Reproductive BioMedicine Online (2015) 31, 681–688

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ARTICLE

Investigation of the Annexin A5 M2 haplotype in 500 white European couples who have experienced recurrent spontaneous abortion

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Abstract Annexin A5 is a placental anti-coagulant protein that contains four nucleotide substitutions (M2 haplotype) in its promoter. This haplotype is a risk factor for recurrent spontaneous abortion (RSA). The influence of the M2 haplotype in the gestational timing of spontaneous abortions, paternal risk and relationships with known risk factors were investigated. European couples ($n = 500$) who had experienced three or more consecutive spontaneous abortions, and two fertile control groups, were selected for this study. The allele frequency of M2 was significantly higher among patients who had experienced early RSA than among controls ($P = 0.002$). No difference was found between controls and patients who had undergone late spontaneous abortions. No difference was found between patients who had experienced RSA who had a live birth or no live births, or between patients who were positive or negative for known risk factors. Male and female partners in each group had similar allele frequencies of M2. The M2 haplotype is a risk factor for early spontaneous abortions, before the 12th week of gestation, and confers about the same relative risk to carriers of both sexes. Having one or more M2 allele(s) in combination with other risk factors further increases the RSA risk.

KEYWORDS: annexin A5, ANXA5, M2 haplotype, recurrent miscarriage, recurrent spontaneous abortion, risk factor

http://dx.doi.org/10.1016/j.rbmo.2015.07.004
1472-6483/

Introduction

Recurrent spontaneous abortion (RSA) is defined as more than three consecutive pregnancy losses, and it affects about 1% of couples trying to conceive (Stirrat, 1990). It is associated with higher rates of morbidity and also with complications later in pregnancy, including fetal growth restriction, prematurity and pre-eclampsia (Rai and Regan, 2006). A number of these spontaneous abortions occur as a result of chromosomal abnormalities in the fetus, and this risk increases as maternal age rises (Nybo Andersen et al., 2000).

Risk factors for RSA include antiphospholipid syndrome (APLS), which is an autoimmune disease caused by the presence of circulating maternal antiphospholipid antibodies, such as anticardiolipin antibodies and lupus anticoagulant. These antibodies cause thrombosis of the placental vessels, which in turn leads to fetal loss (Lim, 2013; Salafia and Cowchock, 1997). Another risk factor includes a point mutation (Arg506→Gln) in the *Factor V* gene referred to as the Factor V Leiden (FVL) mutation. This mutation causes slower cleavage (10-fold) of Factor V by activated protein C (APC) and, therefore, an increased level of thrombin and predisposition to clot formation (Kalafatis et al., 1994). Additionally, a mutation in the prothrombin gene (nt 20210 G→A) that is associated with increased prothrombin levels is a risk factor for venous thrombosis (Poort et al., 1996).

Annexin A5 (ANXA5) is a placental anti-coagulant protein that occurs on normal placental villi. Its ability to bind to anionic phospholipids that are found on platelets causes it to impede aggregation, and hence it is thought to function as an inhibitor of coagulation (Thiagarajan and Tait, 1990). High levels of ANXA5, a ubiquitous, but not abundantly expressed protein, are manifested in the liver, kidney and placenta (Morgan et al., 1998). Abundance of ANXA5 was reduced in the placental trophoblast in the presence of antibodies, characteristic of the APLS (Rand et al., 1994).

The *ANXA5* gene is found on human chromosome 4q27 and consists of 13 exons and 12 introns. The gene spans 29 kb and encodes a single transcript of about 1.6 kb and a protein product of 320 amino acids with a molecular weight of about 35 kDa. The region genomic locus encompassing the promoter is very guanine–cytosine rich (73% guanine–cytosine) (Cookson et al., 1994).

Bogdanova et al. (2007) reported the presence of two variant *ANXA5* promoter haplotypes in addition to the wild type (WT) in the promoter region of this gene; the M1 and M2 haplotypes, which are common in the normal population. M1 haplotype comprises of two nucleotide substitutions (1A→C and 27T→C) and M2 haplotype comprises of four substitutions (-19G→A, 1A→C, 27T→C and 76G→A) that are in linkage disequilibrium (LD) with each other (Bogdanova et al., 2007).

The frequency of the M2 haplotype was found to be significantly higher in patients who had experienced RSA than among controls (Bogdanova et al., 2007). In fact, subsequent studies have shown that the M2 haplotype is present in 11% of fertile Japanese controls and 21% in Japanese patients who had experienced RSA (Miyamura et al., 2011), and in 15% of European populations and 21–30% in patients of European origin who had experienced RSA (Tiscia et al., 2009; Tüttelmann et al., 2013). No significant association of the M1 haplotype was found with RSA. Other studies have also shown an association between the M2 haplotype and pre-eclampsia or gestational hypertension (Tiscia et al., 2009), as well as being a risk factor for fetal growth restriction and small for gestational age newborns (Chinni et al., 2009; Tiscia et al., 2012).

Reporter gene assays have demonstrated a 60% reduction in the *ANXA5* promoter activity when the M2 haplotype was present compared with the wild-type promoter (Bogdanova et al., 2007). Therefore, in patients carrying the M2 haplotype, the anti-coagulant properties of ANXA5 are reduced and may lead to a hypercoagulable state in the intervillous space, potentially explaining an increased risk of RSA. Moreover, a study on *ANXA5* expression in RSA placentas showed that mRNA levels are reduced regardless of the parental origin of the M2 haplotype (Markoff et al., 2010).

To further elucidate the role of the M2 *ANXA5* haplotype and its association with RSA, a large cohort consisting of 500 white European couples who had experienced RSA was genotyped. Our aim was to investigate the influence of the *ANXA5* M2 haplotype on the timing of spontaneous abortions, to assess the male risk and to investigate any interaction with known risk factors, such as APLS FVL and prothrombin mutation.

Materials and methods

Study populations

Patient blood samples used in this study were collected from patients and their male partners who attend the Recurrent Miscarriage Clinic at St Mary's Hospital, Imperial College London, and who agreed to participate in research with signed, informed consent. A total of 996 white European samples (501 female patients and 495 male partners) were used in this study. Patients with uterine anomalies, polycystic ovaries (Rotterdam ESHRE/ASRM-Sponsored PCOS Consensus Workshop Group, 2004) and fetal and parental chromosomal abnormalities were excluded. Confirmation of APLS was by the presence of lupus anticoagulant antibodies, anticardiolipin, or both, and β2-Glycoprotein IgG, IgM autoantibodies, or both (Miyakis et al., 2006) after routine testing by the Clinical Biochemistry service. Data for these tests was therefore extracted from each patient's hospital records. An additional test for the prothrombin variant (G20210A) was conducted as described below.

Patients were broadly divided into different classes according to the gestational period in which the spontaneous abortion occurred. A total of 310 women and 309 male partners who had undergone three or more early spontaneous abortions (before 12th week of gestation) and no late spontaneous abortions were classified as 'spontaneous abortion patients'. The women in this group had an average age at referral of 34.6 ± 4.9 years (mean ± SD). A total of 191 women and 186 men who had at least one late spontaneous abortion (after 12th week of gestation) were classified as 'spontaneous abortion patients'. The women in this group had an average age at referral of 34.0 ± 5.3 years. Still births were not included in this group of patients. The study was approved by the Imperial College Hospital Ethics Committee (REC ref: 12/WA/0196).