The promoter region (−800, −509) polymorphisms of transforming growth factor-β1 (TGF-β1) gene and recurrent spontaneous abortion

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Abstract

Recurrent spontaneous abortion (RSA) is regarded as a common pregnancy complication in southern Iran. The exact causes of RSA are not yet known. Transforming growth factor-β (TGF-β1) is produced by T regulatory lymphocytes (Treg), which play an important role in the physiology of pregnancy. Several polymorphisms of the TGF-β1 gene have been reported, some with important correlation with disease severity. In this investigation, the polymorphism of the TGF-β1 gene at promoter region positions −800 (G/A) and −509 (C/T) was studied in 111 RSA and 110 normal female subjects from southern Iran by PCR-RFLP. Results indicated that at position −800 (G/A) polymorphism, 75.7% of RSA cases and 77.3% of normals were homozygote GG. In addition, 23.4% of cases and 22.7% of normal individuals were heterozygote AG. Only one of the patients appeared to be homozygote AA. None of the normal individuals were found to be homozygote AA at this position. In the case of the −509 (C/T) polymorphism, 38.7% of patients and 28.2% of controls were homozygote CC. While 40.6% of cases and 50.9% of normal individuals were heterozygote CT, 20.7% of RSA cases and 20.9% of controls were homozygote TT. The results indicate that there are no statistically significant
differences of genotype distribution and allele frequency between RSA cases and controls at both polymorphic sites. In conclusion, the promoter region polymorphisms of TGF-β1 at positions −800 (G/A) and −509 (C/T) may not be associated with RSA.

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1. Introduction

Recurrent spontaneous abortion (RSA) is defined by “occurrence of three consecutive pregnancy losses before 20 weeks of gestation, with fetus weighing 500 g or less” (WHO Recommended Definitions, 1997; Garcia-Enguidanos et al., 2002). Approximately one in 300 women worldwide experience RSA. The incidence of RSA among the pregnancies is about 0.5-2%. Although various etiologic factors have been identified, the exact underlying pathophysiologic mechanisms remain elusive in up to 40-50% of cases (Philipp et al., 2003). Studies in murine models have shown that pro-inflammatory cytokines may be detrimental to pregnancy while anti-inflammatory cytokines appear to be advantageous (Wegmann et al., 1993). Also, some women with RSA have increased production of pro-inflammatory cytokines by circulating blood mononuclear cells and decreased levels of anti-inflammatory cytokines (Jenkins et al., 2000; Hefler et al., 2001; Babbage et al., 2001).

Ogasawara et al. (2000) have shown that TGF-β1 level is significantly higher in RSA patients and increased production of this molecule was found to be a risk factor for pregnancy outcome. TGF-β1 is multifunctional cytokine and plays important roles in modulation of cellular growth and differentiation, immunoregulation and extracellular matrix formation (Smith, 1996; Roberts, 1998; Ling and Robinson, 2002; Wennner and Yan, 2003). TGF-β1 is secreted by many cell types, including peripheral blood mononuclear cells and T regulatory lymphocytes. TGF-β1 is synthesized as a latent protein composed of 390 amino acids in which the active molecule is formed by two identical disulfide-linked polypeptide chains consisting of 112 amino acids. The human TGF-β1 gene is located on chromosome 1q13 and contains seven exons (Lawrence, 1996; Clark and Coker, 1998).

Several polymorphisms of TGF-β1 have been reported (Cambien et al., 1996; Awad et al., 1998; Syrris et al., 1998). It has been suggested that certain polymorphisms are associated with an elevated serum level of TGF-β1 (Pelletier et al., 2000; Gewaltig et al., 2002). Other reports indicate that polymorphisms in the promoter region result in an altered transcriptional regulation and thereby influence the development and severity of TGF-β1-related diseases (Cambien et al., 1996; Awad et al., 1998; Gewaltig et al., 2002).

Cytokine gene polymorphisms have previously been studied by several investigators of RSA patients (Hefler et al., 2001; Babbage et al., 2001; Daher et al., 2003). In this investigation, two promoter region polymorphisms of TGF-β1 gene at positions −800 (G/A) and −509 (C/T) were studied in RSA women and normal matched controls.
2. Material and methods

2.1. Subjects

The study group consisted of 111 southern Iranian women (aged 17–38 years, mean 27.2) who had experienced at least three RSA (mean 3.5) and in whom anatomical, hormonal, chromosomal, infectious or autoimmune causes, including anti-phospholipid syndrome, had been excluded. They all attended the Department of Obstetrics and Gynecology Clinic of Shiraz University of Medical Sciences. The control group consisted of 110 ethnic-matched women (aged 22–70 years, mean 38.4) who had at least two children (mean 3.6) and no history of pregnancy losses. The patient and control groups participated in this study after informed consent.

2.2. DNA extraction and TGF-β1 genotyping

Venous blood was collected in EDTA-coated tubes. DNA was extracted from whole blood using the salting-out method. Specific oligonucleotide primers were used as previously described (Luedecking et al., 2000; Cotton et al., 2002). The following primers (MOLBIOL, Germany) were used for amplification of promoter regions of −800 and −509, respectively. Forward primer for position −800: 5′-tgg ggc cga ccg cta tcg, and for reverse primer sequence: 5′-gcc acc cca tac att tac. For the position −509, forward primer: 5′-cag taa atg tat ggg gtc gca g, and the reverse primer sequence: 5′-ggt gtc agt ggg agg agg g. Polymerase chain reaction (PCR) amplification of each polymorphism was performed in a total reaction volume of 20 μl with 300 ng DNA as template. Genotyping was performed by restriction fragment length polymorphism (RFLP) analysis. The following restriction enzymes (Fermentas, Letuvia) were used for the digestion of amplified PCR products. For digestion of PCR products containing position −800, NmuCI and, for the PCR product containing position −509, EcoR I were applied. The digestion conditions were in accordance with the manufacturer’s procedures.

2.3. Statistical analysis

Allele frequencies for each polymorphic site were calculated by allele counting method. Differences in the genotype and allele frequencies between patients and controls were tested by χ² analysis.

3. Results

3.1. Position −800 (G/A)

In this investigation, the change at position −800 (G/A) of the TGF-β1 gene was studied using PCR-RFLP in 111 cases of RSA and 110 normal Iranian female subjects. Results indicated that 84 (75.7%) out of 111 cases, and 85 (77.3%) out of 110 normal subjects, were homozygote GG at this position. In addition, 26 cases (23.4%) and 25 (22.7%) of
normal subjects were heterozygote AG. Only one of the patients appeared to be homozygote AA (Table 1). None of the normal individuals were found to be homozygote AA at this position. There was no statistically significant differences in genotype or allele frequency distributions between RSA patients and controls for the \(-800\) (G/A) polymorphism of the TGF-\(\beta_1\) gene (\(P = 0.808\)). A typical genotyping at this position is represented in Fig. 1a.

### 3.2. Position \(-509\) (G/A)

In addition, the genotype at position \(-509\) (C/T) of TGF-\(\beta_1\) gene was studied in the same study groups. Results indicated that 43 (38.7%) of RSA, and 31 (28.2%) of normal subjects, were homozygotes CC at this position. In addition, 45 (40.6%) of cases and 56 (50.9%) of normal individuals were heterozygote CT. 23 (20.7%) of 111 RSA, and 23 (20.9%) of 110 normal individuals, were homozygote TT. The results indicated that there was no statistically significant difference between RSA cases and normal female individuals in respect to genotype distribution and allele frequency at this position (\(P = 0.208\)). The distribution of TGF-\(\beta_1\) genotype and allele frequency in cases and controls are summarized in Table 1. A typical genotyping at position \(-509\) (C/T) is represented in Fig. 1b.

As shown in Table 1 in the case of \(-509\) (C/T) is represented in Fig. 1b.

A Hardy–Weinberg equilibrium test was performed for the two polymorphisms. The distribution of observed genotypes were not significantly different from the expected distributions according to Hardy–Weinberg equilibrium in both the patient and control groups.

### Table 1

<table>
<thead>
<tr>
<th>Genotype</th>
<th>RSA patients, (N = 111) (%)</th>
<th>Controls, (N = 110) (%)</th>
<th>(P)-value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>(-800)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GG</td>
<td>84 (75.7)</td>
<td>85 (77.3)</td>
<td>0.808</td>
</tr>
<tr>
<td>GA</td>
<td>26 (23.4)</td>
<td>25 (22.7)</td>
<td></td>
</tr>
<tr>
<td>AA</td>
<td>1 (0.9)</td>
<td>0 (0)</td>
<td></td>
</tr>
<tr>
<td><strong>Allele</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>G</td>
<td>0.874</td>
<td>0.886</td>
<td>0.712</td>
</tr>
<tr>
<td>A</td>
<td>0.126</td>
<td>0.114</td>
<td></td>
</tr>
<tr>
<td><strong>(-509)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CC</td>
<td>43 (38.7)</td>
<td>31 (28.2)</td>
<td>0.208</td>
</tr>
<tr>
<td>CT</td>
<td>45 (40.6)</td>
<td>56 (50.9)</td>
<td></td>
</tr>
<tr>
<td>TT</td>
<td>23 (20.7)</td>
<td>23 (20.9)</td>
<td></td>
</tr>
<tr>
<td><strong>Allele</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C</td>
<td>0.59</td>
<td>0.536</td>
<td>0.255</td>
</tr>
<tr>
<td>T</td>
<td>0.41</td>
<td>0.464</td>
<td></td>
</tr>
</tbody>
</table>

*Value are shown in absolute numbers (percentages).*
Fig. 1. PCR-RFLP genotyping of three individuals (duplicate). (a) Position −800 (G/A). PCR product (315 bp) was digested by "NnuCI restriction enzyme. Lane 1–2, homozygote GG; lane 3–4, homozygote AA; lane 5–6, heterozygote GA and lane 7, DNA size marker. (b) Position −509 (C/T). PCR product (153 bp) was digested by "EcoR1 restriction enzyme. Lane 1–2, homozygote TT; lane 3–4, homozygote CC; lane 5–6, heterozygote CT and lane 7, DNA size marker.
4. Discussion

A reduction of pro-inflammatory cytokines by T helper cells, and a Th1/Th2 deviation, has been reported in healthy pregnant women, a circumstance which may be absent in women with RSA (Wegmann et al., 1993). Murine studies indicate that dominance of Th1-type dependent cytokines, e.g. IL-2, TNF-α and IFN-γ, is incompatible with successful pregnancy (Wegmann et al., 1993). Reports of elevated cytokine levels, including IL-1β, in women with RSA are in accordance with these experimental data (Jenkins et al., 2000).

TGF-β1 is an immunoregulatory, and predominantly immunosuppressive, cytokine. This cytokine may contribute to regulation of maternal immune responses directed against the fetal allograft, and thereby prevent immunological rejection of the fetus. In addition, all three isoforms of TGF-β1 are found in pregnancy-associated sites and have numerous potential roles during the gestation. Studies have shown that TGF-β1 negatively regulates the primary immune response induced by Th1 but not Th2 memory cells. In maternal circulation, TGF-β1 has a primary function to limit immune responses and favor a Th2 memory response, thereby explaining the postulated Th2 bias at this time (Wegmann et al., 1993; Power et al., 2002). Studies have shown TGF-β1 may be involved in reproductive-related problems, such as pre-eclampsia and RSA, but existing data are controversial (Ogasawara et al., 2000; Benian et al., 2002; Huber et al., 2002).

Three polymorphisms have been reported in the TGF-β1 promoter region at positions −988, −800 and −509 (Cambien et al., 1996; Awad et al., 1998). It has been reported that the −509 (C/T) polymorphism is significantly associated with the plasma concentration of TGF-β1 (Grainger et al., 1999). Some studies indicate that polymorphisms in the promoter region of this cytokine result in altered transcriptional regulation and thereby might influence the development and severity of TGF-β1-related disease. The −800 (G/A) substitution is thought to disrupt a consensus half-site for binding of the nuclear transcription factor CRE-binding protein, consequently contributing to a lower production of total TGF-β1 in the circulation (Syrris et al., 1998). On the other hand, the T allele of the −509 (C/T) polymorphism has been reported to be associated with a higher transcriptional activity (Laudecking et al., 2000) and therefore higher production of total and active TGF-β1 (Syrris et al., 1998; Grainger et al., 1999).

In our study, the genotype distribution and allele frequencies of polymorphisms at position −800 (G/A) (P = 0.808) and −509 (C/T) (P = 0.208) were not significantly different between RSA patients and controls. However, the −509T allele frequency in controls was higher than patients and this may be consistent with the finding of Grainger et al. (1999) in which individuals carrying T allele at this position, found to be TGF-β1 high producer. Previous studies of cytokine gene polymorphisms in RSA patients have presented controversial results. Helfer et al. (2001) have shown that an IL-1β polymorphism at exon 5 (position +3953) is not associated with RSA. In the case of pro-inflammatory and anti-inflammatory cytokine gene polymorphisms, significant differences were not detected between patients and controls (Babbage et al., 2001; Karhu-Korpi et al., 2001). Contrary to the above finding, Daher et al. (2003) observed an association between IFN-γ and IL-10 gene polymorphism and RSA in a meta-analysis.

Studies of TGF-β1 promoter region polymorphisms in other immunologic diseases have produced different results. Pulley et al. (2001) reported that asthma severity is associated
with the T allele of the −509 polymorphism. Schulte et al. (2001) have shown that the TGF-β1 polymorphism at position −509 (C/T) is not associated with Crohn’s disease. It has been reported that genetic variants of the TGF-β1 gene do not contribute to susceptibility to multiple sclerosis (Weinshenker et al., 2001). The results of this investigation conclude that there are no significant differences between TGF-β1 polymorphisms at positions −800 (G/A) and −509 (C/T) in southern Iranian RSA patients and an ethnic-matched normal population. This conclusion is based on using two known SNP regions of TGF-β1 gene promoter which has been broadly investigated in other autoimmune diseases. We are currently exploring other known SNPs of the TGF-β1 gene in RSA, and also searching for possible TGF-β1 variants in a large Iranian normal population using direct sequencing. In case of finding new SNPs in Iranian ethnic subjects, the DNA samples of the current study will be subjected to reanalysis for possible association of TGF-β1 and RSA susceptibility.

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References


