A functional polymorphism in the promoter region of interleukin-10 gene increases the risk for spontaneous abortions—a triad study

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Abstract

Purpose Spontaneous abortion or miscarriage is the natural death of an embryo or foetus in the early stages of prenatal development. Interleukin-10 is an anti-inflammatory cytokine, produced by human cytotrophoblasts, and defects in its production result in specific pathological conditions during pregnancy. The present study is aimed to evaluate the association of IL-10 -1082G/A polymorphism in spontaneous abortions by comparing foetal, maternal and paternal groups—a triad study. **Methods** A total of 50 families with spontaneous abortions and 60 families with medically terminated pregnancies were considered for the present study. DNA from foetal tissue and parental blood samples were extracted, and the genotype analysis of IL-10 -1082G/A promoter polymorphism was carried out by amplification refractory mutation system-polymerase chain reaction followed by agarose gel electrophoresis. A

statistical analysis was applied to test for the significance of the results.

Results There was a statistically significant difference in the distribution of AA genotypes and A allele of IL-10 -1082G/A between the two family groups among fetuses ($P=0.0002$) and mothers ($P=0.00005$). The paternal group showed no significant difference in the genotype distribution of IL-10 between cases and controls.

Conclusion In conclusion, IL-10 G-1082A gene promoter polymorphism may act as a major genetic regulator in the etiology of spontaneous abortions.

Keywords Interleukin-10 gene · Polymorphism · Spontaneous abortions · Polymerase chain reaction

Capsule This is the first case-control and family-based triad study that has analysed the association between IL-10 gene (-1082G/A) polymorphism and spontaneous abortions. The findings of the present study showed a statistically significant association of IL 10 (-1082 G/A) with spontaneously aborted fetuses and mothers and not fathers with their respective control groups. IL-10 -1082AA genotype is a foetal risk as well as maternal risk but not a paternal risk factor for spontaneous abortions. IL-10 (-1082G/A) promoter polymorphism may act as a major genetic regulator in the etiology of spontaneous abortions.

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Introduction

Spontaneous abortion or miscarriage is defined as “the occurrence of clinically detectable pregnancy loss prior to 20 weeks of gestation” [1]. It refers to a pregnancy that ends spontaneously before the foetus has reached a viable gestational age. It is the most common complication of early pregnancy [2]. Spontaneous abortion is seen in 10–15 % of all clinically recognised pregnancies [3–5]. A spontaneous abortion is as opposed to an induced abortion which is purely accidental, that is spontaneous. The frequency of spontaneous abortions decreases with increasing gestational age [6–8]. It occurs due to failure of foetal and placental angiogenic development which results in unsuccessful embryo implantation. Immunological factors are suggested to play a key role in unexplained spontaneous pregnancy loss [9–12]. Interleukin-10 (IL-10) is an important immunosuppressive and anti-inflammatory cytokine, located on chromosome 1 at 1q31-32 position. IL-10 is produced locally by both T helper (Th1 and Th2) cells in the foeto-placental unit

by cytotrophoblasts [13]. It is also expressed by a variety of haematopoietic cells including macrophages, natural killer cells, B cells and mast cells at the maternal-foetal interface. It serves to protect the foetus from harmful maternal immune response. It acts as a suppressive agent for active maternal immunity in order to accept the foetal allograft. IL-10 functions as a vital bridge that links immunity, placental angiogenesis and hypoxia at maternal-foetal interface [14]. It inhibits the production of various pro-inflammatory cytokines produced by different immune cells, and defects in this production result in specific pathological conditions of pregnancy [15]. On the other hand, its decreased production inhibits angiogenic factors thereby blocking angiogenesis and impairing vascular development, ultimately resulting in abortion. The G/A polymorphism at position -1082 (rs1800896) in the promoter region of IL-10 has been correlated with both upregulation and downregulation of IL-10 which in turn results in normal pregnancy or spontaneous abortion, respectively [16, 17]. The substitution of A allele in place of G allele at -1082 position reduced the production of IL-10, thereby dampening the maternal immune response, enhancing foetal tissue damage and thus resulting in spontaneous abortion.

In view of the above, the present study was aimed to evaluate the role of IL-10 gene promoter polymorphism (rs1800896) as maternal, paternal, embryonic genetic risk factor for susceptibility to spontaneous abortion. To the best of our knowledge, this is the first case-control as well as family-based triad study conducted in our population.

Materials and methods

Sample collection

A total of 150 samples including 50 spontaneously aborted foetal tissues along with blood samples of their parents (mother and father— $50 \times 2 = 100$) referred to the Department of Obstetrics and Gynecology, Government Maternity Hospital, Hyderabad, Telangana State, were considered for the present study. The control group consisted of 180 samples from medically terminated pregnancies including 60 normal foetal tissues together with 60 peripheral blood samples of their parents (mother and father— $60 \times 2 = 120$). The present study was a case-control and family-based triad study with samples collected over a period of 2 years from Feb 2013–Jan 2015. Demographic data regarding age, consanguinity, number of miscarriages and parity were collected from all the couples with the help of standard proforma. This study was approved by the Institutional Ethical Committee. An informed consent was obtained from all the couples before including them in the study.

Aborted tissue of about 50 mg was taken in saline solution from all the females with spontaneous abortion and medically terminated pregnancies. Peripheral blood samples of about 5 ml

each were collected in EDTA vacutainers from all the couples. All the samples were stored at -20°C for further genotyping analysis.

The inclusion criteria

Spontaneously aborted fetuses at <20 weeks of gestational age were obtained as the case group for the present study. Aborted fetuses from medical termination of pregnancies with similar gestational age were taken as the control group for comparative analysis. The mean age of mothers in both controls and cases was 31 years (range 20–35 years), and the mean age of fathers in both controls and cases was 35 years (range 25–40 year). The case group consisted of mothers with at least two recurrent spontaneous abortions and no previous history of successful pregnancy. The cause of these abortions was idiopathic. Couples with at least two healthy live births showing no previous history of abortions and devoid of any infertility problems were considered as control group, and all the fetuses of the medically terminated pregnancies were normal. All the control and case samples were included from the South Indian population.

The exclusion criteria

The subjects with a history of risk factors like chronic infections, thrombosis, autoimmune diseases, endocrinological disorders or congenital anomalies were excluded from the present study. None of the participants included in the study used oral contraceptives, any other medications before or during the course of pregnancy. Pregnant women with preeclampsia and abnormal karyotype were excluded. Males with infertility and other health problems were excluded from the study.

Determination of IL-10 gene promoter polymorphism

Genomic DNA from foetal tissue and peripheral blood of their parents was extracted by modified phenol chloroform method of Blinn and Stafford (1976) [18]. The isolated DNA was genotyped for IL-10 (-1082G/A) polymorphism detection by amplification refractory mutation system-polymerase chain reaction method (ARMS-PCR) [19]. Briefly, two complementary reactions were established for each allele consisting of target DNA: allele-specific ARMS primers (FG for G allele and FA for A allele) and the common primer (CF). A 169-bp region in the IL-10 gene promoter was targeted for amplification. The primers used were as follows: a common (CF) anti-sense primer 5'-GTA AGC TTC TGT GGC TGG AGT C-3'; (FG) sense 5'-AAC ACT ACT AAG GCT TCT TTG GGT G-3' G-primer and (FA) sense 5'-AAC ACT ACT AAG GCT TCT TTG GGT A-3'. The optimised reaction conditions consisted of 40 ng of genomic DNA in a reaction volume of 15 μl containing $1 \times$ reaction buffer, 1.5 μM MgCl_2 , 30 μM of each dNTP, 0.16 μM of each primer and 0.3 U of Taq DNA polymerase. The cycling conditions were as follows: an initial denaturation at

94 °C for 4 min, followed by 35 cycles at 94 °C for 30 s, 61 °C for 20 s and 72 °C for 30 s. The final extension step was at 72 °C for 6 min. The PCR products were visualised on 2 % agarose gel stained with ethidium bromide under ultraviolet light with the help of a 100-bp ladder. All the collected samples (60+120=180 controls and 50+100=150 cases) were genotyped successfully. Results were cross-checked with internal positive (796 bp of HLA gene) and negative controls (Millipore water) [20].

Statistical analysis

Genotype distribution and allele frequencies in the medically terminated pregnancy group (controls) and spontaneously aborted groups (cases) were analysed by chi-square test to assess the Hardy–Weinberg equilibrium. All *P* values were detected by two-tailed Fisher's exact test. Odds ratios (OR) and their 95 % confidence intervals (CI) were calculated to measure the strength of association between IL-10 gene (-1082G/A) polymorphism and spontaneous abortions. For all statistical analyses, *P*<0.05 was considered statistically significant.

Results

The present study was carried out in a total of 180 controls and 150 cases. Demographic details from 60 medically terminated couples and 50 spontaneously aborted couples were mentioned in Table 1. The analysis of the demographic characteristics from control and case groups revealed a statistically significant difference with respect to age, consanguinity and parity. No significant difference was observed in regard to number of miscarriages. An interesting observation was that

there was a fivefold increase in risk for the case group compared to control group with respect to consanguinity.

Genotype and allele frequencies were in Hardy–Weinberg equilibrium. Genotype and allele frequencies of control group and case group are given in Tables 2, 3 and 4, respectively.

We found a significant increased risk for both heterozygous, GA vs GG (OR=3.70; 95 % CI 1.498–9.137; *P*=0.0075) and homozygous, AA vs GG (OR=4.955; 95 % CI 1.724–14.24; *P*=0.004) genotypes in the aborted fetuses compared to medically terminated fetuses (Table 2). Moreover, when the dominant model was applied (GA + AA vs GG), there was a marginally increased risk for abortion (OR=4.137; 95 % CI 1.845–9.274; *P*=0.0008). Further, recessive model also revealed a significant association of the -1082A variant (AA vs GG + GA) in cases compared to controls (OR=2.786; 95 % CI 1.068–7.269; *P*=0.032). Thus case/control analysis in fetuses revealed that SNP rs1800896 was associated with the increased risk of spontaneous abortion, since there was a significant difference in genotype.

A significantly elevated risk of spontaneous abortion was observed in mothers who were heterozygous, GA vs GG for the IL-10 -1082G/A variant (OR=4.138; 95 % CI 1.586–10.79; *P*=0.002) (Table 3). A maternal AA vs GG homozygous genotype was a significant independent contributor to abortion risk (OR=5.257; 95 % CI 1.642–16.84; *P*=0.007). Nevertheless, when a dominant effect (GA + AA vs GG) of the -1082A allele of the mothers was considered, we found a fourfold increased risk for spontaneous abortions (OR=4.537; 95 % CI 1.997–10.31; *P*=0.0004). A significant association was observed when the recessive model was applied (AA vs GG + GA; OR=3.474; 95 % CI 1.131–10.67; *P*=0.023). No significant difference was found for the genotypes of the

Table 1 Demographic details of study subjects (control and case groups)

Variables	Controls, N=50(%)	Cases, N=60(%)	OR	95 % CI	<i>P</i> value
Maternal age (years)					
<30	58(96.66)	35(70)	0.80	0.017–0.373	0.0003*
≥30	2(3.33)	15(30)			
Paternal age (years)					
<30	55(91.66)	12(24)	0.02	0.009–0.088	0.0000001**
≥30	5(8.33)	38(76)			
Consanguinity					
Yes	13(21.66)	30(60)	5.42	2.353–12.5	0.00009**
No	47(78.33)	20(40)			
Number of miscarriages (years)					
2	0(0)	28(56)	1.273	0.024–66.75	0.36
>2	0(0)	22(44)			
Parity (number)					
Nulliparous	0(0)	37(74)	0.002	0.0001–0.050	<0.0000001*
Pluriparous	60(100)	13(26)			

P*<0.05 is statistically significant; *P*<0.001

Table 2 Genotype and allele frequencies of the IL-10 (-1082G/A) polymorphism in control foetuses and case foetuses

IL-10 -1082 A>G	Controls, <i>N</i> =60(%)	Cases, <i>N</i> =50(%)	OR (95 % CI)	<i>P</i> value
GG	37(61.66)	14(28)	3.70 (1.498–9.137)	0.007*
GA	15(25)	21(42)		
AA	8(13.33)	15(30)	4.955 (1.724–14.24)	0.004*
GA + AA vs GG	23(38.33)	36(72)	4.137 (1.845–9.274)	0.0008**
	37(61.66)	14(28)		
AA vs GG + GA	8(13.33)	15(30)	2.786 (1.068–7.269)	0.03*
	52(86.66)	35(70)		
GA vs GG + AA	15(25)	21(42)	2.172 (0.966–4.885)	0.05*
	45(75)	29(58)		
Allele frequency				
G	0.7416	0.49	2.988 (1.696–5.265)	0.0002**
A	0.2583	0.51		

P*<0.05 is statistically significant; *P*<0.001

fathers in any of the genetic model applied (codominant, dominant or recessive) (Table 4).

An increased frequency of A allele was observed in both the foetal group (OR=2.988, *P*=0.002) and the maternal group (OR=3.694, *P*=0.00005) but not in paternal group (OR=1.26, *P*=0.5963).

Discussion

Spontaneous abortions are complex and unexplained phenomenon which is a common most problem of the pregnant women. It involves the interaction of several genetic and environmental risk factors. The main objective of the present study was to determine the effect of IL-10 -1082G/A gene promoter polymorphism in spontaneously aborted foetuses in addition to their parental groups. IL-10 serves a dual role in the production of pro-inflammatory cytokines and acts as an important anti-inflammatory cytokine at foeto-placental interface [21–23]. Pro-inflammatory cytokines (IL-1, IL-2, IL-12, IFN- γ , TNF- α , IF-3) and anti-inflammatory cytokines (IL-4,

IL-13, TGF- β) counter regulate each other such that they maintain a balance in their production resulting in normal pregnancy. In contrast, imbalance among these cytokines will result in pregnancy complications such as spontaneous abortions. IL-10 has its role in the preservation of pregnancy until the completion of gestation. IL-10 production in human placental tissue includes higher levels during the first and second trimesters compared to the third trimester of pregnancy, suggesting an immuno-suppressive role over the course of human gestation. Increased production of IL-10 is found in a variety of haematopoietic cells like macrophages, natural killer cells and T cells at maternal–foetal interface in early and mid-pregnancy. IL-10 maintains the pregnancy by its increased production from the placenta. Women with reduced IL-10 production may be sensitive to the inflammatory effects, in turn prone to increased risk towards several disorders of pregnancy, resulting in adverse pregnancy outcomes. In other words, the decreased production of IL-10 contributes to poor placental developmental process with an increased rate of spontaneous abortions [24]. Finally, IL-10 plays a vital role during pregnancy by dampening the immune response to foetus and

Table 3 Genotype and allele frequencies of the IL-10 (-1082G/A) polymorphism in control mothers and case mothers

IL-10 -1082 A>G	Controls, <i>N</i> =60(%)	Cases, <i>N</i> =50(%)	OR (95 % CI)	<i>P</i> value
GG	46(76.66)	21(42)	4.138 (1.586–10.79)	0.002*
GA	9(15)	17(34)		
AA	5(8.33)	12(24)	5.257 (1.642–16.84)	0.007*
GA + AA vs GG	14(23.33)	29(58)	4.537 (1.997–10.31)	0.0004*
	46(76.66)	21(42)		
AA vs GG + GA	5(8.33)	12(24)	3.474 (1.131–10.67)	0.02*
	55(91.66)	38(76)		
GA vs GG + AA	9(15)	17(34)	2.919 (1.165–7.317)	0.01*
	51(85)	33(66)		
Allele frequency				
G	0.841	0.59	3.694 (1.964–6.947)	0.00005**
A	0.097	0.41		

P*<0.05 is statistically significant; *P*<0.001

Table 4 Genotype and allele frequencies of the IL-10 (-1082G/A) polymorphism in control fathers and case fathers

IL-10 -1082 A>G	Controls, N=60(%)	Cases, N=50(%)	OR (95 % CI)	P value
GG	41(68.33)	32(64)	1.11 (0.463–2.663)	0.99
GA	15(25)	13(26)		
AA	4(6.66)	5(10)	1.602 (0.3975–6.453)	0.75
GA + AA vs GG	19(31.66)	18(36)	1.214 (0.5491–2.683)	0.78
	41(67.33)	32(64)		
AA vs GG + GA	4(6.66)	5(10)	1.556 (0.3945–6.134)	0.77
	56(93.33)	45(90)		
GA vs GG + AA	15(25)	13(26)	1.054 (0.4458–2.492)	0.92
	45(75)	37(74)		
Allele frequency				
G	0.8083	0.77	1.26 (0.6571–2.415)	0.59
A	0.1916	0.23		

* $P < 0.05$ is statistically significant; ** $P < 0.001$

consequently controlling the foetal tissue damage. As IL-10 is encoded by both maternal and paternal genes, this study was also conducted in parents to determine the role of maternal and paternal IL-10 polymorphism in addition to its effect in spontaneously aborted fetuses.

This study revealed a significant association of *AA* genotype and *A* allele in spontaneously aborted fetuses compared to medically terminated normal fetuses, indicating its possible association with spontaneous abortions. On the other hand, the maternal case group compared to maternal control group also showed a strong association with IL-10 -1082 polymorphism. Thus, our data shows that the case mothers with -1082 AA genotype or with A allele were at higher risk for spontaneous abortions when compared to normal control mothers. However, there was no such strong association in paternal groups.

Earlier studies on IL-10 polymorphism and spontaneous abortions revealed conflicting results. In other words, distribution of genotype and allele frequencies of IL-10 (-1082G/A) polymorphism showed a significant variation in different populations. A study by Parveen et al. (2013) showed a significant association of A allele in North Indian women with recurrent pregnancy loss [25]. Medica et al. (2009), who performed a meta-analysis in 635 women with spontaneous abortions, revealed a statistically significant association of A allele with spontaneous abortions [26]. Bohiltea et al. (2014) demonstrated a strong association for -1082G/A IL-10 polymorphism in 69 women of the Romanian population with idiopathic recurrent spontaneous abortion [27]. In contrary, Karhukorpi et al. (2001) showed no significant differences in the genotype and allele frequencies of IL-10 (-1082G/A) polymorphism in Finnish women with spontaneous abortions and normal control women [28]. Alkhuriji et al. (2013) studied 65 Saudi females with recurrent spontaneous abortions and 65 control females and found no significant differences in the distribution of IL-10 (-1082G/A) genotype frequencies in both

groups [29]. Bahadori et al. (2014) observed lack of statistically significant association in the frequencies of IL-10 (-1082G/A) polymorphism among Iranian women with miscarriages [30]. These discrepancies may be explained by ethnic differences of the study groups as well as the variation in the study subjects. None of the studies, however, had triad samples including foetal and paternal genotypes considered, as in this study. The present study revealed a statistically significant higher frequency of *AA* genotype and *A* allele of IL-10 -1082G/A polymorphism in mothers and aborted fetuses compared to their normal counterparts. Finally, our results suggest the importance of IL-10 (-1082G/A) polymorphism as an important risk factor in the etiology of spontaneous abortions. Thus, it can be used as one of the genetic markers for adoption of therapeutic management and counselling.

Conclusion

This is the first case-control and family-based triad study that has analysed the association between IL-10 gene (-1082G/A) polymorphism and spontaneous abortions. The findings of the present study demonstrated that -1082G/A polymorphism in IL-10 gene promoter showed a statistically significant association with spontaneously aborted fetuses and mothers with their respective controls. In contrast, there is no such association observed in case fathers when compared with their control fathers. Thus, *IL-10 -1082AA* genotype is a foetal risk as well as maternal risk but not a paternal risk factor for spontaneous abortions. In conclusion, the data presented in this study provides an evidence to suggest that IL-10 (-1082G/A) promoter polymorphism may act as a major genetic regulator in the etiology of spontaneous abortions. However, a large sample size has to be analysed to confirm the obtained results.

Conflict of interest The authors declare that they have no competing interests.

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