A C-634-G Polymorphism of the Interleukin-6 Gene Promoter as a Possible Risk for Idiopathic Recurrent Miscarriage

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ABSTRACT

Background: Cytokines have been described to play a major role in the pathogenesis of idiopathic recurrent miscarriage (IRM). We investigated the association between IRM and a polymorphism of the interleukin-6 (IL-6). Methods: In a prospective case-control study, we studied 100 women with IRM and 60 healthy controls. Peripheral venous blood sample was taken from each woman for DNA extraction and PCR was employed to genotype women for the presence of a polymorphism at position -634 in the promoter region of IL-6. Results: Allele frequencies among women with IRM and controls were 82.5 and 78.3% respectively for allele C (wild type); 17.5% and 21.6% respectively for allele G (mutant). No association between allele G and the occurrence of IRM was found (odds ratio 1.304; 95% confidence interval = 0.760–2.299; P = NS). Conclusion: To our knowledge this is the first report on IL-6 polymorphism in IRM on Egyptian population, yet the IL-6 polymorphism investigated was not associated with IRM in the studied group of Egyptian population.

INTRODUCTION

Physiologically, the maternal immune system confronts the embryo/fetus with a host-defense reaction, based on the recognition of paternally derived fetal and placental antigens. To avoid rejection of the semi-allogenic embryo/fetus, the maternal immune response is selectively suppressed in physiological pregnancies. While T_{H2} type immunity is believed to contribute to successful pregnancy, T_{H1} type immunity has been shown to be associated with idiopathic recurrent miscarriage (IRM).

IL-6 is a multifunctional cytokine, produced by many different cell types, including immune cells, fibroblasts, endothelial cells, adipocytes and myocytes. Secretion of IL-6 leads to a stimulation of the hypothalamic-pituitary-adrenal axis during inflammatory processes, promotes osteoclastogenesis and participates in the development of osteoporosis associated with estrogen withdrawal. IL-6 is not constitutively expressed, but is highly inducible and produced in response to a number of inflammatory stimuli.

IL-6 is generally considered to be a proinflammatory cytokine. IL-6 also has anti-inflammatory properties, as...
demonstrated in IL-6 gene knock-out mice. The role of IL-6 expression during pregnancy, as well as its predictive value for pregnancy outcome, is unclear. In human studies, IL-6 production has been described in the decidua during early pregnancy. Also, IL-6 has been shown to induce the release of hCG from trophoblasts, leading to a subsequent cascade of progesterone production, release of Th2 cytokines, e.g. IL-6, IL-4, and suppression of Th1 cytokines. This is compatible with an anti-inflammatory role for IL-6 in pregnancy. On the other hand, elevated levels of IL-6 and proinflammatory cytokines, e.g. IL-1, TNF-α, and IL-8 in the placenta, amniotic cells, and decidua have been demonstrated in pregnancies complicated by pre-term premature rupture of the membranes, intrauterine infection and prematurity.

The etiology of recurrent pregnancy loss remains largely unclear. The possible immunologic etiologies of pregnancy failure have been intensively investigated. A shift to type 2 T-helper (Th2) cytokine production with abundant interleukin IL-6 and IL-10 is considered essential for the maintenance of normal pregnancy. There is evidence of a diminished Th2 immune response to placental antigens in women with recurrent pregnancy loss. Plasma levels of IL-6, IL-8, and IL-11 have been found to be decreased in such women compared with those with normal pregnancies. Additionally, IL-6 levels in maternal serum, amniotic fluid, vaginal fluid, and placenta have been found to increase during the process of normal labor compared with the nonlabor state. One study demonstrated an increase in the frequencies of type 1 T-helper (Th1) cytokine IL-1 gene promoter region variants IL-1 -511C and IL-1 –311T in women with recurrent pregnancy loss. One of the Th2 cytokines, IL-10 promoter region variant (-1082G→A), was not associated with recurrent pregnancy loss. However, relationships between many diseases and IL-6 promoter gene polymorphisms, such as IL-6 -174G→C and IL-6 -634C→G, were recently demonstrated. It is also known that the former polymorphism is frequently found in Caucasians and the latter in the Japanese.

MATERIALS & METHODS

The present case–control study was performed in Cairo, Egypt, during the years 2010–2011. We studied 100 cases aged 20–43 years with a history of recurrent pregnancy loss and 60 controls aged 21–48 years who visited the Obstetrics and Gynecology Department at Cairo University Hospital. The characteristics of the study groups are shown in Table 1. Recurrent pregnancy loss was defined as a history of two or more consecutive spontaneous abortions and stillbirths. The primary recurrent pregnancy loss group comprised 89 women with a history of two or more pregnancy losses but no live birth. The 11 women in the secondary recurrent pregnancy loss group experienced three or more pregnancy losses after one live birth. All women with recurrent pregnancy loss were...
subjected to blood analyses for TORCH, antinuclear antibody, anti-DNA antibody, lupus anticoagulant, anticardiolipin antibody, anticardiolipin -2-glycoprotein I complex antibody, and activated partial thromboplastin time, d-dimer, protein S, protein C activity, and factor XII to identify patients with antiphospholipid antibody syndrome and thrombophilia.

Subjects were examined by ultrasound and hysterosalpingography for detection of anatomic abnormalities of the genital tract. All couples were also subjected to chromosome karyotypic analyses of peripheral blood. Couples with balanced type chromosomal translocation and women with recurrent pregnancy loss with uterine conformational abnormalities, such as a septate uterus, were excluded from the study, because these etiologies of recurrent pregnancy loss are known to have a close cause-and-effect relationship. Women with positive test results for autoantibodies and women with antiphospholipid antibody syndrome were included in this study, because immunologic abnormalities, including IL-6, possibly underlie the pathophysiology of recurrent pregnancy loss in these women. Of the 100 women, 13 (13.0 %) had autoimmune disease and/or antiphospholipid antibody syndrome, and 8 (8%) had hematologic abnormalities, but none had a history of thromboembolism. Control women consisted of 60 volunteers who had experienced at least two live births and no abortion and who had no history of infertility. There were no significant differences in age between cases and controls.

Participants in the study gave informed consent and the study was approved by the institutional ethical boards for human gene and genome studies at Cairo University School of Medicine. Genomic DNA was extracted from lymphocytes of peripheral blood samples by the use of standard techniques. Sequence amplification was performed with polymerase chain reaction (PCR). To analyze the -634C→G genotype, PCR amplifications were carried out as described by Ota et al.\(^{(29)}\), with the primers 5'-GAG AGG CCT TGA AGT AAC TG-3' and 5'-AAC CAA AGA TGT TCT GAA CTG A-3' (Nanjing Bio Eng Co). The following PCR protocol was used: 95°C for 3 min, 35 cycles of 95°C for 1 min, 59°C for 1 min, 72°C for 5 min (BIO-RAD, Iapa.) After the PCR product was digested with 2 U of BsrBI endonuclease (New England Biolabs) at 37°C for 5 hours. The digested products were separated in 3.0% agarose gel electrophoresis and identified by ethidium bromide staining. The resulting products of 180-base pair (bp), a 120 and a 60-bp fragments and 180+120+60-bp fragments represented the “CC”, GG, and CG genotypes, respectively.

QUALITY CONTROL: 30% of samples were randomly selected to be genotyped a second time to ensure reproducibility. Genotyping was performed blinded to clinical status.

Statistical Analysis: We calculated age-adjusted odds ratios (OR) and 95% confidence intervals (CI) associated with the IL-6 genotypes by unconditional logistic
regression analysis. All analyses were conducted with SPSS version 13.0 software for Windows (SPSS Inc. Chicago, IL). The differences in allele frequency among groups were examined for statistical significance with chi-square test by SAS 6.12. All tests were 2-tailed and \( p<0.05 \) was taken to denote significance.

**RESULTS**

The characteristics of the study groups are shown in Table 1. The frequency of the \(-634C\rightarrow G\) genotype in 100 cases with recurrent pregnancy loss were compared with those in 60 controls among an Egyptian population (Table 2). There was no significant difference in the \(-634C\rightarrow G\) genotype frequency (CC vs. CG/GG) between the women with recurrent pregnancy loss and the controls. The risk of recurrent pregnancy loss was the same in the carriers of the G allele and in women with the wild type (CC) (OR 0.737; 95% CI - 0.737–2.735).

We next evaluated the \(-634C\rightarrow G\) genotype in both subgroups of women with three or more pregnancy losses and women with two pregnancy losses (Table 3). We found that the allele frequencies as well as the distribution of genotypes were not significantly different between the study and the control groups. Allele frequencies among women with IRM and controls were 82.5 and 78.3 % respectively for allele C (wild type), and 17.5 and 21.6% respectively for allele G (mutant). No association between allele G and the occurrence of IRM was found (odds ratio 1.304; 95% confidence interval = 0.760–2.299; \( P = NS \)).

**Table 1:** Characteristics of 100 cases with recurrent pregnancy loss and 60 controls in an Egyptian population.

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Cases</th>
<th>Controls</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>No</td>
<td>%</td>
</tr>
<tr>
<td>Age (years)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>20-29</td>
<td>35</td>
<td>35</td>
</tr>
<tr>
<td>30-39</td>
<td>62</td>
<td>62</td>
</tr>
<tr>
<td>&gt;40</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td>Number of previous pregnancy losses</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>42</td>
<td>42</td>
</tr>
<tr>
<td>3</td>
<td>40</td>
<td>40</td>
</tr>
<tr>
<td>&gt;4</td>
<td>18</td>
<td>18</td>
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</table>

<table>
<thead>
<tr>
<th>IL-6 genotype</th>
<th>Cases</th>
<th>Controls</th>
<th>OR (95%CI)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>-634C→G</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CC</td>
<td>65</td>
<td>34</td>
<td>1.420(0.737-2.735)</td>
<td>0.189</td>
</tr>
<tr>
<td>CG</td>
<td>35</td>
<td>26</td>
<td>1.20(0.737-2.735)</td>
<td></td>
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Table 3: Distribution of IL-6 genotypes among cases with 3 or more pregnancy loss and 2 pregnancy losses and controls

<table>
<thead>
<tr>
<th></th>
<th>Cases No</th>
<th>Cases %</th>
<th>Controls No</th>
<th>Controls %</th>
<th>OR (95%CI)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>3 or more pregnancy losses</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CC</td>
<td>35</td>
<td>62.5%</td>
<td>34</td>
<td>56.6%</td>
<td>1</td>
<td>0.326</td>
</tr>
<tr>
<td>CG/GG</td>
<td>21</td>
<td>37.5%</td>
<td>26</td>
<td>43%</td>
<td>1.275 (0.606-2.682)</td>
<td>0.326</td>
</tr>
<tr>
<td>2 pregnancy losses</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CC</td>
<td>25</td>
<td>56.8%</td>
<td>34</td>
<td>56.6%</td>
<td>1</td>
<td>0.574</td>
</tr>
<tr>
<td>CG/GG</td>
<td>19</td>
<td>40.2%</td>
<td>26</td>
<td>43%</td>
<td>1.006 (0.459-2.206)</td>
<td>0.574</td>
</tr>
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</table>

Table 4: IL-6 G634C allele polymorphism: allele frequencies among women with recurrent pregnancy losses and controls

<table>
<thead>
<tr>
<th>Allele</th>
<th>Women with IRM No</th>
<th>Women with IRM %</th>
<th>Controls No</th>
<th>Controls %</th>
<th>Odds ratio (95%)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>C (wild)</td>
<td>165</td>
<td>82.5</td>
<td>94</td>
<td>78.33</td>
<td>1</td>
<td>0.219</td>
</tr>
<tr>
<td>G(mutant)</td>
<td>35</td>
<td>17.5</td>
<td>26</td>
<td>21.66</td>
<td>1.304 (0.760-2.299)</td>
<td>0.219</td>
</tr>
</tbody>
</table>

**DISCUSSION**

Many investigators have assessed possible associations between etiologies of recurrent pregnancy loss and gene polymorphisms, including a family of enzymes responsible for metabolism of environmental toxins, glutathione S-transferase (GST)\(^{30,33}\), and associations between etiologies of recurrent pregnancy loss and the GSTP1\(^{31}\) and GSTM1 polymorphisms\(^{33}\) have been demonstrated. Others sought etiologies in gene polymorphisms susceptible to infection in women with recurrent pregnancy loss; these cytokine genes included tumor necrosis factor-\(\alpha\)\(^{26,33,34}\), interferon-\(\alpha\)\(^{26}\), IL-1\(\beta\)\(^{34,35}\), and anti-inflammatory cytokine IL-10\(^{35,36}\). Among these studies, however, only one demonstrated an increase in the frequencies of IL-1\(\alpha\) promoter region variants IL-1 \(\alpha\) 511C and IL-1 \(\alpha\) -311T in women with recurrent pregnancy loss; a IL-1 \(\alpha\) 511C variant was found to be associated with T\(_{H1}\) overimmunity to trophoblast antigens\(^{24}\).

In the present study, we attempted to establish an association between a polymorphism in the promoter region of the IL-6 gene, known to alter IL-6 protein expression, and the occurrence of IRM. Our hypothesis to test the IL-6 gene as a candidate gene for IRM was based on existing evidence that immunological processes are involved in the pathogenesis of this condition\(^4\).

There are now several well documented instances where nucleotide polymorphisms occur
within the regulatory region of cytokine genes, and some of these are associated with an altered rate of gene expression (8). In addition, women with IRM have been found to carry a polymorphic allele of the IL-1 receptor antagonist gene (IL-1 RN*2) more often than women without a compromised reproductive history (8).

Recently, von Wolff and colleagues found an abnormal expression of IL-6 and IL-1β mRNA in endometrium during the mid-secretory phase in women with IRM (37). Others, reported significantly higher serum concentrations of the Th2 cytokines IL-6 and IL-10 at normal delivery than in women with IRM (38). Furthermore, higher IL-6 levels were found in women with IRM who had a successful pregnancy as compared with women with IRM who had another abortion (38). Thus, the dominance of Th2 cytokines seems to be of importance in maintaining pregnancy. Our study, however, falls short of determining a significant effect of the IL-6 genotype on IRM.

Saijo and colleagues (39) have demonstrated an association between recurrent pregnancy loss and the 634 C to G genotype of the IL-6 gene. They also found that recurrent pregnancy loss risk in the carriers of the G allele was lower than that in the wild type (OR=46). Also, Ota et al. (29) reported frequencies of the IL-6 -634C-G allele in a sample of Japanese women: gene frequencies of the C and G alleles were 0.814 and 0.186, respectively.

During pregnancy, IL-6 serum levels are detectable and increase significantly at the time of delivery (38,40). In those patients with intra-amniotic infection, high intra-amniotic IL-6 levels are detectable (12). Thus, based upon these and our findings, a model of the role of IL-6 during pregnancy can be proposed. In normal pregnancy, IL-6 may be regarded as part of an antiinflammatory mechanism aimed at maintaining pregnancy. Based on our data, there seems to be no effect of IL-6 on IRM. At the time of acute inflammatory responses such as in intra-amniotic infection, IL-6 seems to act as an inducer of acute phase reactions and an important player in the elicitation of cellular immune responses.

**CONCLUSION**

Multiple cytokine polymorphisms were reported to be associated with RSA. However, a majority of studies were not confirmed by other investigators or refuted by others. Inconsistent study results might be related to: (i) the production of these cytokines is partly under genetic controls and other factors affect cytokine levels; (ii) ethnic background, environmental factors, and selection criteria for study populations are different and (iii) the possibilities exist that multiple cytokine gene polymorphisms or other genes in linkage disequilibrium may play a role in RSA.

In summary, this is the first report of a genetic variant of the IL-6 C634→G promoter gene among Egyptian women with IRM. We could demonstrate that the polymorphism at position –634 in the promoter region of IL-6 is not associated with altered risk of IRM in a sample of Egyptian
population. Based on our data, IL-6 seems not to be a candidate gene for that condition.

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الملخص العربي

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قسمى البالطولوجيا الأكلينيكية والكيمية، والتوليد وأمراض النساء. كلية الطب، جامعة القاهره

وصفت السيتوكينات بأنها تلعب دوراً رئيسياً في التسبب في فقدان الحمل المتكرر مجهول السبب. ولذا تهدف هذه الدراسة استقصاء مدى علاقة التعدد الشكلي لجين أنترلوكين ۶ وفقدان الحمل المتكرر مجهول السبب.

تم إجراء هذه الدراسة في قسمى البالطولوجيا الأكلينيكية والكيمية، والتوليد وأمراض النساء بكلية الطب جامعة القاهره، وقد شملت الدراسة 100 رضيعة فقدان الحمل المتكرر مجهول السبب ومجموعة ضابطة ضمت 10 أشخاص لا يعانيون من أمراض ولا يعانون من تعارض مع الدراسة. وقد تم اخذ عينة دم من كل من شاركت في البحث، تم استخلاص الحمض النووي من الدم، واستخدام جهاز الفتق البصلي في معرفة المورثات من الـIL-6 في منطقة المرجع من النترلوكين-۶. وكانت ترددات البيئات النشطة (البري، النوع) بين النساء من عانين فقدان الحمل المتكرر مجهول السبب، ومجموعهالضابطة ۶۲.۸٪، على التوالي، و ۱۷.۶٪ و ۲۱.۶٪ على التوالي. (P = NS).

الاستنتاج: هذا هو التقرير الأول على تعدد الشكل الجيني لانترلوكين ۶ في فقدان الحمل المتكرر مجهول السبب في السعودية المدنية من السكان في مصر.

البحث يشير إلى أن الترددات للأنترلوكين-۶ في النساء الحوامل من مصر، متفاوتة جداً، وتشمل صفات السكان المحلي. وقد أظهرت الدراسة تأثيرًا ثابتًا بين عدوى السبب في مجموعات-P = ۱.۷×۱۰⁻۴، p = ۷۷/۵ و ۲۷۹/۲، p = ۰/۰۲۴. في النتائج، فقدان الحمل المتكرر مجهول السبب، يمكن إجراء مزيد من الدراسات على مجموعة أكبر من السكان، لإيجاد حلول علاجية فعالة في هذا المرض.