Journal of Assisted Reproduction and Genetics (2019) 36:979–988
https://doi.org/10.1007/s10815-019-01431-y

GENETICS

Association of genetic polymorphism of vascular endothelial growth factor in the etiology of recurrent pregnancy loss: a triad study

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Received: 17 December 2018 / Accepted: 27 February 2019 / Published online: 15 March 2019

Abstract

Purpose The study estimates the association of VEGF gene polymorphism (-1154 G/A, -2549 I/D, -2578 C/A, and +936 C/T) in recurrent pregnancy loss from South Indian population.

Methods A total of 100 couples with the history of recurrent pregnancy loss and 100 couples with medically terminated pregnancies were considered. Fetal tissues with < 20 weeks of gestation including peripheral blood from case and control couples were collected. VEGF gene polymorphisms were determined by allele-specific polymerase chain reaction. Genotypic distribution and allele frequencies were evaluated by odds ratio with 95% confidence intervals. Haplotype analysis was done to determine the association of specific haplotypes with recurrent pregnancy loss.

Results The VEGF -1154 G/A polymorphism was significantly prevalent in the aborted fetuses and in their mothers whereas -2549 I/D polymorphism was significantly higher in the aborted fetuses while the + 936 C/T polymorphism showed prevalence in the case mothers revealing their statistically significant association to recurrent pregnancy loss. $A_{1154}D_{2549}A_{2578}T_{936}$ haplotype showed an increased risk in case fetuses and mothers whereas $A_{1154}D_{2549}C_{2578}C_{936}$, in case mothers and fathers while haplotype $G_{1154}I_{2549}A_{2578}C_{936}$ found a protective association in the case fetuses compared to controls.

Conclusion This is the first report of family-based triad study revealing a significant association of VEGF gene polymorphism in the etiology of recurrent pregnancy loss.

Keywords Recurrent pregnancy loss · AS-PCR · VEGF · Polymorphism · Fetus

Introduction

A recurrent pregnancy loss is the loss of 3 or more consecutive clinically recognized pregnancies before 20 weeks of gestational age [1]. It is the natural death of the fetus in the mother's womb at prenatal stages of pregnancy. It is a common complication that occurs in at least 15–20% of all recognized pregnancies mostly during the first trimester [2]. The recurrent miscarriages include multifactorial etiology with the involvement of genetic, endocrinological, anatomical, immunological, inflammatory, environmental, and infectious agents. Among several investigated etiologies, genetic factors were found to be majorly associated towards pregnancy loss [3]. Certain growth factors were in connection with the implantation of embryo and placental vasculature maintaining a healthy and successful pregnancy. Vascular endothelial growth factor (VEGF) is an essential growth factor that promotes the process of angiogenesis at the early embryonic stages of pregnancy [4]. The VEGF gene is located on the

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chromosome at 6p21.3 and encodes eight exons and seven introns [5]. It is a glycosylated mitogen secreted by the embryonic trophoblasts during the implantation of embryo thereby maintaining the tissue integrity of the growing fetus [6]. VEGF promotes the development of pre-existing (angiogenesis) and de novo vessels (vasculogenesis) during the growth of embryo. VEGF was mainly involved in the fetal blood vessel formation and maternal vascular development, feto-placental circulation and successful implantation of the embryo. The sufficient blood circulation was needed during placental growth along with the supply of oxygen for the fetal growth. The frequency of VEGF expression increases with the progress of pregnancy suggesting VEGF as an essential factor in the vascular development of placenta. Enhancing VEGF levels during pregnancy maintains the permeability of maternal blood vessels and controls the cardiovascular interactions in the fetus [7]. Thus, VEGF activates angiogenesis, vasculogenesis, endothelial cell proliferation, migration, and permeabilization of blood vessels during pregnancy. VEGF is produced highly during pregnancy whereas its low production is related to first trimester pregnancy loss [8].

The four common VEGF gene polymorphisms -1154 G/A, -2549 I/D, -2578 C/A located at promoter region and +936 C/T at 3′ untranslated region were considered to play an effective role in regulating the VEGF production [9]. The substitution of A allele in place of G allele at -1154 position; D allele in place of I allele at -2549 position; the transversion of A allele in place of C allele at -2578 position and T allele in place of C allele at +936 position were found to alter the production of VEGF thereby involved in the obstruction of placental angiogenesis resulting in abortions [10]. Hence, VEGF gene polymorphisms may act as an important modulator of the fetal as well as the placental development during pregnancy.

In view of the above statement, the present study is aimed to examine the association of four common VEGF gene polymorphisms -1154 G/A (rs1570360), -2549 I/D (rs35569394), -2578 C/A (rs699947), +936 C/T (rs3025039) and their haplotypes in spontaneous abortions by comparing respective fetal, maternal, and paternal groups. To the best of our knowledge, this is the first report of family-based triad study from Telangana State of South Indian population.

Materials and methods

Sample collection

The present study includes 100 case fetuses of recurrent pregnancy loss and 100 medically terminated control fetuses with their respective parents from Modern Government Maternity Hospital, Petlaburz, during 2013 to 2017. The peripheral blood (5 ml) from the parents was collected in sterile lithium and EDTA heparin vacutainers. An abortion can be detected by observing the aborted fetus through the transvaginal ultrasound scan. The aborted tissue sample (50 mg) was collected in a sterile 15-ml falcon tube with normal saline. The tubes were tightly capped. Care was taken to transport the samples immediately to the cytogenetic laboratory to prevent loss of viability of the cells. The study was approved by the Ethical Committee of Institute of Genetics, Osmania University (Approval No: 69/IG/IEC/2014 dt 13/08/2014). Demographic details with an informed written consent were obtained before the sample collection from all the subjects.

The inclusion criteria

Fetuses with < 20 weeks of gestation and their respective parents from controls and cases were considered for the present study. The case group with at least two abortions and no previous history of live births were included. The control group with two normal healthy children and no previous history of abortions were considered. Fetal tissues included in the study were obtained from recurrent pregnancy loss and medically terminated pregnancies.

The exclusion criteria

The parents with a history of thrombosis, chronic infections, autoimmune diseases, hormonal imbalances, or congenital anomalies of both control and case groups were excluded from the study. Maternally contaminated aborted tissues were also excluded from the study. The maternal group using oral contraceptives, any other medications before or during the course of pregnancy were not included. The paternal group with infertility and other health problems was excluded from the study.

Determination of VEGF gene polymorphisms

DNA was extracted from both aborted tissue and peripheral blood samples obtained from control and case triad groups using the phenol-chloroform method of Blinn and Stafford (1976). The isolated DNA was genotyped by AS-PCR (allele-specific polymerase chain reaction) method for the detection of VEGF gene polymorphisms using specific primers as shown in Table 1. The VEGF gene polymorphisms considered in this study were three promoter SNPs (-1154 G/A, -2549 I/D, -2578 C/A) and one 3′ UTR SNP (+936 C/T). The PCR conditions were as follows: 5 min at 96 °C, followed by 35 cycles of denaturation at 95 °C for 45 s, annealing at 62 °C for 45 s and extension at 72 °C for 30 s, and a final extension at 72 °C for 7 min. The PCR products were electrophoresed on a 2% agarose gel and visualized under UV light [11].

Table 1 Primer sequences of VEGF gene Polymorphisms

Polymorphism	AS-PCR primers	Primer sequences (5′–3′)	Allele base pair
VEGF -1154 G/A	Common reverse	5′-CCC CGC TAC CAG CCG ACTT-3′	252 bp
	G-forward	5′-GCC CGA GCC GCG TGT GGAG-3′	
	A-forward	5′-GCC CGA GCC GCG TGT GGAA-3′	
VEGF -2549 I/D	I-sense	5′-GCTGAGAGTGGGGCTGACTAGGTA-3′	211 bp
	D-anti-sense	5′GTTTCTGACCTGGCTATTTCCAGG-3′	229 bp
VEGF -2578 C/A	Common reverse	5′TGCCCCAGGGAACAAAGT3′	160 bp
	C-forward	5′TAGGCCAGACCCTGGCAC3′ 5′	
	A-forward	TAGGCCAGACCCTGGCAA3′	
VEGF +936 C/T	Common reverse	5′GGGTGGGTGTGTCTACAGGA3′	220 bp
	C-forward	5′GGTCGGGTGACCCAGCAC3′ 5′	
	T-forward	GGTCGGGTGACCCAGCAT3′	

Statistical analysis

Allele and genotype frequencies of VEGF promoter polymorphisms were compared between the control and case triad groups using a chi-square test. Statistical analysis was performed with OpenEpi 2 × 2 contingency table. Statistically significant differences were determined by two-tailed Fisher's exact test and p value < 0.05 was taken as statistically significant. Odds ratios (ORs) and 95% confidence intervals (95% CIs) were used to measure the correlation between VEGF genotypes and recurrent miscarriages. A χ^2 test using SNPstat was done to determine the statistical analysis between control and case triad groups [12]. The coefficient (D') of pairwise linkage disequilibrium (LD) between the four SNPs was calculated using the Haploview version 4.2 [13]. Sample size for the present study was determined for an unmatched case-control study with 95% confidence interval and 80% power.

Results

A total of 300 control triad samples (100 × 3 = 300) and 300 case triad samples (100 × 3 = 300) were included in the present study. The results obtained were compared among the fetal, maternal, and paternal groups. A statistical power of more than 80% was attained for an unmatched case-control study with 95% confidence interval for the present study. Demographic details like maternal age, paternal age, consanguinity, the number of miscarriages, and gestational duration was taken from 100 medically terminated couples and 100 couples with the history of recurrent pregnancy loss belonging to the same geographic area with the help of standard pro forma and are presented in Table 2. The demographic analysis showed a significant fourfold increased risk with respect to the maternal age and consanguinity in case group compared to control group. A threefold and a twofold significant risk with the paternal age and early gestational duration were revealed in case group compared to controls respectively.

Thus, the couples (females and males) with ≥ 30 years of age are at an increased risk for abortions compared to < 30 years age group. Couples with consanguineous marriages are more prone to abortions compared to non-consanguineous group. Gestational duration of 6–12 weeks is at an increased risk for abortions compared to 12–20 weeks of gestational duration.

VEGF -1154 G/A polymorphism

The genotype distribution and allele frequencies of -1154 G/A polymorphism at the promoter region of the VEGF gene in aborted fetuses along with their parents compared to respective controls were shown in Table 3. The study revealed an increased prevalence of AA genotype and A allele in the aborted fetuses ($p < 0.001$**) and in their respective mothers ($p < 0.001$**) in comparison to that of control fetuses and their mothers. In the paternal group, insignificant association was observed in all the genetic models applied (co-dominant, dominant, or recessive). The statistically significant 17-fold increased risk was observed in the aborted fetuses (OR = 17.54; 95% CI, 6.19–49.64; $p < 0.001$**) and a 13-fold increased risk in case mothers (OR = 13.7; 95% CI, 5.50–34.34; $p < 0.001$**) compared to their respective controls.

VEGF -2549 I/D polymorphism

The distribution of genotype and allele frequencies of -2549 I/D polymorphism in aborted fetal, maternal, and paternal groups in comparison with their respective controls was presented in Table 4. In the fetal group, the frequency of DD genotype was significantly associated with a sixfold risk in the aborted fetuses compared to control fetuses (OR = 6.60; 95% CI, 2.62–16.61; $p < 0.001$**), whereas the maternal and

Table 2 Demographic details of case and control groups

Variables	Controls ($N = 100\%$)	Cases ($N = 100\%$)	OR	95% CI	p value
Maternal age (years)					
< 30	92 (92%)	70 (70%)			
≥ 30	8 (8%)	30 (30%)	4.36	1.82–10.48	0.001*
Paternal age (years)					
< 30	70 (70%)	24 (24%)			
≥ 30	30 (30%)	76 (76%)	3.5	1.88–6.49	0.0001**
Consanguinity					
Yes	26 (26%)	60 (60%)			
No	74 (74%)	40 (40%)	4.74	2.51–8.96	0.000001**
Number of miscarriages					
< 3	0 (0%)	89 (89%)			
≥3	0 (0%)	11 (11%)	–	–	–
Gestational duration (weeks)					
6–12	40 (40%)	63 (63%)			
12–20	60 (60%)	37 (37%)	2.55	1.44–4.51	0.001*

p value < 0.05* is statistically significant, p value < 0.001**

paternal groups revealed no significant differences in all the genetic models (co-dominant, dominant, or recessive) studied.

VEGF -2578 C/A polymorphism

The prevalence of genotype and allele frequencies of VEGF promoter polymorphism -2578 C/A in triad groups were presented in Table 5. In the fetal group, the variant homozygous genotype (AA) of the -2578 polymorphism found no statistically significant association in cases compared to controls but showed a statistically significant association of A allele in the aborted fetuses (OR = 1.83; 95% CI, 1.00–3.37; $p = 0.04$*) in comparison to that of control fetuses. Insignificant association of AA genotype and A allele was observed in both the maternal and paternal groups. A higher frequency of CA genotype was observed in the fetal and maternal groups whereas CC genotype in the paternal group.

Table 3 Genotype distribution and allele frequencies of the VEGF -1154 G/A polymorphism in control and case group

Genotype	Fetus		Mother		Father	
VEGF -1154 G/A	Controls $N = 100$ (%)	Cases $N = 100$ (%)	Controls $N = 100$ (%)	Cases $N = 100$ (%)	Controls $N = 100$ (%)	Cases $N = 100$ (%)
GG	74 (74%)	27 (27%)	55 (55%)	14 (14%)	64 (64%)	51 (51%)
GA	21 (21%)	41 (41%)	35 (35%)	51 (51%)	29 (29%)	39 (39%)
AA	5 (5%)	32 (32%)	10 (10%)	35 (35%)	7 (7%)	10 (10%)
G	170 (0.85)	96 (0.48)	146 (0.73)	80 (0.40)	156 (0.78)	140 (0.7)
A	30 (0.15)	104 (0.52)	54 (0.27)	120 (0.60)	44 (0.22)	60 (0.3)
HWE	3.93	3.17	1.49	0.45	1.98	0.39
p value	0.04*	0.07	0.22	0.50	0.15	0.53
	OR (95% CI)	p value	OR (95% CI)	p value	OR (95% CI)	p value
GA Vs GG	5.35 (2.69–10.62)	< 0.001**	5.72 (2.76–11.85)	< 0.001**	1.68 (0.92–3.09)	0.08
AA Vs GG	17.54 (6.19–49.64)	< 0.001**	13.7 (5.50–34.34)	< 0.001**	1.79 (0.63–5.03)	0.26
GA + AA Vs GG	7.69 (4.10–14.42)	< 0.001**	7.66 (3.77–14.94)	< 0.001**	1.70 (0.97–3.00)	0.06
AA Vs GG + GA	0.11 (0.04–0.30)	< 0.001**	0.20 (0.09–0.44)	< 0.001**	0.67 (0.24–1.85)	0.65
GA Vs GG + AA	2.64 (1.37–5.08)	0.005*	1.95 (1.00–6.32)	< 0.001**	1.54 (0.83–2.89)	0.22
A Vs G	6.02 (3.75–9.66)	< 0.001**	4.03 (2.65–6.14)	< 0.001**	1.52 (0.97–2.40)	0.06

p value < 0.05* is statistically significant, p value < 0.001**

Table 4 Genotype distribution and allele frequencies of the VEGF -2549 I/D polymorphism in control and case group

Genotype	Fetus		Mother		Father	
VEGF -2549 I/D	Controls $N = 100$ (%)	Cases $N = 100$ (%)	Controls $N = 100$ (%)	Cases $N = 100$ (%)	Controls $N = 100$ (%)	Cases $N = 100$ (%)
II	62 (62%)	27 (27%)	18 (18%)	10 (10%)	29 (29%)	17 (17%)
ID	30 (30%)	50 (50%)	72 (72%)	75 (75%)	66 (66%)	75 (75%)
DD	8 (8%)	23 (23%)	10 (10%)	15 (15%)	5 (5%)	8 (8%)
I	154 (0.77)	104 (0.52)	108 (0.54)	94 (0.47)	124 (0.62)	110 (0.55)
D	46 (0.23)	96 (0.48)	92 (0.46)	106 (0.53)	76 (0.38)	90 (0.45)
HWE	2.34	0	20.18	25.38	16.05	26.24
p value	0.12	1.00	< 0.00001**	< 0.00001**	0.00009**	< 0.00001**
	OR (95%CI)	p value	OR (95%CI)	p value	OR (95%CI)	p value
ID Vs II	3.82 (2.01–7.25)	< 0.001**	1.87 (0.81–4.33)	0.13	0.93 (0.97–3.84)	0.11
DD Vs II	6.60 (2.62–16.61)	< 0.001**	2.7 (0.88–8.21)	0.07	2.72 (0.76–9.69)	0.11
ID + DD Vs II	4.41 (2.42–8.02)	< 0.001**	1.97 (0.86–4.52)	0.10	1.99 (1.01–3.92)	0.04*
DD Vs II+ ID	0.29 (0.12–0.68)	0.003*	0.63 (0.26–1.47)	0.43	0.60 (0.19–1.91)	0.38
ID Vs II + DD	2.33 (1.26–4.30)	0.009*	1.16 (0.59–2.27)	0.77	1.54 (0.45–5.24)	0.70
D Vs I	3.09 (2.00–4.75)	< 0.001**	1.29 (0.87–1.92)	0.19	1.36 (0.91–2.02)	0.12

p value < 0.05* is statistically significant, p value < 0.001**

VEGF +936 C/T polymorphism

The genotype and allele frequencies of the + 936 C/T polymorphism in the 3′ UTR region of the VEGF gene in couples with recurrent pregnancy loss and aborted fetuses compared to respective controls were presented in Table 6. In the maternal group, the prevalence of TT genotype is higher in cases compared to controls and found statistically significant ninefold increased risk (OR = 9.05; 95% CI, 1.96–41.71; $p < 0.0001$**). In the fetal and paternal groups, statistically significant association was not observed in all the genetic models analyzed.

Haplotype analysis of the VEGF gene

Haplotype analyses for the combination of the four polymorphisms, VEGF -1154A/G (rs1570360), -2549I/D (rs35569394),

Table 5 Genotype distribution and allele frequencies of the VEGF -2578 C/A polymorphism in control and case group

Genotype	Fetus		Mother		Father	
VEGF -2578 C/A	Controls $N = 100$ (%)	Cases $N = 100$ (%)	Controls $N = 100$ (%)	Cases $N = 100$ (%)	Controls $N = 100$ (%)	Cases $N = 100$ (%)
CC	38 (38%)	25 (25%)	32 (32%)	35 (35%)	76 (76%)	78 (78%)
CA	48 (48%)	56 (56%)	48 (48%)	50 (50%)	22 (22%)	15 (15%)
AA	14 (14%)	19 (19%)	20 (20%)	15 (15%)	2 (2%)	7 (7%)
C	124 (0.62)	106 (0.53)	112 (0.56)	120 (0.60)	170 (0.85)	174 (0.87)
A	76 (0.38)	94 (0.47)	88 (0.44)	80 (0.40)	30 (0.15)	26 (0.13)
HWE	0.03	1.54	0.07	0.17	0.08	15.61
p value	0.86	0.21	0.79	0.68	0.77	< 0.00001**
	OR (95% CI)	p value	OR (95% CI)	p value	OR (95% CI)	p value
CA Vs CC	1.77 (0.94–3.34)	0.06	0.95 (0.51–1.77)	0.87	0.66 (0.32–1.37)	0.26
AA Vs CC	2.06 (0.87–4.85)	0.09	0.68 (0.30–1.56)	0.36	3.41 (0.68–16.94)	0.11
CA + AA Vs CC	1.83 (1.00–3.37)	0.04*	0.87 (0.48–1.57)	0.65	0.89 (0.46–1.72)	0.7
AA Vs CC + CA	0.69 (0.32–1.47)	0.34	1.41 (0.67–2.95)	0.35	0.27 (0.05–1.33)	0.08
CA Vs CC + AA	1.39 (0.77–2.51)	0.1	1.08 (0.60–1.95)	0.9	0.62 (0.28–1.35)	0.2
A Vs C	1.44 (0.97–2.15)	0.06	0.84 (0.57–1.26)	0.41	1.13 (0.64–2.00)	0.66

p value < 0.05* is statistically significant, p value < 0.001**

Table 6 Genotype distribution and allele frequencies of the VEGF +936 C/T polymorphism in control and case group

Genotype	Fetus		Mother		Father	
VEGF +936 C/T	Controls $N = 100$ (%)	Cases $N = 100$ (%)	Controls $N = 100$ (%)	Cases $N = 100$ (%)	Controls $N = 100$ (%)	Cases $N = 100$ (%)
CC	56 (56%)	47 (47%)	78 (78%)	56 (56%)	62 (62%)	55 (55%)
CT	38 (38%)	38 (38%)	20 (20%)	31 (31%)	25 (25%)	26 (26%)
TT	6 (6%)	15 (15%)	2 (2%)	13 (13%)	13 (13%)	19 (19%)
C	150 (0.75)	132 (0.66)	176 (0.88)	142 (0.71)	148 (0.74)	136 (0.68)
T	50 (0.25)	68 (0.34)	24 (0.12)	58(0.29)	52 (0.26)	64 (0.32)
HWE	0.02	2.35	0.28	5.73	11.7	16.21
p value	0.65	0.12	0.59	0.01*	0.0007**	< 0.00001**
	OR (95% CI)	*p* value	OR 95% CI)	*p* value	OR (95% CI)	*p* value
CT Vs CC	0.76 (0.41–1.43)	0.40	2.15 (1.11–4.17)	0.02*	1.17 (0.60–2.26)	0.63
TT Vs CC	1.91 (0.67–5.42)	0.21	9.05 (1.96–41.71)	< 0.001**	1.64 (0.74–3.64)	0.21
CT + TT Vs CC	0.92 (0.51–1.66)	0.78	2.78 (1.50–5.15)	< 0.001**	1.33 (0.75–2.34)	0.31
TT Vs CC + CT	0.45 (0.17–1.24)	0.11	0.13 (0.03–0.62)	0.003*	0.63 (0.29–1.37)	0.24
CT Vs CC + TT	0.97 (0.53–1.79)	0.4	1.81 (0.92–3.59)	0.05*	1.06 (0.54–2.09)	0.9
T Vs C	1.13 (0.72–1.76)	0.58	2.92 (1.72–4.964)	< 0.001**	1.37 (0.89–2.12)	0.15

p value < 0.05* is statistically significant, *p* value < 0.001**

-2578C/A (rs699947), and +936C/T (rs3025039), in the family-based triad study were given in Table 7. Sixteen possible haplotype frequencies among case and control triad groups were noticed. The study revealed a statistically significant association of specific haplotypes with the susceptibility to abortions. In aborted fetuses, the haplotype analysis revealed the significant association of $A_{1154}D_{2549}A_{2578}C_{936}$ and $A_{1154}D_{2549}A_{2578}T_{936}$, haplotypes with a fivefold and twofold increased risk to recurrent pregnancy loss respectively (OR = 5.37; 95% CI, 1.09–26.55; $p = 0.04$*, OR = 2.94; 95% CI, 1.12–7.68; $p = 0.02$*) while haplotype $G_{1154}I_{2549}A_{2578}C_{936}$ showed a protective role (OR = 0.11; 95% CI, 0.02–0.60; $p = 0.01$*). In the maternal group, haplotypes $A_{1154}D_{2549}A_{2578}T_{936}$ (OR = 6.59; 95% CI, 1.91–22.74; $p = 0.003$*) and $A_{1154}D_{2549}C_{2578}C_{936}$ (OR = 4.66; 95% CI, 1.03–21.14; $p = 0.04$*) showed significant association to recurrent pregnancy loss. In the paternal group, $A_{1154}D_{2549}C_{2578}C_{936}$ (OR = 4.68; 95% CI, 1.58–13.85; $p = 0.005$*) and $G_{1154}D_{2549}C_{2578}C_{936}$ (OR = 5.28; 95% CI, 1.74–15.98; $p = 0.003$*) haplotypes showed an increased risk to abortions compared to their respective controls.

Linkage disequilibrium analysis

Linkage disequilibrium (LD) analysis, defined by the delta coefficient (D'), was estimated for the four SNPs of VEGF (G-1154A, I-2549D, C-2578A, and C+936 T) in both control and case triad groups. Linkage disequilibrium between the four SNPs of VEGF polymorphism in case fetal, maternal, and paternal groups is given in Figs. 1, 2 and 3 respectively. In aborted fetuses, a weak LD was observed between locus -2578 and at locus -2549 ($D' = 0.463$); between locus -1154 and at locus -2578 ($D' = 0.0607$); between locus -1154 and at locus +936 ($D' = 0.18040$); between locus -2549 and at locus -1154 ($D' = 0.1214$); between locus -2578 and at locus

Table 7 Haplotype distribution of VEGF gene polymorphisms in case triad groups and control triad groups

GROUP	S. no.	Haplotype	Controls	Cases	OR (95% CI)	*p* value
Fetus	1	ADAC	0.0252	0.0769	5.37 (1.09–26.55)	0.04*
	2	ADAT	0.0377	0.1334	2.94 (1.12–7.68)	0.02*
	3	GIAC	0.0201	0.0148	0.11 (0.02–0.60)	0.01*
Mother	1	ADAT	0.0354	0.1539	6.59 (1.91–22.74)	0.003*
	2	ADCC	0.0615	0.1246	4.66 (1.03–21.14)	0.04*
Father	1	ADCC	0.0866	0.1218	4.68 (1.58–13.85)	0.005*
	2	GDCC	0.1066	0.1627	5.28 (1.74–15.98)	0.003*

First position: *G*, G allele at -1154 G/A; *A*, A allele at -1154 G/A. Second position: *I*, I allele at -2549 I/D; *D*, D allele at -2549 I/D. Third position: *C*, C allele at -2578 C/A; *A*, A allele at -2578 C/A. Fourth position: *C*, C allele at +936 C/T; *T*, T allele at +936 C/T. *p* value < 0.05* is statistically significant, *p* value < 0.001**

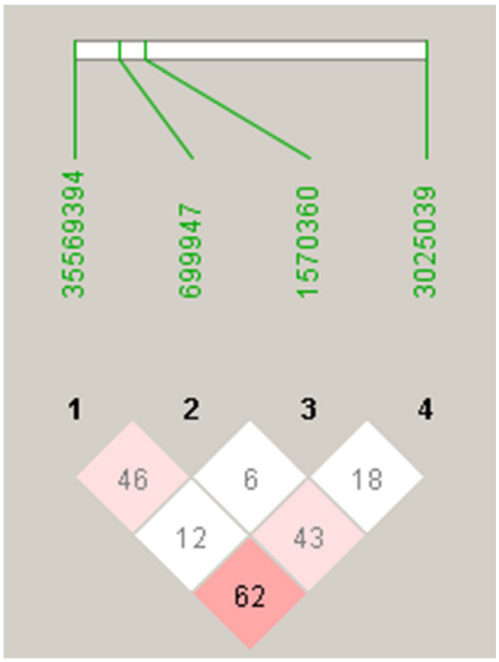

Fig. 1 The pattern of linkage disequilibrium between single nucleotide polymorphisms -1154 G/A, -2549 I/D, -2578 C/A, and +936 C/T of VEGF gene in spontaneously aborted fetuses

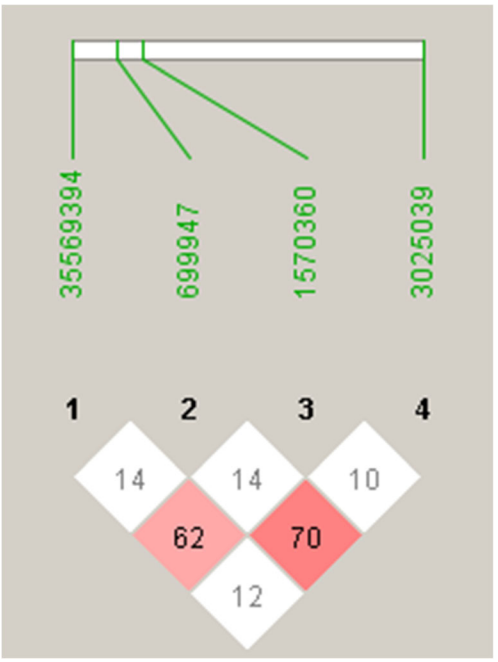

Fig. 3 The pattern of linkage disequilibrium between single nucleotide polymorphisms -1154 G/A, -2549 I/D, -2578 C/A, and +936 C/T of VEGF gene in spontaneously aborted fathers

+936 (D' = 0.4307). A moderate LD was noticed between locus -2549 and at locus +936 (D' = 0.6263). In case mothers, a mild LD was observed between locus -2578 and at locus -2549 (D' = 0.504); between locus -2578 and at locus -1154 (D' = 0.404); between locus -1154 and at locus +936 (D' = 0.2731); between locus -2578 and at locus +936 (D' = 0.3267); between locus +936 and at locus -2549 (D' = 0.4632) and a moderate LD between allele A of locus -1154 and allele D of locus -2549 (D' = 0.7636) was shown. In case fathers, a moderate linkage disequilibrium was observed between locus -2578 and at locus -2549 (D' = 0.732) and between locus -1154 and at locus -2549 (D' = 0.777). A weak LD was observed between locus -2578 and at locus -1154 (D' = 0.222); between locus -1154 and at locus +936 (D' = 0.222); between locus -2578 and at locus +936 (D' = 0.2631); between locus +936 and at locus -2549 (D' = 0.112). Thus, the present family-based triad study observed the strong linkage disequilibrium of these SNPs among the fetal, maternal, and paternal groups indicating the inheritance of risk alleles from the maternal and paternal origin. Linkage disequilibrium among control triad groups (fetal, maternal, and paternal) did not reveal any statistically significant association (figures not presented).

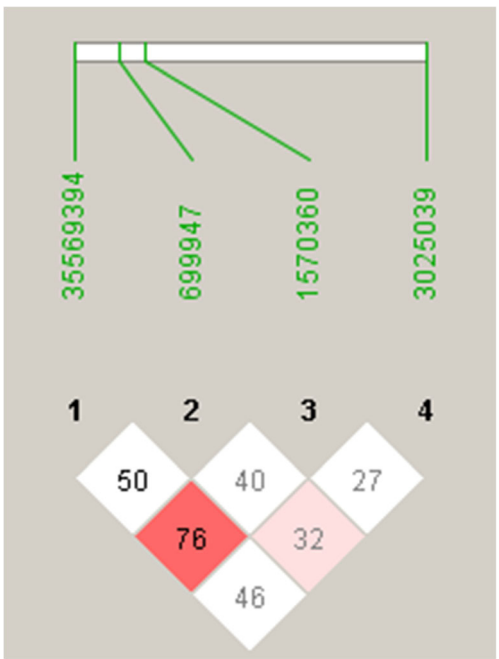

Fig. 2 The pattern of linkage disequilibrium between single nucleotide polymorphisms -1154 G/A, -2549 I/D, -2578 C/A, and +936 C/T of VEGF gene in spontaneously aborted mothers

Discussion

VEGF is an important multifunctional angiogenic and vasculogenesis stimulator at the feto-placental circulation during pregnancy [14]. It is an essential growth factor responsible for the early implantation of the embryo. The alterations in VEGF production during the first trimester of pregnancy inhibit the growth and development of fetal blood vessels leading to early abortions [15, 16].

The analysis of the demographic features suggests a significant association of recurrent pregnancy loss in relation to maternal age, paternal age, consanguinity, and gestational duration in

the case group compared to control group. VEGF gene polymorphisms are mainly correlated with the VEGF expression, particularly during early pregnancy. The transition of A allele in place of G allele at -1154 position reduces the expression of VEGF which results in the impaired vascular and placental development of the embryo leading to abortions [17]. Thus, A allele and AA genotype is considered to be associated with an increased risk towards the pathogenesis of miscarriages. The study reveals the association of VEGF gene -1154 G/A polymorphism in case fetuses and in their mothers compared to controls. According to our results, AA genotype was more frequent in the case fetuses and case mothers which suggests a possible susceptible role of AA genotype in the pathogenesis of recurrent pregnancy loss. G allele has a protective role in the case fetal and maternal group compared to the A allele. Thus, an increased distribution of AA genotype and A allele frequencies were observed among both fetal group and the maternal group but not in paternal group indicating it as a fetal and maternal risk factor.

Moreover, a relationship between VEGF gene polymorphism and recurrent pregnancy loss was reported in different studies. Şamli et al., (2012) revealed the high prevalence of the -1154 G/A polymorphism and A/A genotype were significantly higher in 38 women with the recurrent pregnancy loss from the region of Bursa, Turkey [18]. Xinghua et al. found that the rs1570360 variant was at statistically significant risk in recurrent spontaneous abortions among non-Asian population [19]. Papazoglou et al. focused the association of −1154 G/A with idiopathic recurrent pregnancy loss [20]. In contrast to our findings, Eller et al. found no difference when compared with the American women homozygous -1154 A allele between recurrent spontaneous miscarriage cases and controls from the region of Utah [21]. Xing et al. study revealed that VEGF -1154 G /A polymorphism was not associated with the susceptibility to recurrent spontaneous abortions in Chinese Han women [22]. These discrepancies for the SNP -1154 G/A might be due to the variability of its allele frequencies among different ethnic groups.

The insertion/deletion (I/D) of an 18 bp fragment located at -2549 position of the promoter region is involved in the development of many diseases, particularly those that are related to angiogenesis. The substitution of D allele in place of I allele at -2549 position decreases the VEGF expression progressing the pregnancy to miscarriages. Hence, the D allele and DD genotype are related to that of abortion risk. The present triad study revealed the statistically significant association of VEGF-2549 I/D polymorphism only in spontaneously aborted fetuses compared to control fetuses but no significant differences were observed in maternal and paternal groups. The D allele is associated with an increased risk to aborted fetuses showing VEGF I/D polymorphism, an independent risk factor in aborted fetuses. Even though a higher frequency of the DD genotype in spontaneously aborted mothers compared to control mothers was observed, the difference did not reach statistical significance and no association was noticed under any genetic model. Surprisingly, a higher prevalence of the ID genotype than DD and II genotypes were noticed in the maternal and paternal groups. Limited studies were done to evaluate the association of VEGF -2549 I/D polymorphism in recurrent pregnancy loss. Shagun et al., (2011) found no significant association between -2549 I/D promoter polymorphism and recurrent miscarriages [23]. Nina et al. found no association of -2549 I/D polymorphism in women with idiopathic recurrent spontaneous abortions but revealed the association of -2549 I/D polymorphism in men [24].

The transversion of A allele in place of C allele at -2578 promoter region lowers the expression of VEGF at first-trimester prone to recurrent pregnancy loss showing the risk for the termination of pregnancy in association with the A allele and AA genotype [17]. In the case fetuses, the variant homozygous genotype (AA) of the VEGF -2578 C/A polymorphism found statistically insignificant association compared to control fetuses indicating no association towards recurrent abortions. Similarly, in both the maternal and paternal groups, an insignificant association of AA genotype and A allele was observed. Thus, statistically, an insignificant association of VEGF -2578 C/A polymorphism with the triad study was observed in the present study. The present study results are in support of other findings done in several ethnic populations. Xingua Xu et al., (2015) observed no associations between -2578 polymorphism and recurrent spontaneous abortions [19]. Lee et al. showed that -2578 C/A polymorphism of VEGF were not significantly associated with the risk of recurrent spontaneous abortions in any of the models [25].

The expression of VEGF gets diminished by the transition of T allele in place of C allele at +936 position which blocks the angiogenesis of the embryo leading to pregnancy loss [17]. This confirms that T allele and TT genotype increases the risk of pregnancy loss, particularly during embryogenesis. The case maternal group showed a significant association of VEGF +936 C/T polymorphism compared to controls. Both the case fetal and paternal groups observed insignificant differences compared to respective control groups. Hence, an increased frequency of T allele was shown in the maternal group but not in the fetal group and paternal group indicating it as a maternal risk factor. Sinem et al. showed that the TT genotype and T allele frequencies of +936 C/T polymorphism were found as risk factors for abortion in fetus compared to their mothers and controls [26].

Several studies have been carried out on the association between the different SNPs of the VEGF gene and the susceptibility to abortions, but these studies showed conflicting results due to different geographical areas. The present study revealed the significantly increased risk of -1154, -2549 and +936 polymorphisms of VEGF gene and their haplotypes to the susceptibility of miscarriages. In support of the present study, Brogan et al., (1999) demonstrated the association of the -1154 G/A, -2578 C/A, -2549 I/D and +936 C/T polymorphisms with altered VEGF production and thus to spontaneous abortions [13]. In contrary to results, Papazoglou et al. observed no association of -2578, -634, and +936 polymorphisms with miscarriages [20].

This is the first family-based triad study reporting the haplotype association of VEGF polymorphism with recurrent pregnancy loss. The results found that $A_{1154}D_{2549}A_{2578}T_{936}$ combination was highly susceptible to abortions in the fetal group and the maternal group compared to their respective controls. Haplotype $A_{1154}D_{2549}C_{2578}C_{936}$ showed a statistically significant increased risk of abortions in the maternal group and paternal group. The $G_{1154}I_{2549}A_{2578}C_{936}$ combination confirmed the significant protective association in the aborted fetal group. On considering individual genotype analysis of VEGF polymorphisms, the findings observed an insignificant association in the paternal group but by evaluating combined haplotype analysis of all the four polymorphisms, a statistically significant association in case fathers compared with controls was observed. The study indicated the susceptible role of combined association of -1154,-2549,-2578 and +936 polymorphisms towards recurrent pregnancy loss rather than their individual associations. The VEGF-dependent angiogenesis mechanism is essential for the embryonic and placental vascular development to promote the rapid growth of the fetus during early stages of pregnancy. However, the reduced expression of VEGF gene inhibits the angiogenesis leading to decrease in the feto-placental blood supply resulting in delay in the implantation and development of embryo which further relates to the recurrent pregnancy loss [16]. Thus, this study supports the major role of VEGF in the pathophysiology of recurrent pregnancy loss.

Thus, the present triad study clearly found that VEGF polymorphisms in combination were highly associated with recurrent pregnancy loss suggesting the complex interaction of these four SNPs resulting in lowered expression of VEGF thereby leading to miscarriages. Further, the strong linkage disequilibrium of these SNPs in the present triad study supports the hypothesis that the inheritance of risk alleles of the VEGF gene from the maternal and paternal origin results in the improper development of embryo during pregnancy. However, further analysis with larger sample size needs to be analyzed for the confirmation of the obtained findings.

Conclusion

The present study investigated the genetic role of VEGF gene polymorphisms (-1154, -2549, -2578, and +936) among the fetal, maternal, and paternal groups. In this family-based triad study, for the first time, the haplotype analysis of VEGF polymorphisms with recurrent miscarriages revealed that the VEGF gene promoter polymorphisms confer susceptibility to pregnancy loss. Thus, the VEGF gene promoter polymorphism might act as an important genetic determinant in the etiology of recurrent pregnancy loss.

Funding information The present work was greatly supported by the Senior Research Fellowship granted by the Indian Council of Medical Research, New Delhi (ICMR–45/1/2014-HUM-BMS).

Compliance with ethical standards

The study was approved by the Ethical Committee of Institute of Genetics, Osmania University (Approval No: 69/IG/IEC/2014 dt 13/08/2014). Demographic details with an informed written consent were obtained before the sample collection from all the subjects.

Conflict of interest The authors declare that they have no conflict of interest.

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