GMR Genetics and Molecular Research
Online Journal
ISSN:1676-5680

Vascular endothelial growth factor gene 1154 G/A, 2578 C/A, 460 C/T, 936 C/T polymorphisms and association with recurrent pregnancy losses

H. Şamlı[1], B.Ç. Demir[2], A. Özgöz[3], M.A. Atalay[2] and G. Uncu[2]

[1]Department of Genetics, Faculty of Veterinary Medicine, Uludağ University, Bursa, Turkey
[2]Department of Obstetrics and Gynecology, Faculty of Medicine, Uludağ University, Bursa, Turkey
[3]Department of Nutrition and Dietetics, Fazıl Boyner School of Health, Kastamonu University, Kastamonu, Turkey

Corresponding author: H. Şamlı
E-mail: halesamli@gmail.com

Genet. Mol. Res. 11 (4): 4739-4745 (2012)
Received June 5, 2012
Accepted September 14, 2012
Published December 17, 2012
DOI http://dx.doi.org/10.4238/2012.December.17.6

ABSTRACT. Vascular endothelial growth factor (VEGF) regulates endothelial cell proliferation, migration and differentiation. VEGF plays a critical role in angiogenesis during placenta formation. We investigated whether VEGF gene polymorphisms are associated with recurrent pregnancy loss. Thirty-eight women with recurrent pregnancy loss and 30 control women with live-born children were recruited from 2010 to 2011 in the region of Bursa, Turkey. VEGF gene polymorphisms were assessed with PCR-RFLP analysis of DNA samples obtained from leukocytes. DNA fragments were investigated by using appropriate primers. SNP scanning was performed using *Mnl*I, *Bgl*II, *Bsh*I2361, *Hsp*92II restriction enzymes for 1154 G/A, 2578 C/A, 460 C/T, and 936 C/T polymorphisms, respectively. The frequencies of 2578 C/A, 460 C/T, 936 C/T polymorphisms were not significantly different between the controls and women with recurrent pregnancy loss. However, the

prevalence of the 1154 G/A polymorphism A/A genotype was significantly higher in the recurrent pregnancy loss group (23.7 *vs* 3.4%). One of the four common polymorphisms of the VEGF gene was found to be more frequent in women with recurrent pregnancy loss. It is possible that disruption of VEGF function and placental angiogenesis can contribute to pregnancy loss in women with recurrent pregnancy loss.

Key words: Polymorphisms; Recurrent pregnancy loss; VEGF

INTRODUCTION

The etiology of recurrent pregnancy loss (RPL), which affects around 5% of women of childbearing age, appears to be diverse and controversial (Regan, 1998). Some of the reasons for RPL are chromosomal anomalies, infections, hormonal irregularities, immunological factors, and coagulation protein/platelet disorders, as well as unknown reasons (Wilcox et al., 1988; Li et al., 2002). The success of the implantation of an embryo depends on the formation of its own blood vessels (Torry et al., 2004). Therefore, the successful establishment of feto-placental circulation is essential for the continuation of pregnancy (Letsky and Swiet, 1994; Hellgren, 1996).

The family of vascular endothelial growth factors (VEGF) consists of seven different factors designated VEGF-A, VEGF-B, VEGF-C, VEGF-D, VEGF-E, placenta growth factor, and snake venom VEGF (VEGF-F). The receptors for VEGF are located on the surface of endothelial cells. The VEGF gene is located on chromosome 6p21.3 (NCICB, 2012; Ferrara et al., 2003; Harry and Paleolog, 2003). VEGF, which has a mitogenic effect on endothelial cells and increases their migratory capacity, is secreted from ovarian follicles just before ovulation to increase the formation of new blood vessels. Following ovulation, the corpus luteum takes on this process (te Velde et al., 1997; Zygmunt et al., 2003). Immediately following implantation, VEGF is secreted by the embryonic trophoblasts. Although VEGF secretion decreases towards the end of first stage of embryological development, it increases drastically in organogenesis stages. Polymorphisms in the VEGF genes have been shown to be correlated with variations in the production of the VEGF protein (Brogan et al., 1999; Renner et al., 2000; Watson et al., 2000; Awata et al., 2002; Mohammadi et al., 2003).

The aim of this study was to determine the relationship between polymorphisms in the VEGF gene [1154 G/A and 2578 C/A in the promoter, 460 C/T in the 5'-untranslated region (UTR), 936 C/T in the 3'-UTR] (Brogan et al., 1999; Renner et al., 2000; Awata et al., 2002; Mohammadi et al., 2003) and the healthy development of angiogenesis in the embryo. This is especially important in early implantation and embryonic stages and thereby for the continuation of the pregnancy.

MATERIAL AND METHODS

Ethical approval for this study was obtained from the Local Ethics Committee before the study was started. The principles adopted in the Helsinki Declaration were followed, and written informed consent was also obtained from the participants.

For the study, an experimental group of 38 women with RPL, who had a history of at least two abortions before the 20th week of pregnancy without any other incurring reasons were recruited. The control group comprised 30 women with children and no history of abortion. It was ensured that the two groups had the same frequency of consanguineous marriages. Detailed history was taken from all the subjects by expert physicians at Department of Obstetrics and Gynecology, School of Medicine, Uludağ University, and their pedigree was determined in addition to physical examinations. The study was also carried out at Department of Obstetrics and Gynecology, School of Medicine, Uludağ University, between 2010 and 2011.

Peripheral blood was collected from the subjects in 2-mL EDTA tubes, and genomic DNA was isolated using a Puregene DNA isolation kit. To determine the polymorphisms 1154 G/A, 2578 C/A, 460 C/T, and 936 C/T, genomic DNA was amplified using the primers (F5'-TCCTGCTCCCTCCTCGCCAATG-3') (R5'-GGCGGGGACAGGCGAGCATC-3'), (F5'-GGATGGGGCTGACTAGGTAAGC-3') (R5'-AGCCCCCTTTTCCTCCAAC-3'), (F5'-TGTGCGTGTGGGGTTGAGCG-3') (R5'-TACGTGCGGACAGGGCCTGA-3'), and (F5'-AAGGAAGAGGAGACTCTGCGC-3') (R5'-TATGTGGGTGGGTGTGTCTACAG-3'). The PCR conditions were as follows: 5 min at 95°C, followed by 35 cycles of denaturation at 94°C for 45 s, annealing at 60°C for 45 s, extension at 72°C, and a final extension at 72°C for 7 min (Table 1). The PCR products were detected on a 2% agarose gel. Afterwards, the PCR products were digested with the restriction enzymes *Mnl*I, *Bgl*II, *Bsh*I2361, and *Hsp*92II, and the digested products were detected on a 3% Nusieve agarose gel.

The Statistical Package for Social Sciences program was used for statistical analysis, and all data were compared using the Student *t*-test. Final analysis was carried out using the Fisher chi-square test.

RESULTS

The average age of the subjects in the RPL group was 30.11 ± 6.62 years, while that for the control group was significantly higher (36.77 ± 9.32; $P < 0.05$). From the pedigree investigations, 9 women in each group were found to be in consanguineous marriages [9 in the RPL group (23.7%), 9 in the control group (30%)]. There was no statistically significant difference between the number of women in consanguineous marriages in the two groups ($P > 0.05$).

RFLP produced the following bands for the polymorphisms. For 1154 G/A, homozygote G showed 3 bands with sizes of 150, 34 and 22 bp, homozygote A showed two bands of 184 and 22 bp, and heterozygote G/A showed 4 bands of 184, 150, 34, and 22 bp. For 2578 C/A, homozygote C showed a single band of 324 bp, homozygote A showed two bands of 202 and 122 bp, and heterozygote C/A showed three bands of 324, 202 and 122 bp. For 460 C/T, homozygote T showed a single band of 175 bp, homozygote C showed two bands of 155 and 20 bp, and heterozygote C/T showed three bands of 175, 155 and 20 bp. For 936 C/T, homozygote C showed a single band of 198 bp, homozygote T showed two bands of 112 and 86 bp, and heterozygote C/T showed three bands of 198, 112 and 86 bp (Table 1).

The RFLP-PCR data indicated that the frequency of the 2578 C/A, 460 C/T and 936 C/T polymorphisms of the VEGF gene was statistically insignificant between the two groups. On the other hand, the frequency of the A/A genotype in the 1154 G/A polymorphism was found to be significantly higher in the RPL group than in the control group. Genotypes and their frequencies are shown in Table 2.

Table 1. Primer sequences, restriction enzymes, PCR and enzyme digestion product sizes.

Polymorphism	Primer F	Primer R	PCR product	Restriction enzyme	RFLP products
1154 G/A	5'-TCCTGCTCCCTCCTCGCCAATG-3'	5'-GGCGGGGACAGGCGAGCATC-3'	206 bp	*Mnl*I	(G) 150 bp 34 bp 22 bp (A) 184 bp 22 bp
2578 C/A	5'-GGATGGGGCTGACTAGGTAAGC-3'	5'-AGCCCCCTTTTCCTCCAAC-3'	324 bp	*Bgl*II	(A) 202 bp 122 bp (C) 324 bp
460 C/T	5'-TGTGCGTGTGGGGTTGAGCG-3'	5'-TACGTGCGGACAGGGCCTGA-3'	175 bp	*Bsh*I2361	(C) 155 bp 20 bp (T) 175 bp
936 C/T	5'-AAGGAAGAGGAGACTCTGCGC-3'	5'-TATGTGGGTGGGTGTGTCTACAG-3'	198 bp	*Hsp*92II	(C) 198 bp (A) 112 bp 86 bp

Table 2. VEGF 1154 G/A, 2578 C/A, 460 C/T, 936 C/T polymorphism genotype frequencies in the recurrent pregnancy loss (RPL) and control groups.

Genotype	RPL (N = 38)	Control (N = 30)	Total (N = 68)
VEGF-1154			
G/G	16 (42.1)	17 (56.7)	33 (48.5)
A/A	9 (23.7)	1 (3.4)	10 (14.7)
G/A	13 (34.2)	12 (40)	25 (36.8)
VEGF-2578			
C/C	20 (52.6)	16 (53.3)	36 (52.9)
A/A	8 (21.1)	4 (13.3)	12 (17.65)
C/A	10 (26.32)	10 (33.3)	20 (29.4)
VEGF-460			
C/C	1 (0.38)	-	1 (0.68)
T/T	37 (97.4)	29 (96.7)	66 (97.05)
C/T	-	1 (0.3)	1 (0.68)
VEGF-936			
C/C	27 (10.26)	23 (6.9)	50 (34)
T/T	4 (3.2)	2 (0.6)	6 (4.08)
C/T	7 (2.66)	5 (1.5)	12 (8.16)

Data are reported as number of individuals with percent in parentheses.

Of the 28 subjects who experienced only first-trimester abortion, the genotype frequency of the 1154 G/A polymorphism was found to be 39.3% G/G, 35.7% G/A and 32.14% A/A. The genotype frequency for the 2578 C/A polymorphism was found to be 50% C/C, 32.1% C/A and 17.9% A/A. In these subjects, for the 460 C/T polymorphism, T/T genotype was found for all, and for the 936 C/T polymorphism, the genotype frequency was found to be 75% C/C, 21.4% C/T and 3.6% T/T (Table 3).

Table 3. VEGF 1154 G/A, 2578 C/A, 460 C/T, 936 C/T polymorphism genotype frequencies in 28 recurrent pregnancy loss women with only first-tremester pregnancy losses.

Genotype	N (%)
VEGF-1154 G/G	11 (39.3)
1154 A/A	9 (32.14)
1154 G/A	10 (35.7)
VEGF-2578 C/C	14 (50.0)
2578 A/A	5 (17.9)
2578 C/A	9 (32.1)
VEGF-460 C/C	-
460 T/T	28 (100.0)
460 C/T	-
VEGF-936 C/C	21 (75.0)
936 T/T	1 (3.6)
936 C/T	6 (21.4)

DISCUSSION

VEGF belongs to a multifunctional family of growth factors, and is especially important in endothelial cells (Yancopoulos et al., 2000). It is responsible for the proliferation, migration and differentiation of endothelial cells (Bikfalvi, 2004). VEGF, in both developmental stages and in adults, is necessary for vasculogenesis and angiogenesis (Shalaby et al., 1995). The gene has 8 exons and there are 6 known isoforms, designated $VEGF_{121}$, $VEGF_{145}$, $VEGF_{165}$, $VEGF_{183}$, $VEGF_{189}$, and $VEGF_{206}$. VEGF is synthesized in many different cells in the body such as ovarian follicles, corpus luteum, lung alveolar cells, renal glomeruli, visceral epithelial cells, kidney proximal tubular cells, all cells of the adrenal cortex, Leydig cells, active macrophages, fibroblasts surrounding arterioles, bronchial and choroid plexus epithelial cells, and hepatocytes.

VEGF is especially important for a successful pregnancy. Studies carried out on VEGF knock-out mice, showed that VEGF plays an important role in the early stages of pregnancy during the process of angiogenesis (Ferrara et al., 1996; Rowe et al., 2003). VEGF is responsible for increased vascular permeability and the proliferation of endothelial cells for the successful implantation of the embryo (Brogan et al., 1999; Rowe et al., 2003). Any dysfunction or reduced expression of the gene and its products may lead to failure of implantation.

Recent studies have shown that 30 single nucleotide polymorphisms (SNPs) of the gene, especially VEGF-1154A, VEGF-2578A and VEGF-936T, are related to lowered expression of VEGF and are responsible for the reduced production of the protein (Watson et al., 2000; Awata et al., 2002; Mohammadi et al., 2003; Papazoglou et al., 2005).

Of these SNPs, the polymorphisms 1154 G/A and 2578 C/A, 460 C/T (found in the 5'-UTR), 936 C/T (found in the 3'-UTR), were investigated in 38 Turkish women with a history of RPL and 30 control women who had no history of RPL in the current study.

To reduce any effects of recessive genetic defects on early termination of pregnancy, the percentage of consanguineous marriages in the RPL group (N = 9, 23.7%) and the control group (N = 9, 30%) was kept at levels that were not significantly different (P = 0.559). The age difference between the groups was found to be statistically significant ($P < 0.05$). The reason for this is that efforts were made to choose healthy subjects for the control group who are fertile and about to complete their fertile stage so that any likelihood of RPL could be eliminated.

Various studies on RPL in different communities have shown that the frequency of the A allele in the 1154 G/A polymorphism is significantly higher (Papazoglou et al., 2005; Lee et al., 2010).

In our study, a similar result was observed such that 1154 A/A phenotype in the RPL group was found to be higher than that in the control group (23.7%) ($P < 0.034$). The 1154 A/A polymorphism leads to a reduction in VEGF protein expression by reducing the transcription of the VEGF gene (Watson et al., 2000; Awata et al., 2002; Mohammadi et al., 2003). This results in anomalies in fetal and placental angiogenesis and failure in implantation (Ferrara et al., 1996; Carmeliet et al., 1996). In similar previous studies, the frequency of 1154 A/A was also found to be higher in the RPL group than in the control group (Papazoglou et al., 2005; Coulam and Jeyendran, 2008; Lee et al., 2010).

A complete linkage has been reported between the presence of the 2578 C allele in the promoter region of the VEGF gene and an 18-bp deletion/insertion fragment at position -2549, where this appears to be related to the level of VEGF protein expression. The structure carrying the 18-bp deletion shows a 1.95 times higher transcriptional activity compared to the structure carrying the insert (Yang et al., 2003). The individuals with 2578 A have the 18-bp insert while the individuals with 2578 C lack this insert (Awata et al., 2002; Krippl et al., 2003; Jin et al., 2005; Liu et al., 2009). In our study, the frequency of the 2578 C/C genotype was found to be higher in both study groups, but the difference in the frequency of the 2578 C/A genotype was not statistically significant. Papazoglou et al. (2005) and Lee et al. (2010) reported similar results.

In the 460 C/T polymorphism, the C allele increases basal promoter activity of the gene and it has been reported that the CC genotype is found more frequently (Awata et al., 2002; Liu et al., 2009). Our study showed that the frequency of the C/T genotype was not significantly different between the two groups. The T/T genotype frequency in RPL and control groups was 37 (97.4%) and 29 (96.7%), respectively. We are the first to report on this SNP in a group with RPL.

It has been shown that the 936 C/T polymorphism is correlated with a reduction in gene expression and that it drastically decreases plasma VEGF levels in VEGF 936 T allele carriers. This effect was thought to be due to the loss of a specific region of interaction between VEGF and angiopoetin 4 or due to post-transcriptional regulation, leading to a transformation of the 3'-UTR, which may prolong the half-life of the mRNA (Yancopoulos et al., 2000; Krippl et al., 2003). In our study, the frequency of this genotype did not differ in the two groups, while the 936C allele was found to be higher in both groups. Papazoglou et al. (2005) and Lee et al. (2010) have reported similar results for this polymorphism. In this study, of the 4 polymorphisms that we studied, only the 1154 A/A was found to occur at a higher frequency in the RPL group. Although it is not enough to explain miscarriage, it may be proposed as one of the factors that may lead to miscarriage in pregnancy.

In conclusion, in the light of this and other similar studies, it is possible that these SNPs are not enough to explain all the reasons for miscarriages. The risk of a miscarriage is less likely to result from one specific gene; rather, the likelihood of it occurring due to a combination of thrombophilic mutations and angiogenic polymorphisms appears to be higher.

ACKNOWLEDGMENTS

Research supported by the Commission of Scientific Research Projects of Uludag University (Project #UAP(F)-2009/41).

REFERENCES

Awata T, Inoue K, Kurihara S, Ohkubo T, et al. (2002). A common polymorphism in the 5'-untranslated region of the

VEGF gene is associated with diabetic retinopathy in type 2 diabetes. *Diabetes* 51: 1635-1639.
Bikfalvi A (2004). Recent developments in the inhibition of angiogenesis: examples from studies on platelet factor-4 and the VEGF/VEGFR system. *Biochem. Pharmacol.* 68: 1017-1021.
Brogan IJ, Khan N, Isaac K, Hutchinson JA, et al. (1999). Novel polymorphisms in the promoter and 5' UTR regions of the human vascular endothelial growth factor gene. *Hum. Immunol.* 60: 1245-1249.
Carmeliet P, Ferreira V, Breier G, Pollefeyt S, et al. (1996). Abnormal blood vessel development and lethality in embryos lacking a single VEGF allele. *Nature* 380: 435-439.
Coulam CB and Jeyendran RS (2008). Vascular endothelial growth factor gene polymorphisms and recurrent pregnancy loss. *Am. J. Reprod. Immunol.* 59: 301-305.
Ferrara N, Carver-Moore K, Chen H, Dowd M, et al. (1996). Heterozygous embryonic lethality induced by targeted inactivation of the VEGF gene. *Nature* 380: 439-442.
Ferrara N, Gerber HP and LeCouter J (2003). The biology of VEGF and its receptors. *Nat. Med.* 9: 669-676.
Harry LE and Paleolog EM (2003). From the cradle to the clinic: VEGF in developmental, physiological, and pathological angiogenesis. *Birth Defects Res. C Embryo Today* 69: 363-374.
Hellgren M (1996). Hemostasis during pregnancy and puerperium. *Haemostasis* 26: 224-247.
Jin Q, Hemminki K, Enquist K, Lenner P, et al. (2005). Vascular endothelial growth factor polymorphisms in relation to breast cancer development and prognosis. *Clin. Cancer Res.* 11: 3647-3653.
Krippl P, Langsenlehner U, Renner W, Yazdani-Biuki B, et al. (2003). A common 936 C/T gene polymorphism of vascular endothelial growth factor is associated with decreased breast cancer risk. *Int. J. Cancer* 106: 468-471.
Lee HH, Hong SH, Shin SJ, Ko JJ, et al. (2010). Association study of vascular endothelial growth factor polymorphisms with the risk of recurrent spontaneous abortion. *Fertil. Steril.* 93: 1244-1247.
Letsky EA and Swiet M (1994). Maternal Hemostasis: Coagulation Problems of Pregnancy. In: Thrombosis and Hemorrhage (Loscalzo J and Schafer AI, eds.). Blackwell Scientific Publications, Boston, 965-998.
Li TC, Makris M, Tomsu M, Tuckerman E, et al. (2002). Recurrent miscarriage: aetiology, management and prognosis. *Hum. Reprod. Update* 8: 463-481.
Liu Q, Li Y, Zhao J, Sun DL, et al. (2009). Association of polymorphisms -1154G/A and -2578C/A in the vascular endothelial growth factor gene with decreased risk of endometriosis in Chinese women. *Hum. Reprod.* 24: 2660-2666.
Mohammadi M, Ollier WE and Hutchinson IV (2003). A functional association study of VEGF gene promoter polymorphisms with VEGF expression by stimulated pbm cells. *Hum. Immunol.* 64 (Suppl 10): S125.
NCICB (2012). National Cancer Institute Web Services. Available at [http://omim.org/entry/192240]. Accessed May 15, 2011.
Papazoglou D, Galazios G, Papatheodorou K, Liberis V, et al. (2005). Vascular endothelial growth factor gene polymorphisms and idiopathic recurrent pregnancy loss. *Fertil. Steril.* 83: 959-963.
Regan L (1998). Overview of recurrent miscarriage. *Gynaecol. Forum* 3: 3-7.
Renner W, Kotschan S, Hoffmann C, Obermayer-Pietsch B, et al. (2000). A common 936 C/T mutation in the gene for vascular endothelial growth factor is associated with vascular endothelial growth factor plasma levels. *J. Vasc. Res.* 37: 443-448.
Rowe AJ, Wulff C and Fraser HM (2003). Localization of mRNA for vascular endothelial growth factor (VEGF), angiopoietins and their receptors during the peri-implantation period and early pregnancy in marmosets (*Callithrix jacchus*). *Reproduction* 126: 227-238.
Shalaby F, Rossant J, Yamaguchi TP, Gertsenstein M, et al. (1995). Failure of blood-island formation and vasculogenesis in Flk-1-deficient mice. *Nature* 376: 62-66.
te Velde EA, Exalto N, Hesseling P and van der Linden HC (1997). First trimester development of human chorionic villous vascularization studied with CD34 immunohistochemistry. *Hum. Reprod.* 12: 1577-1581.
Torry DS, Hinrichs M and Torry RJ (2004). Determinants of placental vascularity. *Am. J. Reprod. Immunol.* 51: 257-268.
Watson CJ, Webb NJ, Bottomley MJ and Brenchley PE (2000). Identification of polymorphisms within the vascular endothelial growth factor (VEGF) gene: correlation with variation in VEGF protein production. *Cytokine* 12: 1232-1235.
Wilcox AJ, Weinberg CR, O'Connor JF, Baird DD, et al. (1988). Incidence of early loss of pregnancy. *N. Engl. J. Med.* 319: 189-194.
Yancopoulos GD, Davis S, Gale NW, Rudge JS, et al. (2000). Vascular-specific growth factors and blood vessel formation. *Nature* 407: 242-248.
Yang B, Cross DF, Ollerenshaw M, Millward BA, et al. (2003). Polymorphisms of the vascular endothelial growth factor and susceptibility to diabetic microvascular complications in patients with type 1 diabetes mellitus. *J. Diabetes Complications* 17: 1-6.
Zygmunt M, Herr F, Munstedt K, Lang U, et al. (2003). Angiogenesis and vasculogenesis in pregnancy. *Eur. J Obstet. Gynecol. Reprod. Biol.* 110 (Suppl 1): S10-S18.