The Single Nucleotide Polymorphisms (SNPS) of Vascular Endothelial Growth Factor (VEGF) Gene and Endometriosis

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Abstract—

Aim: *The aim of the present work was to evaluate associations between the risk of endometriosis and -460C/T (rs833061) and +405G/C (rs2010963) polymorphisms in the VEGF gene.*

Methodology: *In the present study, we examined group of 100 patients with endometriosis and 100 controls. Genomic DNA was extracted from peripheral blood. Determination of genes polymorphic variants was made using polymerase chain reaction-restriction fragment length polymorphism technique (PCR-RFLP).*

Results: *Presented study showed statistically significant increase in the endometriosis development risk for the -460T/T genotype (OR 3.39; 95% CI, 1.60-7.13; p = 0.002) and for the -460T allele (OR 2.49; 95% CI, 1.64-3.78; p <.0001), as well as for the +405C/C genotype (OR 2.16; 95% CI, 1.047-4.48; p = 0.035) in patients with endometriosis in comparison with healthy control group. We also observed positive association of the +405C/C genotype (OR 0.26; 95% CI, 0.08-0.79; p = 0.019) as well as the +405C allele occurrence with an increased endometriosis development risk (OR 0.31; 95% CI, 0.19-0.71; p = 0.005), assessed by the degree of rASRM classification stages.*

Conclusion: *The results support the hypothesis that the -460C/T and +405G/C polymorphisms of the VEGF gene may be associated with endometriosis occurrence in Poland.*

Keywords— endometriosis, genetic polymorphisms, VEGF.

I. INTRODUCTION

Endometriosis is a common gynaecological disease of unknown aetiology. Endometriosis is a multifactorial and polygenic disease. Angiogenesis (the growth of blood vessels from pre-existing vasculature) is considered as a major process in the pathogenesis of endometriosis. Many factors are involved in this complex mechanism such as cytokines (interleukins IL-1, IL-6, IL-8, Tumor Necrosis Factor α (TNFα)), metalloproteinases (MMP1, MMP-3, MMP-9) and vascular endothelial growth factors family (VEGF-A, VEGF-B, VEGF-C, VEGF-D, VEGF-E, VEGF-F) [1, 2].

VEGF is one of the most potent and specific angiogenic factors. VEGF is responsible for increased vascular permeability and the proliferation of endothelial cells [3]. There are many causes indicating the role of vascular endothelial growth factor in the development of endometriosis, such as expression of VEGF in stromal and epithelial cells and its regulation by estrogen receptors α and β [4, 5].

Elevated concentrations of VEGF have been detected in the peritoneal fluid of patients with endometriosis [6-9].

VEGF gene has been mapped to chromosome 6p21.3 and is polymorphic in nature [10-12]. Polymorphisms within the 5′-untranslated region (5-'UTR) lead to differences in VEGF expression level between individuals and could influence the aetiology of a variety of pathological conditions with which VEGF has been associated. Several transcription factor-binding sites are found in the *VEGF* 5′-UTR and variation within the region increases the transcriptional activity [13].

Single nucleotide polymorphisms (SNPs) within the *VEGF* gene have been identified, some of which have functional significance [14, 15]. Two common *VEGF* single nucleotide polymorphisms, +405G/C (rs2010963) and -460C/T (rs833061) in the 5-′UTR have been reported to be associated with altered gene transcription [16, 17].

In the literature, many reports confirm the significance *VEGF* gene -460C/T and +405G/C polymorphism, regarding the risk of endometriosis [18-29]. However, the reported results have rather been inconsistent [30, 31].

Little is known on the association between *VEGF* polymorphisms and endometriosis in Polish women. In the present work we analysed an association between endometriosis and two single nucleotide polymorphisms occurring in *VEGF* gene: -460C/T and +405G/C, respectively.

II. MATERIALS AND METHODS

1.1. Patients

In the present study, blood samples were obtained from 100 women with endometriosis, treated at the Department of Gynaecology, Institute of Polish Mother's Memorial Hospital, Lodz, Poland between 2010-2012. The demographic data and the pathologic features of the patients are summarized in Table 1. All the endometriosis samples were staged by a method, based on the criteria of rASRM (The Revised American Society for Reproductive Medicine classification of endometriosis 1996). Blood samples from age- matched, endometriosis-free women (n = 100) served as control. The study was approved by the Local Ethic Committee of the Institute of Polish Mother's Memorial Hospital, Lodz, Poland and each patient gave a written consent.

TABLE 1
THE CHARACTERISTIC OF ENDOMETRIOSIS PATIENTS* AND CONTROL GROUP*

Characteristic	Patients N (%)	Controls N (%)
Age		
Mean ± SD	36.4 ± 6.0	38.3 ± 6.2
Range	23-58	29-61
Sex		
Women	100	100
BMI (body mass index) (kg/m^2)		
<25	44 (44%)	42 (42%)
25≤BMI<30	48 (48%)	49 (49%)
≥ 30	8 (8%)	9 (9%)
Number of birth		
Yes		
1	46 (46%)	49 (49%)
>2	29 (29%)	31 (31%)
No	17 (17%)	18 (18%)
	54 (54%)	61 (61%)
Recurrent pregnancy loss		
Yes	10 (10%)	2 (2%)
No	90 (90%)	98 (98%)
Use of hormone replacement therapy - HRT		
Yes	46 (46%)	21 (21%)
No	54 (54%)	79 (79%)
rASRM classification		
I	11 (11%)	
II	14 (14%)	
III	44 (44%)	
IV	31 (31%)	

n=100

1.2. DNA isolation

Genomic DNA was prepared using QIAmp Kit (Qiagen GmbH, Hilden, Germany) according to the manufacturer instruction.

1.3. Determination of VEGF -460C/T genotype

Polymorphism -460C/T of the *VEGF* gene was determined by PCR-RFLP, using primers: sense 5′-TGTGCGTGTGGGGTTGAGCG-3′, antisense 5′- ACGTGCGGACAGGGCCTGA-3′ [15]. The PCR was carried out in a PTC-100 TM (MJ Research, INC) thermal cycler. PCR amplification was performed in the final volume of 25 µl of reaction mixture, which contained 100 ng of genomic DNA, 0.2 µmol of each primer (ARK Scientific GmbH Biosystems, Darmstad, Germany), 2.5 mM of $MgCl_2$, 1 mM of dNTPs and 1 unit of Taq Polymerase (Qiagen GmbH, Hilden, Germany). PCR cycle conditions were the following: 94°C for 30s, 60°C for 30s and 72°C for 60s, repeated in 30 cycles. PCR products were electrophoresed in a 2% agarose gel and visualised by ethidium bromide staining. The cleavage with *Bst*U1 (New England Biolabs), produced fragments of 175 and 20/155 bp corresponding to the T and C alleles of the VEGF gene, respectively. The enzimatic digestion was controlled by negative controls. Negative controls contained dH_2O (1 µl for 25 µl of PCR Mix).

1.4. Determination of +405 G/C genotype

Polymorphism +405G/C of the VEGF gene was determined by PCR-RFLP, using primers: sense 5′-TTGCTTGCCATTCCCCACTTGA-3', antisense 5′-CCGAAGCGAGAACAGCCCAGAA-3′ [32]. The PCR was carried out in a PTC-100 TM (MJ Research, INC) thermal cycler. PCR amplification was performed in the final volume of 25 µl of reaction mixture, which contained 100 ng of genomic DNA, 0.2 µmol of each primer (ARK Scientific GmbH Biosystems, Darmstad, Germany), 2.5 mM of MgCl2, 1 mM of dNTPs and 1 unit of Taq Polymerase (Qiagen GmbH, Hilden, Germany). PCR cycle conditions were the following: 94°C for 60s, 62°C for 30s and 72°C for 60s, repeated in 30 cycles. PCR products were electrophoresed in a 2% agarose gel and visualised by ethidium bromide staining. The cleavage with BsmFI (New England Biolabs), produced fragments of 469 and 196/273 bp corresponding to the C and G alleles of the VEGF gene, respectively. The enzimatic digestion was controlled by negative controls. Negative controls contained dH_2O (1 µl for 25 µl of PCR Mix).

1.5. Statistical analysis

Logistic regression analysis was used to compute odds ratio (OR) and associated 95% confidence interval (95% CI) relating each of the SNPs as well as combinations of SNPs and another analysed factors presented in Table 1 to the risk of endometriosis. The observed numbers of each *VEGF* genotype were compared with those expected for a population in Hardy-Weinberg equilibrium by using the Chi-square test. Genotype frequencies in the study cases and the controls were compared by the Chi-square test. Wild type genotypes and alleles were used as reference groups. P-values < 0.05 were considered significant. All the statistical analyses were performed, using the STATISTICA 6.0 software (Statsoft, Tulsa, Oklahoma, USA).

III. RESULTS

Table 2 shows genotype distribution values of *VEGF* +405G/C polymorphisms in endometriosis patients and controls. An association was found between the 405C/C genotype of the +405G/C polymorphism of *VEGF* gene and endometriosis occurrence. In the examined patients, the observed frequencies of G/G, G/C and C/C genotypes differed significantly ($p < 0.05$) from the distribution range, as expected from the Hardy-Weinberg equilibrium.

TABLE 2
DISTRIBUTION OF GENOTYPES AND ALLELES FREQUENCY OF THE +405 G/C POLYMORPHISM OF THE *VEGF* GENE IN PATIENTS WITH ENDOMETRIOSIS AND CONTROLS

	Patients (N = 100) N (%)	**Controls (N = 100) N (%)**	**OR (95% CI)[A]**	***P*[B]**
VEGF +405 G/C				
G/G	24 (24)	26 (26)	1.00 Ref.	
G/C	24 (24)	48 (48)	0.54 (0.25-1.13)	0.149
C/C	52 (52)	26 (26)	**2.16 (1.04-4.48)**	**0.035**
G	72 (36)	100 (50)	1.00 Ref.	
C	128 (64)	100 (50)	0.90 (0.58-1.39)	0.729

Data in bold font are statistically significant
[a]Crude odds ratio (OR), 95% CI = confidence interval at 95%, [b]Chi square

Table 3 shows genotype distribution of *VEGF* -460C/T polymorphism, illustrating the difference between the patients with endometriosis and the controls. It can be seen from the Table 3 that there are significant differences in the frequency of *VEGF* -460 C/T genotypes ($p < 0.05$) between the two investigated groups. A weak association was observed between endometriosis occurrence and the presence of T/T genotypes. Variant 460T allele of *VEGF* increased endometriosis risk. In case of the -460C/T polymorphism of *VEGF* gene the distribution of the genotypes in the patients differed significantly from one expected from the Hardy-Weinberg equilibrium ($p < 0.05$).

TABLE 3
DISTRIBUTION OF GENOTYPES AND ALLELES FREQUENCY OF THE -460 C/T POLYMORPHISM OF THE *VEGF* GENE IN PATIENTS WITH ENDOMETRIOSIS AND CONTROLS

	Patients (N = 100) N (%)	**Controls (N = 100) N (%)**	**OR (95% CI)[A]**	***P*[B]**
VEGF -460 C/T				
C/C	16 (16)	28 (28)	1.00 Ref.	
C/T	22 (22)	40 (40)	0.96 (0.43-2.15)	0.920
T/T	62 (62)	32 (32)	**3.39 (1.60-7.13)**	**0.002**
C	54 (27)	96 (48)	1.00 Ref.	
T	146 (73)	104 (52)	**2.49 (1.64-3.78)**	**<.0001**

Data in bold font are statistically significant
[a]Crude odds ratio (OR), 95% CI = confidence interval at 95%, [b]Chi square

We also analyzed combined genotype of all polymorphism pairs. Table 4 shows the haplotypes distribution of *VEGF*. The haplotypes analysis according to wild-type of G405G–C460C showed high frequency C405C-T460T, genotype. The combined C405C-T460T genotype increased the risk of endometriosis (OR=18, p=0.0001).

TABLE 4
HAPLOTYPES DISTRIBUTION AND FREQUENCIES OF VEGF GENE POLYMORPHISMS IN ENDOMETRIOSIS PATIENTS AND THE CONTROLS

Haplotypes *VEGF*-405-460	**Patients (N = 100) N (%)**	**Controls (N = 100) N (%)**	**OR (95% CI)[A]**	***P*[B]**
G/G-C/C	3 (3)	10 (10)	1.00 Ref.	
G/C-C/C	5 (5)	12 (12)	1.38 (0.26-7.39)	0.514
C/C-C/C	8 (8)	3 (3)	**8.88 (1.39-56.57)**	**0.043**
G/G-C/T	3 (3)	8 (8)	1.25 (0.19-7.95)	0.589
G/C-C/T	3 (3)	16 (16)	0.62 (0.10-3.72)	0.469
C/C-C/T	14 (14)	13 (13)	3.58 (0.80-16.00)	0.082
C/C-T/T	27 (27)	5 (5)	**18.00 (3.61-89.58)**	**0.0001**
G/C-T/T	16 (16)	17 (17)	3.13 (0.72-13.05)	0.213
G/G-T/T	21 (21)	16 (16)	**4.37 (1.03-18.55)**	**0.037**

Data in bold font are statistically significant
NE - Not estimated.
[a]Crude odds ratio (OR), 95% CI = confidence interval at 95%, [b]Chi square

Endometriosis staging was related to *VEGF* polymorphisms. Endometriosis stage were evaluated in all the cases (n = 100). Stages I+II and III+IV were accounted together for statistical analysis (see Table 5).

TABLE 5
DEPENDENCE OF GENOTYPES AND FREQUENCIES OF VEGF GENE POLYMORPHISM ALLELES ON ENDOMETRIOSIS STAGE IN PATIENTS[a]

PATIENTS				
stage[b]	**I+II (n = 25)**	**III+IV (n = 75)**	**OR (95% CI)[C]**	**P[D]**
VEGF - 460C/T	Number (%)	Number (%)		
C/C	4 (16)	15 (20)	1.00 Ref	
C/T	8 (32)	17 (23)	1.76 (0.44-7.06)	0.639
T/T	13 (52)	43 (57)	1.13 (0.31-4.01)	0.560
C	16 (32)	47 (31)	1.00 Ref	
T	34 (68)	103 (69)	0.96 (0.48-1.92)	0.920
VEGF +405G/C				
G/G	9 (36)	13 (17)	1.00 Ref	
G/C	8 (32)	17 (23)	0.68 (0.20-2.24)	0.740
C/C	8 (32)	45 (60)	**0.26 (0.08-0.79)**	**0.019**
G	26 (52)	43 (29)	1.00 Ref	
C	24 (48)	107 (71)	**0.31 (0.19-0.71)**	**0.005**

Data in bold font are statistically significant
[a]n = 100; [b]according to rASRM classification; [c]Crude odds ratio (OR), 95 % CI = confidence interval at 95 %, [d]Chi square

No differences were observed in those groups, regarding either *VEGF* -460C/T genotype or allele distributions. Some correlation was observed between the genotypes of *VEGF* +405G/C polymorphisms and endometriosis. A statistically significant decrease was observed, regarding 405C allele frequency (OR 0.31; 95% CI 0.19 – 0.71, p = 0.005) in stage III+IV patients, according to rASRM classification. A tendency for an increased risk of endometriosis was observed with the occurrence of 405G allele of *VEGF* polymorphism.

No statistically significant differences were observed in the alleles or in the genotype frequencies of the *VEGF* gene polymorphisms between risk factors of endometriosis such as BMI (body mass index), HRT (hormone replacement therapy) and recurrent pregnancy loss and the women with endometriosis (data not shown).

IV. DISCUSSION

In the present study we genotyped two common polymorphisms of the *VEGF* gene and tested the association between the distributions of their genotypes with endometriosis. The following SNPs were considered in the angiogenesis pathway: VEGF -460C/T (rs833061) and +405G/C (rs2010963). The study was performed on an ethnically homogenous population, which may improve our knowledge, regarding to what an extent the genotype-phenotype relationship variations are population-related.

An important aspect of the association between endometriosis and VEGF, also for possible future therapeutic application is that the single nucleotide polymorphisms in vascular endothelial growth factor gene are associated with the risk of endometriosis.

VEGF gene consists of 8 exons. *VEGF* gene transcript undergoes alternative splicing processes to produce a protein family [33]. There are a few transcription factor binding sites in 5'- UTR in *VEGF* gene [34]. From here, polymorphisms in this region result in different levels of gene expression, causing various ranges of diseases such as endometriosis.

A G to C substitution at the position +405 and C to T substitution at the position -460 of the *VEGF* gene have been describing as a single nucleotide polymorphisms. Both polymorphisms are located in the regulatory element (5'-untranslated region) of the *VEGF* and are suggested to be associated with messenger RNA stability and expression [14, 15].

The –460C/T and +405G/C functional *VEGF* polymorphisms have been found to be associated with variation in VEGF protein production [26, 15] and have been related to several diseases in which angiogenesis is involved [35-37].

From a review of the literature we learned that *VEGF* –460C/T and +405G/C polymorphisms were investigated in diverse populations with endometriosis. Many experimental studies confirm the significant role the influence of specific genetic variants on endometriosis risk [18-20]. However the contribution of polymorphisms of *VEGF* gene in developing endometriosis is controversial [29, 30].

This is the first study which attempted to analyse the incidence of alleles of the *VEGF* -460C/T and +405G/C polymorphisms in samples from Polish women with endometriosis. In the presented study, *VEGF* +405C/C and -460T/T genotype were associated with an elevated risk of endometriosis in the Polish population. There was an 18-fold increased risk of endometriosis for C405C-T460T genotype carriers. Moreover +405G/C polymorphism was related to endometriosis stage. We have also found that variant -460T allele of *VEGF* increased endometriosis risk. It is possible that the presence of the -460T allele is in linkage disequilibrium with another, so far unknown, mutation located outside the coding region in the *VEGF* gene, which may be of importance for the VEGF concentration in plasma and better endometriosis development.

Our results are in line with the data from other reports.

Analysis of the *VEGF* +405 G/C polymorphism in a Korean population showed that the single nucleotide polymorphism was associated with the risk of advanced stage endometriosis [38].

The study of *VEGF* +405G/C in an Indian population identified a haplotype associated with endometriosis. Vanaja et al. showed that the *VEGF* 405G/G genotype is associated with the risk of endometriosis in Indian women [18].

Emamifar et al. [19] found the correlation between the *VEGF* +405G/C polymorphism and endometriosis. The *VEGF* 405C allele was associated with a significantly increased risk of endometriosis in northern Iran.

Similar results were obtained by Gentilini et al. in the Italian population [15]. Also, the analysis of Turkish women suggests that *VEGF* gene polymorphisms in 5'-UTR are correlated wit endometriosis [20, 17].

However, other literature data were also found. No significant associations were observed between the *VEGF* -460C/T and +405G/C polymorphism and endometriosis in Asians and Caucasians population [29, 30].

V. CONCLUSION

In conclusion, the presented study implies that -460C/T and +405G/C polymorphisms of the *VEGF* gene may be associated with endometriosis in Polish women. The study was carried out on a relatively small patient population, thus the obtained results cannot be considered as definitive and require further, more extensive evaluations, performed on bigger groups of patients. However, our preliminary results are fairly promising, indicating a significant role of the polymorphisms of *VEGF* genes for endometriosis development. It also appears from a thorough review of the medical literature that the polymorphisms in *VEGF* gene, involved in the angiogenesis pathway, have for the first time been analyzed in endometriosis patients in Poland. Thus we feel that our observations may be an important signal, prompting to appreciate the role of *VEGF* in endometriosis occurrence and likely triggering further studies on this interesting subject.

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CONFLICTS OF INTEREST

The authors declare no conflicts of interest.

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