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Association between ANXA5 haplotypes and the risk of recurrent pregnancy loss

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Abstract

Background: Annexin A5 (ANXA5) haplotypes can increase the risk of recurrent pregnancy loss (RPL). This study aimed to investigate the effect of ANXA5 haplotypes on ANXA5 expression in patients with RPL.

Methods: Female subjects with RPL, parous controls (those who intentionally aborted without medical conditions or complications), and population controls (normal delivery) were studied. Real-time polymerase chain reaction was carried out to evaluate ANXA5 expression in the placenta and peripheral blood. Western blotting and immunohistochemistry were used to assess ANXA5 protein expression. The luciferase assay was performed to detect the effect of M1 and M2 haplotypes on transcription efficiency of the ANXA5 promoter.

Results: We found that the percentage of the M2 carrier was highest in the RPL group. ANXA5 expression in the placenta and peripheral blood in subjects with RPL was significantly inhibited. Furthermore, ANXA5 expression in subjects carrying the M2 haplotype was remarkably suppressed compared with that in carriers of other haplotypes. Finally, the M2 haplotype decreased the transcription efficiency of the ANXA5 promoter.

Conclusion: Our findings show that ANXA5 expression is decreased in carriers of the M2 haplotype and that M1/M2 haplotypes in the ANXA5 gene are associated with an increased risk of RPL.

Keywords

Annexin A5, recurrent pregnancy loss, single nucleotide polymorphism, haplotype, placenta, transcription efficiency

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Introduction

Recurrent pregnancy loss (RPL) refers to ≥ three consecutive miscarriages occurring within 22 weeks of gestation. RPL affects up to 5% of pregnancies and can be triggered by multiple factors, including obesity, metabolic disorder, autoimmune disorder, endocrine factors, and genetic diseases.[1]

Annexin A5 (ANXA5) helps to prevent coagulation by binding to the membrane of syncytiotrophoblasts in the placenta.[2] The M2 haplotype reduces ANXA5 expression to block M2/ANXA5 signaling and causes placental complications.[3–5] Moreover, during RPL, the anti-thrombotic protein layer formed by ANXA5 is impaired by antibodies targeting ANXA5, including anti-phospholipid antibodies, to prevent the activation of anticoagulation function of ANXA5, thus resulting in the onset of RPL.[6] Therefore, patients with RPL commonly display high titers of anti-phospholipid antibodies in the placenta.[7]

Genetic studies have shown a group of four single nucleotide polymorphisms (SNPs), which are rs113588187, rs28651243, rs28717001, and rs112782763, in the ANXA5 promoter.[3] Additionally, the minor alleles of these SNPs, c.-373G>A, c.-422T>C, c.-448A>C, and c.-467G>A, form a haplotype termed M2, which increases the risk of RPL in Austronesian, Asian, and European populations.[3] Accordingly, the M1 haplotype in the ANXA5 promoter shares two SNPs (i.e., SNP3 and SNP2) with the M2 haplotype. Among the alleles M1, M2, and N, the transcription level of the N allele is the highest, while the transcription level of the M2 haplotype is the lowest.[3] Therefore, the N allele has an advantage over the M1 and M2 haplotypes in inducing placental villi to release a higher level of anti-coagulation factors, thus decreasing the risks of obstetric complications. Additionally, the M2 haplotype is implicated in multiple obstetric complications, while ANXA5 functions as an anti-coagulation factor in the placenta by shielding the membrane of syncytiotrophoblasts in placental villi.[8] Therefore, reduced ANXA5 expression increases the risk of obstetric complications.[5,9] Moreover, bisulfite modifications observed in the motif region of G-quadruplex suggest that the structural change in the G-quadruplex induced by M1, M2, and N alleles in the promoter can affect the transcription of ANXA5. This study aimed to investigate the effect of the ANXA5 haplotype on ANXA5 expression and their association with RPL.

Materials and methods

We enrolled 105 subjects in our study during May 2013 to August 2017. The subjects comprised female patients with RPL, parous control (PAC) subjects (those who had an intentional abortion without medical conditions or complications), and population control (POC) subjects. Patients with RPL were seeking medical at our hospital and were diagnosed with two or more miscarriages at < 20 weeks of gestation. Participants in the PAC group had an intentional abortion. Patients in the POC group had a history of delivery and were selected from those who were treated by other departments in our hospital. The exclusion criteria of the study were as follows: patients who were positive for anti-phospholipid antibodies or other known reasons for RPL; those who had pregnancy complications; and those who had cardiac dysfunction, pulmonary disease, renal failure, or cirrhosis. The demographic and clinicopathological characteristics of the participants are shown in Table 1. This study was approved by the First People's Hospital of Wenling Affiliated to Wenzhou Medical University Ethics Committee (approval number: ZJWZWL938201X03) and was conducted in compliance with the Declaration of Helsinki. All participants signed consent forms before the study

Table 1. Demographic data of the subjects in this study.

Characteristic	RPL (n = 35)	PAC (n = 35)	POC (n = 35)
Age (years), median (range)	33 (26–43)	32 (24–46)	35 (26–41)
Gravidity, median (range)	5 (3–11)	5 (3–9)	/
Parity, median (range)	7 (1–8)	6 (1–9)	/
Number of fetal losses, median (range)	5 (2–8)	6 (3–9)	/
Weeks of early fetal losses, median (range)	8 (5–16)	9 (5–17)	/
Weeks of late fetal losses, median (range)	16 (10–18)	16 (10–19)	/
GDM	4	5	/
GHT	1	0	/
Genotype			
N/N	18 (51.4)	17 (48.6)	17 (48.6)
N/M2	11 (31.4)	11 (31.4)	10 (28.6)
M2/M2	6 (17.2)	7 (20.0)	8 (22.8)
M2 AF	0.235	0.253	0.263
M2 carriage	17 (45.7)	18 (45.7)	18 (48.6)

Values are median (range), number, or number (%).
RPL, recurrent pregnancy loss; PAC, parental control; POC, population control; GDM, gestational diabetes mellitus; GHT, gestational hypertension.

Samples of placental tissue and peripheral blood were collected for subsequent study. Sampling in the PAC group was achieved by chorionic villus sampling at delivery at full term. Sampling in the POC/RPL group was performed by chorionic villus sampling immediately after abortion. To rule out anti-phospholipid antibodies as a confounding factor, none of the participants of this study were positive for anti-phospholipid antibodies.

Total RNA of tissue and blood samples was isolated using an RNA extraction kit (Thermo Fisher Scientific, Waltham, MA, USA) by following the manufacturer's instructions. Extracted RNA was then converted into cDNA via reverse transcription and used for subsequent procedures of real-time polymerase chain reaction (PCR) in conjunction with SYBR Green Master Mix (Roche, Mannheim, Germany) and corresponding primers for ANXA5 mRNA using the 2^{DDCt} approach. Human umbilical vein endothelial cells were used in this small-scale association study. A pDNA3.1 basic luciferase reporter plasmid was used to create luciferase reporter constructs containing the wild-type, M1 haplotype, and M2 haplotype of the ANXA5 promoter. Luciferase activity of lysate was determined using a luciferase assay kit (Roche) according to kit instructions. Western blot analysis was performed to measure tissue ANXA5 protein expression following common protocols. Immunohistochemistry was carried out with a Histo-staining Assay kit (Maixin, Fuzhou, China) by following the manufacturer's protocol to determine ANXA5 protein expression in collected tissue samples. For peripheral blood samples, an ELISA assay kit (Thermo Fisher Scientific) was used to measure ANXA5 protein expression.

All results are shown as mean ± standard deviation. Correlations between ANXA5 haplotypes and the risk of RPL were determined using the chi-square test. The threshold of $p < 0.05$ was chosen to determine statistical significance. All statistical tests

were double-sided. IBM SPSS 19.0 software (IBM Corp., Armonk, NY, USA) was used for analysis.

Results

There were 35 patients in each group. The demographic and clinicopathological characteristics of the participants were not significantly different among the RPL, PAC, and POC groups (Table 1). There were 35 patients in each group. The carrier rate of M2 was significantly higher in the RPL group than in the PAC and POC groups ($p < 0.05$, Figure 1a).

Quantitative reverse transcription PCR showed that ANXA5 mRNA expression was significantly lower in the RPL group than in the PAC group ($p < 0.05$), while no difference was found between the PAC and POC groups (Figure 1b). Furthermore, ANXA5 mRNA expression was significantly lower in M2 carriers than in carriers of other haplotypes in all three groups

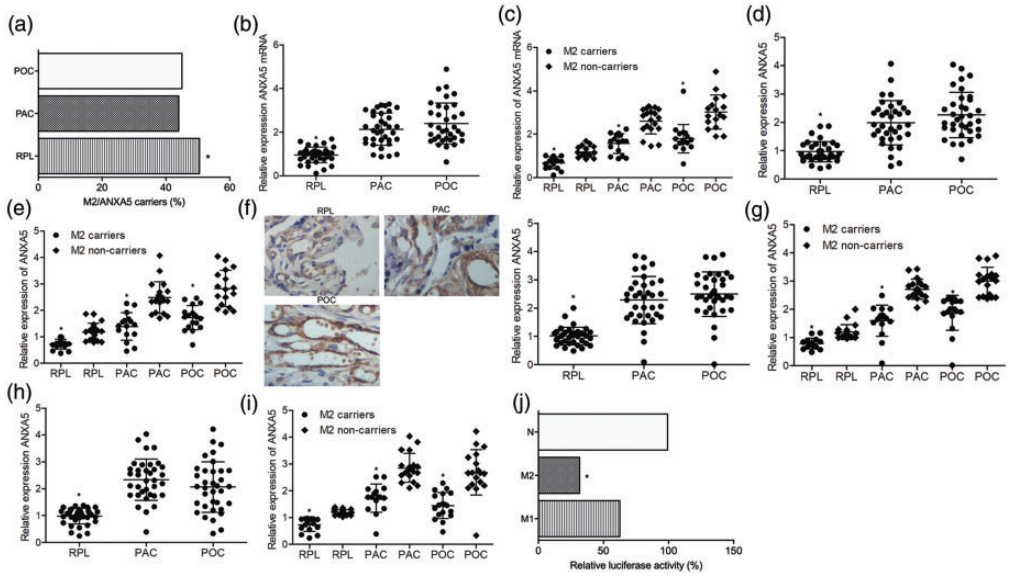

Figure 1. a: Histogram shows the percentage of M2 carriers in the RPL (n = 35), PAC (n = 35), and POC (n = 35) groups (*$p < 0.05$ vs. the PAC and POC groups). The percentage of M2 carriers was similar among the groups. b: ANXA5 mRNA expression in the placenta was decreased in the RPL group (*$p < 0.05$ vs. the PAC group). c: ANXA5 mRNA expression in the placenta was lower in M2 carriers than in carriers of other haplotypes in the RPL, PAC, and POC groups (*$p < 0.05$ vs. M2 non-carriers). d: Placental ANXA5 protein expression in the RPL group was decreased (*$p < 0.05$ vs. the PAC group). e: Placental ANXA5 protein expression in M2 carriers was lower than that in carriers of other haplotypes in the RPL, PAC, and POC groups (*$p < 0.05$ vs. M2 non-carriers). f: Immunohistochemistry analysis showed that placental ANXA5 protein expression in the RPL group was reduced (*$p < 0.05$ vs. the PAC group). g: Placental ANXA5 protein expression in M2 carriers was lower than that in carriers of other haplotypes in the RPL, PAC, and POC groups (*$p < 0.05$ vs. M2 non-carriers). h: An ELISA assay showed that peripheral blood ANXA5 protein expression in the RPL group was reduced (*$p < 0.05$ vs. the PAC group). i: Peripheral blood ANXA5 protein expression in M2 carriers was lower than that in carriers of other haplotypes in the RPL, PAC, and POC groups (*$p < 0.05$ vs. M2 non-carriers). j: The M2 haplotype suppressed the transcription efficiency of the ANXA5 promoter (transcription efficiency of the N haplotype was set to 100% for normalization; *$p < 0.05$ vs. the N haplotype, **$p < 0.05$ vs. the M1 haplotype).
RPL, recurrent pregnancy loss; PAC, parental control; POC, population control; ANXA5, annexin A5.

($p < 0.05$, Figure 1c). Similarly, western blotting analysis of placental samples indicated that ANXA5 protein expression was significantly lower in the RPL group than in the PAC group ($p < 0.05$, Figure 1d) and ANXA5 protein expression in M2 carriers was significantly lower than that in carriers of other haplotypes ($p < 0.05$, Figure 1e). Moreover, ANXA5 protein expression levels in placental tissue were similar among the groups (Figure 1f and 1g). In peripheral blood, ANXA5 protein expression was significantly lower in the RPL group than in the PAC group ($p < 0.05$, Figure 1h) and M2 carriers showed significantly lower ANXA5 protein expression than carriers of other haplotypes in all three groups ($p < 0.05$, Figure 1i).

Luciferase activity at 48 hours post-transfection showed that the M1 and M2 haplotypes of the ANXA5 promoter had significantly reduced transcription efficiency compared with the N haplotype (both $p < 0.05$), and the M2 haplotype showed the lowest transcription efficiency (Figure 1j).

Discussion

ANXA5 is enriched in the placenta and acts as an anti-coagulant.[10] Moreover, ANXA5 plays a critical role in cell membrane repair to maintain the integrity of the placenta.[11] As a highly enriched protein in the placenta, ANXA5 helps to generate a layer of lateral aggregates in cell membrane repair and contributes to complications, such as RPL.[12,13] In this study, we found that the carrier rate of M2 was higher in the RPL group compared with the PAC and POC groups, and we also found an inhibitory role of the M2 haplotype in ANXA5 expression in the placenta.

Luciferase reporter assays have shown that HeLa and BeWo cells have a lower expression level of c.-467G>A SNP than that of the M1 allele. Reduced ANXA5 mRNA expression has been detected in patients carrying the M2 haplotype[14] and this change is specific for haplotypes.[5,9] Additionally, reduced ANXA5 protein levels have been detected in patients with pre-eclampsia carrying the M2 allele. The transcription efficacy of the M2 haplotype is lower compared with that of the N and M1 haplotypes.[3] Therefore, women who carry the SNP5 minor allele and M2 haplotype might have low ANXA5 protein expression and a greater risk of RPL.[8] Interestingly, the M2 haplotype increases the risk of other complications, including venous thromboembolism and pre-eclampsia. Additionally, the M1 haplotype in the ANXA5 promoter shares two SNPs (SNP3 and SNP2) with the M2 haplotype, although the transcription level of the M1 haplotype is higher than that of the M2 haplotype.[3] Women carrying the M2 haplotype of ANXA5 have a higher risk of RPL, although the results vary with ethnicity.[15] Nevertheless, SNPs located in the ANXA5 promoter appear to affect the risk of RPL because the risk of RPL in women carrying the M2 haplotype is two-fold higher than in non-carriers.[3] In this study, we found that ANXA5 protein expression was lower in patients carrying the M2 haplotype than in those without the M2 haplotype.

There are limitations to our study. In this small-scale association study, the sample size was relatively small and the nationality of all recruited participants was the same. A further study is required to confirm our findings in a larger sample size with women of various nationalities.

In summary, our study shows that ANXA5 expression is decreased in carriers of the M2 haplotype and that M1/M2 haplotypes in the ANXA5 gene are associated with an increased risk of RPL. Our findings suggest that the presence of the M2 haplotype in ANXA5 might be used as a novel biomarker to predict the risk of RPL.

Declaration of conflicting interest

The authors declare that there is no conflict of interest.

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