Investigation of Vascular Endothelial Growth Factor (VEGF) Polymorphism in Patients With Idiopathic Heavy Menstrual Bleeding

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Abstract

Purpose: To investigate whether there is a relationship between the VEGF gene polymorphisms and idiopathic heavy menstrual bleeding (HMB-E).

Methods: 65 patients diagnosed with HMB-E according to the FIGO classification system and 65 female healthy volunteers were included in the study. Detection of rs699947 (-2578C>A), rs1570360 (-1154G>A), rs2010963 (+405G>C), rs3025039 (+936C>T), rs25648 (c534C>T) polymorphic regions of the VEGF gene was performed by Next Generation DNA Sequencing method.

Results: the -2578C>A polymorphic region CC, CA+AA genotypes, and C allele, as well as the -1154G>A polymorphic region AA genotype, and A allele were associated with increased risk of HMB-E (all p<0.05). However, no statistically significant difference was found between the patient group and the control group in terms of genotype and allele distributions in the 405G>C, +936C>T, c534C>T polymorphic regions (all p>0.05). While the -2578/-1154/+405/c534 AGGC haplotype decreased the risk of HMB-E, CAGC haplotype was found to increase the risk of HMB-E.

Conclusion: VEGF -2578C>A and -1154G>A polymorphisms were significantly associated with the risk of HMB-E in the Turkish population.

Introduction

Heavy menstrual bleeding (HMB) is an important health problem defined as menstruation at regular intervals but with excessive flow and duration. Clinically, it is defined as a blood loss of more than 80 ml per cycle. It is a common gynecological problem among women of reproductive age and accounts for more than 20% of outpatient visits to gynecologists [1]. Although HMB is often associated with uterine pathology (e.g., fibroid, polyp, adenomyosis, carcinoma), abnormal blood clotting or disruption of hormonal regulation, approximately 50% of HMB cases occur in the absence of recognized uterine pathology [2,3], suggesting a defect in the cellular processes and regulatory mechanisms of menstruation. Such idiopathic HMB of endometrial origin (HMB-E) has been estimated to account for 9-14% of gynecological outpatient visits [4,5].

HMB can lead to iron deficiency anemia. Current treatment options often compromise fertility, and may require hysterectomy in patients who do not respond to the treatments [6]. Another problem is increased maternal morbidity and mortality for pregnant women who has HMB-associated anemia. Heavy bleeding can affect daily activities. Need for long-term medication, the side effects of these therapies, and the intolerance can cause psychological and social discomfort. Also for numerous female individuals, problems related to employment have led to the designation of HMB as a public health problem [4].

Disturbance of endometrial angiogenesis is one of several proposed underlying mechanisms that have been suggested to play a role in HMB [4,5]. In the formation of functional and stable micro vessels, covering the luminal surface of endothelial tubes with mesenchymal pericytes is an important step. The pericytes interact tightly and adhere to the endothelial cells with the pores in the basal layer [7]. Pericytes may induce angiogenesis with Vascular Endothelial Growth Factor (VEGF) production which stimulates endothelial cell proliferation and tube formation. Previous studies have shown that The biopsy specimens of women with HMB have revealed that number of capillaries expressing VEGF-A and its receptors (VEGFR-1 and VEGFR-2) were significantly higher in patients with HMB compared to healthy controls [1]. It is known that glandular luminal surfaces of patients with HMB are wider and this finding is highly correlated with overexpression of VEGF-A and VEGFR-1 [8]. It is suggested that the mesenchymal pericytes acting on the basis of angiogenesis may contribute to the vascular fragility. This leads to excessive blood
loss due to extensive endothelial cell gaps and abnormal vascular remodeling as a result of inappropriate coverage of
the laminal surface of endothelial tubes [8,9].

In the literature, there is no study investigating the VEGF polymorphism in patients with idiopathic HMB-E. The
evidence of the presence of VEGF polymorphism in patients with HMB-E may provide new opportunities for future
research and treatment options. Thus, patients and doctors will avoid the risks of unnecessary surgeries. We therefore
planned to investigate the relationship between VEGF polymorphism and idiopathic HMB-E.

Materials And Methods

Our study is a cross-sectional study conducted from February 2019 to March 2020. It was approved by the Ankara
Yıldırım Beyazıt University Yenimahalle Training and Research Hospital Clinical Research Ethics Committee on the
date of 29.01.2019 and with the decision number of 2019/01/01 and complied with the Helsinki Declaration,
including current revisions and the Good Clinical Practice guidelines.

Power analysis of the study showed that 128 patients were needed to gain 80% power when α error was set at 0.05, β
error at 0.05, and effect size at 0.50.

A total of 65 patients between the ages of 18-45 who were admitted with the complaint of abnormal uterine bleeding
and diagnosed with idiopathic HMB according to the FIGO Classification System [10] and 65 voluntary women with
regular menstruation were included in the study. All participants were informed about the study and consent forms
were signed.

The participants were selected according to the defined inclusion criteria: 18 to 45 years old, having a regular
menstrual cycle between 24 - 38 days, not using hormonal or intrauterine contraception for at least three months prior
the study, no abnormal ultrasonographic or hysteroscopic findings, not pregnant and not lactating.

Women were excluded from the study if they had a uterine pathology (such as polyp, adenomyosis, leiomyoma,
malignancy, hyperplasia), had a history of significant medical problems (such as coagulopathies, hypothyroidism,
hyperprolactinemia, Polycystic ovary syndrome, endometriosis), smokers, taken any medicines that increases the
tendency to bleeding.

For patient selection according to these criteria all participants were evaluated based on a complete medical,
gynecological and obstetric history and gynecological examination with ultrasound assessment. Additionally,
hysteroscopy or dilatation and curettage was performed in the presence of suspicion of endometrial pathology.

Menstrual blood loss was evaluated with Pictorial Blood loss Assessment Chart (PBAC) which has a a positive
predictive value of 85.9% in diagnosing menorrhagia and with a good correlation to alkaline hematin method [11,12].
Self-evaluated PBAC consists of diagrams representing different soiled pads or tampons each day and pads were
scored according to the degree of staining and the size of the clots on it. Women with a PBAC score > 100 were
included in the HMB group and healthy, ovulating women with a PBAC score < 100 were considered in the control
group.

Single nucleotide polymorphism (SNP) selection: Five highly plausible candidate SNPs in the VEGF gene were
selected including rs699947 (-2578C>A, in the promoter region), rs1570360 (-1154G>A, in the promoter region)
rs2010963 (+405G>C, at 5'UTR), rs3025039 (+936C>T, at 3'UTR), rs25648 (c534C>T, at exon1) and checked in
Ensemble, dbSNP database.
Peripheral venous blood samples were collected from all subjects and transferred to EDTA tubes. Samples were stored frozen at -20°C until they were studied.

Laboratory studies were carried out in the INTERGEN Genetic and Rare Diseases Diagnosis Research and Application Center.

**DNA extraction and genotyping:** DNA extraction was performed from 200 µl peripheral blood with spin column method using HibriGen Blood DNA isolation kit (Istanbul, Turkey). The isolates were measured with Nanodrop 1000 (Thermo Inc.) microvolume spectrophotometer and the study continued with samples containing 10 ng / µl and more.

Primer design was made for PCR amplification of all coding region and exon-intron junction regions of VEGFA gene and 4 polymorphisms (rs699947, rs1570360, rs2010963, rs3025039). Primers, amplicon size and usage purposes are shown in table 1.

Amplification of the regions was achieved by using the isolated DNA and the designed primers in the PCR reaction. Amplification efficiency was evaluated by visualizing the reaction results with 2% agarose gel electrophoresis. PCR pools were created by combining the PCRs of each sample and then purified by NucleoFast® 96 PCR kit (MACHEREY-NAGEL GmbH). The purified samples were quantitated by a spectrophotometer (Nanodrop N1000, Thermo Inc.). The amount of DNA was determined and diluted to 5 ng / µl.

Detection of polymorphisms was performed by NGS (Next Generation DNA Sequencing) method using Miseq device (Illumina Inc. San Diego, CA, USA). Standardized samples were prepared for next generation sequencing using the Nextera XT sample preparation kit (Illumina Inc.). DNA Library preparations were made according to the determined instructions. A sample worksheet file was created to introduce the study to the device. All of the prepared samples were loaded into the sample loading compartment in the Miseq Reagent Kit v2 2x150 (MS-102-2002, Illumina Inc.) cartridge. After all the preparations and controls were made, the Miseq device was started. Approximately 24 hours later, study results were taken and analyzed. Alignment of the obtained readings was done with Miseq Reporter (illumina Inc.) software on human genome hg19 version. Analysis of the aligned bam files was performed using IGV 2.3 (Broad Institute) software.

**Statistical Analyses**

Continuous variables were first inspected for normality of statistical distribution graphically and by Shapiro–Wilk test. Data were reported as mean ± standard deviation (SD), median with interquartile ranges (IQRs) or numbers and percentages, as appropriate. The data were analyzed using the Student’s t test, Mann–Whitney test or chi square test to determine the significance of the differences between the groups. The Chi-square test was used to assess the variation in each SNP frequency from Hardy–Weinberg equilibrium (HWE). Logistic regression analysis models were used to calculate the odds ratio (OR) and 95% confidence interval (CI), thereby assessing the effect of genotypes on HMB risk. These analyzes were calculated with IBM SPSS 25 and the epitools package in R 3.6.1 program. The haplotype of VEGF polymorphisms was analyzed by using SHEsis. The linkage disequilibrium of genotype and allele pairs was calculated via the Haploview version 4.2 (Broad Institute, Cambridge, MA, USA). Statistical significance was set at P ≤ 0.05.

**Results**

**Baseline characteristics**
The baseline characteristics of the subjects are summarized on Table 2. There were no differences in age, Body Mass Index (BMI), marital status, gravidity, parity, age at menarche, length of menstrual cycle between the analyzed groups. Compared with the control group, as aspected the patients in HMB group presented significant increases in menses duration and PBAC score.

**Genotype and allele distribution of VEGF polymorphisms**

The genotype frequency distribution of the selected population were found to be compatible in Hardy-Weinberg equilibrium (p > 0.05), suggesting it represents the population.

As shown in Table 3, the risk of having HMB in individuals with the VEGF gene −2578A>C polymorphic region CC genotype was 3.05 times higher, the AC + CC genotype was 2.35 times higher compared with the AA genotype, C allele was 1.75 times higher compared with A allele (CC vs AA, OR = 3.05, 95% CI = 1.11-8.84, p = 0.025; AC + CC vs AA, OR = 2.35, 95% CI = 0.97-6.0, p = 0.051; C vs A, OR = 1.75, 95% CI = 1.07-2.88, p = 0.024). In comparison with the −1154G>A polymorphic region GG genotype, AA genotype is related to 3.49 times higher risk of HMB and in comparison with G allele, A allele is related to 1.74 times higher risk of HMB (AA vs GG, OR = 3.49, 95% CI = 0.89-18.10, p = 0.06; A vs G, OR = 1.74, 95% CI = 1.01-3.01, p = 0.04). However, the genotype and allele frequency distributions of rs2010963, rs3025039 and rs25648 were not statistically different between groups (all p > 0.05).

**Haplotype analysis for the association between VEGF gene polymorphisms and HMB risk**

VEGF gene polymorphisms in linkage disequilibrium analysis including four SNP (-2578C>A, -1154G>A, +405G>C and 534C>T) revealed one SNP block (Figure 1).

We detected four main haplotypes with frequencies greater than 0.03. CACC, AGGC, CAGC, AAGT. These haplotypes were analyzed with the online software SHEsis and compared between the HMB and the control group (Table 4). The AGGC haplotype was found to be statistically significantly lower in the HMB group with a percentage of 23.8% than in the control group 38.5% (OR = 0.505, 95% CI = 0.295-1.517, p = 0.012). The CAGC haplotype was statistically significantly higher in the HMB group with 25.4% and 9.2% in the control group (OR = 3.387, 95% CI = 0.481-2.116, p = 0.0005). As a result, it was concluded that AGGC haplotype decreased the risk of HMB and CAGC haplotype increased the risk of HMB.

**Discussion**

In this study we aimed to investigate the significance of SNPs of the VEGF gene (rs699947 (-2578C>A), rs1570360 (-1154G>A) rs2010963 (+405G>C), rs3025039 (+936C>T), rs25648 (c534C>T) in HMB. We have found increased risk for HMB in patients with CC, AC+CC genotype, and C allele of the -2578C>A polymorphic region and AA genotype and A allele of the -1154G>A polymorphic region (p < 0.05). However, we have not observed any associations between HMB and other three candidate polymorphisms of +405G>C, +936C>T, and c534C>T (p > 0.05).

In the literature, we have not encountered any studies emphasising on the relationship between SNPs and HMB. Nevertheless, there are several studies investigating the effect of VEGF gene polymorphisms in cardiovascular diseases, essential hypertension, certain cancer types, psoriasis, and endometriosis. Although being a unique study represents strength of our work, it also enabled us to discuss or compare our results with similar papers. Therefore, we compared our findings with articles about endometriosis, which defines a gynecologic disease that endometrial tissue is present outside the uterus.
Perini et al. [13] evaluated -2578C>A, -460T>C, -1154G>A, +405G>C, and +936C>T polymorphisms in 182 Brazilian women with endometriosis and reported that variant allele -1154A was had significant relationship with endometriosis. They found AA genotype in 9.3% of patients and 1.9% of controls while the frequency of GA+AA genotype was 44.1% in patients and 30.2% in controls, and frequency of A allele was 26.7% in patient group and 16.0% in controls (p < 0.05). In our study, the AA genotype was found in 56.9% of patients and 38.5% of controls while the frequency of GA+AA genotype was 95.4% for patients and 87.7% for controls and the frequency of A allele was 76.1% in HMB patients and 61.5% in controls (p < 0.05). Perini et al. [13] reported an approximate two-fold increased risk for endometriosis in individuals with GA+AA genotype of the -1154G>A polymorphic region and an approximate 6-fold increased risk for AA genotype. Similarly, we have observed an approximate two-fold increased risk for HMB in individuals with GA+AA genotype (p > 0.05), an approximate 3.5 fold increase in AA genotype (p = 0.06), and an approximate two fold increase in case of harbouring A allele (p = 0.04). Although these two studies presents distinct genotype and allele frequencies, individuals having A allele in 1154G>A polymorphic region have been found to have significantly increased risk for the defined diseases.

On the other hand, in another study from Northern China on 344 women with endometriosis and 360 healthy controls by Liu et al. [14], AA genotype of the -1154G>A polymorphic region was alleged to decrease the risk of endometriosis. The frequency of AA genotype of the -1154G>A polymorphic region was 1.7% in patients and 5.8% in controls while the frequency of harboring A allele was 16.1% in patients and 22.2% in controls concluding a statistically significant reduction of endometriosis risk in case of A allele of -1154G>A polymorphism.

Perini at al. [13] did not report an association between endometriosis and VEGF gene -2578C>A polymorphisms. Similarly, in our study the frequency of CC genotype of -2578C>A polymorphism was 38.5% in patients and 24.6% in controls, and the frequency of AC + CC genotype was 86.2% in patients and 72.3% in controls. Despite being more frequent in patients, the difference was not statistically significant (p = 0.083). As for the C allele, the frequency was 62.3% in patients and 48.5% in controls which established a statistically significant difference (p < 0.05). Therefore, we have found an approximate 3 fold increased risk for HMB in individuals with CC genotype of the -2578C>A polymorphic region and an approximate 2 fold increased risk in AC+CC genotype and C allele. Also, Liu et al. [14] reported frequencies of AA, CA, and CC genotypes and A allele frequency of -2578C>A polymorphic region in patient and control groups respectively; 3.2% - 8.1%; 32.0% - 36.4%, and 64.8% - 55.6%, 19.2% - 26.3% concluding a statistically significant difference in terms of genotype and allele harbouring (p <0.004, p = 0.002 respectively). Similarly, having C allele has been associated with HMB in our study as in case with endometriosis.

With regards to the +405G>C, +936C>T polymorphic region our results suggest no significant effect on the susceptibility to HMB. It is noteworthy that our results are consistent with Perini's study [13] and Zhao and colleagues [15] which consist a larger number (958 cases and 959 controls) of Australian women. Similarly Liu et al. [14] found that the + 936C>T polymorphic region does not have a significant relationship with the risk of developing endometriosis.

The main reason for these inconsistent results may be the differences between ethnic groups. For example, the Ensembl database shows that the frequency of the VEGF -2578 A allele was 27.2% with the CHB (Han Chinese in Beijing), 34.1% with AMR (American), and 45.5% with CEU (Utah Residents with Northern and Western European Ancestry).

In the literature, we found two studies from our country evaluating the effect of VEGF gene +405G>C polymorphism on endometriosis risk with controversial results. Altinkaya et al. [16] evaluated the genotype frequencies of VEGF +405G>C polymorphic regions on 98 endometriosis patients and 94 controls. They detected GC genotype in 58.2% of
patients and 10.6% of controls and G allele in 45.4% of patients and 5.3% of controls. They suggested that GC genotype and G allele may be associated with the advanced grade endometriosis risk in the Turkish population. In another study from Turkey, Attar et al. [17] also proposed that CC genotype of +405G>C polymorphisms may be related to endometriosis whereas G allele has a protective role against endometriosis. The small sample sizes impede with reflecting data of the whole Turkish population and may partially explain the conflicting results.

The meta-analyses also yielded conflicting results. In a meta-analysis evaluating 14 eligible studies on a total of 3313 endometriosis patients and 3393 controls, VEGF gene -1154G>A polymorphic region GA genotype and A allele and -2578C>A polymorphic region CC genotype and C allele were found to reduce endometriosis risk whereas +936C>T polymorphic region CT genotype and T allele increased the risk similar to our results on HMB [18]. No effect of the +405G>C polymorphism was reported in line with our findings. On the contrary, Jiang et al. [19] stated that VEGF +405G>C and +936C>T SNPs may be associated with the risk of endometriosis in their meta-analysis, in which they investigated 9 studies including 1610 endometriosis patients and 1643 control cases.

The most important limitation of the current study is the small sample size which can also be held responsible for the controversial results in other studies in the literature. Besides, the facts that these studies have been performed on distinct ethnic groups and different diseases and having identified various haplotypes may have led to the conflicting results. These diverse results suggest that disease tendency may stem from not solely a single polymorphism but a combined effect of polymorphisms and that haplotype effect may play a more significant role in disease tendency than single polymorphisms.

In our study in the examination of the -2578C>A, -1154G>A, +405G>C and 534C>T polymorphic regions of the VEGF gene we have identified 4 haplotypes: CACC (38.1%), AGGC (31.2%), CAGC (17.3%), AAGT (12.3%). AGGC haplotype was less prevalent in HMB patients (23.8% vs 38.5%) while CAGC haplotype was more frequent in the HMB group (25.4% vs 9.2%). Perini et al. [13] emphasized that CCGG haplotype derived from four polymorphisms (-2578C>A / -460T>C / -1154G>A / +405G>C) has a protective effect against the development of endometriosis (3% in cases, 6.7% in the control group). Liu et al. [14] stated that the VEGF gene -460C>T -1154G>A, -2578C>A regions included CAA, TAA and TAC haplotypes can significantly reduce the risk of developing endometriosis, and the CAC haplotype can significantly increase the risk of developing endometriosis. The polymorphic regions included in the studies produced different linkage disequilibrium results and different haplotypes were obtained in each study making it difficult to compare study results.

Our study on VEGF gene polymorphisms in Turkish women with HMB suggests increased risk for C allele, CC, and AC+CC genotypes of -2578A>C polymorphic region, A allele, AA genotype, and CAGC haplotype of the -1154G>A polymorphic region whereas protective role of the AGGC haplotype. However, further studies with a larger sample size are required to figure out a more certain association.

**Declarations**

**Author Contribution**

İ Kaygusuz: Project development, Data collection, Data analysis, Manuscript writing, editing

N Semerci Gündüz: Project development, reduction, editing

**I. Ethical approval:** The study was approved by the Ankara Yıldırım Betazıt University Human Ethical Committee

**II. Funding details (in case of Funding):** No funding
III. **Conflict of interest:** The authors declare that they have no conflict of interest.

IV. **Informed Consent:** Signed informed consent was obtained from all patients before starting study.

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Tables

**Table 1.** Primers, amplicon size and usage purposes
| Primer         | Orientation | Sequencing | Genotyping | Amplicon size (bp) | Sequence (5'→3') |
|---------------|-------------|------------|------------|-------------------|------------------|
| VEGFA_SNP2_F  | Forward     | Exon 1     | SNP 2      | 1500              | CGGATGGGTAATTTCAGGCTGTG |
|               |             |            | SNP 3      |                   |                  |
|               |             |            | SNP 5      |                   |                  |
| VEGFA_1R      | Revers      |            |            |                   | CCTCCCCCTGCACCTAAAGACGAC |
| VEGFA_2F      | Forward     | Exon 2     | -          | 468               | GGGAAAGTGGGGGAGGTACAGG |
| VEGFA_2R      | Revers      |            |            |                   |                  |
| VEGFA_3F      | Forward     | Exon 3     | -          | 433               | GAAGCTCAAAGAGTGCCATTACAG |
| VEGFA_3R      | Revers      |            |            |                   |                  |
| VEGFA_4-5F    | Forward     | Exon 4     | -          | 975               | AGGAGTCAGGGTAAGCTAGAGAG |
| VEGFA_4-5R    | Revers      | Exon 5     |            |                   |                  |
| VEGFA_6F      | Forward     | Exon 6     | -          | 398               | AGAGACCACCTGCCTCTGACAC |
| VEGFA_6R      | Revers      |            |            |                   |                  |
| VEGFA_7F      | Forward     | Exon 7     | -          | 429               | CTGTAAGCTCGGATCCTTCCAG |
| VEGFA_7R      | Revers      |            |            |                   |                  |
| VEGFA_8F      | Forward     | Exon 8     | SNP 4      | 463               | CCTTCCGTCTCCTCCTTATCG |
| VEGFA_8R      | Revers      |            |            |                   |                  |
| VEGFA_SNP1_F  | Forward     | -          | SNP 1      | 250               | CTGGAGCGTTTTGTGTTAAATTGAGG |
| VEGFA_SNP1_R  | Revers      |            |            |                   |                  |

Table 2: Baseline characteristics of study subjects
| Variables                | HMB group (n=65) | Control group (n=65) | t/z c² | p     |
|-------------------------|------------------|----------------------|--------|-------|
| Age (year)              | 30.58 ± 7.06     | 29.08 ± 6.56         | t=1.261| 0.210 |
| Body Mass Index (kg/m²) | 22.35 ± 2.57     | 22.77 ± 2.65         | t=0.923| 0.358 |
| Marital status          |                  |                      |        |       |
| Married                 | 43 (% 66.2)      | 40 (% 61.5)          | c² =0.300| 0.584 |
| Unmarried               | 22 (% 33.8)      | 25 (% 38.5)          |        |       |
| Gravidity               | 1 (2)            | 1 (1)                | Z=-1.361| 0.180 |
| Parity                  | 1 (2)            | 1 (2)                | Z=-1.185| 0.290 |
| Age at menarche (year)  | 13 (2)           | 13 (1)               | Z=-1.311| 0.190 |
| Menses duration (days)  | 7 (2.5)          | 5 (2)                | Z=5.339 | <0.001*|
| Length of Menstrual cycle (day) | 28 (3) | 28 (2) | Z=0.320 | 0.749 |
| PBAC score              | 258 (246)        | 50 (44)              | Z=9.937 | <0.001 |

Abbreviations: PBAC, Pictorial Blood Loss Assessment Chart

Data are mean ± SD, median (IQR) or numbers (n) and percentages (%)

t= Student’s t test, Z=Mann–Whitney U-test, c² = Chi-square

* p values ≤ 0.05 were considered statistically significant

**Table 3.** Logistic regression analysis of associations between VEGF polymorphisms and risk of HMB
| Markov Takımı  | AMK-E Grup, n (%) | Kontrol Grup, n (%) | OR (95% CI)       | p   |
|----------------|------------------|------------------|------------------|-----|
| rs699947 (-2578A>C) |                  |                  |                  |     |
| AA             | 9 (13.8)         | 18 (27.7)        | Referans         |     |
| AC             | 31 (47.7)        | 31 (47.7)        | Referans         |     |
| CC             | 25 (38.5)        | 16 (24.6)        | 3.05 (1.11-8.84) | 0.03*|
| AC+CC          | 56 (86.2)        | 47 (72.3)        | 2.35 (0.97-6.0)  | 0.05*|
| A Alel         | 49 (37.7)        | 67 (51.5)        | Referans         |     |
| C Alel         | 81 (62.3)        | 63 (48.5)        | 1.75 (1.07-2.88) | 0.02*|
| rs1570360 (-1154G>A) |                  |                  |                  |     |
| GG             | 3 (4.6)          | 10 (15.4)        | Referans         |     |
| GA             | 25 (38.5)        | 30 (46.2)        | 2.13 (0.53-11.20) | 0.26 |
| AA             | 37 (56.9)        | 25 (38.5)        | 3.49 (0.89-18.10) | 0.06*|
| GA+AA          | 62 (95.4)        | 57 (87.7)        | 2.07 (0.56-10.32) | 0.26 |
| G Alel         | 31 (23.8)        | 50 (38.4)        | Referans         |     |
| A Alel         | 99 (76.1)        | 80 (61.5)        | 1.74 (1.01-3.01) | 0.04*|
| rs2010963 (+405G>C) |                  |                  |                  |     |
| CC             | 9 (13.8)         | 9 (13.8)         | Referans         |     |
| CG             | 30 (46.2)        | 33 (50.8)        | 0.91 (0.31-2.66) | 0.85 |
| GG             | 26 (40.0)        | 23 (35.4)        | 1.12 (0.37-3.41) | 0.82 |
| CG+GG          | 56 (86.2)        | 56 (86.2)        | 0.99 (0.33-2.78) | 1.00 |
| C Alel         | 48 (36.9)        | 51 (39.2)        | Referans         |     |
| G Alel         | 82 (63.1)        | 79 (60.8)        | 1.10 (0.66-1.82) | 0.70 |
| rs3025039 (+936C>T) |                  |                  |                  |     |
| CC             | 49 (75.4)        | 43 (66.2)        | Referans         |     |
| CT             | 15 (23.1)        | 20 (30.8)        | 0.66 (0.29-1.45) | 0.29 |
| TT             | 1 (1.5)          | 2 (3.1)          | 0.46 (0.01-5.98) | 0.49 |
| CT+TT          | 16 (24.6)        | 22 (33.9)        | 0.64 (0.29-1.37) | 0.24 |
| C Alel         | 113 (86.9)       | 106 (81.5)       | Referans         |     |
| T Alel         | 17 (13.1)        | 24 (18.5)        | 0.66 (0.33-1.30) | 0.23 |
| rs25648 (c.534C>T) |                  |                  |                  |     |
| CC             | 49 (75.4)        | 51 (78.5)        | Referans         |     |
| CT             | 16 (24.6)        | 12 (18.5)        | 0.72 (0.31-1.68) | 0.18 |
Table 4. Haplotype distributions of VEGF gene polymorphisms rs699947, rs1570360, rs2010963, and rs25648

| Haplotype | Frequency (%) | HMB group, n=65 (%) | Control group, n=65 (%) | P   | OR (95% CI)    |
|-----------|---------------|---------------------|-------------------------|-----|----------------|
| CAAC      | 38.1          | 48 (36.9)           | 51 (39.2)               | 0.737 | 0.918 (0.555-1.517) |
| AGGC      | 31.2          | 31 (23.8)           | 50 (38.5)               | **0.012** | 0.505 (0.295-0.864) |
| CAGC      | 17.3          | 33 (25.4)           | 12 (9.2)                | **0.0005** | 3.387 (1.659-6.917) |
| AAGT      | 12.3          | 16 (12.3)           | 16 (12.3)               | 0.981  | 1.009 (0.481-2.116)  |

Abbreviations: OR, odds ratio; CI, confidence interval.

* p values ≤ 0.05 were considered statistically significant
Figure 1

Linkage disequilibrium plot created by Haploview 4.2