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ORIGINAL ARTICLE

Genetic variants of vascular endothelial growth factor-634 and vascular endothelial growth factor-936 in Circassians and Chechens subpopulations in Jordan

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Abstract *Background:* Vascular endothelial growth factor (VEGF) is a signaling protein that promotes the growth of new blood vessels in vasculogenesis and angiogenesis. The most important member of VEGF family is VEGF-A which bind to VEGFR-1 and VEGFR-2 and plays a major role in diseases that involve blood vessels such as Tumor Angiogenesis, Age-related Macular Degeneration (AMD) and Diabetic Retinopathy (DR).

Objective: Studying the interindividual variability in VEGF by determining the allele frequency for certain genetic polymorphisms of *VEGF-936* and *VEGF-634* genes in two subpopulations in Jordan; Circassians and Chechens, as well as comparing the allele frequencies with other populations, including Jordanian Arabs.

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Methods: 319 unrelated healthy Circassian and Chechen individuals were genotyped for *VEGF-936* and *VEGF-634* by using PCR and RFLP.

Results: We found that Circassians did not have any significant difference in allele frequencies of *VEGF-634* compared to the Jordanian Arab population and all three populations had similar frequencies of *VEGF-936*.

Conclusion: These findings provide genetic information that may serve as a basis for larger studies designed to assess variability associated with *VEGF* polymorphisms. They also provide important data for the implementation of personalized medicine in Circassians and Chechens populations living in Jordan.

1. Introduction

During physiological angiogenesis namely, embryogenesis, reproductive functions and, skeletal growth, the vascular endothelial growth factor (VEGF) is an essential regulator. VEGF was also implicated in many pathological conditions like tumors, cardiovascular disease, degeneration of muscles, rheumatoid arthritis and endometriosis. The human VEGF family includes five growth factors: VEGF-A, VEGF-B, VEGF-C, VEGF-D and placental growth factor (PGF) and three tyrosine kinase receptors (VEGFR1, known as Fms-like tyrosine kinase-1 (FLT1)), VEGFR2 also known as Kinase-insert Domain containing Receptor (KDR), VEGFR3, also known as (FLT4), which differ significantly in signaling properties (Zhang et al., 2011). All of the five growth factors bind to these receptors located on the cellular surface leading to an induction then dimerizing and eventually activated by the means of transphosphorylation (Ferrer et al., 1998). *VEGF* gene is located on chromosome 6 at 6p21.1, extremely polymorphic, consists of eight exons and seven introns and its coding region is approximately 14 kilobases (Vincenti et al., 1996). Several studies have documented polymorphisms within the promoter (-2578C > A, −2489C > T,-1498C > T and −1154G > A), 5′-UTR (634G > C and 7C > T) and 3′-UTR (936C > T and 1612G > A) for the *VEGF* gene (Jain et al., 2009).

Current studies are mainly focusing on the *VEGF* 634G > C polymorphism in the 5′-untranslated region (UTR) and 936C > T polymorphism in 3′-UTR (Kim and Hong, 2015).

VEGF family has a key role in cancerous growth and metastasis and considered to play as the main factor in angiogenesis processes, it is the main mediator of angiogenesis (Zhang et al., 2011). As tumor proliferates, a hypoxic environment is being created due to lack of enough blood supply generates, this in turn leads to enhancing the release of hypoxia inducible factor (HIF) − 1α, and thus stimulating the angiogenesis processed by the means of starting the transcription of *VEGF*. Furthermore, overexpression of *VEGF* binding to its receptors are usually seen in many resistant to treatment cancers, including but not limited to the breast, colorectal (CRC) and prostate cancers (Jain et al., 2009). In these types of cancers, the inter-individual discrepancy developed from the change of VEGF protein concentrations resulting from Single nucleotide polymorphisms (SNPs). It was found that vascular density as well as *VEGF* expression were significantly elevated in cancerous tissue of prostate versus normal prostate tissue or even benign prostate hypertrophy (BPH) (Lurje et al., 2008). Moreover, for prognosis in colorectal cancers, *VEGF* has shown to be a crucial predictor especially in cases of advanced types. Some monoclonal antibodies and small molecule kinase inhibitors are well- known to interfere with the normal *VEGF* signaling pathway, include bevacizumab, sunitinib, sorafenib, andaflibercept, axitinib, brivanib, cilengitide, vandetanib and motesanib (Maitland et al., 2010).

The Circassians and Chechens are populations of the North Caucasus, many of whom were forced to migrate from their homeland because of the fear of prosecution by the Russian over powering in the nineteenth century (Hamed-Troyansky, 2017). Currently, approximately 100,000 Circassians and 8,000 Chechens live in Jordan. This population has undergone a limited genetic exchange with other population, as Circassians and Chechens people remained endogamous (Shami, 1994) (Zhemukhov, 2008). This study aimed to determine whether the frequencies of the *VEGF* 634G > C polymorphism in the 5′-untranslated region (UTR) and 936C > T polymorphism consistently differ between Circassians and Chechens in comparison to Jordanian Arabs.

2. Material and method

2.1. Study design

Our study is a cross-sectional study that included a total of 319 DNA samples of random unrelated Circassians and Chechens living in Jordan; 129 Chechens and 190 Circassians.

The samples were obtained from the Circassian and Chechen DNA bank; a project started by Professor Rana Dajani and colleagues during 2009–2014, to collect DNA samples of subpopulations in Jordan. The collection of samples was approved by the Institutional Review Board (IRB) of the University of Jordan Hospital and the DNA was isolated from 9 mL of whole blood.

Each participant in the study completed a survey that contained family information; identity of the previous three generations; parents, grandparents, great grandparents from both sides (maternal and paternal) were documented. Any participant with non-Circassian or non-Chechen heritage was ruled out from the study.

The sample size was calculated according to this equation:

$$n = \frac{z^2_{\left(1-\frac{\alpha}{2}\right)} * p * (1-p)}{d^2}$$

Where $z(1- \propto /2)$ is the standard normal variant (at 5% type 1 error ($p < 0.05$) it is 1.96 in this study), p is the proportion in population based on previous studies (the allele frequencies in the Jordanians were used; for *VEGF-634* = 0.52 and for *VEGF-936* = 0.14), d = absolute error or precision (0.05 in this study). So, 196 and 95 samples are needed for the two genes, respectively (Charan and Biswas, 2013).

2.2. Primer used for DNA replication

The primers were designed to study the different polymorphisms of interest as shown in Table 1. The sequence of the primers used to study *VEGF-634* gene was described previously

Table 1 The sequence of primers used for genotyping.

Gene	Type of Primer	Primer Sequence
VEGF-634	Forward	5′-CGACGGCTTGGGGAGATTGC-3′
	Reverse	5′-GGGCGGTGTCTGTCTGTCTG-3′
VEGF-936	Forward	5′-AAGGAAGAGGAGACTCTGCGCAGAGC-3′
	Reverse	5′-TAAATGTATGTATGTGGGTGGGTGTGTCTACAGG-3’

by (Ding et al., 2016) and the sequence of the primers for *VEGF-936* gene was described by (Zhang et al., 2014).

2.3. Protocol for VEGF gene

The master mix calculations were done; the final amount of reagents were calculated by multiplying the amount per reaction by the number of reactions (samples to be examined). All the components were then added to a 1.5 mL Eppendorf tube.

PCR products were visualized using 2.5% agarose gel run in Tris-Borate-Ethylenediaminetetraacetic acid (TBE) buffer to determine the size of the DNA fragment and we made sure that we had the desired DNA fragment and no non-specific amplification. The band size of PCR product was 274 bp for *VEGF*-634 and 208 bp for *VEGF*-936.

2.4. Genotyping and PCR-RFLP product

By using PCR-Restriction Fragment Length Polymorphism (RFLP) technique, two SNPs were found in this study. Amplification of a precise site in *VEGF* gene that contains the polymorphic alleles of (*VEGF*-634G > C and *VEGF*-936C > T) was accomplished. The PCR products were digested with *BsmF1* and *NIaIII* restriction enzymes, respectively.

For the restriction enzyme *BsmF1*, the PCR product and the enzyme were incubated at 37℃ for 16 h and heat inactivated at 80℃ for 15 min. For restriction *NIaIII* enzyme the PCR product and the enzyme were incubated at 37℃ for 16 h and heat inactivated at 80℃ for 15 min.

2.5. Statistical analysis

Nonparametric Chi-square Test and Hardy–Weinberg Equilibrium.

For comparison of allele frequency between other populations, chi-square ($\chi 2$) nonparametric tests was used. The Chi-square was calculated using the online calculator for 2×2 contingency table at:

https://www.socscistatistics.com/tests/chisquare/default2.aspx.

Chi-square test for independence was used to compare between Circassians and Chechens allele frequencies reported in this study, and because one of the assumptions of the Pearson's test, which is that any cell should have a lowest expected frequency of 5 or more, was not met in our data the Fisher Freeman-Halton test was used instead. Also, it was used to study the impact of gender on different genotypes.

All nucleotide polymorphisms were evaluated for the Hardy–Weinberg equilibrium and heterozygosity was accordingly determined. First, the actual allele frequencies were obtained from RFLP data, and then using Hardy–Weinberg equation, expressed below, we calculated the expected allele's frequencies. If the actual and expected frequencies match then the allele follows Hardy–Weinberg equilibrium (Gaedigk, 2013).

The Hardy-Weinberg equation:

$$p2 + 2pq + q2 = 1$$

Where p is the frequency of the first allele and q is the frequency of the second allele in the population. Thus the homozygous genotype frequency of the first allele is represented by p2, while q2 represents the frequency of the homozygous genotype for the second allele and ultimately 2pq represents the frequency of the heterozygous genotype (Gaedigk, 2013).

One important assumption of the Hardy-Weinberg equilibrium is random mating of samples (Rykman and Williams, 2008), and that does not apply to our ethnic group's data when combined together and thus the assumption will be violated. Therefore, we analyzed the data of each population separately.

3. Results

3.1. Population demographics

Our cross-sectional study involved a total of 319 volunteers of random unrelated Circassians and Chechens living in Jordan. For *VEGF-936* polymorphism, 190 Circassians (Females 61% (115), Males 39% (75)) and 129 Chechens (Females 32% (41), Males 68% (88)) were included in the study, while for *VEGF-634* polymorphism, 138 Circassians samples were involved. Table 2 indicates the population demographics in our study.

Table 2 Demographics of Circassians and Chechens included in the study of *VEGF*.

Age range	11–77	years old
Age mean	37.9	years old
Standard Deviation	16.9	
Gender % (n)	Females 49.2% (158)	
	Males 50.8% (163)	
Total participants (n)	129 Chechens and 190 Circassians	

Any participant with any non-Chechen or non-Circassian ethnic background was excluded from the study. DNA extraction was done for all samples and were amplified by PCR. The quality of PCR samples was identified by 2.5% agarose gel electrophoresis then RFLP was conducted for *VEGF* polymorphisms.

Additionally, RFLP data from a previous study (Al Saffarini and Zihlif, 2017), of 150 Jordanian samples were used in the statistical analysis for *VEGF-936* and *VEGF-634*. *VEGF-936* and *VEGF-634* alleles in Circassians and Chechens follow the Hardy-Weinberg equilibrium in our study as the actual and expected frequencies match each (Gaedigk, 2013).

3.2. Results of VEGF-936 polymorphisms

Table 3 shows the way genotypes determination was done depending on the number of fragments resulted after PCR-RFLP product.

3.2.1. Effect of ethnicity

Table 4 shows the frequencies of different *VEGF-936* genotypes (Wild, Heterozygous, Mutant), Fisher's exact test analysis revealed no statistically significant difference in *VEGF-936* polymorphism between the Circassian and Chechen populations (p value = 0.662). Also, Table 5 shows Allele Frequencies for *VEGF-936* in Circassians and Chechens.

3.2.2. Effects of gender

Fisher's exact test was used to discover the impact of gender on different genotypes, there was no significant difference at the $p < 0.05$ level between the groups with p value = 0.603.

3.3. Results of VEGF-634 polymorphisms

3.3.1. Representative results of VEGF-634 (rs2010963)

Table 6 shows the way genotypes determination was done depending on the number of fragments resulted after PCR-RFLP product.

Table 7 shows different genotypes for *VEGF-634*. We only analyzed 138 Circassians samples only. We could not get a band in the PCR for the DNA samples of Chechens (129) and the rest of Circassians samples (52), this can be considered as one of the limitations of our study. When we compared the mutant frequency of *VEGF-634* with that of the Jordanian Arabs, no significantly difference was seen (p value = 0.157).

3.3.2. Effect of gender

Fisher's exact test was used to explore the impact of gender on different genotypes, there was no significant difference at the $p < 0.05$ level between the groups with p value = 0.702.

Table 3 RFLP product for *VEGF-936*.

VEGF-936 genotypes	Number of fragments
Wild (CC)	One (208 bp)
Mutant (TT)	Two (122 bp & 86 bp)
Heterozygous (CT)	Three (208 bp, 122 bp &86 bp)

Table 4 Genotypes frequencies of *VEGF-936* C > T in Circassians and Chechens.

Population (n)	Genotype n (%)		
	C/C Wild	**C/T**	**T/T Mutant**
Circassians (190)	139 (73.16%)	49 (25.79%)	2 (1.05%)
Chechens (129)	94 (72.89%)	32 (24.81%)	3 (2.33%)

Statistical results
X^2 (2, N = 319) = 0.8245, p = 0.662172.

Table 5 Allele frequencies of minor allele variant *VEGF-936* in Circassians and Chechens.

Population	Minor allele variant frequency	p value
Circassians (n = 190)	0.140	0.702
Chechens (n = 129)	0.226	

Table 6 RFLP product for *VEGF-634*.

VEGF-936 genotypes	Number of fragments
Wild (GG)	Two (166 bp & 108 bp)
Mutant (CC)	One (274 bp)
Heterozygous (GC)	Three (274 bp, 166 bp &108 bp)

4. Discussion

VEGFA is a signaling protein that motivates the growth of new blood vessels. The overexpression of *VEGFA* is a main factor for the development of diseases such as in tumors (Breast, Non-Small Cell Lung Cancer (NSLC), CRC and Prostate cancer), AMD, and DR.

Evaluation of *VEGFA* polymorphisms can be used for the recognition of patients appropriate for anti-VEGFA therapy. Some SNPs are linked to a susceptibility of many disorders;

Table 7 Genotypes frequencies of *VEGF-634* G > C in Circassians.

Population (n)	Genotype n (%)		
	G/G Wild	**G/C**	**C/C Mutant**
Circassians (138)	44 (32%)	73 (53%)	21 (15%)

however, the results are not always consistent in all the studied populations. *VEGF-634* and *VEGF-936* are the most prevalent polymorphisms that have been associated with some diseases risk.

After analyzing 319 of blood samples through PCR-RFLP we found that there is no significant difference between (Circassians, Chechens and Jordanian Arabs) with p value = 0.702 for *VEGF-936*. Table 8 shows genotypes for *VEGF-936* in Circassians, Chechens and Jordanian Arabs. It can be observed that Circassians and Chechens have similar levels of mutant genotype compared to the Jordanian Arabs. In this study, we found for *VEGF-936*, in Circassians the allele frequency is 0.14 and in Chechens is 0.15. In term of ethnicity as shown in Table 9 and Table 10.

As it can be seen from the Table 9, there is no significant difference between Circassians and populations mentioned regarding *VEGF-936* polymorphisms. This means that they will have similar risk level of many diseases that are related to *VEGF-936* polymorphisms such as osteosarcoma, prostate cancer, lung adenocarcinoma in male, DR, AMD and others. This also applies when choosing anti *VEGF-A* therapy. Regarding the allele frequency of *VEGF-936,* the Circassians have lower allele frequency than Korean and Chinese (Kataoka et al., 2006) (Kim et al., 2008) while Circassians have higher allele frequency than Caucasians and Swedish. Conversely, the allele frequency of *VEGF-936* is similar to that in Jordanian Arabs and Iranian. Finally, Circassians have almost similar allele frequency to Chechens and Tunisians.

In Table 10, it can be seen that there is no significant difference between Chechens, Swedish, Korean, Chinese, and Caucasians in the *VEGF-936* allele frequency. This implies a similar risk level for the diseases that are related to *VEGF-936* polymorphisms. Regarding *VEGF-936* allele frequency, Chechens are almost similar to Circassians, Jordanian Arabs, Iranians, and Tunisians (Table 10). While Chines and Korean have a higher allele frequency of VEGF-936 compared to Che-

Table 11 Genotype Results for *VEGF-634*.

Genotype	Circassians (%)	Jordanian Arabs (%)
Wild	(32%)	(20.06%)
Heterozygous	(53%)	(54.66%)
Mutant	(15%)	(24.66%)

Table 8 Genotype Results for *VEGF-936*.

Genotype	Circassians (%)	Chechens (%)	Jordanian Arabs (%)
Wild	(73%)	(72.9%)	(76.66%)
Heterozygous	(26%)	(24.8%)	(18.66%)
Mutant	(1.05%)	(2.32%)	(4.66%)

Table 9 Comparison between *VEGF-936* allele frequency in Circassians with other populations.

Population	Sample size	% C-allele	% T-allele	p value	Reference
Circassian	190	86	14	–	Current study
Chechen	129	85	15	0.841	Current study
Jordanian	150	86	14	1.00	(Al Saffarini and Zihlif, 2017)
Caucasian	1458	88	12	0.674	(Zhai et al., 2008)
Korean	413	79	21	0.192	(Chae et al., 2008)
Swedish	934	88	12	0.674	(Jin et al., 2005)
Chinese	1233	81	19	0.341	(Kataoka et al., 2006)
Tunisian	100	86	14	1.00	(Sfar et al., 2006)
Iranian	215	86	14	1.00	(Rezaei et al., 2016)

Table 10 Comparison between *VEGF-936* allele frequency in Chechens with other populations.

Population	Sample size	% C-allele	% T-allele	p value	Reference
Chechen	129	85	15	–	Current study
Circassian	190	86	14	0.841	Current study
Jordanian	150	86	14	0.841	(Al Saffarini and Zihlif, 2017)
Caucasian	1458	88	12	0.535	(Zhai et al., 2008)
Korean	413	79	21	0.269	(Chae et al., 2008)
Swedish	934	88	12	0.535	(Jin et al., 2005)
Chinese	1233	81	19	0.451	(Kataoka et al., 2006)
Tunisian	100	86	14	0.841	(Sfar et al., 2006)
Iranian	215	86	14	0.841	(Rezaei et al., 2016)

Table 12 Comparison between *VEGF-634* allele frequency in Circassians with other populations.

Population	Sample size	% G-allele	% C-allele	*p* value	Reference
Circassian	138	58	42	–	Current study
Jordanian	150	48	52	0.157	(Al Saffarini and Zihlif, 2017)
Korean	413	53	47	0.477	(Chae et al., 2008)
Swedish	941	72	28	**0.037**	(Jin et al., 2005)
Tunisian	100	67	33	0.189	(Sfar et al., 2006)

chens. On the contrary, Swedish and Caucasian have lower allele frequency compared to Chechens (Table 10).

Table 11 shows the genotype results for *VEGF-634* in Circcasians and Jordanian Arabs. We only analyzed *VEGF-634* 138 Circassians samples. The DNA samples of Chechens (129) and the rest of Circassians samples (52) showed no bands in the PCR. This can be considered as one of the limitations of our study. When we compared the mutant frequency of *VEGF-634* with that of the Jordanian Arabs, no significant difference was seen (p value = 0.157).

As it is shown in Table 12, there was no significant difference between Circassians and Jordanian Arabs, Korean, and Tunisians in the allele frequency for *VEGF-634* polymorphisms. That means a similar risk level for osteosarcoma, prostate cancer, lung adenocarcinoma in males, DR, AMD, and others. The only significant difference (p value = 0.037) was between Circassians and Swedish population.

Earlier studies concerning risk of cancer in Circassians and Chechens subpopulations living in Jordan have found that these populations have higher crude rates of different types of cancers than the Arab population living in Jordan (Fathallah and Dajani, 2013) (Han et al., 2015). Our results may explain, at least in part, these findings. As we found a lower frequency although not significant of the mutant genotype of *VEGF-936* which has a protective role in breast cancer.

Fathallah and Dajani, 2013 conducted a study of the cancer risk in Circassians and Chechens and concluded that females had higher crude rates of breast cancer in Circassians and Chechens than in Arabs. In males, lung cancer was the most common cancer in Arabs and Chechens with crude rates of 4.2 and 8.0 per 100,000, respectively. The lung cancer crude rate in Circassians was 6.5 per 100,000 and the colorectal cancer crude rates in Circassians was twice as high as in Chechens and Arabs (Fathallah and Dajani, 2013). The relative genetic homogeneity of the Circassian and Chechen populations in Jordan results in incidences of cancer that differ from the general Jordanian population, who are mostly Arabs.

Abudahab et al., (2019) conducted a study on Circassians, Chechens and Jordanian Arabs by genotyping 20 different SNPs in *UGT1A* gene. Glucuronidation, is a phase II metabolic pathway, of many medications which is catalyzed by UGT1A1 and UGT1A7. The inter-ethnic variation in *UGT1A* alleles frequencies may affect drug response and susceptibility to so many cancer types among different subethnic groups in Jordan (Abudahab et al., 2019).

Remarkably, at the level of *UGT1A7* Circassians and Chechens showed very similar patterns of differences with other ethnicities; for example, they are both different from Arab Jordanians at the level of all the three *UGT1A7* variants, they are also different from all other ethnicities at the *UGT1A7*4* variant. This in turn makes Circassians and Chechens genetically different from the Jordanian Arabs living in Jordan at the level of *UGT1A7* gene, which could potentially lead to different methods of personalized medicine in these populations, particularly when using certain chemotherapeutic agents (Abudahab et al., 2019).

Therefore, Circassians and Chechens are expected to have the same levels of risk of certain diseases which are related to *VEGF-936* polymorphisms and similar responses to anti-*VEGF* therapy. Circassians and Jordanian Arabs living in Jordan have comparable levels of the *VEGF-634* gene, this can be used to predict the efficacy and safety of anti VEGF therapy, especially in cancer patients in Jordan.

5. Conclusion

In this study we examined *VEGF* variability by calculating the allele frequency for certain genetic polymorphisms of *VEGF-634* and *VEGF-936* genes in two subpopulations in Jordan; Circassians and Chechens, then were compared with the allele frequencies to other populations, including the Jordanian Arabs living in Jordan. We noticed that Circassians and Chechens had similar frequencies of *VEGF-936* and *VEGF-634* polymorphisms with the Arab population. Finally, the knowledge of the allele frequency of *VEGF* polymorphisms in the Circassian and Chechen population in Jordan could help in recognizing possible risk groups for adverse drug reactions and optimizing doses for the therapeutic efficacy of anti-*VEGF* therapy.

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Conflict of Interest

The authors declare that there are no conflicts of interest.

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