital of Wenzhou Medical University approved this research (approval no. 153368XX06) and all methods were performed in accordance with the last vision of the Declaration of Helsinki. Written informed consent was obtained from all participants before the initiation of this study.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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