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RESEARCH ARTICLE

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Haplotype-based association of Vascular Endothelial Growth Factor gene polymorphisms with urothelial bladder cancer risk in Tunisian population

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Background/Aim: Accumulated data suggested that Vascular Endothelial Growth Factor is a major mediator in vasculogenesis, angiogenesis and recently in tumorigenesis. Therefore, we aimed to investigate for the first time the association between VEGF gene variants (-2549I/D (rs35569394), -2578C/A (rs699947), and +936C/T (rs3025039)) with urothelial bladder cancer (UBC) in Tunisian population.

Methods: A total of 218 UBC patients and 204 controls were recruited and genotyped by Polymerase Chain Reaction technique. Odds ratios (OR) and 95% confidence intervals (CIs) were used to access the association between the VEGFA gene polymorphisms and UBC.

Results: We found a significant decreased risk association of -2578 C/A polymorphism with UBC (OR (95% CI), 0.62 (0.41-0.94), $P = .026$) for CA genotype and (OR (95% CI), 0.40 (0.21-0.76), $P = .005$) for double homozygous mutant genotype. No associations were found in case of both polymorphic sites of VEGF, vis. -2549I/D and +936C/T, respectively. Haplotype analysis revealed a strong linkage disequilibrium between -2578C/A and -2549I/D and CIC combination is the significant haplotype associated with increased risk of UBC (OR (95% CI), 3.63 (1.47-8.97), $P = .005$). Regarding tumor grade/stage and family history of cancer, no associations were found for -2578C/A polymorphism.

Conclusion: CIC haplotype of VEGF gene may be important risk factor for UBC development in Tunisia.

KEYWORDS

association, bladder cancer, case control study, haplotype, polymorphism, VEGF

1 | INTRODUCTION

It is now acknowledged that angiogenesis is important for the development of cancer essentially in tumor growth, invasiveness, and metastasis.[1] Several factors modulate angiogenesis, including Vascular Endothelial Growth Factor (VEGF) gene (OMIM +192240), a heparin binding protein and an important initiator and regulator of angiogenesis and vascularization.[2] VEGF human gene is located on chromosome 6.p12-p21 and consists of 8 exons and 7 introns. At least 30 single-nucleotide polymorphisms (SNPs) in this gene have been identified. Two polymorphisms were rs699947 (-2578C/A), rs35569394 (-2549I/D) located both in the promoter region and rs3025039 (+936C/T) situated in the 8-exon corresponding the 3′-untranslated region were correlated with variations in gene

expression and VEGF protein production and were reported in various types of cancer. In Tunisia, bladder cancer is the most common urological carcinoma, especially in men.[3–5] Data gathered that cancer is a multistep process involving complex interactions between environmental and genetic factors[6] and recent studies have reported the associations between VEGFA gene polymorphisms and the occurrence of cancer[7,8] but no study has reported their associations with UBC in Tunisia yet. Hence, this study aimed to evaluate; firstly the possible associations between VEGF gene polymorphisms with UBC risk. Secondly, to evaluate the persistence of the associations after stratification by stage, grade, and family history of cancer and thirdly to determine the possible associations of the haplotype combination of VEGF gene polymorphisms in our based case-control study.

2 | MATERIALS AND METHODS

2.1 | Patients

A total of 218 UBC patients (ICD-10 C67) (Sex ratio: 185/33; mean age 64.8 ± 11.9 years) recruited from the Department of urology at Rabta hospital and 204 unrelated subjects with no history of cancer (Sex ratio: 171/33; mean age 62.5 ± 11.6 years) were included in this study. All cases have been classified according to American Joint Committee on Cancer's 2002 TNM[9] and all the methods were in accordance with Declaration of Helsinki. This study was approved by the Ethics Committee of Rabta hospital and all participants made written informed consent. The data collection tool was a questionnaire including demographic (age, gender, tobacco smoking, and alcohol consumption) and clinical information (tumor grade/stage/family history of cancer). Ever smokers were defined us smoking more than 100 cigarettes in their lifetime and alcohol drinkers if they drank at least once per week.

TABLE 1 Demographic and clinical characteristics of urothelial bladder cancer cases and controls

Variables	Patients (n = 218)	Controls (n = 204)	*P* value[a]
Gender, n (%)			
Male	185 (84.9)	171 (83.8)	.436
Female	33 (15.1)	33 (16.2)	
Age (y)	64.8 ± 11.9	62.5 ± 11.6	**.049**
Smoking status, n (%)			
Ever	176 (80.7)	110 (53.9)	**<.001**
Never	42 (19.3)	94 (46.1)	
Alcohol consumption, n (%)			
Yes	40 (18.4)	15 (7.4)	**<.001**
No	177 (81.6)	189 (92.6)	
Tumor stage, n (%)			
NMIBC (Tis-T1)	146 (67.0)	—	—
MIBC (T2-T4)	72 (33.0)	—	
Tumor Grade, n (%)			
G1	76 (34.9)	—	—
G2	73 (33.5)	—	
G3	69 (31.7)	—	

NMIBC, non-muscle invasive bladder cancer; MIBC, muscle invasive bladder cancer.
Data presented as mean ± SD or number (%). Boldface indicates statistically significant differences.
[a]Pearson's chi square test (categorical variables).

2.2 | Genotyping

Genomic DNA was isolated from peripheral blood leukocytes using a Salting-out method[10] and genotyping of VEGF gene polymorphisms was performed using polymerase chain reaction method (PCR). The PCR primers (Bio Basic Canada Inc.) were sense 5′GCTGAGAGTGGGGCTGACTAGGTA3′ and antisense 5′ GTTTCTGACCTGGCTATTTCCAGG3′ for the -2549 I/D polymorphism,[11] sense 5′AGGGTTCGGGAACCAGATC3′, and antisense 5′CTCGGTGATTTAGCAGCAAG 3′ for the +936 C/T polymorphism[12] and sense 5′GGCCTTAGGACACCATACC3′ and antisense 5′CACAGCTTCTCCCCTATCC3′ for -2578 C/A polymorphism.[12] PCR reactions were performed on 50-µL reaction volumes containing 200 ng of genomic DNA, 25 pmol of each primer, 200-µmol dNTPs mix and 1.5-U Taq polymerase (Promega, Madison, WI) in PCR buffer with 1.5-mmol $MgCl_2$. PCR was performed for 35 cycles. Each cycle consisted of denaturation at 95°C for 60 seconds, annealing for 60 seconds (at 67°C for -2549I/D, 58°C for +936C/T, 62°C for -2578C/A) and an extension at 72°C for 30 seconds. Predenaturation was performed at 94°C for 5 minutes and further extension was at 72°C for 2 minutes. The PCR products were mixed with 2 U of restriction enzymes. In -2549 I/D, 2 bands were shown, the 18 bp D allele yield a 211 bp band while the insertion allele yield 229 bp band. In -2578 C/A polymorphism, A allele would be digested into 2 fragments of 202 bp and 122 bp while the C allele wouldn't, which is 324 bp band. In +936 C/T SNP, C allele is undigested and yield a 266 bp but T allele was cut into 211 and 55 bp fragments. Electrophoresis gels (3% agarose gel) were treated with ethidium bromide (0.2 mg/L) and DNA fragments were visualized by ultraviolet illumination. To ensure that the genotyping was adequate quality, all gels were reread blindly by 2 persons without any change, and 20% of the analyses was repeated randomly.

2.3 | Statistical analysis

Statistical analysis was performed by SPSS 18.0 software. Continuous variables are compared using independent student's *t* test. Categorical variables analyses were evaluated by Pearson Chi-square test. The Hardy-Weinberg equilibrium was tested by a goodness-of-fit χ^2 test in each group and stratified analyses were performed to assess the association of -2578C/A polymorphism in VEGF Gene with tumor grade/stage and family history of cancer. Haplotype analysis was done using THESIAS software (http://

TABLE 2 Genotypic and allelic distribution of VEGF polymorphisms in urothelial bladder cancer cases and controls

	Patients (n = 218)		Controls (n = 204)			
	n (%)	HWE, *P*	n (%)	HWE, *P*	OR (95% CI)	*P*
-2549I/D (rs35569394)						
DD	74 (33.9)	1.10, .577	61 (29.9)	1.17, .556	1	
ID	120 (55.0)		111 (54.4)		0.89 (0.58-1.36)	.596
II	24 (11.0)		32 (15.7)		0.61 (0.33-1.15)	.134
ID+II vs DD	144 (66.0)		143 (70.1)		0.83 (0.55-1.25)	.374
ID+DD vs II	194 (88.9)		172 (84.3)		0.66 (0.37-1.17)	1.159
D allele	.61		.57			
I allele	.38		.42		0.83 (0.63-1.09)	.207
+936C/T (rs3025039)						
CC	130 (59.6)	0.40, .816	129 (63.2)	0.42, .809	1	
CT+TT	88 (40.3)		75 (36.8)		1.16 (0.78-1.72)	.448
C allele	.79		.81			
T allele	.20		.18		1.13 (0.80-1.59)	.488
-2578C/A (rs699947)						
CC	97 (44.5)	4.60, .100	64 (31.4)	4.91, .086	1	
CA	100 (45.9)		106 (52.0)		0.62 (0.41-0.94)	**.026**
AA	21 (9.6)		34 (16.7)		0.40 (0.21-0.76)	**.005**
CA+AA vs CC	121 (55.5)		140 (68.7)		0.57 (0.38-0.84)	**.006**
CA+CC vs AA	197 (90.4)		170 (83.4)		1.87 (1.04-3.58)	**.034**
C allele	.67		.57			
A allele	.32		.42		0.64 (0.49-0.85)	.002

OR, Odds Ratio; CI, Confidence interval, HWE, Hardy-Weinberg equilibrium.
Boldface indicates statistically significant differences.

genecanva.ecgene.net) and haplotype reconstruction was performed using haploview 4.1 (http://www.broad.mit.edu/mpg/haploview). A *P* value <.05 based on two-sided calculation was considered significant.

3 | RESULTS

3.1 | Study subjects

Demographic and clinical characteristics of each group were shown in Table 1. No significant differences were observed between UBC patients and controls in gender. However, we found significant associations in age ($P = .049$), smoking and drinking status ($P < .001$) between the UBC patients and the control subjects. Among the 218 UBC patients, we found that 146 had Non Muscle Invasive Bladder Cancer (NMIBC) and 72 had Muscle Invasive Bladder Cancer (MIBC). Tumors were presented as grade 1 in 76 patients, grade 2 in 73 patients and grade 3 in 69 patients.

3.2 | Association of VEGF polymorphisms with UBC

The genotype and allele frequencies of VEGF-2578C/A, +936C/T and -2549 I/D polymorphisms in UBC patients and controls were presented in Table 2. Genotype distributions were in agreement with the Hardy-Weinberg equilibrium in UBC patients and in control subjects. We found a statistically significant difference in -2578 C/A polymorphism, showing frequencies of 44.5% vs 31.4%, 45.9% vs 52.0% and 9.6% vs 16.7% of CC, CA, and AA genotypes in patients and controls, respectively. There was significant decrease in A allele frequency in the patients as compared with controls (0.32 vs 0.42). The AA genotype (OR (95% CI), 0.40 (0.21-0.76), $P = .005$), the CA genotype (OR (95% CI), 0.62 (0.41-0.94), $P = .026$), and A allele (OR (95% CI), 0.64 (0.49-0.85), $P = .002$) were associated with decreased risk of UBC (Table 2).

In contrast, both -2549I/D and +936C/T polymorphisms did not differ significantly among cases with UBC in our study ($P > .05$). The genotype and allele frequencies were also investigated in order to determine their associations with stage or grade and family history of cancer but no associations were found (Table 3).

3.3 | Haplotype analysis

Haplotypes were constructed for -2578C>A, +936C/T and -2549 I/D using THESIAS software to evaluate the combined effect of them. Linkage Disequilibrium was seen for -2578 C/A and-2549I/D ($D' = 0.94$, $r^2 = 0.87$, $P < .01$) among controls and ($D' = 0.96$, $r^2 = 0.71$,

TABLE 3 Association of VEGF rs699947 polymorphism and clinicopathological parameters in UBC cases

	-2578C/A (rs699947)			
Parameters	**CC n (%)**	**CA n (%)**	**AA n (%)**	***P***
Stage				
NMIBC	63 (64.9)	71 (71.0)	12 (57.1)	.400
MIBC	34 (35.1)	29 (29.0)	9 (42.9)	
Grade				
G1	29 (29.9)	40 (40.0)	7 (33.3)	.690
G2	35 (36.1)	31 (31.0)	7 (33.3)	
G3	33 (34.0)	29 (29.0)	7 (33.3)	
Family history of cancer				
Yes	8 (6.5)	5 (3.4)	0 (0.0)	.203
No	116 (93.5)	140 (96.6)	34 (100)	

NMIBC, non-muscle invasive bladder cancer; MIBC, muscle invasive bladder cancer.

P < .01) among patients. The +936C/T was not linked to the other polymorphisms (Figure 1). CIC haplotype was the only haplotype combination which showed a high risk of 3.63-fold with UBC predisposition (Table 4).

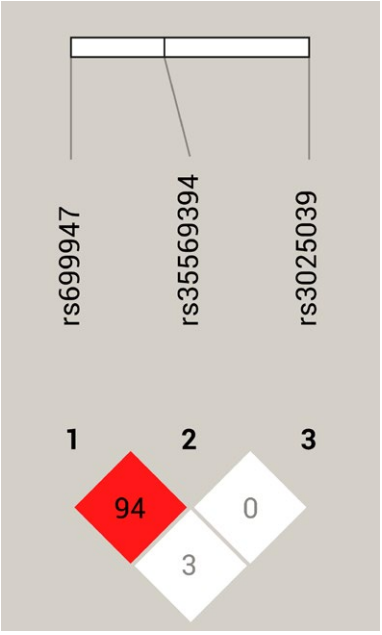

FIGURE 1 Linkage disequilibrium (LD) map of VEGFA SNPs genotyped by Haploview. The positions of the SNPs are displayed above the Haploview output. The relative LD between specific pair of SNPs is indicated by the color scheme representing LD relationships, which is based on D' values (normalized linkage disequilibrium measure or D) multiplied by 100; D′ is calculated as D divided by the theoretical maximum for the observed allele frequencies. Values approaching zero indicate absence of LD, and those approaching 100 indicate complete LD. The square colored red represent varying degrees of LD <1 and LOD (logarithm of odds) >2 scores; darker shades indicating stronger LD. For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this Article

4 | DISCUSSION

It is well known that tumors released angiogenic factors such as VEGF. We conducted a hospital-based case-control study to assess for the first time the possible associations between 2 promoter polymorphisms and one 3′UTR VEGF region (-2549 I/D, -2578C/A and +936C/T, respectively) and UBC risk in a Tunisian population.

The VEGF gene is highly polymorphic and there are variations in the distribution of its polymorphisms in different populations and also in various disease states. In this study, we found a significant association between -2578 C/A polymorphism and UBC risk, but no associations for -2549 I/D and +936C/T SNPs.

For -2578C/A polymorphism, the frequency of the minor allele A was 0.32. This result is comparable to the frequencies seen in Caucasians; 0.37 in Tunisians[13] and 0.45 in Europeans.[14] In addition, we found that AA genotype is associated with decreased risk of UBC (OR (95% CI), 0.40 (0.21-0.76), *P* = .005). Researchers correlated the inhibition of tumor development with A allele which prevent the creation of GATA-2 binding site and then reduces the transcriptional activity and negatively affects the VEGF protein levels.[15] Different studies showed that associations were variables and differed according to tumor type. Data from case-control studies revealed similar results with cervical cancer (OR (95% CI), 0.39 (0.16-0.96), *P* = .040),[16] prostate cancer (OR (95% CI), 0.16 (0.04-0.70), *P* = .007)[17] and hepatocellular carcinoma (OR (95% CI), 0.51 (0.28, 0.92), *P* = .024).[18] Mishra et al[19] also found that CA genotype was associated with decreased risk of Gallbladder cancer risk. In another case-control study, a significant association was observed with carriers of CC genotype in patients with increased nasopharyngeal cancer risk in Tunisian[20] and with invasive breast cancer in an American cohort (OR (95% CI), 1.46 (1.00-2.14), *P* = .049).[21] Furthermore, other contrasting results were reported; Ruhi Kapahi found a significant increase of AA genotype frequency in patients with breast cancer disease with 2.87-fold risk.[22] Same risk associations were reported with cervical cancer,[23] thyroid carcinoma,[24] bladder cancer,[25] and breast cancer risk.[26] But it's noteworthy that others studies failed to found any significant associations with different cancer type including gastric,[7] bladder[27,28] breast,[29] renal,[30] and Gallbladder cancer.[19] Quanchi Chen et al[31] in their meta-analysis study found that this polymorphism reduced risk of cancer in Asian population and was not associated neither in Caucasians (OR (95% CI), 0.92 (0.76-1.11)) nor with bladder cancer risk (OR (95% CI), 1.06 (0.74-1.53)).

For -2549I/D polymorphism, we did not found any significant association. Same result was found in Poland, Germany and Iranian populations.[29,32] Others found a significant protective association with bladder cancer disease in Indian population (OR (95% CI), 0.58 (0.40-0.84), *P* = .004).[28] It has been previously reported the relation between I allele frequency and behcet disease in Italian patients[33] and with its severity

TABLE 4 Distribution of VEGF Haplotypes in urothelial bladder cancer patients and controls

Haplotypes	-2578C/A	-2549I/D	+936C/T	Frequencies		OR (95% CI)	*P*
				Patients	Controls		
H1	C	D	C	0.466	0.454	1	1
H2	C	D	*T*	0.140	0.101	1.33 (0.71-2.51)	.363
H3	**C**	***I***	***C***	0.067	0.017	3.63 (1.47-8.97)	**.005**
H4	*A*	D	C	0.007	0.015	0.52 (0.11-2.39)	.407
H5	*A*	*I*	C	0.252	0.326	0.74 (0.51-1.09)	.139
H6	*A*	*I*	*T*	0.065	0.085	0.77 (0.38-1.54)	.473

OR, Odds Ratio.
The haplotype CDC is chosen to be the reference haplotype. Boldface indicates statistically significant differences while italics indicated minor allele.

in Tunisian patients.[34] Significant increased frequency of II genotype of -2549I/D polymorphism was observed in patients with breast cancer risk in North India as compared to controls (OR (95% CI), 2.76 (1.55-4.92); *P* = .0005).[22] However, D allele and ID genotype were also been related to renal cell carcinoma[35] and prostate cancer, respectively.[36]

The result of +936C/T polymorphism exhibited a non-significant association with UBC in our study. In fact, this SNP was studied in different cancers types. Same results were found in Chinese,[37] Spanish,[8] Kashmir[38] and Iranian studies.[29] However, other researchers found significant associations with decreased cancer risk in Han Chinese,[39] Spanish,[40] and Australian populations.[41]

Furthermore, we examined the combined effects of the promoter polymorphisms in the context of their haplotypes. Six combinations (C-D-C, C-D-T, C-I-C, A-D-C, A-I-C, and A-I-T) for -2578C/A, -2549I/D, and +936C/T VEGF gene polymorphisms, respectively, were determined (Table 4). Therefore, we observed one C-I-C haplotype, including wild allele of the functional -2578C/A SNP, associated with the disease. In table 2, the combined results showed significant decreased risk associations in carriers of minor -2578A allele within VEGF gene. But interestingly, we also found that the major C allele carriers (CA+CC) genotypes had 1.87-fold risk association with UBC compared to AA genotype (OR (95% CI), 1.87 (1.04-3.58), *P* = .034). As result, the haplotype $C\text{-}_{2578}$ - $I\text{-}_{2549}$ - C_{+936} was associated with increased risk of UBC (OR (95% CI), 3.63 (1.47-8.97), *P* = .005) compared to common haplotype C-D-C (Table 4).

The linkage disequilibrium test showed a strong LD associations between -2578C/A and -2549I/D polymorphisms with (D'=0.94, r^2 = 0.87, *P* < .01) among controls and (D' = 0.96, r^2 = 0.71, *P* < .01) among patients.

Both polymorphisms are located in the promoter region affecting the transcriptional activity of VEGF gene. For C-2578A polymorphism, A allele could be involved in prevention of the development of a variety of pathologies. In vitro, carriers of AA and II genotypes reduced the secretion of VEGF serum levels by lipopolysaccharides in peripheral blood mononuclear cells than carriers of CC and DD genotypes, respectively.[35]

Haplotype analyses in different studies were heterogeneous and some of them have demonstrated the linquage between these 3 SNPs together. Jaiswal et al[28] analyzed VEGF haplotype of -2578C/A, - 7C/T, -2549I/D, and -1001G/C SNPs and found that C-T-I-G combination is associated with 2.19-fold bladder cancer risk. Kapahi et al[22] also found that -2578C/A and -2549I/D were in complete LD (D' = 0.99, r^2 = 0.97) but II-AA genotype combination was associated with increased risk of breast cancer disease. Another study of Maryam et al[29] did not show any significant associations in all combination including our SNPs. Prakash et al[42] also reported that $T_{+936}\text{-}A_{1154}\text{-}C_{\text{-}2578}\text{-}I_{\text{-}2549}$ haplotype combination was associated with increased risk of end stage renal disease (OR (95% CI), 24.10 (3.22-180.2), *P* = .0001).

Considering +936C/T SNP, it's always reported to be in weak LD with both -2578C/A and -2549I/D polymorphisms.[25]

The reason for these discrepancies remains unclear. These could be a result of the effect of ethnic differences related to the distribution of VEGF polymorphisms in these populations, as well as, sex distribution and interactions with other genetic or environmental factors involved in the pathogenesis of different cancers. Other explanation for the diversity of the results are selection criteria adopted for patients and controls, in particular age, extent of disease, concomitant environmental risk factors like differences in the lifestyles (smoking and diet and type of activity). Unfortunately, our study is not without limitations and these should be mentioned: Firstly, this is a hospital-based case-control study, so the selection bias may not be avoidable and the subjects may not be representative of the general population. Secondly, we did not also measure the VEGF production in our population and evaluate the relationships of these variants with the plasma levels of VEGF, which may potentially reflect the disease state of patients.

Further prospective and multiethnic studies, including a large sample size, are required to confirm our finding with a particular attention to the role of gene-gene and gene-environment interaction.

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