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RESEARCH ARTICLE

Association between lower frequency of R381Q variant (rs11209026) in IL-23 receptor gene and increased risk of recurrent spontaneous abortion (RSA)

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Abstract

Recurrent spontaneous abortion (RSA) is defined as three or more consecutive spontaneous abortions before the 20th week of gestation. The purpose of the present study was to investigate the association between a functional single nucleotide polymorphism (SNP) in the interleukin (IL)-23 receptor gene (*IL-23R*; rs11209026, 1142 G wild type → A reduced function, Arg381Gln, R381Q) and RSA. For the study, 200 RSA patients (confirmed using established diagnostic criteria) and 200 normal individuals in fertility and infertility centers in the cities of Yazd and Isfahan were recruited during a period from 2012–2013. Using PCR-RFLP, the R381Q variant was screened for in the *IL-23R* gene of the patients and controls. The results indicated there were significant differences in the frequency of this genetic variant in the patients versus the healthy controls, i.e. 2% and 7.5%, respectively (p value = 0.01; odds ratio = 0.25; CI = 95%). No significant difference was found for the G allelic frequency in patients with RSA and in the control group ($p = 0.60$). The A allelic frequency was significantly different between the two groups ($p = 0.01$). Based on these findings, it is concluded that the frequency of single nucleotide polymorphism in the IL-23 receptor (R381Q) in patients with recurrent spontaneous abortion (RSA) is less than that found in normal control women.

Keywords

IL-23R, R381Q, recurrent spontaneous abortion

History

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Introduction

Spontaneous abortion is a frequent complication of pregnancy. Recurrent spontaneous abortion (RSA), defined as the occurrence of three or more sequential spontaneous abortions before the 20th week of gestation, occurs due to several identifiable causes, i.e. anatomic, genetic, and infectious etiologies. However, a major proportion of RSA (50–60%) is due to unexplained etiologies (Dehghani Firoozabadi et al., 2006; Ghasemi et al., 2010; Liu et al., 2010; Raghupathy, 2003). Recently, the potential roles of cellular immune effectors and cytokines in RSA have been the focus of intense investigation. It appears certain cytokines may be beneficial to pregnancy (cytokines derived from T-helper [T_H] type-2 cells), while some are actually antagonistic to pregnancy (cytokines derived from T_H1 and T_H17 cells) (Larid et al., 2003; Liu et al., 2010; Piccinni, 2006; Raghupathy, 2003).

It is well known that pro-inflammatory cytokines such as interleukin (IL)-1, tumor necrosis factor (TNF)-α, IL-6, IL-17 (cytokines produced by immune cells, decidua, and trophoblast cells of placenta) exert an adverse effect on pregnancy (Kwak-Kim et al., 2009). Accordingly these cytokines have been proposed as candidate agents involved in the pathogenesis of RSA (Pongcharoen et al., 2007; Wang et al., 2010b). Human T_H17 cells producing IL-17 may play a major role in rejecting conceptus antigens and, therefore, also may be harmful to the maintenance of pregnancy (Fina et al., 2008; Lee et al., 2012; Peng et al., 2010; Safrany et al., 2013; Sheibanie et al., 2007).

Pro-inflammatory IL-23 is composed of two subunits, i.e. p19 and p40. The p40 subunit is shared with IL-12, a key cytokine that induces development of T_H1 cells (Duvallet et al., 2011; Lee et al., 2012; Wang et al., 2010a). The pro-inflammatory features of IL-23 have been linked with T_H17 cell responses through the expansion and maintenance of T_H17 cells (Gyulveszi et al., 2009; Guan et al., 2012; Hedrick et al., 2009; Peng et al., 2010; Sheibanie et al., 2007). IL-23 is also involved in the differentiation and maintenance of $CD4^+$ T_H17 and $CD8^+$ T_C17 cells (Sarin et al., 2011). As such, IL-23 has been recognized as a central cytokine in autoimmunity and a highly promising target for treatment in inflammatory diseases, including potentially RSA.

IL-23 acts by a specific receptor-complex, IL23 receptor (IL-23R). The *IL-23R* gene is located on chromosome 1p31, and the encoded protein including two subunits that form the receptor, together with the B1 subunit of the IL-12 receptor (IL-12RB1).

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These two subunits comprise the IL23R receptor-complex (Safrany et al., 2013). Upon ligand binding, JAK2 phosphorylates IL23R at Tyr705, thereby recruiting STAT3. JAK2 then phosphorylates STAT3 which then homodimerizes and translocates to the nucleus and triggers expression of cytokines, including IL-17A and IL-17F in T_H17 cells. Thus, IL-23 acts as a mediator of T_H17 cytokine production (Pidasheva et al., 2011; Safrany et al., 2013).

Recent studies have shown that a functional single-nucleotide polymorphism (SNP) (Arg381Gln;R381Q; rs11209026; 1142 G wild type → A reduced function) in the *IL-23R* gene led to decreased IL-23-dependent IL-17 production relative to values in cells from wild-type (WT) IL-23R individuals. This SNP was also associated with a lower percentage of circulating T_H17 and T_C17 cells. Further, R381Q $CD8^+$ T-cells underwent decreased IL-23- and STAT3-dependent expansion and STAT3 activation as compared to that by the WT cells. The protective R381Q IL23R variant leads also to selective loss of function in primary human $CD4^+$ and $CD8^+$ T-cells (Guan et al., 2012).

Some studies have indicated that R381Q variants confer protection from inflammatory diseases, including ankylosing spondylitis, psoriasis, and inflammatory bowel disease (Liu et al., 2010; Pidasheva et al., 2011). With this regards, because RSA is related to inflammatory factors (IL-17 and IL-23) and T_H17 cells and the R381Q SNP can act as a regulator of inflammation, it occurred to us that there could be a relationship between expression of this SNP and the incidence of RSA in women. Indeed, *IL-17*, *IL-23*, *STAT3*, and *RORC* (RAR-related orphan receptor C, encoded by *RORγt* (RAR-related orphan receptor gamma transcription factor) gene) mRNA expressions were all significantly higher in a group of RSA women than in normal pregnant women (Wang et al., 2010b). To date, there have been no studies evaluating the frequency of the IL-23R Arg381Gln polymorphism in patients with RSA. Thus, the aim of the present study was to evaluate of frequency of functional R381Q variants in the *IL-23R* gene of normal and RSA women and to determine if there was any association between functional R381Q variant expression in this gene and the occurrence of RSA.

Materials and methods

Patients and controls

For this case-control study, individuals were recruited from the Fertility and Infertility Centers in the cities of Yazd and Isfahan during the period from 2012–2013. For the study, 200 RSA patients (confirmed using established diagnostic criteria) and 200 normal individuals (with no history of abortion) were recruited. The patients did not have any other known medical conditions apart from the RSA. Male partners underwent semen analyses and were found to be normal. From each patient/control subject, the following data was obtained: age, age at each spontaneous abortion, number of spontaneous abortions, time of spontaneous abortion during pregnancy, and occurrence of bleeding and pain during the event. The Ethics Committee of the Shahid Sadoughi University of Medical Sciences approved this study; all patients/subjects provided informed consent.

Genotyping of the Arg381Gln IL-23R variant

To provide materials for analysis, each study subject provided a sample of blood. Specifically, 2 ml of fasting blood was collected into EDTA-coated Vacutainer tubes. The timing of the sample collection among the RSA patients was that the previous RSA episode had to have occurred within 3 months prior to the sample collection.

Molecular analyses were performed using DNA extracted from peripheral blood leukocytes. These cells were isolated from each sample by density centrifugation over Ficoll-Hypaque. Once the leukocytes had been isolated, a salting-out protocol was used to isolate the sample DNA (Miller et al., 1988). The quality of each sample of DNA was confirmed spectrophotometrically and then the R381Q polymorphism was genotyped using a polymerase chain reaction (PCR) followed by a restriction fragment length polymorphism assay (PCR-RFLP). The latter tested for the R381Q polymorphism (GenBank NW 921351) using the primers 5′-CTTTT-CTGGCAGGGTCATTTTG-3′ (sense) and 5′-AAGT TGTTTCCTGGGGTAGTTGTG-3′ (anti-sense).

PCR was performed with a DNA Polymerase Master Mix kit (Bie & Berntsen AS, Åbyhøj, Denmark) in a 96-well plate using a programmable thermal cycler (ABI; Foster City, CA) at 94 °C for 5 min, followed by 30 cycles at 94 °C for 30 s, at 55 °C for 30 s, 72 °C for 60 s, and 72 °C for 5 min. Each total 25 µl reaction system contained: 1 µl forward primer (5 µM), 1 µl reverse primer (5 µM), 12.5 µl MasterMix 2X, 2 µl DNA template, and 8.5 µl distilled water. The 508-bp PCR product was then resolved over a 1% agarose gel and the quality was confirmed using ethidium bromide stain and a UV transiluminator (Figure 1). For the RFLP assay, Hpy188i restriction endonuclease (New England Biolabs, Beverly, MA) was used. Specifically, 10 µl of PCR product were digested with 10 U Hpy 188i in a 2 h period at 37 °C. The digestion of a PCR product containing the R381Q polymorphism would result in 82, 103, and 323 bp bands (with a heterozygous pattern [AG]); conversely, presence of the wild-type pattern in the gene would be indicated by the presence of 35, 82, 103, and 288 bp digestion products (Figure 2).

Statistical analysis

All data were analyzed using a Chi-square test in the presence and absence of R381Q IL-23R–expressing individuals. Odds ratio values were calculated with a confidence interval (CI) of 95%. A *t*-test was used to compare the mean ages in the RSA subject and control groups. All data were processed using SPSS 16 software (IBM, Armonk, NY). Significance was assigned when p values < 0.05 were obtained.

Results

The analyses showed there was no significant ($p = 0.40$) difference between mean ages in the case (RSA) and control groups. Mean age in the case women was 35.3 [±5.8] years (range = 19–43), while mean age in the control women was 34.9 [±3.2] years (range = 20–41).

The frequency of R381Q variant in the case and control group, and the association between R381Q variant and recurrent spontaneous abortion was also investigated. A significant difference was found in the presence of the genetic variant between the

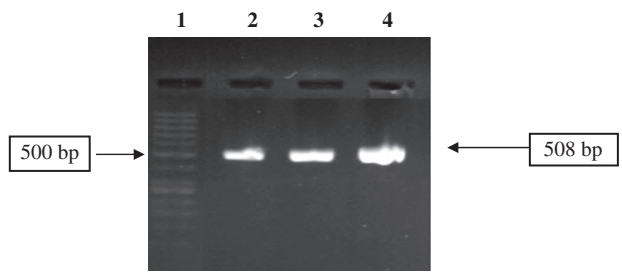

Figure 1. PCR assay for R381Q variant in IL-23R (G1142A). Agarose gel electrophoresis from case subjects showing the 508 bp [base pair] PCR amplicons of *IL-23R* gene. Lane 1: 50 bp DNA molecular weight marker. Lanes 2, 3, and 4: samples.

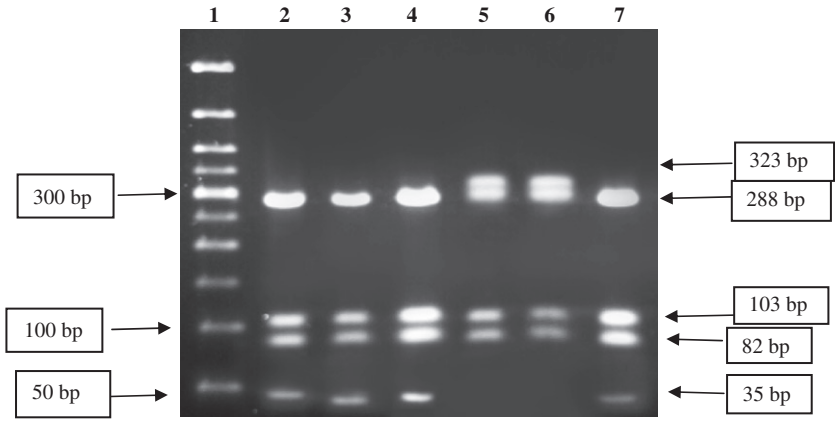

Figure 2. RFLP assay for R381Q variant in IL-23R (G1142A). Electrophoretic pattern of RFLP after digestion with Hpy 188I from individual subjects. Lane 1, 50 bp [base pair] DNA molecular weight marker. Lanes 5 and 6: samples with heterozygous patterns (381Q; AG; 323, 103, and 82 bp). Lanes 2, 3, 4, and 7: samples with wild-type pattern (homozygote pattern; GG; 288, 103, 82, and 35 bp).

case and control groups. Specifically, 2% of RSA patients had the R381Q variant, while the control value was 7.5% ($p = 0.01$; odds ratio = 0.25; CI = 95%). When the frequency of the alleles was compared in the two groups, no significant differences were found for the G allelic frequency in patients with RSA and in the controls ($p = 0.60$). However, the A allelic frequency was significantly different between the two groups ($p = 0.01$). Genotypes and allelic frequencies of R381Q for the case and control groups are presented in Table 1.

The results also indicated there was a significant inverse correlation between time of spontaneous abortion during pregnancy and number of spontaneous abortions ($p = 0.002$, Pearson correlation = −0.22). This meant the highest number of spontaneous abortions inversely correlated with the earliest timepoints of spontaneous abortion. Clinical characteristics of the studied patients are shown in Table 2. There was no significant correlation between presence of the R381Q variant and the variables shown in Table 2. The case and the control groups were compared due to the presence of R381Q variant (Figure 3).

Table 1. Genotypes and allelic frequencies of R381Q for the case and control groups.

Type of genotype/allele	Case group	Control group
AA	0 (0%)	0 (0%)
GG	196 (98.0%)	185 (92.5%)
AG	4 (2.0%)	15 (7.5%)
Sum	200 (100%)	200 (100%)
A	4 (1.0%)	15 (3.8%)
G	396 (99.0%)	385 (96.2%)
Sum	400 (100%)	400 (100%)

Table 2. Clinical characteristics of patients with RSA.

Patients ($n = 200$)	n (%)
Number of spontaneous abortions	
3	125 (62.6%)
4	55 (27.6%)
5	20 (9.8%)
Time of abortion (week of gestation)	
1–5	83 (41.5%)
6–10	90 (45.0%)
11–15	22 (11.0%)
16–19	5 (2.5%)
Bleeding/pain during the event	
Yes	160 (80%)
No	40 (20%)

Discussion

It is well known that pro-inflammatory cytokines exert an adverse effect on pregnancy (Kwak-Kim et al., 2009) and that there are mutations in genes for certain pro-inflammatory cytokines (Liu et al., 2010). Normally, during conception/pregnancy, the normal dominant T-helper (T_H)-1 inflammatory immune response (tumor necrosis factor [TNF]-α, interferon [IFN]-γ) is switched to a T_H2 interleukin (IL-4, IL-10)-driven immune response that permits induction of maternal immune tolerance to the allogenic fetus and assures successful implantation and reproductive outcomes (Kwak-Kim et al., 2009; Soares, 2004).

Previous studies showed that the proportions of T_H17 ($CD4^+IL\text{-}17A^+$) cells in peripheral blood and decidua were significantly higher in unexplained RSA subjects than in normal pregnant women (Mandal et al., 2011). In fact, levels of T_H17 cells were relatively enriched in the decidua of unexplained RSA patients, suggesting these cells may play a key role in the maternal–fetal local immunological rejection (Lee et al., 2012).

IL-17 secreted by T_H17 cells is also suggested to have a major role in angiogenesis and immune regulation (Numasaki et al., 2003). IL-17 is localized in both cyto- and syncytio-trophoblasts (Pongcharoen et al., 2007) and expression of IL-17A by $CD4^+$ T- cells is significantly higher in RSA than in normal pregnant women (Liu et al., 2011; Ng et al., 2002). In the context of pregnancy, T_{reg} and T_H17 cells are two lymphocyte subsets with opposing actions. In normal pregnancy, T_{reg} cells prevent generation of an immune response against fetal tissue and a decrease in the number of T_{reg} cells is associated with abortion (Saito et al., 2005). In contrast, T_H17 cells promote inflammation and increased levels of T_H17 cells – accompanied by a decrease in T_{reg} cell levels – were reported to occur in association with unexplained RSA events (Santner-Nanan et al., 2009).

Determining regulatory mechanisms that could suppress T_H17 cells might lead to novel approaches in the prevention of RSA. IL-23 has been linked with T_H17 cell responses, mainly through its effects on the expansion and maintenance of these cells (Peng et al., 2010; Sheibanie et al., 2007). IL-23 is also involved in the differentiation and maintenance of $CD4^+$ T_H17 and $CD8^+$ T_C17 cells (Sarin et al., 2011). This concept builds on the fact that serum levels of IL-17 (secreted by T_H17 cells) and IL-23 (inducer of inflammatory responses mediated by T_H17 cells) were significantly higher in patients with unexplained RSA than in controls (Pongcharoen et al., 2007).

Considering that different genetic variants of the IL-23 receptor (IL-23R) have been repeatedly implicated in a number

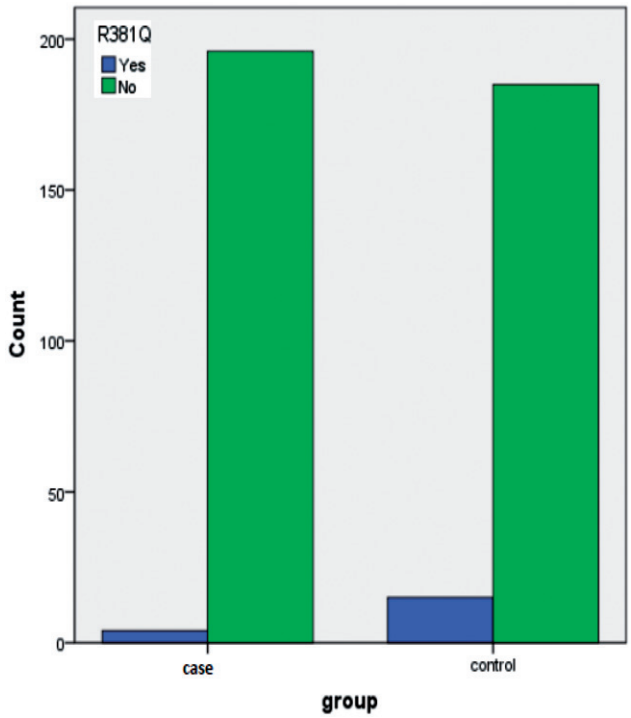

Figure 3. Frequency of 381Q variant (AG genotype; heterozygote pattern) in RSA and control groups.

of inflammatory diseases, this suggested to us that the T_H17 pathway has a determining role in these pathologic states, including RSA. Therefore, characterizing association patterns of IL23R to specific pre-disposing/protective variants might help in the elucidation of the etiology of RSA and other autoimmune diseases. Duerr et al. (2006) reported on an association between Crohn's Disease (a chronic inflammatory disease of gastrointestinal tract) and variants in the *IL-23R* gene at 1p31 subsequently discovered in a genome-wide association analysis (Lakatos et al., 2008). Other investigations suggested that *ATG16L1* (an autophagy gene) and *IL-23R* were susceptibility loci in Crohn's Disease in Hungarian and Czech patients (Hradsky et al., 2011). Genome-wide association studies (GWAS) in several populations have demonstrated significant association of changes in the IL-23R gene with Crohn's Disease, ulcerative colitis (UC), and psoriasis, suggesting that perturbations in the IL-23 signaling pathway was likely relevant to the pathophysiology of these inflammatory-based diseases. Interestingly, one particular variant – R381Q (rs11209026) – seems to confer a measure of protection against development of Crohn's Disease and psoriasis.

Some studies have analyzed the frequency of R381Q in several inflammatory diseases with pathologies similar to RSA (i.e. in which T_H17 cells have an important role in adverse inflammatory events); one reported frequencies of 6% and 25% for R381Q in, respectively, Crohn's Disease patients and control subjects, illustrating that its presence appeared to confer some sort of protection (Venegas et al., 2008). The A allele (coding for Gln, protective allele) was much less common with an allele frequency of 1.9% in Crohn's Disease patients and 7.0% in matching controls (Hradsky et al., 2011). There are different frequencies of R381Q variant in populations of patients with different diseases and, thus, in the control groups in different studies. For example, in the above-noted Crohn's study, the difference (7 versus 25%) seemed to suggest a closer link or relevance; the difference in the present study was just 2 versus 7.5%.

Some studies also indicated that the A allele frequency of R381Q was lower in ankylosing spondylitis (AS) patients than in healthy controls (Duan et al., 2012; Rahman et al., 2008). Another study indicated that a R381Q polymorphism modulated IL-17A expression in patients with rheumatoid arthritis (Hazlett et al., 2012). Those authors suggest that, in patients with the 381Gln allele, higher IL-23 concentrations may be needed to produce IL-17A concentrations similar to those in patients with the 381Arg allele. To our knowledge, no study has yet to specifically evaluate the frequency of the IL-23R R381Q variant in patients with RSA.

To date, there has yet to be a similar set of studies to investigate if the presence/absence of this variant (i.e. the IL-23R R381Q variant) might be an important marker in RSA patients. The results of the present study showed that in fact there was a tendency for RSA patients to carry a lower frequency of the R381Q variant in their *IL-23R* gene.

Conclusions

This is the first report on the association between polymorphisms in the IL-23R gene (i.e. as related to the R381Q variant specifically) and RSA. The data clearly showed that the occurrence of the R381Q polymorphism was lower in patients with RSA compared to in healthy controls. As with other inflammation-based pathologies, it would again appear that having this specific polymorphism could help confer a protective effect against potential RSA in women. While further studies in different populations are needed to confirm our findings, these outcomes provide insight into a potential immuno-dysregulatory basis for RSA.

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Declaration of interest

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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