

## The Role of +405 G/C VEGF A Polymorphism in Women with Recurrent Implantation Failure, Endometriosis and Miscarriages after ICSI-ET Cycles

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### Abstract

**Background:** Approximately one of six couples in reproductive age worldwide experience different difficulties in conceiving. In such cases different types of assisted reproductive techniques (ART) could be performed in order to deliver healthy child. Unfortunately large percent of the IVF/ICSI-ET attempts finish without positive pregnancy test or with spontaneous miscarriage. The successful implantation of embryos depends on trophoblast proliferation, migration and invasion in the endometrium. This is mediated by locally produced molecular factors and hormones. Vascular Endothelial Growth Factor (VEGF) is one of these factors that play pivotal role in human angiogenesis and embryogenesis.

**Methods:** Blood samples were collected from control women with natural conceived successful pregnancy and patients from three groups of reproductive failure: (i) RIF - Recurrent/Repeated Implantation Failures; (ii) endometriosis; (iii) RSM - Recurrent Spontaneous Miscarriages. Genomic DNA was isolated from blood samples using standard protocol with columns according to manufacturer's instructions. In order to detect the VEGF +405 G/C allelic state PCR reaction forward by Restriction Fragment Length Polymorphism assay (PCR-RFLP) by BsmFI restrictase was performed. To confirm the results Real Time PCR genotyping with 5' exonuclease technology and Sanger capillary sequencing as a gold standard was used.

**Results:** The C allelic frequency was statistically higher in patients group compared to controls (53.6% vs 24%, OR = 3.68, 95% CI,  $p < 0.002$ ). In addition, we detected statistically significant incidence of the +405 C/C genotypes in women from the patient group than in control group (37% vs 4%, OR = 16.2, 95% CI,  $p < 0.02$  when calculating against GG genotype).

**Conclusion:** The results of the present study revealed association between the VEGF A +405 C/C genotype and the risk of development of endometriosis and spontaneous miscarriages. In the RIF the results did not reveal such correlation. This test could be offered to patients with presented indications and special attention to be paid on their clinical management. The study group should be expanded and include more genetic polymorphisms in association with the mentioned reproductive pathologies.

**Keywords:** Implantation; miscarriage; VEGFA; endometriosis; angiogenesis

**Abbreviations:** RIF: Recurrent/Repeated Implantation Failures; ART: Assisted Reproductive Technologies; VEGFA: Vascular Endothelial Growth Factor A; VEGFR: Vascular Endothelial Growth Factor Receptor; RSM: Recurrent Spontaneous Miscarriages; POC: Product of Conception; IVF: *In Vitro* Fertilization; ICSI: Intra Cytoplasmic Sperm Injection; ET: Embryo Transfer; OHSS: Ovarian Hyper Stimulation Syndrome; COS: Controlled Ovarian Stimulation; SNP: Single Nucleotide Polymorphism; UTR: Un Translated Region; PCR: Polymerase

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Chain Reaction; RFLP: Restriction Fragment Length Polymorphism; FGF-4: Fibroblast Growth Factor 4; IGF-I: Insulin Like Growth Factor; LIF: Leukemia Inhibitory Factor; CSF-1: Colony Stimulating Factor 1; WNT7a: wingless-type MMTV integration site family, member 7A

### Introduction

One of the most important molecular processes in the female reproductive system is the angiogenesis. This is a key biological phenomenon for the development and differentiation of the human endometrium. For the proper supply of this dynamic tissue a new blood vessels every month are required. The interaction between the endometrium and the trophoctodermal cells of the blastocyst is one of the crucial moments for the beginning and maintenance of successful pregnancy [1]. The regulation of this complex process requires the coordination of a number of different molecular factors [2]. This coordinate the exact time and duration of the so called “implantation window” when the endometrium is highly responsive for the epiblast invasion [3]. The crosstalk effects many events on DNA, RNA or protein level via different interactions and cell signaling pathways [2,3]. In cases of deregulation different pathologies in human reproduction could be presented such as recurrent implantation failures, recurrent spontaneous miscarriages (RSMs) or endometriosis. RIFs refers to failure to achieve clinical pregnancy after embryo transfer (ET) of good quality embryos in a minimum three IVF/ICSI cycles [4]. RSMs affect 1-3% of the couples trying to conceive and in general they are defined as at least three miscarriages prior to 20<sup>th</sup> gestational week [5]. Endometriosis is a proliferative disorder which affects 25-30% of the women with chronic pains in the pelvis or infertility [6,7].

One of the major molecular factors in the angiogenesis regulation is the VEGFA [8]. In the female reproductive tract this protein is expressed from mesenchymal Hofbauer cells as N-glycosilated homodimer [9]. VEGF is a mitogen which induces the proliferation and migration of endothelial cells, increase permeability and inhibits the mechanisms of the apoptosis. It activates different pathways after the recognition with VEGF receptors (VEGFRs) which affects important signaling molecules like MAP kinases (MAPKs), Caspases, etc [10-12].

The gene encoding VEGFA is situated in cytogenetic locus 6p21.3, with length 16 303 base pairs (bp) and it consist eight exons. The protein has 6 determinate functional regions - signal sequence (exon 1), N-terminal domain (exon 2), VEGFR1 binding place (exon 3), VEGFR2 binding place (exon 4), Plasmin cutting region (exon5) and heparin binding place (exon 6 and 7) [13].

There are a number of diseases which are tightly associated with angiogenesis deregulation. Such pathologies include different disorders with excessive or insufficient angiogenesis - oncological diseases, rethinopathies, cardiovascular diseases, psoriasis, etc. [14-17]. Concerning female reproductive system endometriosis, RIFs and RCMs are the main clinical phenotypes associated with VEGF deregulation. There are different genetic variants of VEGF, mainly single nucleotide polymorphisms (SNPs), which affects the gene expression or protein structure and has negative impact on human reproduction [4,5,18].

One of these genetic variants is in the 5' untranslated region (5'-UTR) in place +405. The allelic state is the presence of guanine or cytosine (G/C). This polymorphism is associated with altered gene expression of the VEGFA which has as a consequence different angiogenesis response [19]. The presence of C-allele of +405 G > C play important role in the reproductive medicine because it is associated with the pathogenesis of several female reproductive tract diseases such as pre-eclampsia, endometriosis, recurrent pregnancy loss and ovarian hyper stimulation syndrome (OHSS) after controlled ovarian stimulation (COS) for IVF [20,21].

Given these data, in the present study we aimed to perform genetic analysis of patient and control group of samples in order to detect the association of VEGF +405 G > C SNP and RIFs, RCMs and endometriosis in Bulgarian subjects. In addition, we made laboratory validation of our results, which could help use such genetic markers as indication for personalized IVF or other treatment dependent on the women clinical history.

### Materials and Methods

In the present study we included women after IVF/ICSI-ET treatment in the assisted reproduction unit of Medical Center “Repro-BioMed”, Sofia, Bulgaria and Medical Center “Repromed”, Pleven, Bulgaria. All the women from the patient and control groups provided their prior, written informed consent to participation. The samples were collected between July 2014 and February 2015. The patients

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were 41 between the age of 23 and 35 years. A peripheral blood for DNA extraction was taken as a clinical material. All of the samples were divided according to the pathological phenotype (Table 1). The control group was consisted of women (n = 21) between the age of 25 and 42 years with natural conceived successful pregnancy. For the VEGFA +405 G/C SNP detection a polymerase chain reaction followed by restriction analysis (PCR-RFLP) was used. In order to prove the results genotyping via Real Time PCR was performed. For the laboratory validation capillary Sanger sequencing was used as a gold standard in SNP detection.

Pathological Phenotypes	Patients Number (n)
Endometriosis	11
Recurrent Implantation Failure (RIF)	15
Recurrent Spontaneous Miscarriages (RSM)	15

**Table 1:** Distribution of the patient group according to the different reproductive pathology.

The used protocols for ovarian stimulation, embryo cultivation and transfer were similar for all patients. Genomic DNA was isolated from peripheral blood with standard column method according manufacturer’s instructions (NucleoSpin Blood, Macherey-Nagel Inc., Germany). The amount and the purity of the extracted DNA were evaluated via spectrophotometer (Jenway, Genova Life Science, and Germany). The measurement shows DNA concentration between 85 and 115 ng/μl and log ratio A260/280 between 1.7 and 2.0 for all samples.

For the primer design free online software (Primer3 <http://bioinfo.ut.ee/primer3-0.4.0/>) was used. The allele specific primer pairs were synthesized from Metabion (Metabion International AG, Germany). After the delivery they were diluted to 100 pg/μl and stored at -20°C until required. Work dilutions were with concentration 6 pg/μl for each primer. For the PCR optimization we used different annealing temperatures and PCR additives like Betain (Affymetrix, Inc., USA). For the specific amplification Kappa ready mix (Kappa Biosystems, Inc., USA) was used. The exact PCR mixture and reaction conditions are given in Tables 2 and 3. The desired allele specific amplicon was 469 bp in length. In order to detect and distinguish the VEGF +405 G/C alleles a specific restriction enzyme was used. The BsmFI (New England Biolabs Inc., USA) enzyme was selected with NEB cutter software (New England Biolabs Inc., USA <http://nc2.neb.com/NEBcutter2/>). Restriction digestion was performed overnight at 65°C followed by enzyme inactivation at 80°C for 20 minutes. When the G allele is present the enzyme recognizes the restriction place specifically and cut the amplicon in two fragments - 273 bp and 196 bp. In case of a presence of the C-allele the BsmFI does not cut the 469 bp fragment. In heterozygous samples we had three bands - 469, 273 and 196 bp. The fragment length detection was performed on standard horizontal agarose electrophoresis. The agarose gel (Lonza, Switzerland) was prepared at 4% in 1 X TBE buffer (Applichem Inc., Germany).

Reaction Component	Initial Concentration	Reaction Concentration	Volume per 50 μl Reaction
Kapa Ready Mix	2X	1X	25 μl
Forward primer	100 pmol/μl	6 pmol/μl	1 μl
Reverse primer	100 pmol/μl	6 pmol/μl	1 μl
Betain	5M	1M	10 μl
DNA template	90-160 ng/μl	50 ng/μl	1 μl
dH <sub>2</sub> O			Up to 50 μl

**Table 2:** PCR reaction components.

PCR stage	Temperature (°C)	Stage Duration	Number of Cycles
Initial Denaturation	96°C	5 min	1
Template Denaturation	96°C	30 sec	40
Primer Annealing	62°C	30 sec	
Synthesis	72°C	60 sec	
Final synthesis step	72°C	10 min	1

**Table 3:** PCR reaction conditions.

Real Time PCR genotyping was performed with allele specific probes (Primer Design, UK) on the Eco Real Time instrument (Illumina Inc., USA). The laboratory protocol was performed according to manufacturer’s instructions.

For laboratory validation and substantiation of the protocol and the results capillary Sanger sequencing was used. Allele specific amplification was performed according to the PCR protocol for the PCR-RFLP assay. The amplified products were enzymatically purified ExoSAP (Affymetrix, Inc., USA). After that a standard sequencing reaction was performed with BigDye Terminator v3.1 Cycle Sequencing kit (Life Technologies, Inc., USA). For every sample a two sequencing reactions were established - one with the forward and one with the reverse primer. The reaction products were loaded on a capillary electrophoresis sequencer ABI3130xl Genetic Analyzer (Life Technologies Inc., USA). For the visualization of the amplicon sequences and data analysis FinchTV (Geospiza, PrekinElmer Inc., USA) and SeqScape (Life Technologies Inc., USA) software applications were used.

For the statistical analysis of the data a standard t-test was used. Allele ratios and genotype distributions in the patient and control groups were analyzed by logistic regression. GG genotype and G alleles were assumed as reference group in the analysis. The odds ratio (OR), its standard error and 95% confidence interval are calculated according to Altman, 1991. P-values less than 0.05 were considered statistically significant.

**Results and Discussion**

A total 41 patients and 23 controls consented to participate in the present study. All of the samples and the controls were subjected to three different genotyping methods. The results were compared between the patients group and the control group. In addition a comparison between the three different methods was made. The genotypes detected via PCR-RFLP, Real Time PCR and Sanger sequencing were in absolute concordance.

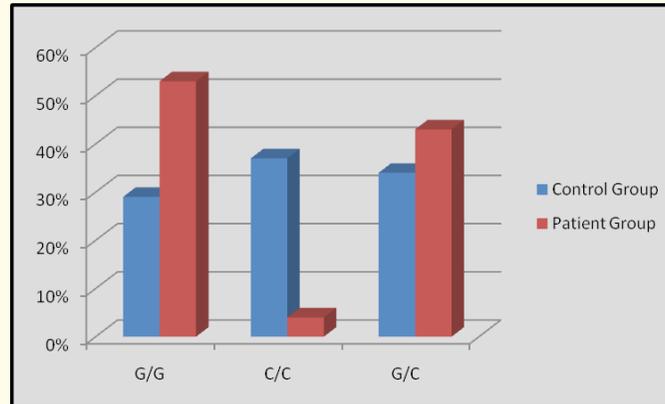
Table 4 and 5 represent the alleles and genotypes frequencies among patients and controls. There was statistically significant difference in the frequency of C allele between patients and control group (OR = 3.68, 95% CI, p < 0.002). Concerning genotypes, we detected significantly higher incidence of CC genotype in patients group compared to the control group - 37% and 4% respectively (OR = 16.2, 95% CI, p < 0.02 when calculating against GG genotype) (Figure 1). Comparison of heterozygote genotype CG to homozygote GG genotype revealed OR = 1.68, 95% CI, p = 0.37.

Genotype	Cases (n = 41)	Controls (n = 23)	p-value
CC	15 (37%)	1 (4%)	0.04
GC	14 (34%)	9 (43%)	n.s.
GG	12 (29%)	13 (53%)	n.s.

**Table 4:** Distribution of the three genotypes in the patients and controls group.

Allele	Cases (n = 82)	Controls (n = 46)
C	44 (53.6%)	11 (24%)
G	38 (46.4%)	35 (76%)

**Table 5:** Genotype distribution in cases and controls.



**Figure 1.** Graphical distribution of the frequencies of the three genotypes in patients and controls groups in percentages.

In case when the patients are separated according to the presented pathology (RIF group, Endometriosis group and RSM group) the observations reveals association between the VEGFA +405 C/C genotype and the clinical phenotype but without statistical significance probably because of the small number of the patients in the subgroups.

VEGF +405 C/C genotype VEGF, the key regulator for such fundamental biological process as angiogenesis, is responsible for the proper activity of many molecular signaling pathways in different tissues and organs in the human body [8]. The presence of some dysregulation in the VEGF production or function leads to variety of pathological phenotypes. There is data about the association of the VEGFA +405 C/C genotype and the higher incidence of rethinopathy in patients with diabetes. Other disease which is connected with this SNP is the psoriasis [16-18].

Vascular endothelial growth factor is a highly potent pro-angiogenic protein and it is strongly involved in vasodilatation, endothelial permeability, proliferation and inhibition of apoptosis. It is key factor for the human reproduction because a lot of data reveals its crucial role in oocyte maturation in the ovaries, the angiogenesis in the decidua, placenta and early gestation [2,5]. Dysregulation in these processes is known to be associated with RIF and RSM. Some studies suggest also the involvement of the VEGFA in the development of endometriosis. Some authors even associate +405 G > C polymorphisms with familial cases of the disease in different populations [22,23].

In the present study, women with mentioned above reproductive pathologies had a higher frequency of the (resulting in lower VEGF expression, according to literature) than control patients did. According to our data it increases about 16 times the risk for these complications compared to women with VEGF +405 G/G genotype. There is data that the higher VEGF expression is detected in pregnant patients. This observation suggests that higher VEGF levels are required for successful implantation and pregnancy. This hypothesis is supported also from the genotyping of aborted fetuses after spontaneous miscarriages. A higher frequency of the VEGF +405 C/C genotype is observed in the product of conception (POC) samples [24]. The up-regulation of the VEGFA gene transcription is associated with +405 G/G genotypes [25]. The distribution of the heterozygous genotypes was without any significant difference between the patients

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and controls -32% and 43% respectively. These tendencies are also observed when we compare the three groups of patients separated in RIF group (n = 15, 33%, p > 0.05), RSM group (n = 15, 33%, p > 0.05) and endometriosis group (n = 11, 36%, p > 0.05). This may reveal a compensatory effect and phenotype domination of the +405 G-allele above the C-allele. In the patient group with recurrent implantation failures we have prevalence of the VEGFA +405 G/G genotype which makes the meaning of this polymorphism controversial according to the implantation potential of the endometrium. The trophoblast invasion in the endometrium is a complex process and it depends from the mother's condition as well as the vitality and the developmental potential of the embryo. During the molecular signaling from the endometrium to blastocyst and inversely (so called cross-talk) a large number of different factors plays role [2]. In addition to VEGF important roles in implantation plays molecular signaling proteins like FGF-4, IGF-I, LIF, CSF-1, WNT7a, etc. The fine balance between different angiogenic factors is needed. This makes the diagnostics and therapy of such pathological conditions extremely complicated. The future requires the establishment of different infertility genetic panels which could include many polymorphisms in different genes. This will require a very precise and hard analysis according to the growing knowledge in the databases. In addition to this discrepancy about the VEGF + 405 C/C genotype and RIF group we should consider also the small amount of the collected samples.

The future research work in this direction must be focused in collection of more samples and also the including of more VEGF polymorphisms and variants in different genes which are involved in the complex process of implantation and successful pregnancy.

### Conclusion

During the last two decades there is a huge progress of infertility diagnosis and treatment. Nowadays the reproductive medicine is well established multidisciplinary modern field of the biomedical science. Unfortunately there are still many ongoing questions about the molecular mechanisms of embryo implantation and the key factors of successful pregnancy. In the present study we tried to reveal the association of one single nucleotide polymorphism VEGFA +405 G > C with reproductive failure in women. We supported the evidence for correlation between VEGFA +405 C/C genotype and infertility connected to endometriosis and recurrent miscarriages. According to our data the association between RIFs and the C/C genotype is questionable, but this could be artifact because of the small number of the samples in this particular patient group.

The results demonstrate that the optimized PCR-RFLP analysis is reliable as Real Time PCR genotyping and Sanger sequencing for diagnostic assays.

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