The M2 haplotype in the ANXA5 gene is an independent risk factor for idiopathic small-for-gestational age newborns

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Abstract: Hereditary thrombophilias can impair vascular placental functions and predispose to the birth of small-for-gestational age (SGA) babies. The placental anticoagulant protein annexin A5 (ANXA5) may contribute to this process. A functional haplotype (M2) within the ANXA5 gene is associated with fetal loss and venous thrombosis. This study investigated the prevalence of the M2 haplotype in a group of women with idiopathic SGA newborn babies. Seventy-eight women with at least one previous unexplained SGA birth and 195 controls all from Southern Italy were investigated. Hereditary thrombophilia was found in 13 (16.5%) cases and 21 (11%) controls (P, 0.05). The M2 haplotype was found in 29% of cases (n = 23) and 15% of controls [n = 30; P = 2.3, 95% CI (1.17–4.48)]. Within the case group, 82.5% of the M2 haplotype carriers gave birth to babies with a birthweight below the 3rd percentile [P = 0.01; OR = 2.4, 95% CI (1.26–4.73)]. A logistic regression, corrected for age, parity and gravity showed that the M2 haplotype was independently associated with the delivery of an SGA newborn [P = 0.029; OR = 2.6, 95% CI (1.1–6.0)]. In conclusion, the M2 haplotype of the ANXA5 gene confers a risk of delivering SGA babies.

Key words: pregnancy / polymorphism

Introduction

It is well established that the term ‘small-for-gestational age’ (SGA) refers not to fetal growth but to the size of the infant at birth, whereas the term ‘fetal growth restriction’ (FGR) suggests a reduced growth velocity in the fetus (Iams, 2010). The risk of perinatal morbidity and mortality is greater in SGA newborns and the risk for chronic diseases in later life, such as glucose intolerance, type 2 diabetes mellitus and cardiovascular diseases is also higher (Barker, 1997).

Although SGA and FGR are distinct and independent adverse pregnancy outcomes, the former might represent a clinical consequence of the latter (Verkauskiene et al., 2007) and be only the tip of an iceberg.

FGR is associated with maternal factors, such as fibroma (Aydeniz et al., 1998), alcohol intake, smoking (Jackson et al., 2007), pregnancy-induced hypertension (Liu et al., 2008), pre-eclampsia (Odegård et al., 2000), malnutrition (Hendrix and Berghella, 2008) and with fetal factors, such as multiple pregnancies, chromosomal abnormalities and malformations (Tyson and Staat, 2008). In the absence of such common causes, an impaired fetal growth is hypothesized to be due to placental insufficiency, involving any mismatch placental perfusion and fetal oxygenation. Only a third of FGR cases can be accounted for by obvious maternal, fetal and placental causes (Brodsky and Christou, 2004), the remainder being idiopathic.

Hereditary thrombophilias, such as anti-thrombin, protein C and protein S deficiency, factor V Leiden (FVL) and prothrombin G20210A (PTm) mutations are factors predisposing to the birth of SGA babies (Martinelli et al., 2001; Roqué et al., 2004; Sabadell et al., 2010). We previously reported that common hereditary thrombophilias and hyperhomocisteinaemia in the amniotic fluid are associated with intrauterine growth restriction (Grondone et al., 2002; Grondone et al., 2006). However, the relationship between hereditary thrombophilia and SGA births remains controversial (Rodger et al., 2010).

Defects in the placental anticoagulant protein annexin A5 (ANXA5) have been suggested as contributing factors to the occurrence of SGA newborns. Recently, a common haplotype (M2) within the ANXA5 has been demonstrated to be an independent risk factor for both
The M2 haplotype in the ANXA5 gene for SGA newborns

The presence of the M2 haplotype (a set of four consecutive nucleotide substitutions in the ANXA5 gene promoter: 19G/A[rs112782763], +1A/C[rs28717001], 27T/C [rs28651243] and 76G/A [rs113588187]) was investigated as described by Bogdanova et al. (2007). When only two of the four variants (+1A/C, 27T/C) were present, the haplotype was defined as M1.

Statistical analysis

All the analyses were performed using the Statistical Package for Social Science (SPSS 6.1 for Macintosh). The difference in means was compared by a non-parametric test, whereas the significance of any difference in proportions was tested by the $\chi^2$ statistic. Allele frequencies were estimated by gene counting in cases and controls; genotypes were scored for each subject. The significance of the difference of alleles and genotypes observed between the groups was tested using the $\chi^2$ analysis, after grouping homozygous and heterozygous carriers of the FVL and PTm. $\chi^2$ (2 degree of freedom) Hardy-Weinberg equilibrium (HWE) assay was performed for all variants (Rodriguez et al., 2009). Departures from the HWE equilibrium for relevant $\chi^2$ values obtained were evaluated from calculated $P$ values with a $P$ value < 0.05 indicating a significant deviation. Proportions were compared using the Fisher exact test where appropriate. Odds ratios (OR) and 95% confidence intervals (CI) were calculated. Adjusted ORs and 95% CI were calculated by logistic regression models. Potential confounding variables were age, parity, gravidity.

Results

The clinical data of cases and controls are set out in Table I. As far as a smoking habit is concerned, 12/78 (15%) cases smoked 1–10 cigarettes/day, 1 (1.3%) 10–20/day; among controls 15 (11.3%) smoked 1–10 cigarettes/day and 7 (3.5%) 10–20/day. These differences were not statistically significant ($P > 0.05$). The median BMI was 22.6 (range: 17–33) in cases and 24 (range: 18–32) in controls ($P > 0.05$). Sixty-two (79.5%) newborns were delivered by means of Caesarean section. Overall, a history of at least one previous fetal loss was recorded in 20 (25.5%) cases and 11 (5.5%) controls. A status of hereditary thrombophilia (Table I) was found in 13 (16.5%); 8 FVL, 5 PTm, $P > 0.05$ cases and 21 controls (11%; 10 FVL, 11 PTm, $P > 0.05$). Sixty-seven (77%) out of 78 cases gave birth to newborns below the 3rd percentile (31 males, 29 females), whereas the remaining 18 (23%) had a newborn with a percentile between the 10th and 3rd (7 males, 11 females). Features of the newborns according to different percentiles and SGA-related weight according to gender and gestational weeks of index-pregnancy are shown in Table II.

Sample collection, plasma- and DNA-based tests

Blood samples were collected in a 1:10 ratio in sodium citrate of 0.1 M and centrifuged at 1841 g for 10 min (min) at room temperature. Plasma samples were separated and stored at −80 °C until assayed for protein C, free protein S and antithrombin levels, as well as for antiphospholipid antibodies, confirmed presence of lupus anticoagulant (LA), IgG and IgM anticardiolipin antibodies (aCL), IgG and IgM anti-beta-2-glycoprotein (β2GPI). Platelet-free plasmas for LA testing were obtained by prefiltration through a 0.22 μm disposable filter. LA, IgG and IgM aCL, IgG and IgM β2GPI (QUANTA Lite™, INOVA Diagnostics, San Diego, CA, USA), antithrombin and protein C (Berichrom™ Antithrombin and Protein C amidolytic assays, Behring, Germany), and total and free protein S antigen (IMMUCOLONE™ Protein S ELISA, American Diagnostica, Stamford, CT) were determined in all the patients. The inter-assay and intra-assay coefficients of all the variables never exceeded 8.0 and 5.0%, respectively.

Leukocyte DNA was obtained from frozen blood by the use of standard techniques. FVL and FII G20210A (PT) gene variants were tested by a TaqMan® SNP Genotyping Assay (PE; Applied Biosystems, Foster City, USA) via a Real-Time PCR System ABI PRISM® 7700 Sequence Detection System (PE; Applied Biosystems).

<table>
<thead>
<tr>
<th>Table I</th>
<th>Main characteristics of enrolled subjects.</th>
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</thead>
<tbody>
<tr>
<td><strong>Cases, n = 78</strong></td>
<td><strong>Controls, n = 195</strong></td>
</tr>
<tr>
<td>Age, median (range)</td>
<td>32 (16–39)</td>
</tr>
<tr>
<td>Gravidity, median (range)</td>
<td>2 (1–5)</td>
</tr>
<tr>
<td>Parity, median (range)</td>
<td>1 (0–3)</td>
</tr>
<tr>
<td>Smoking habit, n (%)</td>
<td>13 (16.5)</td>
</tr>
<tr>
<td>Miscarriages, n (%)</td>
<td>20 (25.5)</td>
</tr>
<tr>
<td>FV Leiden, n (%)</td>
<td>8 (10)</td>
</tr>
<tr>
<td>FII A20210, n (%)</td>
<td>5 (6.5)</td>
</tr>
</tbody>
</table>
The presence of deviations from HWE was assayed for the expected and the observed frequency for each of them (Table III). For the four SNPs: no significant difference was found between the M2 haplotype, which gave birth to newborns below the 3rd percentile for the M1 haplotype. Nineteen (82.5%) out of the 23 cases, carrying the M2 haplotype, gave birth to newborns below the 3rd percentile. Five cases (6%) and 11 controls (6%) had the variants. Controls were found to be carriers of genotypes defining the M2 haplotype. As can be seen in the same table, 23 (29.5%) cases and 30 (23%) controls were found to be carriers of genotypes defining the M2 haplotype. Five cases (6%) and 11 controls (6%) had the variants +1A/C[r’s28717001] and 277T/C[r’s28651243] which are responsible for the M1 haplotype. Nineteen (82.5%) out of the 23 cases, carrying the M2 haplotype, gave birth to newborns below the 3rd percentile [Fisher exact test \( P = 0.01, \text{ OR} = 2.4, 95\% \text{ CI} (1.26–4.73)\)]. In addition, haplotype frequencies are reported in Table III.

Using logistic regression analysis the M2 haplotype was independently associated with the delivery of an SGA newborn \([P = 0.029; \text{ OR} = 2.6, 95\% \text{ CI} (1.1–6.0)]\) after correction for age, parity and gravidity.

### Discussion

The ubiquitous expression of ANXA5 (Kräkken et al., 1994) in syncytiotrophoblast, where the protein plays a well-known thrombomodulatory role (Ornaghi et al., 2011), supports the idea that ANXA5 represents a functional placental-related factor. This study demonstrates a significantly higher prevalence of the M2 haplotype in a group of women with a history of idiopathic SGA babies. Women carrying the M2 haplotype have a 2-fold higher risk of giving birth to an SGA newborn. In addition, all the M2 homozygotes (no homozygote was found among controls) had a history of a severe (below the 3rd percentile) SGA. Normal fetal growth is strongly linked to the adequate transfer of solutes from maternal to fetal circulation, including oxygen and necessary nutrients. We believe that the M2 haplotype, responsible for lowering the transcriptional efficiency of the promoter, might determine a reduction in ANXA5 levels in trophoblasts, thus exposing pregnant carriers to gestational vascular complications.

Our findings confirm and extend previous data (Chinni et al., 2009; Markoff et al., 2010; Sifakis et al., 2010). Sifakis et al. compared ANXA5 mRNA and protein levels in placenta from FGR and normal pregnancies, and found significant differences in mRNA expression (3-4 fold lower in FGR placenta) but not in protein levels. In addition, our group (Chinni et al., 2009) showed that mRNA levels in placenta are significantly lower in women carrying the M2 haplotype, independent of the presence of an obstetric complication. Thus, it is conceivable that placenta from women carrying the M2 haplotype had significantly lower mRNA levels than those from women without this haplotype. The investigation of other ANXA5 variants as risk factors for FGR/SGA has been reported. In a case–control study, a polymorphism (-1C>T), known to be associated with a better translation efficiency and to an increase of protein levels (González-Conejero et al., 2002), has been evaluated as a protective factor for FGR (Franchi et al., 2006), although the authors failed to show a protective effect in this group of patients. More recently, the same gene variant has been shown to be in linkage disequilibrium with the M2 haplotype and in association with fetal loss in Japanese RPL population (Miyamura et al., 2011). We suggest that the four variants within the ANXA5, inherited as the M2 haplotype, might represent an independent risk factor for otherwise unexplained SGA. Further studies on larger populations are required before considering the M2 haplotype.

### Table II Newborn data according to percentiles and gender.

<table>
<thead>
<tr>
<th>Gender</th>
<th>SGA &lt;3rd percentile, n = 60</th>
<th>SGA &gt;3th&lt;10th percentile, n = 18</th>
</tr>
</thead>
<tbody>
<tr>
<td>Males/females</td>
<td>31/29</td>
<td>7/11</td>
</tr>
<tr>
<td>Birthweight (g), males/females, median (range)</td>
<td>1820 (750–2700)/1775 (550–2570), overall: 1800 (550–2700)</td>
<td>750 (670–2450)/2400 (820–2960), overall: 2190 (670–2960)</td>
</tr>
<tr>
<td>Gestational age at delivery, males/females, weeks median (range)</td>
<td>37 (29–41)/37.5 (25–41), overall: 37 (25–41)</td>
<td>28 (26–38)/39 (29–42), overall: 37 (26–42)</td>
</tr>
</tbody>
</table>

### Table III SNP information and frequencies related to alleles, genotypes and haplotypes.

<table>
<thead>
<tr>
<th>SNP Information</th>
<th>Cases (n = 78)</th>
<th>Controls (n = 195)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Allelic frequencies</td>
<td></td>
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</tr>
<tr>
<td>rs11278763 G/A</td>
<td>G, 0.82; A, 0.18</td>
<td>G, 0.92; A, 0.08</td>
</tr>
<tr>
<td>rs28717001 A/C</td>
<td>A, 0.77; C, 0.23</td>
<td>A, 0.90; C, 0.10</td>
</tr>
<tr>
<td>rs28651243 T/C</td>
<td>T, 0.77; C, 0.23</td>
<td>T, 0.90; C, 0.10</td>
</tr>
<tr>
<td>rs113588187 G/A</td>
<td>G, 0.82; A, 0.18</td>
<td>G, 0.92; A, 0.08</td>
</tr>
<tr>
<td>Genotypes</td>
<td></td>
<td></td>
</tr>
<tr>
<td>NN</td>
<td>49 (62%)</td>
<td>154 (79%)</td>
</tr>
<tr>
<td>NM1</td>
<td>5 (6%)</td>
<td>11 (6%)</td>
</tr>
<tr>
<td>M1M1</td>
<td>1 (1%)</td>
<td>0</td>
</tr>
<tr>
<td>NM2, M1M2a</td>
<td>18 (23%)</td>
<td>30 (15%)</td>
</tr>
<tr>
<td>M2M2</td>
<td>5 (6%)</td>
<td>0</td>
</tr>
<tr>
<td>Haplotypes</td>
<td></td>
<td></td>
</tr>
<tr>
<td>N</td>
<td>120/156 (77%)</td>
<td>349/390 (89.5%)</td>
</tr>
<tr>
<td>M1</td>
<td>8/156 (5%)</td>
<td>11/390 (3%)</td>
</tr>
<tr>
<td>M2</td>
<td>28/156 (18%)</td>
<td>30/390 (7.5%)</td>
</tr>
</tbody>
</table>

*aObserved in one individual in case’s group.*

SNP information and allelic frequencies in cases and controls are found in Table III. The presence of deviations from HWE was assayed for the four SNPs: no significant difference was found between the expected and the observed frequency for each of them (Table III). As can be seen in the same table, 23 (29.5%) cases and 30 (15%), Fisher exact test \( P = 0.011; \text{ OR} = 2.3, 95\% \text{ CI} (1.17–4.48)\) controls were found to be carriers of genotypes defining the M2 haplotype. Five cases (6%) and 11 controls (6%) had the variants +1A/C[r’s28717001] and 277T/C[r’s28651243] which are responsible for the M1 haplotype. Nineteen (82.5%) out of the 23 cases, carrying the M2 haplotype, gave birth to newborns below the 3rd percentile [Fisher exact test \( P = 0.01, \text{ OR} = 2.4, 95\% \text{ CI} (1.26–4.73)\)]. In addition, haplotype frequencies are reported in Table III.
diagnostics in the risk assessment algorithm of women who previously suffered from an idiopathic SGA.

**Acknowledgement**

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**Authors’ roles**

G. T. and D. C. enrolled patients and performed genetic analyses. G.F. and P.M. performed the statistical analysis; E.G., M.M and G.T. designed and supervised the experimental strategy and wrote the manuscript.

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**Conflict of interest**

None declared.

**References**


