

Polymorphisms rs2010963 and rs833061 of the VEGF gene in polycystic ovary syndrome

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SUMMARY

OBJECTIVE: The polycystic ovary syndrome is the most common endocrine disorder, characterized by the dysregulation of ovarian angiogenesis. This alteration can be related to changes in the activities of the vascular endothelial growth factor (VEGF) gene. Single-nucleotide polymorphisms have been observed in the promoter, intronic, and untranslated regions of the VEGF gene, and several studies have suggested that these polymorphisms may be associated with the risk of polycystic ovary syndrome. This study aimed to investigate the association between rs2010963 and rs833061 polymorphisms and haplotypes of VEGF in the etiology of polycystic ovary syndrome.

METHODS: A total of 210 women, 102 diagnosed with polycystic ovary syndrome and 108 controls, participated in this study. The genotyping of the samples was performed by PCR-RFLP and real-time PCR for rs2010963 and rs833061 polymorphisms, respectively. The statistical analyses were performed by the chi-square test and logistic regression model.

RESULTS: The clinical characteristics of the patients showed that 75.8% of the patients did not become pregnant, 36.3% had a family history of polycystic ovary syndrome, 58.6% were obese, and about 60% had clinical characteristics of hyperandrogenism. There were no associations between the distribution of rs2010963 (OR 1.24; 95%CI 0.60–2.57; p=0.56) and rs833061 (OR 0.78; 95%CI 0.32–1.92; p=0.59) in patients and controls.

CONCLUSIONS: The patients with polycystic ovary syndrome have similar rates of VEGF polymorphisms rs2010963 and rs833061 on the general population.

KEYWORDS: Polycystic ovary syndrome. Polymorphism, genetic. Haplotypes. Vascular endothelial growth factor A.

INTRODUCTION

Polycystic ovary syndrome (PCOS) is the most common endocrine disorder, and its clinical features include hirsutism, infertility, acne, alopecia, oligo-anovulation, and metabolic abnormalities such as insulin resistance, excessive weight or obesity, type 2 diabetes, dyslipidemia, and an increased risk of cardiovascular disease^{1,2}.

Dysregulation of ovarian angiogenesis contributes to abnormal follicular development in women with PCOS. This alteration may contribute to the ovarian features of PCOS, such as abnormal follicular development, increase in the number of small follicles, and failure in the selection of the dominant follicle, with anovulation and cyst formation³.

Modifications in the vascular endothelial growth factor (VEGF) family are associated with the ovarian angiogenesis³. In the ovary, this gene is expressed in theca cells, granulosa lutein cells, and interstitial tissues and may be involved in the physiological regulation of ovarian angiogenesis, in a manner that suggests a role of this growth factor in both cyclic angiogenesis and regulation of vascular permeability, both critical for ovarian folliculogenesis and for normal reproductive function⁴. The VEGF gene is located in the chromosome region 6p21.3⁵. Single-nucleotide polymorphisms (SNPs) have been observed in the promoter, intronic, and untranslated regions of the VEGF gene, and several studies have suggested VEGF gene polymorphisms may be associated with PCOS risk⁶⁻⁸.

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VEGF rs833061 T and rs2010963 G alleles appear to correlate with altered VEGF expression levels. The increased levels of VEGF have been reported in PCOS⁶. The rs2010963 and rs833061 SNPs VEGF have recently been investigated in five independent studies⁶⁻¹⁰. One of them evaluated the association of VEGFA SNPs (nine tested variants) with altered VEGF secretion level and PCOS among ethnically matched control women. This study showed that VEGF levels in rs833061 genotypes were significantly higher in PCOS⁹. The other study also published in 2019 investigated SNPs rs2010963 and rs833061 and showed that the first one may be associated with the risk of PCOS in Chinese women¹⁰. The studies published in 2020⁶⁻⁸ are of the meta-analysis type and confirm associations of polymorphisms in the VEGF gene with susceptibility to PCOS, with emphasis on rs2010963^{7,8}.

The rs2010963 polymorphism had been described as C→T exchange at nucleotide position 936, in the 3'-UTR of the VEGF gene, and was associated with lower VEGF plasma levels¹¹. Therefore, the rs833061 is located in the promoter region and has been associated with increased VEGF expression levels¹².

In the literature, there are several studies on polymorphisms in the VEGF gene in patients with PCOS from different populations; however, no research was conducted on these two polymorphisms in Brazilian women.

The objective of this study was to investigate the association of the VEGF polymorphisms rs201093 and rs833061 and to identify the frequency of haplotypes with the risk of developing PCOS in women compared with control group.

METHODS

Subjects

This study was approved by the Research Ethics Committee of the Federal University of Triângulo Mineiro (UFTM), protocol 1796, and all participants signed an informed consent form.

Participants' inclusion in the study occurred in the period from 2012 to 2016. This case-control study included 102 patients with a clinical diagnosis of PCOS and 108 control women. The PCOS diagnosis was based on Rotterdam criteria, and the patients who visited the Endocrinology and Gynecology Outpatient Clinic of the UFTM were selected. In the control group, women at reproductive age who had no history of hyperandrogenism, menstrual dysfunction, infertility, or sonographic sign of PCOS, and those who sought medical care for gynecological routine were selected

for the study. All participants answered a questionnaire about the risk factors.

Molecular analysis

Genomic DNA from the peripheral blood was extracted by a salting-out method.

Genotyping of the rs2010963 polymorphism was performed in 106 PCOS patients and 97 controls using polymerase chain reaction restriction fragment length polymorphism (PCR-RFLP) analysis.

The PCR was carried out in a total volume of 30 µL containing approximately 100 ng genomic DNA, 1× PCR buffer, 1 mM MgCl₂ (25 mM), 2 µM of dNTP, 20 pmol of each primer (F: 5'-CCGACGGCTTGGGGAGATTG-3'; R: 5'-CGGCGGTACCCCCAAAAG-3'), 1 U of Taq DNA polymerase, and 5% of glycerol. The amplification program consisted of an initial denaturation step at 94°C for 10 min and 40 cycles (denaturation at 94°C for 45 s, annealing for 45 s at 62°C, extension at 72°C for 30 s) and final extension for 10 min at 72°C.

The PCR products were digested with 0.2 U of the BsmFI restriction enzyme. Digested fragments were analyzed by 10% polyacrylamide gel electrophoresis. After the restriction enzyme treatment, the CC genotype was visualized as a single 197 bp fragment; the CG genotype as three fragments of 197, 167, and 30 bp; and the GG genotype was visualized as two fragments of 167 and 30 bp.

The real-time PCR allelic discrimination technique was used to analyze the rs833061 polymorphism in 94 patients and 87 controls, on the ABI PRISM 7500 Sequence Detection System (Applied Biosystems) using TaqMan Minor Groove Binder (MGB) probes. Primers and probes were designed by Life Technologies (ID: C_1647381_10).

Statistical analysis

The chi-square test was used for the statistical analysis of the genotypic and allelic distribution of the polymorphisms, as well as to verify the Hardy-Weinberg equilibrium (HWE). A statistical power of 95% was tested using the G* Power program 3.1.9.2. In addition, a post-hoc test with the total sample (n=210) was performed, with an effect size of 0.27 and an alpha significance level of 0.05. The multiple logistic regression model was used to determine the effect of risk factors (e.g., family history, smoking, alcohol consumption, and the presence of polymorphism) in PCOS. The SNPStats program was used for the logistic regression model adjusted for age. The

effect of polymorphisms was evaluated by the following inheritance models: codominance, dominance, recessive, and overdominance.

The haplotype from VEGF gene polymorphisms was inferred using the SNPStats program, checking the estimated population frequency of the haplotypes.

The results were presented in the odds ratio (OR) and 95% confidence interval (95%CI), with the level of statistical significance being defined as $p < 0.05$. A post-hoc analysis was performed, and the statistical power for association tests was found to be 98%.

RESULTS

The clinical characteristics of the patients showed that 75.8% of the patients did not become pregnant, 36.3% had a family history of PCOS, 58.6% were obese, and about 60% had clinical characteristics of hyperandrogenism.

In the univariate analysis, no statistically significant differences were observed between the two groups for polymorphisms rs2010963 and rs833061 ($\chi^2=0.38$, $p=0.83$ and $\chi^2=0.86$, $p=0.65$, respectively). For the allele frequencies, no differences were found between the groups ($\chi^2=0.18$, $p=0.67$ and $\chi^2=0.02$, $p=1.00$, respectively).

The genotype distributions of the rs2010963 and rs833061 polymorphisms were in Hardy-Weinberg equilibrium in both patient ($\chi^2=2.35$; $p=0.12$; $\chi^2=0.05$; $p=0.82$) and control ($\chi^2=1.05$; $p=0.31$; $\chi^2=1.04$; $p=0.31$) groups.

The genotypes of 90 women with PCOS and 80 controls were adjusted for age according to the heritable models and showed no association between the polymorphisms and PCOS (Table 1).

The haplotypes that were constructed with the analysis of the two VEGF gene polymorphisms were evaluated in this study. All estimated haplotypes had similar frequencies between the two groups.

Table 1. Association of rs2010963 and rs833061 polymorphisms of the vascular endothelial growth factor gene with polycystic ovary syndrome, adjusted for age (used in case-control study).

Model	Genotype	Case n (%)	Control n (%)	OR (95%CI)	p
rs2010963					
Codominance	C/C	29 (32.2)	27 (33.8)	1.00	0.88
	C/G	40 (44.4)	36 (45.0)	1.16 (0.56–2.41)	
	G/G	21 (23.3)	17 (21.2)	0.98 (0.41–2.35)	
Dominance	C/C	29 (32.2)	27 (33.8)	1.00	0.78
	C/G-G/G	61 (67.8)	53 (66.2)	1.10 (0.56–2.17)	
Recessive	C/C-C/G	69 (76.7)	63 (78.8)	1.00	0.78
	G/G	21 (23.3)	17 (21.2)	0.90 (0.42–1.92)	
Overdominance	C/C-G/G	50 (55.6)	44 (55.0)	1.00	0.62
	C/G	40 (44.4)	36 (45.0)	1.17 (0.62–2.22)	
rs833061					
Codominance	C/C	30 (33.3)	25 (31.2)	1.00	0.58
	C/T	44 (48.9)	43 (53.8)	1.32 (0.65–2.68)	
	T/T	16 (17.8)	12 (15.0)	0.86 (0.32–2.27)	
Dominance	C/C	30 (33.3)	25 (31.2)	1.00	0.62
	C/T-T/T	60 (66.7)	55 (68.8)	1.19 (0.60–2.34)	
Recessive	C/C-C/T	74 (82.2)	68 (85.0)	1.00	0.47
	T/T	16 (17.8)	12 (15.0)	0.73 (0.30–1.73)	
Overdominance	C/C-T/T	46 (51.1)	37 (46.2)	1.00	0.32
	C/T	44 (48.9)	43 (53.8)	1.38 (0.73–2.61)	

OR: odds ratio; CI: confidence interval. Significant $p < 0.05$.

The multiple logistic regression data are shown in Table 2, considering the risk factors (e.g., family history, smoking, and alcoholism) and the two polymorphisms studied in PCOS patients (n=88) and controls (n=81). It was evidenced that the family history is more frequent in patients with PCOS, smoking is more frequent in controls, and there are no differences in relation to alcoholism and in the distribution of the rs2010963 and rs833061 polymorphisms.

DISCUSSION

VEGF alterations characterize numerous pathologies, either with increased, decreased, or abnormal angiogenesis. Therefore, it has been suggested that these alterations may be associated with the decreased, or lack of, ovulation rates and with the formation of many antral follicles in the PCOS ovaries. According to the literature, further studies are required to clarify the role of angiogenesis in PCOS and to develop new potential therapies^{2,3}, such as bromocriptine, metformin, and melatonin¹³⁻¹⁵.

The VEGF is the main angiogenic factor that promotes endothelial cell proliferation and migration and vascular permeability³. Thus, genetic analysis in the VEGF gene may help clarify the pathogenesis of PCOS.

To the best of our knowledge, this is the first molecular study to investigate the association between rs2010963 and rs833061 polymorphisms and PCOS susceptibility in Brazilian women. With regard to VEGF, our group evaluated the polymorphisms rs3025039, rs1570360, and rs699947 and showed that the polymorphism rs1570360 is associated with PCOS and that the T-G-C haplotype could be associated with protective factors¹⁶.

Several VEGF SNPs are associated with various conditions in women, including endometriosis^{17,18}, recurrent miscarriage¹⁹, and preeclampsia²⁰. These results together suggest that VEGF SNP can contribute to the pathogenesis of female reproductive diseases.

In the sample analyzed, the rs2010963 and rs833061 polymorphisms are not associated with PCOS. The lack of significance in this study may be due to the sample size, sample stratification, and ethnic issues related to the Brazilian population. However, they have been extensively studied with conflicting results (Table 3)^{9,10,12,21-24}, probably due to different ethnicities. It is worth mentioning that PCOS is a multifactorial disease and environmental factors and polymorphisms in genes other than VEGF might play an important role in women's susceptibility to its occurrence.

Table 2. Distribution of the polymorphisms rs2010963 and rs833061 of vascular endothelial growth factor gene and risk factor in cases (n=88) and controls (n=81).

Evaluated variable	Cases n (%)	Controls n (%)	OR (95%CI)	p
Smokers				
Yes	07 (7.9)	22 (27.2)	0.20 (0.08–0.55)	<0.05
No	81 (82.1)	59 (72.8)		
Alcoholism				
Yes	21 (23.9)	22 (27.2)	1.11 (0.51–2.41)	0.79
No	67 (76.1)	59 (72.8)		
Family history of PCOS				
Yes	35 (39.8)	17 (21.0)	2.70 (1.30–5.62)	<0.05
No	53 (60.2)	64 (79.0)		
rs2010963				
CC	61 (69.3)	27 (33.3)	1.24 (0.60–2.57)	0.56
CT/TT	27 (30.7)	54 (66.7)		
rs833061				
CC	16 (18.2)	12 (14.8)	0.78 (0.32–1.92)	0.59
CT/TT	72 (81.8)	69 (85.2)		

OR: odds ratio; CI: confidence interval; PCOS: polycystic ovary syndrome. Bold value indicates significant p<0.05.

Table 3. Summary of the mains results of previous studies that investigated rs2010963 and rs833061 single-nucleotide polymorphisms of vascular endothelial growth factor gene in polycystic ovary syndrome.

Study	VEGF gene polymorphisms	Sample	Main results and/or conclusions
Bao et al. ⁹	rs1547651, rs1570360, rs2010963, rs3025020, rs3025039, rs699947, rs833061, rs833058, and rs833068	55 women with PCOS and 52 control subjects.	VEGF levels in rs699947 (AA-major homozygous), rs3025039 (CC-major homozygous), and rs833061 (TT & CC-major & minor homozygous) genotypes were significantly higher in PCOS. The study results evidently proved that the allelic variants in genes may be a factor for PCOS and VEGF serum levels with respect to few SNP variants only.
Huang et al. ¹⁰	rs2010963, and rs833061	118 women with PCOS and 130 controls.	Our study shows for the first time that the rs2010963 polymorphism may be associated with a risk of PCOS in Northern Chinese women.
Almawi et al. ²¹	rs833052, rs1547651, rs699947, rs833061, rs1570360, rs2010963, rs25648, rs833068, rs833070, rs3025020, rs3025026, and rs3025039	382 women with PCOS, and 393 control subjects.	Among the 12 tested VEGFA SNPs, minor allele frequency of only rs3025020 was significantly higher in PCOS cases than control women. Increased and reduced PCOS risk was seen with rs3025020 and rs2010963 genotypes, respectively. Increases and reduction in VEGF levels were associated with rs3025020 and rs2010963, respectively. Our study also confirmed the association of CAACAGCGA haplotype with increased risk of PCOS.
Ben Salem et al. ²²	rs699947, rs833061, rs1570360, rs833068, rs3025020, and rs3025039	118 PCOS patients and 150 controls.	We observed 10 haplotypes in our studied cohort where H1 (ACGG), H2 (ACAG), H7 (CTGG), and H8 (CTGA) were the most frequent. We observed the association of the genotype CT of the SNP rs3025039 with PCOS phenotype.
Guruvaiah et al. ²³	-460C/T and +405C/G	126 PCOS patients and 130 controls.	The frequencies of +405G/G genotype (p=0.03) and +405G alleles (p=0.006) were significantly higher in patients compared to controls. Whereas the genotype and allele frequencies of -460C/T SNP were not significantly different between patients and controls. Our findings suggest that the VEGF +405C/G polymorphism may constitute an inheritable risk factor for PCOS in South Indian women.
Vural et al. ¹²	-2578A/C, -460T/C, and +405C/G	137 patients with PCOS and 155 healthy women.	We did not find any evidence for association between PCOS and the three individual SNPs. The haplotype -2578C/-460T/+405G was significantly overexpressed in the PCOS group in comparison with controls (p=0.019).
Lee et al. ²⁴	-2488C>T, -634G>C, -7C>T, +3436G>C, +6112C>A, +6594C>T, +9374G>A, +9812C>T, +13125C>T, and +13553C>T	134 patients with PCOS and 100 healthy women as controls.	We concluded that one novel SNP at +9812 site, one known SNP at +13553 site, and one selected haplotype in the VEGF gene have a high possibility of significant associations with the pathogenesis of PCOS in a Korean population.

There are seven studies that investigated the polymorphisms rs2010963 and rs833061 of the VEGF gene. Three studies did not confirm any association between PCOS and the polymorphisms investigated^{17,19,20}, a result similar to that found in this study. In contrast, other studies found an association of polymorphism rs2010963^{17,22,23} and rs833061¹⁰ with PCOS.

In relation to haplotype analysis, two studies showed a relationship between haplotypes and PCOS^{21,24}. However, there is no significant difference in the occurrence of the four haplotypes

between the controls and cases, in relation to the polymorphisms rs2010963 and rs833061¹⁷, which is in agreement with our results.

A meta-analysis of seven studies showed there is little association between PCOS risk and the VEGF gene polymorphisms rs2010963, rs833061, and rs699947 in the general populations, whereas the genotype CC (rs2010963) might decrease the risk of PCOS among Asian women⁸. Another recent meta-analysis included 10 relevant case-control studies, involving 1347 PCOS cases and 1378 controls. The VEGF rs2010963 polymorphism was associated

with decreased PCOS risk in the whole population and the Asian populations⁹.

This study demonstrated that family history is more frequent in patients with PCOS. According to the literature, multiple familial and twin studies confirmed the role of genetics in the etiology of PCOS with high heritability of 70%²¹.

There are some limitations to our study. The serum levels of the VEGF are not measured. However, it is also worth mentioning that our study so far is the only one to evaluate these polymorphisms in the Brazilian population.

CONCLUSIONS

The PCOS patients have similar rates of VEGF polymorphisms rs2010963 and rs833061 on the general population.

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AUTHORS' CONTRIBUTIONS

ALSAV: Data curation, Writing – original draft. **ABTM:** Data curation, Writing – review & editing. **MKOG:** Methodology, Writing – review & editing. **AVAL:** Methodology, Writing – review & editing. **MASB:** Methodology, Writing – review & editing. **SCSVT:** Methodology, Writing – review & editing. **EAMRR:** Methodology, Writing – review & editing. **MFPL:** Methodology, Writing – review & editing. **MTRC:** Conceptualization, Data curation, Formal analysis, Writing – original draft, Writing – review & editing, Supervision, Project administration.

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