Polymorphisms in the Interleukin-12/18 Genes and Recurrent Spontaneous Abortion

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Problem
Interleukin (IL) IL-12/IL-18 are involved in uterine NK cells control of uterine vascular development. Polymorphisms in the IL-12/IL-18 genes could modify the cytokine balance, which might result in an increased susceptibility to recurrent spontaneous abortion (RSA).

Method of Study
A case–control study was conducted to determine the association between the IL12 (I/D) and IL18 (*−607C>A*, *−137G>C*) gene polymorphisms and the risk of RSA in 125 women with RSA and in 136 controls.

Results
The frequencies of DD, ID, II for IL-12 were, 25.6%, 52.8% and 21.6% respectively, in patients *versus* 21.3%, 51.5% and 27.2% respectively in controls; the frequencies of CC, CA, AA genotypes for IL-18 (−607) were, 34.4%, 54.4% and 11.2% respectively in patients *versus* 30.1%, 58.1% and 11.8% respectively in controls; the frequencies of GG, GC, CC genotypes for IL-18(−137) were 47.2%, 43.2% and 9.6% respectively in patients and 45.6%, 46.3% and 8.1% respectively in controls.

Conclusion
IL-12B and IL-18 promoter gene polymorphisms were not associated with RSA in our women.

Introduction

Recurrent spontaneous abortion (RSA), the repetition of three or more consecutive abortions before the 24th week of pregnancy, occurs in approximately 0.5–3% of reproductive-aged women.[1] Despite several well established etiological factors, the cause of RSA cannot be determined in almost 50% of cases. Immunologic factors might be involved in the etiology as the distribution of the immune cells and the production of cytokines appear to be critical for pregnancy outcome.[2–4] Maternal genetic determinants might modify the balance in cytokines' profile and thus influence the susceptibility to abortion.

The maternal–fetal relationship is not simply maternal tolerance to a foreign tissue, but a series of intricate mutual cytokine interactions governing selective immune regulation and also control of the adhesion and vascularization processes during this dialogue.[5] Studies in murine abortion models have shown that pro-inflammatory Th1 cytokines may be detrimental to pregnancy, whereas Th2 cytokines suppress the cellular responses that may endanger

the fetus and thus improve fetal survival.[6] Recently, it has been suggested[5,7] that the Th1/Th2 paradigm, as useful as it has been to explain pregnancy, is no longer sufficient in view of the emerging complexity of the cytokine network at the materno-fetal interface.

There are several studies on peripheral blood or endometrial Th1/Th2 cytokine profiles in women with RSA; some support the Th1/Th2 hypothesis,[8–11] while others show conflicting results.[12,13]

The IL-12/IL-18 cytokine system seems to regulate the maternal-embryo interactions during the implantation process in a stage-specific fashion and is expressed in murine and the human uterus.[14] It was also suggested that uterine NK cells (uNK) may have a role in the control of implantation and transformation of uterine vasculature.[15,16] It was hypothesized that some cases of recurrent implantation failure are probably related to the absence of an adequate vascular remodelling,[17] especially in the case of lack of IL-18 and angiopoietin 2 (Ang2) expression, leading to incomplete spiral artery transformation, and thus inducing an inadequate uterine receptivity, whereas too high IL-12 secretion is probably abortifacient because it promotes or co-induces (with IL-18) activation of the uNK cells, engaging them towards a cytotoxic/cytostatic pathway, overriding the physiological control of their cytokine production by KIR/trophoblast MHC interaction, and disregulating their IFN-gamma production.[14] Interestingly, both cytokines were apparently required at lower doses for the successful development of local vascular remodelling.[18] These data suggest that an abnormal cytokine profile might cause defective local vascularization and affect trophoblast cell growth and function.

Furthermore, we have demonstrated the importance of the role of IL-12/18 in the murine model of abortion.[19] These data suggest that abnormal cytokine profiles might cause defective local vascularization and affect trophoblast cell growth and function. IL-12 has an important immunoregulatory function, as it promotes Th1 cells/cytokines differentiation with downregulation of Th2 cytokines. IL-12 acts as an NK cytotoxicity activator, IFN-γ inducer and inhibitor of angiogenesis.[20] IL-12 is a dimer composed of IL-12A subunit encoded by gene located on 3p12-q13.2 (OMIM 161560) and IL-12B subunit, encoded by gene located on 5q31.1-q33.1 (OMIM 161561). In the promoter region of the IL-12B gene, an insertion/deletion polymorphism has been identified.[21] Interleukin-18 is a proinflammatory cytokine, which promotes a Th1 response, primarily by its ability to induce IFN-γ production.[22,23] IL-18 is encoded by a gene located on 11q22.2-q22.3 (OMIM 600953). Two polymorphisms in the promoter region (−607C>A, −137G>C) have been identified and IL-18 expression analyses showed that the presence of wild type alleles had higher levels of IL-18 mRNA compared with other alleles.[24]

The aim of this study was to test the hypothesis that genetic variabilities in the *IL-12* and *IL-18* genes are risk factors for RSA in humans. For this purpose, we analyzed gene polymorphisms in the IL-12B and IL-18 (−607 and −137) in a group of 125 Caucasian women with RSA and in 136 controls without any previous record of pregnancy loss.

Methods

Subjects

The study group consisted of 125 Caucasian unrelated women with a history of three or more spontaneous abortions before the 22nd week of gestation of unexplained etiology with the same partner ascertained through the Division of Medical Genetics, Department of Obstetrics and Gynecology in Ljubljana. Karyotypes were normal, at a 500 level band of resolution, in women with RSA and their male partners. Women with a history of endocrine or metabolic disorder, autoimmune disease, venous thrombosis, or uterine anatomical abnormalities, were excluded from the study. The control group comprised 136 age-matched unrelated women, with at least two live births, and no history of pregnancy loss. Informed consent was obtained from all participants. The study was approved by National Ethics' Committee.

Genotyping

Genomic DNA was extracted from peripheral white blood cells using a commercially available kit (Qiagen® FlexiGene DNA kit; Qiagen GmbH, Hilden, Germany). Polymerase chain reactions were performed to analyze polymorphisms in the interleukin 12, using primers IL12-P8F TGGATTGTGAAGTGGG ACAT and IL12-P7R TAATGTGGTCATTGGCAGGT, as described,[21] with minor modifications. Briefly, the PCR products of interleukin 12B promoter polymorphism were run on Spreadex® gels EL600 Mini (Elchrom Scientific AG, Cham, Switzerland) for

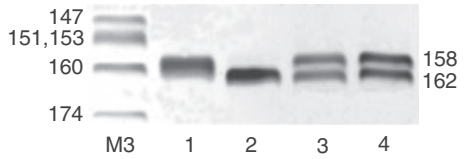

Fig. 1 The electrophoresis image of the PCR products for IL12 polymorphism on Spreadex gel EL600; the sizes of the PCR products (158 bp, 162 bp) were determined in comparison with DNA molecular weight marker M3 (Elchrom Scientific AG, Switzerland) in bp on the left side of the image. Genotypes of the patients are as follows: 1 – DD, 2 – II, 3,4 – ID.

83 min at 55°C and 120 V according to the manufacturer's recommendations (available at: http://www.elchrom.com/technic/manuals/asp, ELQuant™ software), stained by SYBR Gold (Molecular Probes, Inc., Leiden, the Netherlands) and visualized under the UV light (Fig. 1). The PCR products of interleukin 12B insertion/deletion polymorphism were 162 bp (allele I) and 158 bp (allele D). To analyze polymorphisms in the interleukin 18, polymerase chain reactions were performed as described previously[24]. The PCR products of interleukin 18 promoter polymorphisms IL18 (*−607C>A*) and (*−137 G>C*) were analyzed by electrophoresis on 2% agarose gels (Sigma Chemical Co., St Louis, MO, USA) containing ethidium bromide (0.1 mg/mL) and visualized under the UV light (Figs 2 and 3).

Statistic Analysis

Based on the number of cases in the study group (125) and control subjects (136), the statistical power was 80% to find a 1.3-fold difference in the frequency of *IL12B* risk genotypes (percentage of carriers in control group was set at 51.5%), For the *IL18 (−607, −137)* risk genotypes (percentage of carriers in control subjects was set at 16% and 11%, respectively), the statistical power to find a twofold difference in frequency was 80%.

The χ^2 test was used to compare the genotype distributions for the polymorphisms with those expected if the alleles were in Hardy–Weinberg equilibrium. Computations were carried out with the SPSS statistical package 13.0 for Windows (SPSS Inc., IL, USA). Allele and genotype frequencies were compared between groups using the χ^2 test. *P*-values less than 0.05 were considered statistically significant.

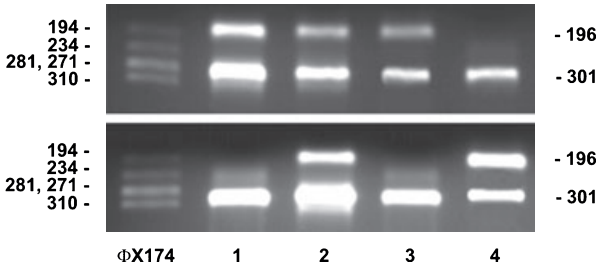

Fig. 2 The electrophoresis image of the PCR products for IL18 (−607C>A) polymorphism with sequence specific primers (196 bp) for allele C (bottom line) and allele A (upper line) and internal positive control (301 bp); the sizes of the PCR products were determined in comparison with DNA molecular weight marker V (MV, Roche Diagnostics, GmbH, Mannheim, Germany) in bp on the left side of the image. Genotypes of the patients are as follows: 1,3 – AA, 2 – CA, 4 – CC.

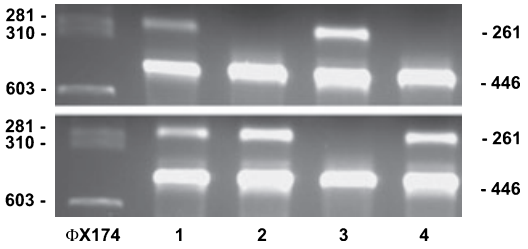

Fig. 3 The electrophoresis image of the PCR products for IL18 (−137G>C) polymorphism (261 bp) with sequence specific primers for G (bottom line) and C allele (upper line) and internal positive control (446 bp); the sizes of the PCR products were determined in comparison with DNA molecular weight marker V (MV, Roche Diagnostics, USA) in bp on the left side of the image. Genotypes of the patients are as follows: 1 – GC, 2,4 – GG, 3 – CC.

Results

The characteristics of the women with RSA are shown in Table I. The frequencies of the IL12B, IL18 (*−607C>A*) and IL18 (*−137G>C*) promoter insertion/deletion polymorphisms in 125 women with RSA were compared with those in 136 controls in our population. There were no differences in the distribution of tested polymorphism frequencies between the group of women with RSA and controls (Table II).

The analyses of combined risk genotypes of the interleukin promoter polymorphisms showed no association with RSA, nor for the IL-12 (DD) and *IL-18 (−607AA)*.

[χ^2 (1) =0.087, P = 0.766] nor for the IL-12 (DD) and IL-18 (*−137CC*) [χ^2 (1) =0.049, P = 0.824].

Table I Characteristics of the Studied Women with Recurrent Spontaneous Abortion (RSA)

	Cases	
Characteristics	*n*	(%)
Age (years)		
20–29	33	(26.4)
30–39	77	(61.6)
≥40	15	(12.0)
Number of RSA		
3	107	(85.6)
4	11	(8.8)
≥5	7	(5.6)

Table II Distribution of Genotype Frequencies for the IL-18 and IL-12 Polymorphisms in Women with SA and in Controls

Polymorphism	RSA (125), *n* (%)	Controls (136), *n* (%)	*P*-value
IL-18 (−607)			
CC	43 (34.4)	41 (30.1)	0.763
CA	68 (54.4)	79 (58.1)	
AA	14 (11.2)	16 (11.8)	
IL-18 (−137)			
GG	59 (47.2)	62 (45.6)	0.841
GC	54 (43.2)	63 (46.3)	
CC	12 (9.6)	11 (8.1)	
IL-12B			
DD	32 (25.6)	29 (21.3)	0.505
ID	66 (52.8)	70 (51.5)	
II	27 (21.6)	37 (27.2)	

Discussion

We found no significant association between IL-12 (ins/del) and IL-18 (−607 and -137) gene promoter polymorphisms nor between the combined risk genotypes for *IL-12 (DD)* and *IL-18* (*−607AA* and *−137CC*) and susceptibility to RSA in our studied women.

The possible explanation for our non-significant finding could be that the association between IL-12/18 gene polymorphisms and RSA is too small to be detected with our sample size (the power of the study was 80% to detect a 1.3-fold difference in the frequency of *IL12B* risk genotypes and twofold difference in the frequency of *IL18 (−607* and *−137)* risk genotypes). Additional studies comprising of larger numbers of women with RSA from different populations are needed to exclude possible minor effects of the IL-12 or IL-18 gene polymorphisms and RSA susceptibility.

Nevertheless, several lines of evidence prompted us to test the IL-12 and IL-18 genes as candidate genes for RSA susceptibility.

First, it has been discovered, that the expression profiles of IL12/18 in women with repeated implantation failure markedly differed when compared to a standard profile in the control group of fertile women and that abnormal cytokine profiles correlated with abnormal angiogenesis.[18]

Second, in the murine abortion model (CBA × DBA/2 mating combination), it has been shown that IL-12/18 were expressed at lower/different levels in feto-maternal interface compared to 'non-abortion' mating combination (CBA × BALB/c).[25]

Third, as a major cytokine inductor of the Th1 immune response, IL-12 (ins/del) genetic variant might be involved in abnormal uterine vascularization, which affects growth and function of trophoblast cells.[14] Moreover, both wild type alleles of the single nucleotide polymorphisms in the promoter region of the IL-18 gene (at positions −607 and −137) result in higher IL-18 transcription activity of the gene[24] and might shift the polarization to Th1 cytokine response, which is thought to be disadvantageous for pregnancy outcome.[24,26]

Our results confirmed and extended the recent report[27] in which no association between the IL-18 gene promoter polymorphisms (at positions −607 and −137) and susceptibility to RSA in Southern Iranian patients was found. In addition, it seems that no differences exist between the Iranian and Slovenian groups as regards IL-18 gene polymorphism distribution. Such a lack of association of the IL-18 gene promoter polymorphism with RSA may be explained by the significant effect of other modifier genes on IL-18 behavior.[27] In women with a RSA history, who miscarried again in the next pregnancy, the pre-pregnancy IL-18 levels were significantly higher than those in women who had a successful pregnancy.[28] Likewise, we have not found any association between IL12B ins/del gene polymorphism and women with RSA. To the best of our knowledge this is the first report on the common IL-12 promoter genetic variant in women with RSA. Other studies analyzing the expression profiles of IL-12 observed a significant association between higher levels of IL-12 in peripheral blood and endometrium in women with RSA.[28,29]

The crucial question is what are the boundaries/fine lines between 'enough' or 'too much' IL-12/IL-18 production in a normal pregnancy.[30] It has been demonstrated that IL-18 is positively involved in the implantation process in mice[19] and in humans.[14] Moreover, IL-18 may be involved in termination of the pregnancy by shifting the Th1/Th2 balance to a Th1 dominant state. IL-18 is considered a unique cytokine with a capacity for both Th1 and Th2 polarization depending on the genetic background.[31] The presence, quantity and ratio of IL-12 level might therefore determine whether IL-18 shifts the immune systems towards a Th-1 type profile or towards Th-2 cell differentiation.[32]

Thus, distribution of IL-12/IL-18 genetic variants should be regarded in the context of other key molecules in pregnancy, as well as their gene polymorphisms and other modifier genes.

In conclusion, we provide evidence that the genetic variation in the IL12B and IL18 promoter genes is not significantly associated with RSA in Slovenian women.

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