Association study of vascular endothelial growth factor gene polymorphisms with ectopic pregnancy in Chinese women

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Summary

Objective: The purpose of this study is to evaluate potential associations between vascular endothelial growth factor (VEGF) gene polymorphisms and ectopic pregnancy (EP) in Chinese women. *Materials and Methods:* This was a case–control study wherein 192 women with a history of EP were compared to 210 post-menopausal controls with two pregnancies and no EP for the genotyping of VEGF polymorphisms. Genotyping of the VEGF gene polymorphisms at -460C/T, -1154G/A, -2578C/A and +936C/T were performed by polymerase chain reaction-restriction fragment length polymorphism. *Results:* No significant differences were found in genotype and allele distributions of the -460C/T, +936C/T polymorphisms between cases and controls. Compared with the -1154G/G genotype, the -1154(A/A+G/A) genotype could significantly reduce the risk of developing EP. For the -2578C/A polymorphism, the A/A+C/A genotype could significantly decrease the risk of developing EP, compared with the C/C genotype. The haplotype analysis suggested that the TAA (VEGF -460/-1154/-2578) and CAA haplotypes could significantly decrease the risk of developing EP compared with the haplotype of TGC. *Conclusion:* The -1154A or -2578A alleles of VEGF gene could significantly decrease the risk of EP and might be potentially protective factors for EP development in Chinese women.

Key words: VEGF; Polymorphism; Ectopic pregnancy; Susceptibility.

Introduction

The incidence of ectopic pregnancy (EP) has increased and accounts for 2% of all pregnancies [1]. Ectopic pregnancy is a major cause of maternal morbidity and mortality, accounting for 9-13% of the first trimester pregnancy-related deaths [2]. It is an important cause of maternal deaths in early pregnancy because most fatal cases result from delayed diagnosis and inappropriate investigation [3]. In spite of high-resolution vaginal ultrasound and high sensitive quantitative beta human chorionic gonadotropin (beta-hCG) assays, at the first presentation, nearly 40-50% of EPs might be initially misdiagnosed [4]. For this reason, some serum markers have been investigated for early diagnosis of EP, such as, vascular endothelial growth factor (VEGF)[5].

VEGF is a well-known angiogenic factor, which may play a key role in the establishment of a viable pregnancy, participating in the processes of implantation and placentation [6], contributing to arterial remodeling and increase of permeability in endometrium, decidua, and trophoblast, leading to vascular development of the embryo [7,8]. The secretion and expression of VEGF is dependent on local conditions, such as hypoxia [9], and it has been observed that the cellular VEGF production is increased in hypoxic conditions [7,10]. The low basal expression of VEGF messenger RNA under normoxic conditions in the cytotrophoblast and the syncytrophoblast (in vitro) supports the lower levels of VEGF in patients with intrauterine pregnancy compared with that in patients with EP [11]. The production and secretion of VEGF seem to be elevated in EP because the implantation environment in the oviduct is very different from the well-vascularized endometrium [5, 7, 12]. Besides, several studies have demonstrated that VEGF seems to be an important serum marker for the diagnosis of EP [5, 12].

The VEGF gene is located in the chromosome region 6p21.3 and consists of eight exons and seven introns, which is a highly polymorphic gene, and a number of single nucleotide polymorphisms (SNPs) have been reported [13]. Numerous studies have shown that several polymorphisms were associated with the production of the VEGF protein, and suggested that the regulation of VEGF expression occurred primarily at a transcriptional level [13-17]. Four single nucleotide polymorphisms (-460C/T, -1154G/A, -2578C/A, +936C/T) of VEGF gene, which locate in the promoter and 5'- and 3'-untranslated regions, may alter VEGF expression [13-19]. Indeed, several studies have investigated the association between the above four single nucleotide polymorphisms and various diseases, including recurrent pregnancy loss, pre-eclampsia, and preterm delivery [20-22].

Considering the important roles of VEGF in pregnancy, the present authors are interested in studying the polymorphism of the VEGF gene, as this marker appears to be very important in the determinism of EP. Therefore they investigated whether -460C/T, -1154G/A, -2578C/A, and +936C/T VEGF polymorphisms alone or haplotypes with other VEGF polymorphisms were a risk factor for EP in a hospital-based case-control study.

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Table 1. — *PCR conditions for VEGF -2578C/A, -1154G/A, -460C/T, and +936C/T restriction fragment length polymorphisms.*

Polymorphism	Primer	Product length (bp)	Restriction enzyme	Fragment length
VEGF -2578C/A	5'-GGATGGGGCTGACT AGGTAAGC-3'(F)	324	BglII	324 bp(C)
rs699947	5'-AGCCCCCTTTTCCT CCAAC-3'(R)			202 + 122 bp(A)
VEGF -1154G/A	5'-TCCTGCTCCCTCCT CGCCAATG-3'(F)	206	MnlI	184 + 22 bp(A)
rs1570360	5'-GGCGGGGACAGGC GAGCATC-3' (R)			150 + 34 + 22 bp(G)
VEGF -460C/T	5'-TGTGCGTGTGGGGT TGAGCG-3' (F)	175	Bsh1236I	175 bp(T)
rs833061	5'-TACGTGCGGACAGG GCCTGA-3' (R)			155 + 20 bp(C)
VEGF +936C/T	5'-AAGGAAGAGGAGAC TCTGCGC-3' (F)	198	Hsp92II	198 bp(C)
rs3025039	5'-TATGTGGGTGGGT GTGTCTACAG-3' (R)			112 + 86 bp(A)

Materials and Methods

Study subjects

The case group comprised of 192 women with a history of EP (mean age 29.4 ± 4.3 years, mean amenorrhea 7.1 ± 2.6 weeks, previous EP 15.2%, and EP after in vitro fertilization 5.4%). The treatment of EP was: salpingectomy in 46.6%, salpingostomy in 12.4%, single dose of intramuscular methotrexate 50 mg/m^2 in 30.3%, and expectant management in 10.7% of the cases. These women were recruited from the Gynaecology Department of Zhujiang Hospital of Southern Medical University. The diagnosis of EP was performed by transvaginal ultrasound and confirmed during the surgery. The control group consisted of 210 healthy, post-menopausal controls with at least two live births and no history of EP, miscarriage, preeclampsia or preterm delivery. All case and control subjects were of Han nationality from South China. The Ethics Committee of Southern Medical University approved the study and informed consent was obtained from all recruited subjects.

DNA extraction

Venous blood (five ml) was drawn from each subject into Vacutainer tubes containing EDTA and stored at 48°C. Genomic DNA was extracted within one week after sampling using proteinase K digestion followed by a salting out procedure according to the previously described method [23].

VEGF -460C/T, -1154G/A, -2578C/A, +936C/T genotyping

Genotypes were determined by polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) method. The PCR was performed in a 20 µl volume containing 100 ng of DNA template, 1.6 µl of 10×PCR buffer for -460C/T (rs833061) and -1154G/A (rs1570360), 2.4 µl of 10×PCR buffer for -2578C/A (rs699947) and +936C/T (rs3025039), 1U of Taq DNA polymerase, 0.4 µl of ten mmol/l dNTPs, and 200 nM of each primer. The PCR cycling conditions were five minutes at 94°C followed by 35 cycles of 45 seconds at 94°C, 45 seconds at 61°C for -460C/T and -1154G/A, 60.5°C for -2578 C/A, 63°C for +936C/T, and 45 seconds at 72°C, with a final step at 72°C for seven minutes to allow for the complete extension of all PCR fragments. PCR products (six to eight ml of reaction) were digested overnight at 37°C in a ten µl reaction with restriction enzyme as follows: 5U Bsh1236I for the -460 C/T polymorphism, 5 U MnlI for -1154G/A, 5U Hsp92II for +936C/T and 10U BglII for -2578C/A. After digestion, the products were separated on a 4% agarose gel that was stained with ethidium bromide. The primers, length of PCR products, restriction enzymes and fragments length are summarized in Table 1.

For a negative control, distilled water was used instead of DNA in the reaction system for each panel of PCR. The PCR reactions of 15% of the samples were run in duplicate for quality control, with a reproducibility of 100%.

Statistical Analysis

Statistical analysis was performed using the Statistical Package for the Social Sciences software package (version13.0; SPSS). Hardy-Weinberg equilibrium (HWE) analysis was performed to compare the observed and expected genotype frequencies using the Chi-square test. The data of age and amenorrhea weeks in the case group were presented as mean ±SD. Comparison of the VEGF -460C/T, -1154G/A, -2578C/A, and +936C/T genotype distributions in the study groups was performed by means of two-sided contingency tables using Chi-square test. The VEGF -460C/T, -1154G/A, -2578C/A, and +936C/T haplotype frequencies and linkage disequilibrium coefficient were estimated using the EH linkage software (version 1.2) and 2LD program, respectively. The odds ratio (OR) and 95% confidence interval (CI) were calculated using an unconditional logistic regression model. A probability level of 5% was considered significant.

Results

Association of VEGF -460C/T, -1154G/A, -2578C/A and +936C/T polymorphisms with the risk of EP

The distributions of the VEGF -460C/T, -1154G/A, -2578C/A, and +936C/T genotypes in the control groups did not significantly deviate from that expected for a Hardy-Weinberg equilibrium (all p values > 0.05). The genotype and polymorphic allele frequencies of the four polymorphisms among the cases and controls and their associations with EP risk are shown in Table 2. There were no significant differences in the genotype distributions and allele frequencies of VEGF -460 C/T and +936 C/T polymorphisms between the cases and controls (p = 0.564, 0.884 and 0.540, 0.294, respectively) (Table 2). Compared with the genotypes of -460T/T and +936C/C, the genotypes of -460C/C+C/T and +936C/T+T/T did not significantly influence the risk of EP (OR = 1.11, 95%CI = 0.74 - 1.66; OR = 1.22, 95%CI = 0.81 - 1.86, respectively) (Table 2).

The genotype frequencies of the VEGF -1154 A/A, G/A, and G/G in cases and controls were 2.1% / 6.2%, 26.6% / 33.8% and 71.4% / 60.0%, respectively; the A and G allele frequencies in the two groups were 15.4% / 23.1% and 84.6% / 76.9%, respectively. There was a significant difference in genotype and allele distributions of the VEGF -1154G/A between two groups (p = 0.021 and

Table 2. — *Genotype distributions and allele frequencies of the four polymorphisms.*

Group	Control n (%)	Case n (%)	*p* value	OR (95%CI)
VEGF -2578C/A genotypes				
C/C	116 (55.2)	125 (65.1)		1.00 (reference)
C/A	75 (35.7)	60 (31.3)	0.034[a]	0.74 (0.49–1.13)
A/A	19 (9.1)	7 (3.6)		0.34 (0.14–0.84)
C/A + A/A	94 (44.8)	67 (34.9)		0.66(0.44–0.99)
Allele				
C	307 (73.1)	310 (80.6)	0.010	1.00 (reference)
A	113 (26.9)	74 (19.4)		0.65 (0.47–0.91)
VEGF -1154G/A genotypes				
G/G	126 (60.0)	137 (71.4)		1.00 (reference)
G/A	71 (33.8)	51 (26.6)	0.021[b]	0.66 (0.43–1.02)
A/A	13 (6.2)	4 (2.1)		0.28 (0.09–0.89)
G/A + A/A	84 (40.0)	55 (28.6)		0.60 (0.40–0.91)
Allele				
G	323 (76.9)	325 (84.6)	0.006	1.00 (reference)
A	97(23.1)	59 (15.4)		0.61 (0.42–0.87)
VEGF -460C/T genotypes				
T/T	132 (62.9)	116 (60.4)		1.00 (reference)
C/T	67 (31.9)	69 (35.9)	0.564[c]	1.17 (0.77–1.78)
C/C	11(5.2)	7 (3.6)		0.72 (0.27–1.93)
C/T + C/C	78 (37.1)	76 (39.6)		1.11 (0.74–1.66)
Allele				
T	331 (78.8)	301 (78.4)	0.884	1.00 (reference)
C	89 (21.2)	83 (21.6)		1.03 (0.73–1.44)
VEGF +936C/T genotypes				
C/C	146 (69.5)	125 (65.1)		1.00 (reference)
C/T	60 (28.6)	61 (31.8)	0.540[d]	1.19 (0.77–1.82)
T/T	4 (1.9)	6 (3.1)		1.75 (0.48–6.35)
C/T + T/T	64 (30.5)	67(34.9)		1.22 (0.81–1.86)
Allele				
C	352 (83.8)	311 (81.0)	0.294	1.00 (reference)
T	68 (16.2)	73 (19.0)		1.22 (0.84–1.75)

VEGF: vascular endothelial growth factor; OR: odds ratio; CI: confidence interval. a: *p* value was estimated using X^2-test for the C/C, C/A, and A/A genotype of -2578C/A SNP. b: *p* value was estimated using X^2-test for the G/G, G/A, and A/A genotype of -1154G/A SNP. c: *p* value was estimated using X^2-test for the T/T, T/C, and C/C genotype of -460T/C SNP. d: *p* value was estimated using X^2-test for the C/C, C/T, and T/T genotype of +936C/T SNP.

0.006, respectively) (Table 2). Compared with the G/G genotype, the A/A+G/A genotype could significantly decrease the risk of developing EP (OR = 0.61, 95%CI = 0.42 - 0.87) (Table 2).

The genotype frequencies of the VEGF -2578 A/A, C/A and C/C in cases and controls were 3.6% / 9.1%, 31.3% / 35.7% and 65.1% / 55.2%, respectively; the A and C allele frequencies in the two groups were 19.4% / 26.9% and 80.6% / 73.1%, respectively. There was a significant difference in genotype and allele distributions of the VEGF -2578C/A between the two groups (*p* = 0.034 and 0.010, respectively) (Table 2). Compared with the C/C genotype, the A/A+C/A genotype could significantly reduce the risk of developing EP (OR = 0.66, 95%CI = 0.44-0.99) (Table 2).

Table 3. — *Haplotypes of VEGF -460C/T, VEGF -1154G/A and VEGF -2578C/A polymorphisms with the pisk of EP.*

VEGF -460/ -1154/-2578	Control (%)	Case (%)	P-value	OR (95%CI)
TGC	150 (35.7)	160 (41.9)		1.00 (reference)
CGC	74(17.6)	62 (16.1)	0.241	0.79 (0.52–1.18)
TGA	61(14.6)	59 (15.3)	0.649	0.91 (0.60–1.38)
TAC	40 (9.6)	39 (10.2)	0.721	0.91 (0.56–1.50)
CGA	29 (6.9)	28 (7.2)	0.730	0.91 (0.51–1.59)
CAC	27 (6.4)	21(5.4)	0.311	0.73 (0.40–1.35)
TAA	23 (5.4)	10 (2.7)	0.020	0.41 (0.19–0.89)
CAA	16 (3.8)	5 (1.2)	0.014	0.29 (0.11–0.82)

Association of haplotypes of VEGF polymorphisms with the risk of EP

The results of the 2LD program analysis revealed that the VEGF -460C/T and -1154G/A, -1154G/A and -2578C/A, -2578C/A and -460C/T polymorphisms displayed linkage disequilibrium (D' = 0.80, 0.47, 0.37, respectively; r^2 = 0.47, 0.19, 0.13, respectively). The VEGF +936C/T polymorphism located on the 3'-untranslated region and did not exhibit linkage disequilibrium with the other three polymorphisms. Therefore, haplotype analysis was only conducted between VEGF -460C/T, -1154G/A, and -2578C/A polymorphisms. The results of the EH linkage software analysis showed that the haplotype distributions derived from the three polymorphisms differed between the cases and controls (*p* = 0.000). The TGC(-460/-1154/-2578) was the most common haplotype in the controls (35.7%), followed by the CGC (17.6%), TGA (14.6%), TAC (9.6%), CGA (6.9%), CAC (6.4%), TAA (5.4%), and CAA (3.8%) (Table 3). Compared with the haplotype of TGC, the TAA, and CAA haplotypes could significantly decrease the risk of EP development (*p* = 0.020, OR = 0.41, 95%CI = 0.19 - 0.89; *p* = 0.014, OR = 0.29, 95%CI = 0.11-0.82, respectively) (Table 3). However, the others (CGC, TAC, CAC, TAA, and CAA) could not significantly modify the risk of developing EP (Table 3).

Discussion

The reason for studying a genetic predisposition for EP is that several patients who develop the disease do not present any risk factor. Besides, recent evidences have shown that VEGF values increase in EP when compared with that in normal intra-uterine pregnancy [5, 7, 10, 12]. Both aspects instigated the present authors to hypothesize that a polymorphism of the VEGF gene could be associated with EP. In this study, the four important functional polymorphisms of the VEGF gene -460C/T, -1154G/A, -2578C/A, and +936C/T were investigated, and which were selected for studying because they have a well-established laboratorial methodology and also because they present a direct correlation with the production of VEGF [13-19]. At present,

there has been no previous Chinese case-control study exploring this relationship.

In the present study, the results suggest that no significant association is found between the -460C/T and +936C/T polymorphisms and the risk of developing EP in Chinese women (p = 0.564 and 0.540, respectively). By contrast, a significant association is indicated between the VEGF-1154G/A, -2578C/A polymorphisms and the risk of developing EP in Chinese women (p = 0.021 and 0.034, respectively). Besides, the haplotype analysis suggest that the TAA(-460/-1154/-2578) and CAA haplotypes could significantly decrease the risk of developing EP compared with the haplotype of TGC in Chinese women (OR = 0.41, 95%CI = 0.19 - 0.89: OR = 0.29, 95%CI = 0.11 - 0.82; respectively).

Both the polymorphisms of -1154G/A and -2578C/A are located in the promoter region of the VEGF gene. The mechanism of VEGF -2578 is well known, which is in complete linkage with deletion/insertion of an 18-bp fragment at the -2549 region, and the construct containing the 18-bp deletion (linkage with the C allele) shows a 1.95-fold increase in transactivation [24]. VEGF -1154G/A is part of a predicted binding site for myeloid zinc finger-1 (MZF1) in which the MZF1 binding site is substituted for the Pax2 or Sp1 binding site by -1154A. Considerable evidences have suggested that the two polymorphisms could affect the expression of VEGF, such as, Koukourakis *et al.* [25] have reported that the -1154G/G genotype in the VEGF gene was linked with protein overexpression of VEGF in non-small cell lung cancer; Shahbazi *et al.* [16] have also demonstrated that the -2578C/C and -1154G/G genotypes were correlated with higher levels of VEGF production by lipopolysaccharides (LPS) stimulated human peripheral blood mononuclear cells (PBMCs).

The -2578C/A and -1154G/A polymorphisms of VEGF gene have been investigated in a number of human diseases. However, the results are inconsistent. Some studies have shown that the VEGF -2578C/C and -1154G/G genotype were associated with increased risk of some diseases, such as acute rejection in renal transplant recipients [16], recurrent pregnancy loss [20], invasive breast cancer [26], and renal cell carcinomas [27]. On the contrary, other studies failed to show a significant association with diabetic retinopathy in type 2 diabetes [15] and severe preeclampsia [28]. However, the present results demonstrated that the -2578A/A+C/A and -1154A/A+G/A genotypes could significantly reduce the risk of developing EP in Chinese women, which were consistent with findings in other studies [29, 30]. This discrepancy might be explained by the following reasons: first, the frequencies of the VEGF -2578C and -1154G allele of the controls are different in the various populations. Such as the frequency of -2578C allele is 0.722 with the HapMap-HCB (Han Chinese in Beijing), and 0.592 with HapMap-CEU (Utah Residents with Northern and Western European ancestry); -1154G allele is 0.5-0.6 in Caucasians and 0.8 in Asians. The present data are 0.731(-2578C) and 0.769(-1154G) which are similar to that in China and Asians, respectively. It was validated that the present data were relatively reliable and indicated that ethnicity might play a critical factor in the manifestation of the effects of the polymorphic alleles. Second, previous evidences have shown that production and secretion of VEGF seemed to be elevated in EP [5, 12]. It has been manifested that VEGF -2578CC and -1154GG genotypes increase VEGF secretion compared with carriage of a rare allele (A; A) [16,19]. Therefore, these results further support the present authors' conclusion that VEGF -2578A and -1154A allele may decrease the risk of EP development which is comparably reasonable. Perhaps, different molecular pathogeneses in different diseases might cause the discrepancy, which would contribute to further study.

The -460C/T is a common polymorphism in the 5'-untranslated region of the VEGF gene. The -460C/T and -405G/C polymorphisms of VEGF gene are in strong linkage disequilibrium and haplotypes containing -460C and -405G have a 71% higher basal promoter activity when compared with the wild-type sequence [18]. Whereas the effect of -460C/T polymorphism on the expression of VEGF remains unclear. Hsieh *et al.* [31] showed a significant association between -460C/T polymorphism and susceptibility to endometriosis, however, opposite results were reported by Zhao *et al.* [32]. In the present study, the -460 polymorphism did not modify the risk of EP development, in agreement with the report by Elito *et al.* in another study of Brazilian with EP [33]. These discrepancies may be due to sample size, participant selection criteria, difference in ethnicity or in the study design.

The +936C/T polymorphism locates on the 3'-untranslated region of the VEGF gene, and the +936T allele is related to low levels of VEGF plasma and the decreased risk of breast cancer [14]. The mechanism by which the VEGF +936T allele leads to lower VEGF plasma levels may be the loss of a potential binding site for activator protein 4 (AP-4) by the +936C->T transition in 3'-untranslated region and AP-4 is a helix-loop-helix transcription factor, enhancing expression of several viral and cellular genes by binding to specific enhancer sites; the loss of this potential binding site could be responsible for decreased VEGF expression by the T-allele [14]. Krippl *et al.* [17] have reported that the carriers of +936T allele was associated with decreased risk of breast cancer. However, the present study failed to show a significant relationship between the +936C/T polymorphism and the risk of developing EP. This result is consistent with previous published studies, which have shown no significant association of VEGF +936C/T polymorphism with EP in the Brazilian population [33]. Besides, the C and T alleles of +936 polymorphism were found at 84% and 16% in white population, respectively [14], frequencies similar to those found in the present study.

In addition, the present study has found that the three promoter region polymorphisms (VEGF -460C/T, -1154G/A,

and -2578C/A) were in linkage disequilibrium. The haplotype analysis suggested that the TAA (-460/-1154/ -2578) and CAA haplotypes could significantly decrease the risk of developing EP compared with the haplotype of TGC. At present, no study has yet been published that investigated the effect of the haplotypes of VEGF -460/-1154/-2578 polymorphisms on VEGF production and their relationship to the development of EP. Therefore, further studies on the functional relevance of the VEGF polymorphisms and haplotypes in EP are required to confirm these results.

To the present authors' knowledge, Elito *et al.* [33] were the first to demonstrate that there was no association between EP and -634C/G, -460T/C and +936C/T VEGF polymorphisms in the Brazilian population. Among them, the effects of -460T/C and +936C/T polymorphisms on EP in the Brazilian population are in agreement with the present results, but -1154G/A and -2578C/A polymorphisms show a significant association with EP of Chinese women in the present study. In comparison, the results of this study may be more reliable, the reasons are as follows: (I) in the present study, the authors included some important functional SNPs (-460C/T, -1154G/A, -2578C/A and +936C/T) in the genotype/haplotype analysis, and these tagging SNPs approach was used to cover the whole genomic region of the target gene (VEGF). Therefore, the present authors' approach should allow to capture more comprehensive information on the VEGF gene block. (II) all participants were of Chinese ethnicity, because of its relatively homogenous ethnic origin, which stands in contrast with the more heterogeneous characteristics of the Brazilian women who were examined in the previous study. (III) the sample size was larger in the present study than in Elito *et al.* study: case group 192 vs 74, control group 210 vs 134.

From the present authors' point of view, care should be taken to analyze these results, because of the limitation of the sample size, ethnical diversity, different compositions of case and control groups, the number of polymorphisms related to the gene in this disease, and whether confounding variables were considered. Nonetheless, further studies are required to replicate the present finding in different ethnic groups with a larger sample size.

In conclusion, the present authors conclude through their results that the -2578A or -1154A alleles of VEGF gene could significantly decrease the risk of EP development and might be potentially protective factors for EP development. Besides, the haplotypes of VEGF -460/-1154/-2578 polymorphisms may reduce the risk of EP development.

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References

[1] Tulandi T., Sammour A.: "Evidence-based management of ectopic pregnancy". *Curr. Opin. Obstet. Gynecol.*, 2000, *12*, 289.
[2] Nederlof K.P., Lawson H.W., Saftlas A.F., Atrash H.K., Finch E.L.: "Ectopic pregnancy surveillance, United States, 1970-1987". *MMWR CDC Surveill. Summ.*, 1990, *39*, 9.
[3] Farquhar C.M.: "Ectopic pregnancy". *Lancet*, 2005, *366*, 583.
[4] Abbott J., Emmans L.S., Lowenstein S.R.: "Ectopic pregnancy: ten common pitfalls in diagnosis". *Am. J. Emerg. Med.*, 1990, *8*, 515.
[5] Daniel Y., Geva E., Lerner-Geva L., Eshed-Englender T., Gamzu R., Lessing J.B., et al.: "Levels of vascular endothelial growth factor are elevated in patients with ectopic pregnancy: is this a novel marker?" *Fertil. Steril.*, 1999, *72*, 1013.
[6] Nowacek G.E., Meyer W.R., McMahon M.J., Thorp J.R., Wells S.R.: "Diagnostic value of cervical fetal fibronectin in detecting extrauterine pregnancy". *Fertil. Steril.*, 1999, *72*, 302.
[7] Torry D.S., Torry R.J.: "Angiogenesis and the expression of vascular endothelial growth factor in endometrium and placenta". *Am. J. Reprod. Immunol.*, 1997, *37*, 21.
[8] Kucera-Sliutz E., Schiebel I., Konig F., Leodolter S., Sliutz G., Koelbl H.: "Vascular endothelial growth factor (VEGF) and discrimination between abnormal intrauterine and ectopic pregnancy". *Hum. Reprod.*, 2002, *17*, 3231.
[9] Ladoux A., Frelin C.: "Hypoxia is a strong inducer of vascular endothelial growth factor mRNA expression in the heart". *Biochem. Biophys. Res. Commun.*, 1993, *195*, 1005.
[10] Evans P.W., Wheeler T., Anthony F.W., Osmond C.: "A longitudinal study of maternal serum vascular endothelial growth factor in early pregnancy". *Hum. Reprod.*, 1998, *13*, 1057.
[11] Shore V.H., Wang T.H., Wang C.L., Torry R.J., Caudle M.R., Torry D.S.: "Vascular endothelial growth factor, placenta growth factor and their receptors in isolated human trophoblast". *Placenta*, 1997, *18*, 657.
[12] Chou T., Yokoyama A., Yoshizawa H., Hoshino M., Ebe T., Kurita Y., *et al.*: "Mega-dose chemotherapy with peripheral blood stem cell transplantation (PBS-CT) for small cell lung cancer (SCLC)". *Gan To Kagaku Ryoho*, 1995, *22*, 1741.
[13] Rygaard K., Nakamura T., Spang-Thomsen M.: "Expression of the proto-oncogenes c-met and c-kit and their ligands, hepatocyte growth factor/scatter factor and stem cell factor, in SCLC cell lines and xenografts". *Br. J. Cancer*, 1993, *67*, 37.
[14] Renner W., Kotschan S., Hoffmann C., Obermayer-Pietsch B., Pilger E.: "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.*, 2000, *37*, 443.
[15] Awata T., Inoue K., Kurihara S., Ohkubo T., Watanabe M., Inukai K., *et al.*: "A common polymorphism in the 5'-untranslated region of the VEGF gene is associated with diabetic retinopathy in type 2 diabetes". *Diabetes*, 2002, *51*, 1635.
[16] Shahbazi M., Fryer A.A., Pravica V., Brogan I.J., Ramsay H.M., Hutchinson I.V., et al.: "Vascular endothelial growth factor gene polymorphisms are associated with acute renal allograft rejection". *J. Am. Soc. Nephrol.*, 2002, *13*, 260.
[17] Krippl P., Langsenlehner U., Renner W., Yazdani-Biuki B., Wolf G., Wascher T.C., et al.: "A common 936 C/T gene polymorphism of vascular endothelial growth factor is associated with decreased breast cancer risk". *Int. J. Cancer*, 2003, *106*, 468.
[18] Stevens A., Soden J., Brenchley P.E., Ralph S., Ray D.W.: "Haplotype analysis of the polymorphic human vascular endothelial growth factor gene promoter". *Cancer Res.*, 2003, *63*, 812.
[19] Lambrechts D., Storkebaum E., Morimoto M., Del-Favero J., Desmet F., Marklund S.L., *et al.*: "VEGF is a modifier of amyotrophic lateral sclerosis in mice and humans and protects motoneurons against ischemic death". *Nat. Genet.*, 2003, *34*, 383.
[20] Lee H.H., Hong S.H., Shin S.J., Ko J.J., Oh D., Kim N.K.: "Association study of vascular endothelial growth factor polymorphisms with the risk of recurrent spontaneous abortion". *Fertil. Steril.*, 2010, *93*, 1244.

[21] Shim J.Y., Jun J.K., Jung B.K., Kim S.H., Won H.S., Lee P.R., *et al.*: "Vascular endothelial growth factor gene +936 C/T polymorphism is associated with preeclampsia in Korean women". *Am. J. Obstet. Gynecol.*, 2007, *197*, e271.

[22] Papazoglou D., Galazios G., Koukourakis M.I., Kontomanolis E.N., Maltezos E.: "Association of -634G/C and 936C/T polymorphisms of the vascular endothelial growth factor with spontaneous preterm delivery". *Acta Obstet. Gynecol. Scand.*, 2004, *83*, 461.

[23] Miller S.A., Dykes D.D., Polesky H.F.: "A simple salting out procedure for extracting DNA from human nucleated cells". *Nucleic Acids Res.*, 1988, *16*, 1215.

[24] Yang B., Cross D.F., Ollerenshaw M., Millward B.A., Demaine A.G.: "Polymorphisms of the vascular endothelial growth factor and susceptibility to diabetic microvascular complications in patients with type 1 diabetes mellitus". *J. Diabetes Complications,* 2003, *17*, 1.

[25] Koukourakis M.I., Papazoglou D., Giatromanolaki A., Bougioukas G., Maltezos E., Sivridis E.: "VEGF gene sequence variation defines VEGF gene expression status and angiogenic activity in non-small cell lung cancer". *Lung Cancer*, 2004, *46*, 293.

[26] Jacobs E.J., Feigelson H.S., Bain E.B., Brady K.A., Rodriguez C., Stevens V.L., *et al.*: "Polymorphisms in the vascular endothelial growth factor gene and breast cancer in the Cancer Prevention Study II cohort". *Breast Cancer Res.*, 2006, *8*, R22.

[27] Kawai Y., Sakano S., Korenaga Y., Eguchi S., Naito K.: "Associations of single nucleotide polymorphisms in the vascular endothelial growth factor gene with the characteristics and prognosis of renal cell carcinomas". *Eur. Urol.*, 2007, *52*, 1147.

[28] Banyasz I., Szabo S., Bokodi G., Vannay A., Vasarhelyi B., Szabo A., *et al.*: "Genetic polymorphisms of vascular endothelial growth factor in severe pre-eclampsia". *Mol. Hum. Reprod.*, 2006, *12*, 233.

[29] Kang S., Zhao J., Liu Q., Zhou R., Wang N., Li Y.: "Vascular endothelial growth factor gene polymorphisms are associated with the risk of developing adenomyosis". *Environ. Mol. Mutagen*, 2009, *50*, 361.

[30] Liu Q., Li Y., Zhao J., Sun D.L., Duan Y.N., Wang N., *et al.:* "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.,* 2009, *24*, 2660.

[31] Hsieh Y.Y., Chang C.C., Tsai F.J., Yeh L.S., Lin C.C., Peng C.T.: "T allele for VEGF gene-460 polymorphism at the 5'-untranslated region: association with a higher susceptibility to endometriosis". *J. Reprod. Med.,* 2004, *49*, 468.

[32] Zhao Z.Z., Nyholt D.R., Thomas S., Treloar S.A., Montgomery G.W.: "Polymorphisms in the vascular endothelial growth factor gene and the risk of familial endometriosis". *Mol. Hum. Reprod.,* 2008, *14*, 531.

[33] Elito J., Jr., Daher S., Fernandes da Silva M.O., Marconi N.M., Pendeloski K.P., *et al.:* "Association study of vascular endothelial growth factor and polymorphisms of its gene with ectopic pregnancy". *Am. J. Reprod. Immunol.,* 2010, *63*, 120.

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