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Research Article

A Triad Study in South Indian Population of Telangana: On the Association of Cytokine Gene Polymorphisms in the Aetiology of Spontaneous Abortions

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Abstract

Background: Spontaneous abortion is defined as the loss of two or more consecutive pregnancies before 20th week of gestation. About 50% of the cases are remained with unexplained etiology. Potential immune cell differentiation and cytokine profile is detrimental for fetal-maternal communication and maternal recognition of pregnancy. An imbalance in the immune modulatory profile results in consecutive loss of pregnancies. In view of the above, the present study is taken up to understand the role of immune- modulators and their gene polymorphism in the etiology of spontaneous abortion.

Methods: In the present case-control triad study genotyping for IFN γ+874 T/A, IL6-137 G/C, IL18-174G/C polymorphisms were done by Amplified Refractory Mutation System polymerase chain reaction method (ARMS-PCR) and statistically analyzed.

Result: A retrospective analysis for association of gene polymorphisms in the present study showed that

maternal and fetal TT genotype of IFN γ+874 polymorphism showed a twofold increased risk (OR - 2.22, 95%CI= 1.29-3.84, p=0.004) whereas in IL 18 - 174G/C gene polymorphism and their association study, the case mother subjects that represented CC genotypes contributed a onefold increased risk towards the development of spontaneous abortion. (OR -2.17, 95%CI- 1.250-3.788, p=0.005)

Conclusion: The proinflammatory and anti-inflammatory gene polymorphisms may contribute towards the susceptibility for spontaneous abortion.

Keywords: IFN γ; Interleukins; Spontaneous Abortion; Polymorphism; Triad Group; Cytokine

1. Introduction

Spontaneous abortion is defined as the unexpected intrusion of pregnancy before 20th week of gestation [1]. It has been estimated that 10-15% of clinically recognized pregnancies in India undergo spontaneous abortion [1, 2]. A number of factors such as chromosomal abnormalities, endocrine disorders, maternal factors, coagulation disorders, reproductive anomalies and immunological factors has been attributed to the aetiology of spontaneous abortion [3]. Despite a number of factors has been attributed to the aetiology of spontaneous abortion, the exact cause for 50 % of the cases is still obscure. Several investigations on the etiopathogenesis of spontaneous abortion revealed that a balance between pro-inflammatory and anti-inflammatory cytokines are crucial during different stages of pregnancy. It has been well demonstrated that the pro inflammatory cytokines are detrimental whereas anti-inflammatory cytokines are more advantageous for the maintenance of pregnancy [4-7]. It has been well postulated that the fetal rejection by the maternal immune system is due to abnormal immune cell recruitment and production of various immune modulatory molecules like cytokines. These immune-modulatory cytokines are expressed by a range of immune cells like peripheral lymphocytes, macrophages, natural killer cells and uterine NK cells in the maternal endometrium [8-10]. The IL6, IL18, IFN γ inflammatory molecules are multifunctional biomolecules that mediates a variety of biological activities in different types of cells, with pivot role in implantation and differentiation of T cell in adaptive immunity. Their involvement in placental maintenance during pregnancy has been demonstrated by initiating vasculature remodeling and angiogenesis at implantation sites [5, 11-13].

The levels of cytokine vary among women with spontaneous abortion compared to women with successful pregnancies. This variation in immune response between individuals might be due to genetic variation and single nucleotide polymorphisms (SNPs) in the immune modulatory genes [14, 15]. The alterations in the cytokine profile can demarcate various biological processes right from implantation to parturition and thereby resulting in rejection of semi allogeneic fetus. The pro inflammatory cytokines are being first dominated during the initial phase of pregnancy that enables implantation of the semi allogenic conceptus, which is then followed by a shift to anti-inflammatory cytokines that helps protect the fetus by suppressing the maternal immune system. The earlier miscarriage studies mainly focused either on women or couples with spontaneous abortion as study subjects, only few studies investigated the role of these gene polymorphisms in the fetal subjects along with the couples in the causation of spontaneous abortion.

We hypothesized that as the fetus is a unique combination of maternal and paternal genes, can play an important role in the immunogenetics of pregnancy.

Taking into consideration, the role played by IFN γ, IL6, IL18 in the maintenance and development of the embryo at the maternal-fetal interface, and to overcome the limitations posed by the previous studies, the present study has attempted a trio-design where mother-father-fetus are enrolled to evaluate the involvement of IFN γ, IL6, IL18 and their polymorphisms towards the susceptibility of spontaneous abortion.

2. Materials and Methods

2.1 Study subjects

The present case-control trio study design includes mother/father/fetus (120×3=360) of spontaneously aborted pregnancies as cases and medically terminated pregnancies (120×3=360) as controls. The study subjects referred to the Department of Obstetrics and Gynecology, Government Modern Maternity Hospital, Hyderabad, Telangana were enrolled for the study. The present study has been approved by the institutional ethical committee for biomedical research Osmania University, Hyderabad. Prior informed consent was obtained from all the subjects recruited for the study. The selection criteria for the case study groups were defined as patients with a history of at least two miscarriages, without any known risk factors such as genetic abnormalities, endocrine disorders, uterine defects, thrombosis, diabetics, anti-phospholipid syndrome.

The inclusive criteria for control groups, considered the couples undergoing medical termination of pregnancies devoid of a history of miscarriage's and at least with two healthy live births. Demographic details of the case-control couples recruited for the triad study were obtained with the help of a standard proforma. A standard operating procedure were followed for the collection of blood and tissue samples from the study subjects. A total of 5 ml of peripheral blood samples from the couples and 100mg of respective fetal tissue was collected from both the groups and stored at -20ºC until DNA isolation and genotyping.

2.2 Genotyping

Extraction of genomic DNA from the peripheral blood and fetal tissues was performed as per phenol chloroform protocol [16]. The DNA isolated was genotyped for IFN γ +874 T/A, IL6 137 G/C, IL18 174 G/C polymorphism by Amplification Refractory Mutation System - polymerase chain reaction (ARMS-PCR) using allele specific primers as shown in Table 1. For 50 µl reaction, 5 µl of 10X abm Taq buffer, 5 µl of $MgSO_4$ (25mM), 1 µl of Promega DNTPS mix (10mM), 1µl of 10pmol of the primers, 1µl for genomic DNA (40ng), 1µl of abmTaq polymerase (5U/µl), 36.5µl of sterile water were added and PCR reaction was performed according to the following conditions as represented in Table 2. Further, the amplified products were separated by agarose gel electrophoresis on a 2.5% agarose gels stained with ethidium bromide and observed under UV light.

2.3 Statistical analysis

The data generated in the current study was analyzed by using online statistical software Open Epi tool. Further using online chi-square calculator (http://www.quantpsy .org/chisq/chisq.htm), the difference in the distribution of genotype frequencies in case-control groups and their consistency with Hardy Weinberg equilibrium was performed by an online HWE calculator(http://www.dr-petrek.eu/documents/HWE.xls). The strength of association and the level of significance for cytokines gene polymorphism towards spontaneous abortion susceptibility was analyzed by estimating their Odds Ratio (OR) at 95% CI and considering the level of significance at (≤0.05) 'p' detected by two- tailed Fisher's exact test. Logistic regression analysis was

performed to test the effect for confounding factors towards development of spontaneous abortion.

Gene	Primer sequence	Product size	Annealing Tm
IFNγ rs2430561		262	49°C
Wildtype Forward Primer	5'TTCTTACAACACAAAATCAAATCT3'		
Mutant Forward Primer	5'TTCTTACAACACAAAATCAAATCA3'		
Common Reverse Primer	5'TCAACAAAGCTGATACTCCA3'		
IL6 rs1800795		270	49°C
Wildtype Forward Primer	5'CCCTAGTTGTGT5CTTGTC3'		
Mutant Forward Primer	5'CCCTAGTTGTGTCTTGTG3'		
Common Reverse Primer	5'ACTTGTGGAGAAGGAGTTCA3'		
IL18 rs187238		194	52°C
Wildtype Forward Primer	5'TTGCTGTATTTGTATAGTCA3'		
Mutant Forward Primer	5'TTGCTGTATTTGTATAGTCC3'		
Common Reverse Primer	5'ACTGATGTACTTGCAGCCT3'		

Table 1: List of Primer sequence and Reaction conditions.

3. Results

A comparative analysis of the demographic details of 120 case couples of spontaneously aborted pregnancies and 120 control couples with medically terminated pregnancies is documented in Table (2). The study revealed that there was no significant difference in the paternal and maternal age groups between the case and control subjects. The gestational period and the spontaneous abortion onset revealed that, although there was a one- fold increase in the number of cases with spontaneous abortion during early gestational period, but no statistically significant differences were demonstrated between the groups. (OR=1.78, CI=0.986-3.22, p=0.0539). The genotypic and the allelic distribution of mother- father- fetus case and control study subjects for the genes IL 18, IL6, IFN γ were assessed to determine their contribution towards the risk of spontaneous abortion is shown in Table (4, 5, 6). All case- control triad study groups enrolled were consistent with Hardy Weinberg equilibrium. In the present study, IL18"CC" maternal genotype, IFN γ "TT" maternal and fetal genotypes in the case groups with spontaneous abortion showed a significant difference when compared to their respective control groups. IL 18C allele from case maternal groups showed a 2-fold risk (OR=1.79, CI=1.244- 2.58 p =0.0016) whereas IFN γ T allele showed a one-fold increased risk in both maternal (OR=2, CI=1.339- 2.82, p =0.004) and fetal subjects (OR=1.71, CI=1.17- 2.36, p =0.012) respectively. The genotypic and allelic distribution for IL 6 gene revealed no significant difference within the groups. Although paternal, maternal and fetal GG genotypes demonstrated a slight increase in their frequencies, no significant difference in genotypic nor either allelic distribution was observed in case- control parental and their fetal study groups.

Further, various genetic models were evaluated for the genes under study to know the role of gene polymorphism in the etiology of spontaneous abortion as represented in the table (4, 5, 6). The maternal and fetal IFN γ TT genotypes conferred an increased risk towards the susceptibility in case groups (OR=2.25, CI=1.342-3.77, p =0.002) (OR=2.122, CI=1.263- 3.566, p =0.004) respectively, under the recessive genetic model. The allelic model also indicated that the, T allele in fetus and mother of case study group have an increased risk (OR=1.71, CI=1.17-2.367, p =0.01259) (OR=2.00, CI=1.39- 2.82, p=0.0039) compared to their respective control subjects. The genetic model analysis and frequency distribution of IL 18G/C genotypes established that the frequency of the homozygous CC genotype was higher in the mother case subjects compared to the mothers in the control group through the recessive mode of inheritance. Even the allelic mode of inheritance showed that, the risk of association increased by one-fold through the C allele in the maternal group when compared to the G allele, but no such differences was observed in the fetal and paternal groups. Logistic regression was carried out to analyse the effect of confounding factors such as age, consanguinity, gestational period and gene polymer-phism. The results further supported that there was no significant association between confounding factors and the gene polymorphisms.

Demographic details		**Case**	**Control**	χ^2	**p value**	**Odds ratio (CI 95%)**
Maternal age (Years)	Less than 35	84	91	1.034	0.3112	0.7436 (0.4197-1.317)
	More than 35	36	29			
Paternal age (Years)	Less than 35	69	72	0.1547	0.6940	0.902 (0.5394, 1.508)
	More than 35	51	48			
Gestation (Weeks)	<12	96	83	3.715	0.0539	1.78 (0.986-3.22)
	≥12	24	37			
Consanguinity	Yes	16	8	2.963	0.08521	0.4643 (0.1907-1.13)
	No	104	112			

Table 2: Demographic characteristics of Control and Case study groups.

Genotype	Fetus		Mother		Father	
IL6(-174G/C)	Cases N (%)	Controls N (%)	Cases N (%)	Controls N (%)	Cases N (%)	Controls N (%)
GG	44 (36.6)	36 (30)	42 (35)	40 (33.3)	48 (40)	46 (38.3)
CG	52 (43.4)	65 (54)	50 (41.6)	55 (45.8)	52 (43.)	59 (49.2)
CC	24 (20)	19 (16)	28 (23.3)	25 (20.8)	20 (17)	15 (12.5)
G	140 (0.6)	137 (0.57)	134 (0.56)	135 (0.56)	148 (0.615)	151 (0.63)
C	100 (0.4)	103 (0.43)	106 (0.44)	106 (0.44)	92 (0.385)	89 (0.37)
Association Models	OR (CI 95%)	P Value	OR (CI 95%)	P Value	OR (CI 95%)	P Value
CC Vs GG	0.967 (0.45-2.03)	0.93	0.9375 (0.4695 to 1.8722)	0.8549	0.8924 (0.4072 to 1.9559)	0.7762
CG Vs GG	1.52 (0.862-2.71)	0.14	1.1550 (0.6479 to 2.0589)	0.6251	1.3611 (0.7865 - 2.3558)	0.2706
CC+GC Vs GG	1.35 (0.7-2.31)	0.27	1.0769 (0.6316 to 1.8363)	0.7855	1.2468 (0.7451 - 2.0865)	0.4011
GG+GC Vs CC	1.3289 (0.68- 2.58)	0.4	1.1565 (0.6279 to 2.1302)	0.6408	1.3168 (0.6346 - 2.7326)	0.4599
GC Vs GG +CC	1.5455 (0.928- 2.57)	0.0938	1.1846 (0.7110 to 1.9738	0.5154	1.4015 (0.8412 - 2.3350)	0.195
G Vs C	1.053 (0.7327,1.512)	0.7816	0.9832 (0.6857, 1.41)	0.9267	0.9482 (0.6555, 1.372)	0.7775

OR: odds ratio, CI: confidence interval, *: p value significant

Table 3: Distribution of genotypic and allelic frequencies of IL6 rs1800795 among the triad groups.

Genotype	Fetus		Mother		Father	
IL18(-174C/G)	Cases	Controls	Cases	Controls	Cases	Controls
	N (%)	N (%)	N (%)	N (%)	N (%)	N (%)
CC	40 (33.3)	33 (27)	29 (24)	43 (36)	33 (27)	31 (26)
CG	53 (44.2)	50 (42)	49 (41)	50 (42)	48 (33)	47 (39.)
GG	27 (22.5)	37 (31)	42 (35)	27 (22)	39 (32)	42 (35)
G	133 (0.55)	116 (0.52)	108 (0.56)	136 (0.43)	114 (0.515)	109 (0.455)
C	107 (0.45)	124 (0.48)	132 (0.44)	104 (0.57)	126 (0.485)	131 (0.545)
Association Models	OR (CI 95%)	P Value	OR (CI 95%)	P Value	OR (CI 95%)	P Value
CC Vs GG	1.6611 (0.8439 - 3.2695)	0.1419	2.3065 (1.1744 - 4.5298)	0.0152*	1.1464 (0.5949 - 2.2091)	0.6831
CG Vs GG	1.4526 (0.7745 - 2.7242)	0.2445	1.5873 (0.8507 - 2.9616)	0.1465	1.0998 (0.6078 - 1.9902)	0.7531
CC+GC Vs GG	1.5355 (0.8617 - 2.7360)	0.1457	1.8547 (1.0494 - 3.2779)	0.0335*	1.1183 (0.6547 - 1.9103)	0.6822
GG+GC Vs CC	0.7586 (0.4368 - 1.3174)	0.3266	0.5707 (0.3259 - 0.9993)	0.0497	0.9183 (0.5181 - 1.6277)	0.7704
GC Vs GG +CC	1.0355 (0.6172 - 1.7372)	0.895	1.0350 (0.6190 - 1.7305)	0.8957	1.0355 (0.6172 - 1.7372)	0.895
C Vs G	1.329 (0.928- 1.903)	0.1205	1.793 (1.244- 2.584)	0.001682	1.087 (0.7595- 1.557)	0.6473

OR: odds ratio, CI: confidence interval, *: p value significant

Table 4: Distribution of genotypic and allelic frequencies of IL18 (rs187238) among the triad groups.

Genotype	Fetus		Mother		Father	
IFNγ+874	Cases	Controls	Cases	Controls	Cases	Controls
	N (%)	N (%)	N (%)	N (%)	N (%)	N (%)
TT	64 (53)	42 (35%)	71 (59%)	47 (41%)	70 (58)	59 (49)
TA	42 (35)	60 (50%)	40 (33%)	59 (49%)	39 (33)	44 (37)
AA	14 (12)	18 (15%)	9 (8%)	14 (12%)	11 (9)	17 (14)
T	170 (0.7)	144 (0.6)	182 (0.8)	153 (0.64)	179 (0.75)	162 (0.67)
A	70 (0.3)	96 (0.4)	58 (0.2)	87 (0.36)	61 (0.25)	78 (0.33)
Association Models	OR	P Value	OR	P Value	OR	P Value
	CI 95%		CI 95%		CI 95%	
TT Vs TA	1.17 (1.251-3.78)	0.005*	2.22 (1.29-3.84)	0.004*	1.33 (0.770- 2.32)	0.3014
TT Vs AA	0.9 (0.403-2.00)	0.796	2.34 (0.91-5.86)	0.062	0.7471 (0.429-1.29)	0.0825
TA+ TT Vs AA	1.33 (0.631- 2.56)	0.44	1.62 (0.676-3.92)	0.27	1.63 (0.731-3.657)	0.2276
TT Vs TA+AA	1.53 (1.15-2.827)	0.045*	2.2 (1.342 – 3.77)	0.0021*	1.447 (0.869-2.409)	0.155
TA Vs TT + AA	1.857 (1.106, 3.119)	0.01875	1.934 (1.148, 3.259)	0.01273	1.202 (0.7059, 2.048)	0.4974
T Vs A	1.71 (1.17-2.367)	0.012*	2 (1.39- 2.82)	0.0039*	1.412 (0.950-2.10)	0.08714

OR: odds ratio, CI: confidence interval, *: p value significant

Table 5: Distribution of genotypic and allelic frequencies of IFNγ rs2430561 among the triad groups.

4. Discussion

Spontaneous abortion is one of the most important areas of concern with both emotional and financial concerns for the couples. In spite of a lot of research done in this particular area, the exact cause of spontaneous abortion is unknown in 50% of the cases. The present study focused on the predisposing effect of cytokine IFN γ+874 T/A, IL6-137 G/C, IL18-174G/C gene polymerphism in the south Indian population of Telangana state towards spontaneous abortion susceptibility. Cytokines are inflammatory molecules that are secreted by T-lymphocytes and transported from the site of secretion through the circulatory system to ensure immunity from disease, infection, and injury [17, 18]. Many studies have examined the existence of a Th1-Th2 cell dichotomy during the progression from implantation to parturition [19]. These Th1 and Th2cells secret pro inflammatory and anti-inflammatory cytokines respectively in a balanced ratio that is essential for the maintenance of pregnancies [20, 21]. It is well noted that for the establishment of a normal pregnancy an increased production of anti-inflammatory cytokines is essential. Any imbalance in anti-inflammatory cytokine levels further leads to a proinflammatory dominance resulting in fetal rejection [22, 23]. Mosmann and Coffman have shown that pro-inflammatory cytokines IFN γ, IL2, TNF have deleterious effects on the conceptus [19]. Hence, an imbalance in pro and anti-inflammatory cytokines ratio which might be due to genetic variation such as, copy number variation (CNV) and single nucleotide polymorphism (SNP) can alter the function of regulatory regions of a gene further leading to an aberrant gene product expression [24, 25]. The role of IFN γ in successful pregnancies has been observed and implicated in a dose-dependent manner by inhibiting angiogenesis, remodeling vascular permeability and leukocyte trafficking [26, 27]. Murphy et al., showed that IFN γ is crucial for trophoblast invasion, growth and embryo differentiation [26]. The IFN γ gene bears a DNase I hypersensitive site with CA-repeat motifs at the position +874 T/A and the DNA sequence containing the +874T allele is the specific binding site (5′-ttcttacaacacaaaatcaaatct-3′) for the NF-kB transcription factor within the first intron correlated with the gene's potential for expression. The +874 T allele correlates with high-*IFN* production and +874 A allele correlates with low-*IFN* γ production as a response to stimuli [24, 28].

The present study in IFN γ (+874A/T) gene polymerphism and their association study with respect to spontaneous abortion revealed that TT genotype and T allele conferred an increased risk towards spontaneous abortion in maternal and fetal case group compared to control mother and fetal group Which can be further explained by genomic imprinting and maternal-fetal genotype incompatibility during the course of pregnancy [29]. James et al., Pilsner and Hu revealed that maternal genotype influence the intrauterine environment and may alter the fetal environment as well as the genetic makeup [30, 31]. Whereas, the paternal group does not show any association in both the cases and controls. Our results were in concordant with the findings of Parveen et al., Al Timimi., et al and Daher., et al, which also clearly showed TT genotype confers an increased risk for recurrent pregnancy loss [28, 32, 33]. From previous reports, it has been suggested that the T allele of IFN γ gene at +874 position is responsible for higher levels of IFN γ in serum, which further results in angiogenesis inhibition, suppression of Treg and Th_{17}polarization, inhibition of extravillous trophoblast invasion by and subsequently leading to the loss of pregnancy in humans and animal models [34-38]. Prignoshin et al., reported that the IFN γ (+874) A/A and T/A genotype as a risk factor for recurrent pregnancy loss of unknown cause in the Argentine

population [38]. However, studies in Iranian and Caucasian women could not establish any significant association between IFN γ (+874) gene polymorphism and recurrent pregnancy loss [39, 40].

The investigation for the role of IL 18 137G/C polymerphism in the present population revealed that the CC genotype was frequently represented in case mother subjects and conferred susceptibility towards the risk for spontaneous abortions. Our results are concordant with the study conducted in the Chinese Hans population by Jun Yue et al., which revealed a significant association towards spontaneous abortion susceptibility through recessive genetic model [41]. The previous reports also demonstrated that CC genotype led to low production of IL18 in patients with recurrent miscarriages. This effect of SNP can be explained by the changes that come at the DNA level and binding of H4TF-1nuclear factor binding site that further expressed in their transcription activity profile [42]. In contradictory to our results, previous studies showed lack of association between IL 18 and recurrent spontaneous abortions [43, 44]. Our investigation on the association between IL 16 gene polymorphism and spontaneous abortion revealed lack of association in all the case-control study groups which were in line with the findings of Prigoshin et al., Daher,et al. [38, 33]. However, few other studies revealed role of IL6 gene polymorphism towards the susceptibility of miscarriages [45-47].

In conclusion, to the best of our knowledge no studies have demonstrated a triad approach (fetal, maternal and paternal) in association with IFN γ +874, IL6 137 G/C, IL18 174 G/C gene polymorphism and spontaneous abortion. Thus, our study not only attempted to reveal the association of parental genotypes but also fetal genotypes and their association towards susceptibility of spontaneous abortion.

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Disclosure Statement

The authors report no conflict of interest.

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