Polymorphisms in the annexin A5 gene promoter in Japanese women with recurrent pregnancy loss

Hironori Miyamura¹,², Haruki Nishizawa², Sayuri Ota¹, Machiko Suzuki¹, Ayaka Inagaki¹,², Hiromi Egusa¹,², Sachie Nishiyama¹,², Takema Kato¹, Kanako Pryor-Koishi², Isao Nakanishi³, Tomio Fujita³, Yuzo Imayoshi³, Arseni Markoff⁴, Itaru Yanagihara³, Yasuhiro Udagawa², and Hiroki Kurahashi¹,*

¹Division of Molecular Genetics, Institute for Comprehensive Medical Science, Fujita Health University, 1-98 Dengakugakubo, Kutsukake-cho, Toyoake, Aichi 470-1192, Japan ²Department of Obstetrics and Gynecology, Fujita Health University School of Medicine, Toyoake, Aichi 470-1192, Japan ³Osaka Medical Center and Research Institute for Maternal and Child Health, Izumi, Osaka 594-1101, Japan ⁴Institute of Medical Biochemistry, Center for Molecular Biology of Inflammation, University of Muenster, D-48149 Muenster, Germany

*Correspondence address. Tel: +81-562-93-9391; Fax: +81-562-93-8831; E-mail: kura@fujita-hu.ac.jp

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ABSTRACT: Recent findings have raised the possibility that polymorphisms within the annexin A5 gene (ANXA5) promoter contribute to the etiology of recurrent pregnancy loss (RPL). In our present study, 243 Japanese women who had suffered more than three fetal losses and a group of 119 fertile controls were genotyped for four ANXA5 gene promoter single-nucleotide polymorphisms (SNPs; SNP1-4: g.-467G>A, g.-448A>C, g.-422T>C, g.-373G>A) previously reported to be associated with this disorder. An additional two SNPs located within the 5′-untranslated region of the ANXA5 (SNP5 and 6: g.-302T>G, g.-1C>T) were also evaluated. Our case–control study revealed that the minor allele was significantly more frequent in the RPL group than controls for all six of these SNPs, among which SNP5 showed the highest significance (P = 0.002). As with the M2 haplotype for SNP1-4 (A-C-C-A) for a western population in previous reports, a haplotype comprising all of the minor alleles for SNP1-6 (A-C-C-A-G-T), the third major haplotype in the Japanese population, showed a significantly higher frequency in our current RPL subjects than in controls (P = 0.025). In addition, the second major haplotype (G-A-T-G-G-C) was found to confer a significant risk of RPL (P = 0.036), implicating SNP5 as a major risk determinant for this disease. Our present findings support the hypothesis that genomic variations within the ANXA5 gene upstream region impact upon the disease susceptibility to RPL. Our data indicate that SNP5 is a novel risk factor for this disease in the Japanese population.

Key words: recurrent pregnancy loss / annexin A5 / promoter / SNP / haplotype

Introduction

Recurrent pregnancy loss (RPL) is a serious reproductive problem affecting ~5% of couples trying to conceive (Sierra and Stephenson, 2006). This disorder is caused by many etiologies, including structural, infective, endocrine, immune and coagulation disorders (Rai and Regan, 2006). However, most of the cases are of an unexplained cause, and this has hindered the development of therapeutic interventions for this serious condition. Couples have thus been treated only empirically, based on anecdotal evidence (Rai and Regan, 2006).

To date, a considerable body of evidence has indicated that genetic factors significantly contribute to the etiology of RPL (Ward, 2000; Sierra and Stephenson, 2006). It is thus possible that RPL develops as a single gene disorder. We recently reported that two women with RPL had a heterozygous mutation within the meiosis-expressing gene, SYCP3, indicating that a small subset of cases is caused by a recurrent chromosomal segregation error during meiotic division in the oocytes (Bolor et al., 2009). However, another prevailing hypothesis is that most instances of RPL are due to a polygenic disorder and are associated with multiple gene mutations (Coulam et al., 2006).

Annexin A5 (ANXA5) is both a Ca²⁺ and phospholipid-binding protein that is localized at the surface of the placental syncytiotrophoblast layer and performs a vital anticoagulant function in the maternal blood at the intervillous space (Gerke and Moss, 2002). It has recently
been shown that polymorphisms in the promoter region of the ANXA5 gene are significantly associated with RPL, and that women with an M2 haplotype have more than a 2-fold higher risk of fetal loss than non-carriers (Bogdanova et al., 2007). In our present study, we further examined upstream ANXA5 gene variants among a cohort of Japanese RPL cases.

Materials and Methods

Patients
A total of 243 Japanese women with a history of RPL (more than three consecutive pregnancy losses) of unknown cause were recruited from Fujita Health University Hospital and the Osaka Medical Center and Research Institute for Maternal and Child Health (aged 34.15 ± 4.27 years; gravidity, 3.14 ± 0.51; parity, 0.083 ± 0.319; fetal losses, 3.06 ± 0.30). None of these individuals had any family history of birth defects. RPL cases of known cause such as hormonal, structural, immunological and coagulation disorders were excluded. All of these women were examined by ultrasonography or hysterosalpingography to detect any possible anatomic abnormalities of the genital tract, and had blood drawn to examine for immunologic risk factors, including anti-nuclear antibodies, and anti-phospholipid antibodies such as lupus anticoagulant. Cases with either inherited or acquired thrombophilia, as determined by measurements of the plasma levels of antithrombin III, and proteins C and S, were excluded. Screening for the Leiden mutation (1691G>A) within the F5 gene and the PTm mutation (20210G>A) of the F2 gene was performed using the Taqman assay (rs6025 and rs1799963, respectively) but no positive cases were found. Blood tests for hyperthyroidism, diabetes mellitus, hyperprolactinemia and infections such as chlamydia were also performed. Cytogenetic analyses demonstrated a normal karyotype in all of these individuals as well as in their partners, but were not performed in all of the abortuses. Peripheral blood samples were obtained at the clinic after appropriate informed consent was obtained. A total of 119 blood samples from Japanese women with at least one child and no history of infertility or miscarriage were used as controls (age at sampling, 46 ± 13.5 years). This study was approved by the Ethical Review Board for Human Genome Studies at Fujita Health University (Accession numbers 214-2, approved on 12 February 2010).

Isolation of genomic DNA and analyses of variants
Genomic DNA was extracted from peripheral blood samples using PureGene (Genta Systems, Minneapolis, MN). The primers used to amplify the promoter region of the human ANXA5 gene (GenBank Accession Number: AC096730) were as follows: Fw, CCGAGGCCCTGGACA GCTCCCCCA; Rv, AGAGGAGAGCGTGTCGCGGGGC (Bogdanova et al., 2007). PCR was performed for 45 cycles of 10 s at 98°C and 5 min at 68°C. After the removal of PCR primers and excess dNTPs by exonuclease I and shrimp alkaline phosphatase (ExoSAP-IT, USB Corp., Cleveland, OH), the amplified products were directly sequenced using the primers and the ABI Prism Sequencer 3130XL (Applied Biosystems, Foster City, CA). Taqman primers and probes were purchased to genotype the −1T> C single-nucleotide polymorphism (SNP) of the ANXA5 gene (rs11575945) in accordance with the manufacturer’s protocol (Applied Biosystems).

Statistical analysis
Deviations from a Hardy–Weinberg equilibrium (HWE) among the genotypes were first evaluated using the χ2 test. Genotype and allele frequency differences between the RPL and control groups were then evaluated by χ2 analysis. Linkage disequilibrium (LD) was evaluated for all SNPs as were the Lewontin’s D’ (|D’|) and the LD coefficient r2 between all pairs of biallelic loci. We estimated the haplotype frequencies among the RPL and control groups using the maximum-likelihood method employing an expectation-maximization algorithm. Haplotype-wise association analysis was evaluated using a likelihood ratio test. All statistical analyses were performed by univariate analyses. In addition, multivariate logistic analysis was also performed for the determination of epistasis. All of these calculations were performed using SNPAlyze software (Dynacom, Chiba, Japan).

Results
We sequenced the promoter region of the ANXA5 gene in 243 Japanese women with RPL and 119 fertile controls and thereby identified four variations that had been previously reported by Bogdanova et al. (2007), i.e. SNP1 (g.-467G>A) and SNP2 (g.-448A>C, rs28717001) in the untranscribed promoter region, and SNP3 (g.-422T>A, rs28651243) and SNP4 (g.-373G>A) within the 5’ untranslated exon (exon 1; Fig. 1). SNPS (g.-302T>G, rs1050606) is another

Figure 1  ANXA5 gene promoter variants. Exons are indicated by boxes and the coding region is denoted by a black box. Vertical arrows indicate the location of six common SNPs (thick arrows) and three rare SNPs (thin arrows). Triangles indicate the position recognized by primers used to amplify the promoter region. The horizontal short bar indicates the regions associated with the Taqman primers and probe.
common polymorphism located within exon 1. We performed further genotyping for another common ANXA5 SNP, g.1C>T (SNP6, rs11538099), which is located one nucleotide upstream of the initiation codon in exon 2. The genotype frequencies for all of the six SNPs examined were found to be in HWE, suggesting neither sampling bias nor mistyping of genotypes (Table I). In addition, we identified three rare SNPs that have not been previously reported. A single nucleotide deletion was identified upstream of SNP1 (SNP7, g.-682delT) but this variation was found in only one RPL case as a heterozygote. A further SNP (SNP8, g.-435G>C) was identified between SNP2 and SNP3 in two RPL subjects and one control also as a heterozygote. The third rare variant was found at one nucleotide downstream of SNP4 within exon 1 (SNP9: g.-372G>A) in a control subject as a heterozygote. These rare variants were excluded from further study on the basis of the common disease–common variant hypothesis.

We performed a case-control study for the six common ANXA5 SNPs in the RPL and fertile control groups. The carrier frequency for the minor alleles was found to be significantly higher in the RPL group for all six SNPs ($P < 0.05$) in both allele-wise and genotype-wise (dominant model) analyses (Table II). Our statistical analyses further indicated that the most significant difference between the groups was for SNP5 (genotype-wise: $P = 0.007$, allele-wise: $P = 0.002$). Women carrying this minor allele were found to have about a 2-fold higher risk of fetal loss than non-carriers. However, SNP4 conferred the highest risk of RPL [odds ratio (OR) = 2.369 (Table II). Since the minor allele frequency was relatively high for SNP5, we also performed recessive model analysis for this polymorphism. Homozygotes of the SNP5 minor allele were more frequent in the RPL group ($P = 0.02$), and this genotype conferred a 7-fold higher risk of RPL (OR = 7.76).

We next performed LD analysis among the six ANXA5 SNPs (Fig. 2). All except for SNP5 manifested a strong LD as was previously reported for SNP1–4 (Bogdanov et al., 2007). We further performed haplotype-wise analysis for these six SNPs, which indicated the presence of three major haplotypes in a Japanese population (Table III). G-A-T-G-T-C and G-A-T-G-G-C were the two major haplotypes and accounted for 90% of the subjects. The first four (G-A-T-G) group for all six SNPs (P < 0.05) in both allele-wise and genotype-wise (dominant model) analyses (Table II). Our statistical analyses further indicated that the most significant difference between the groups was for SNP5 (genotype-wise: $P = 0.007$, allele-wise: $P = 0.002$). Women carrying this minor allele were found to have about a 2-fold higher risk of fetal loss than non-carriers. However, SNP4 conferred the highest risk of RPL [odds ratio (OR) = 2.369 (Table II). Since the minor allele frequency was relatively high for SNP5, we also performed recessive model analysis for this polymorphism. Homozygotes of the SNP5 minor allele were more frequent in the RPL group ($P = 0.02$), and this genotype conferred a 7-fold higher risk of RPL (OR = 7.76).

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SNPs constitute the N haplotype described previously by Bogdanova et al. (2007). The haplotype comprising all six minor alleles (A-C-C-A-G-T) was found to be the third most common. The first four SNPs (A-C-C-A) constitute the so-called M2 haplotype, the frequency of which is 5.4% in the general Japanese population. This is lower than the rate reported for western countries (~16%). The M1 haplotype (G-C-C-G) was found to be rare in Japanese subjects (data not shown). We subsequently performed a case-control study for these haplotypes, and found that A-C-C-A-G-T was significantly more frequent in RPL samples (10.5 versus 5.5%, \(P = 0.025\)). This haplotype also appeared to pose a 2.35-fold higher risk of RPL compared with non-carriers. This is consistent with the previous finding that the M2 haplotype is more frequent in RPL samples (10.7 versus 5.5%, \(P = 0.021\)).

It was of interest that the G-A-T-G-G-C haplotype, which comprises all major alleles except SNP5, was also found to be more frequent in RPL samples (10.7 versus 5.5%, \(P = 0.021\)). This haplotype appeared to pose a 1.84-fold higher risk of RPL compared with non-carriers. The fact that not only the A-C-C-A-G-T haplotype that includes M2, but also the G-A-T-G-G-C haplotype was found to be more frequent in the RPL group suggests that SNP5 is a major risk determinant for this disorder. Since SNP5 and the other five SNPs are in weak LD, SNP5 and M2 haplotype may have confounding effects. However, based on the results of our logistic regression analysis, it is not certain whether SNP5 is epistatic to the M2 haplotype (\(P = 0.086\) for SNP5 versus \(P = 0.292\) for M2). Taken together, however, and regardless of whether it is independent of or dependent on the M2 haplotype, the minor allele of SNP5 may potentially contribute to the etiology of RPL in the Japanese population.

**Discussion**

The M2 haplotype of the ANXA5 gene promoter was recently reported to associate with RPL in a population of 70 women with more than two fetal losses (18 in 70 RPL cases versus 41 in 500 controls) (Bogdanova et al., 2007). In another study of larger cohorts, the M2 haplotype was recorded in 35 of 103 women with RPL and in 30 of 154 controls, and the frequency was reported to be significantly higher in the RPL group (\(P < 0.001\)) (Tiscia et al., 2009). In our current study, we found that the A-C-C-A-G-T haplotype of the ANXA5 gene promoter is associated with RPL in a Japanese population, which is consistent with previous data reported for western populations. Each of the variants tested in our analysis was found to be located within the upstream regulatory region of the gene.
ANXA5 variants in recurrent pregnancy loss

ANXA5 gene. Reporter assays have previously indicated that the transcription efficiency from susceptible ANXA5 variants is lower than that in the major variants (Bogdanova et al., 2007). This suggests that these SNPs are functional and can decrease the expression of ANXA5, thereby impacting upon the etiology of RPL.

We further found that among the polymorphisms tested in our current analysis, SNP5 (g.-302T>G) is also associated with RPL. Our LD analyses indicated that this SNP is not tightly linked to the M2 haplotype and our results raise the possibility that it may be the primary determinant of the risk of RPL in Japanese women. Since SNP5 was not analyzed in previous reports, we are unsure at present whether this polymorphism is also associated with RPL in western cohorts. Whereas the National Center for Biotechnology Information database reports an allele frequency of 15/85 for G/T in the Japanese population, this rises to as high as 54/46 for western populations (Centre d’Etudes du Polymorphisme Humaine). On the other hand, the frequency of the M2 haplotype is 5.5% in the Japanese population, but 16% in western cohorts, which is similar to the rate of the g.-302T>G SNP in Japan. A more thorough analysis using different cohorts of RPL cases would reinforce our conclusion that the SNP5 might be the primary risk determinant.

The ANXA5 protein is localized at the apical surfaces of the syncytiotrophoblast layer in chorionic villi (Krikun et al., 1994). Interestingly, the infusion of anti-ANXA5 antibodies induces coagulation at the surfaces of the syncytiotrophoblast layer, leading to fetal loss, which indicates that ANXA5 protects the feto—maternal interface from the coagulation of maternal blood (Wang et al., 1999). Further, experimental data have indicated that anti-phospholipid antibodies facilitate coagulation by reducing the levels of ANXA5 at the surface of the trophoblasts in antiphospholipid syndrome (APS), a well-established condition that manifests as RPL (Rand et al., 1997; Shapiro, 1996). Transcription of the ANXA5 gene from the M2 haplotype appears to be at a lower level than the standard haplotype (Bogdanova et al., 2007). It is thus reasonable to speculate that women carrying the M2 haplotype, and possibly the minor allele of SNPs, have low levels of ANXA5 protein at the surfaces of their trophoblasts. This will induce a greater susceptibility to coagulation and thus RPL. In this context, RPL in women with the M2 haplotype and APS may belong to the same clinical entity, annexinopathy (Rand, 2000).

Indeed, frequent M2 haplotype has been reported in other obstetric complications such as pre-eclampsia and pregnancy-related venous thromboembolism, which may also belong to annexinopathy (Tiscia et al., 2009; Grandone et al., 2010a, b).

We genotyped DNA from maternal leukocytes in our current analysis, as has been done previously (Bogdanova et al., 2007; Tiscia et al., 2009). It was also reported earlier that the placental expression of ANXA5 at the mRNA level is related to the maternal genotype (Chinni et al., 2009). This is an intriguing finding since the syncytiotrophoblasts that carry ANXA5 protein at their surface are of fetal origin and have a half-maternal and half-paternal genome. Although the ANXA5 gene is expressed abundantly in the placenta, somewhat low level of expression is observed ubiquitously. It is not yet certain whether ANXA5 protein at the syncytiotrophoblast surfaces originates from the maternal circulation or is produced by the fetal syncytiotrophoblasts. Further investigations, including the genotyping of the fetus or placenta from RPL cases, will be necessary to elucidate this phenomenon.

Although a recent study has suggested no role for anticoagulation therapy such as low molecular weight heparin or low-dose aspirin in the treatment of RPL, the selection of appropriate patients using SNP analysis might enhance the therapeutic efficacy of such treatments (Kaandorp et al., 2010). Targeted drug delivery technologies might also yield more effective yet safer chemotherapeutics for RPL in the future.

Authors’ roles

H.M. played a role in execution, analysis, manuscript drafting and critical discussion. H.N. was involved in analysis, manuscript drafting and critical discussion; S.O., M.S., A.I., H.E., S.N., T.K. and K.P.-K. contributed to execution and analysis. I.N., T.F. and Y.I. played a role in execution, analysis and critical discussion. A.M., I.Y. and Y.U. were involved in study design and critical discussion. H.K. contributed to study design, manuscript drafting and critical discussion.

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