Human Immunology 83 (2022) 528–537

Contents lists available at ScienceDirect

journal homepage: www.elsevier.com/locate/humimm

Research article

Association of VEGF-A and KDR polymorphisms with the development of schizophrenia

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ARTICLE INFO

Article history:
Received 3 January 2022
Revised 5 April 2022
Accepted 6 April 2022
Available online 12 April 2022

Keywords:
VEGF-A
KDR
Polymorphism
Haplotype
Schizophrenia

ABSTRACT

Aim: Several approaches indicate different blood flow disturbances in schizophrenia. *VEGF-A* is widely recognized as one of the key molecules involved in the angiogenesis process through mainly its receptor *KDR*. The current work was designed to investigate the potential association between three polymorphisms (*rs699947*; *rs833061* and *rs3025039*) in *VEGF-A* gene and two SNPs (*rs2305948* and *rs1870377*) within *KDR* gene and predisposition to schizophrenia among the Tunisian cohort.
Methods: We carried-out a case-control study composed of 200 patients with schizophrenia and 200 healthy subjects using PCR-RFLP.
Results: Of all analyzed polymorphisms, only *rs833061*, *rs3025039* and *rs1870377*) showed a significant risk for schizophrenia ($P_{Adjusted}$ = 0.04, $P_{Adjusted}$=<0.001, $P_{Adjusted}$ = 0.005 respectively). Following the stratified analysis, *rs3025039* was more prevalent among undifferentiated form ($P_{Adjusted}$=<0.001) and more specifically with male sex ($P_{Adjusted}$=<0.001). Yet, *rs1870377* was correlated with paranoid subtype ($P_{Adjusted}$ = 0.002) and particularly with male sex ($P_{Adjusted}$ = 0.005). We found also that *rs699947* is associated to negative symptoms before and after treatment (P = 0.004; P = 0.001 respectively) and *rs3025039* had an impact on positive and negative symptoms only after treatment (P = 0.03; P = 0.008 respectively). Haplotype analysis revealed a strong LD between *rs833061* and *rs3025039* only for controls and undifferentiated patients (P = 0.005). Moreover, the *rs699947*C* ~ *rs833061*T* ~ *rs3025039*T* haplotype, with only one mutated allele *rs3025039*T*, conferred a high risk to schizophrenia (P = 0.016) and, in particular, to undifferentiated and paranoid forms (P = 0.03; P = 0.02 respectively). Among the last-mentioned subgroup, we noticed another overrepresented haplotype (*rs699947*A* ~ *rs833061*T* ~ *rs3025039*T*; P = 0.01). Furthermore, the *rs2305948*G* ~ *rs1870377*T* haplotype carrying the minor allele *rs1870377*T* displayed increased frequencies in the whole group of patients and particularly among paranoid subtype (P = 0.013; P=<0.001 respectively).
Conclusion: Our results show that all SNPs associated with the development or the severity of schizophrenia, were subsequently correlated with a decrease in the *VEGF-A* levels or influence *KDR* binding affinity. These data need to be strengthened by further independent analyses.

1. Introduction

Schizophrenia (Scz) is a complex neuropsychiatric illness characterized by an admixture of positive, negative and cognitive symptoms. According to the variability of the clinical features, several subtypes have been defined as paranoid type (characterized by preoccupation with delusions or hallucinations), disorganized type (marked by a disordered behavior) and undifferentiated type (considered when a patient exhibits Scz symptoms that do not fit with any specific subtype) [1]. The worldwide prevalence is approximately 0.7% [2] and in Tunisia is around 0.6% [3]. The etiology remains poorly elucidated despite extensive research.

Accumulating data have indicated over-expressed genes associated with vascular function, vasoregulation, shear stress, cerebral ischemia, neurodevelopment and post-ischemic repair among genes that might predispose the risk for the development of Scz [4]. Moreover, neuroimaging approaches have identified dilated lateral ventricles and smaller tissue volumes in various brain regions which can possibly be explained by hypoperfusion or

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https://doi.org/10.1016/j.humimm.2022.04.003
0198-8859/

diminished blood circulation observed in subjects with Scz [5,6]. On the other hand, cognitive deficits were corrected after injection of recombinant human erythropoietin, known to stimulate the process of angiogenesis [7,8]. Aside from being a potent angiogenic factor, vascular endothelial growth factor (*VEGF*) plays a relevant role in the CNS [9] and is secreted not only by endothelial cells, but also by astrocytes and neurons of any degree of maturity [10]. The VEGF family includes VEGF-A, VEGF-B, VEGF-C, VEGF-D, VEGF-E, and placental growth factor (PlGF) [11]. They act via three specific tyrosine kinase receptors: VEGF-R1, VEGF-R2 and VEGF-R3 [12]. *VEGF-A* is the best characterized and the *VEGF-R2* is recognized as the major signal transducer [13].

In Scz patients, changes in cerebral blood circulation caused by *VEGF-A* system disturbance affect cognitive ability and brain activity [14]. Moreover, *post-mortem* results revealed lower *VEGF-A* mRNA levels in the dorsolateral prefrontal cortex (DLPFC) [15]. Other studies reported that *VEGF-A* gene variation were correlated with reduced hallucinations, greater DLPFC and parahippocampus volume [16] and smaller hippocampal volume [17]. The latter brain area has long been regarded a key region in the pathophysiology of this psychosis [5,18] and physiological abnormalities of the hippocampus [19,20] are well established in individuals suffering from Scz. Additionally, the inflammatory mediators may alter the integrity of the blood- brain barrier through reactivation of *VEGF-A* signaling [21] and several pro-inflammatory cytokines, which are assumed to play a role in the vulnerability to Scz, might impact *VEGF-A* levels [22].

The *VEGF-A* gene is mapped to chromosome 6 (6p21.3) [23] and includes several polymorphisms. Three of them, namely *rs699947*; *rs833061*; *rs3025039* were of particular interest to us as they have been suggested to influence *VEGF-A* expression [24,25,26]. Besides, the gene encoding *VEGF-R2*, alternatively designated as kinase insert domain receptor (*KDR*), is located on chromosome 4 (4q11-q12) [27]. Two common substitutions (*rs2305948*; *rs1870377*)) were reported to affect *KDR* binding affinity [28].

The aim of the present study was therefore to evaluate the relationship between the above-mentioned polymorphisms and Scz, and to assess the possible relationships between genotypes and clinical characteristics of this psychotic disorder.

2. Materials & Methods

2.1. Study participants

A group of 200 patients with the diagnosis of Scz were enrolled in the current work. The onset age, which corresponds to the first occurrence of psychotic symptoms ranged from 10 to 48 (25.44 ± 7. 83) years. Showing continuous signs of the disturbance for minimum six months with active symptoms (hallucinations, delusions…) for at least one month is an essential condition to be admitted as true schizophrenic. The diagnosis of the pathology was based on the criteria mentioned in the DSM-IV axis I [29] with an accuracy of subclinical type. All of the cases were treated with either typical (Haloperidol, Fluphenazine, Chlorpromazine), atypical antipsychotics (Risperidone, Olanzapine, Amisulpride) or both. Anticholinergic drugs were prescribed for more than half of the patients (56.7%), as well.

At the time of hospital admission, illness severity was assessed using the Brief Psychiatric Rating Scale (BPRS) and the Positive (SAPS) and Negative (SANS) Syndrome Scale. The BPRS is a rating scale used in clinical psychopharmacology research to characterize psychopathology and to measure change [30]. Moreover, SANS uses a 25-item, 6-point scale to assess negative symptoms. The items are classified into five categories: affective blunting, alogia, avolition/apathy, anhedonia/asociality, and attentiveness. While, SAPS uses a 34-item, 6-point scale to assess positive symptoms. Hallucinations, delusions, odd behavior, and positive formal thought disorder are all covered [31].

The sociodemographic data including birthplace, residence area, marital status, occupation, and educational level were gathered from health record and via interviews with patients. Our study omitted subjects with acute illness (allergic, autoimmune or malignant disorders) or other comorbidities such as epilepsy, bipolar and depression. All patients were recruited from the Psychiatry Department of Monastir Hospital, Tunisia whereas healthy volunteers were selected from the regional blood transfusion center of the same University Hospital. The control group included 200 subjects. The main exclusion criteria were as follows: current neurological or chronic physical problems, family history of psychiatric disease and substance abuse. The demographic data of all participants of both groups are summarized in Table 1.

The study was authorized by the Research in Life Sciences and Health (CER-SVS) ethical committee of the Higher Institute of Biotechnology of Monastir. Informed consent was obtained from each participant or a family member prior to blood sampling.

2.2. Genomic DNA analysis:

Blood samples from all participants were collected in an EDTA-containing tube and the genomic DNA was extracted using the salting-out technique [32]. Regions covering targeted polymorphisms were amplified by the PCR-RFLP. Based on prior reports, PCR primers, restriction enzymes and product sizes were designed [33,34,28], with details listed in Table 1.

The PCR cycling conditions included an initial denaturation step of 5 min at 94 °C followed by 35 cycles of 94 °C for 30 s, specific annealing temperature (Table 1) for 45 s and extension at 72 °C for 1 min. The run was ended by a final extension at 72 °C for 10 min.

PCR was performed in 25 µl reaction volumes containing 100 ng of genomic DNA, 0.2 mM of each dNTP, 2.6 mM MgCl2, 0.2 mM of each primer, 1xTaq polymerase buffer (Biobasic) and 1U Taq DNA polymerase (Biobasic). PCR products were incubated overnight at 37 °C with the appropriate restriction enzymes (Table 2). Afterwards, the digested PCR products were then analyzed on 4% agarose gel and stained with ethidium bromide for visualization under UV light.

2.3. SNP selection criteria

The selection criteria were essentially based on the functionality and the minor allelic frequency (MAF) of the investigated polymorphisms. SNPs information were retrieved from the National Center for Biotechnology Information, dbSNP, http://www.ncbi.nlm.nih.gov/SNP/ and literature references were strongly taken under consideration.

Table 1
Demographic data of all participants.

		Healthy controls	Patients
Gender	Female	21.5%	17.8 %
	Male	78.5%	82.2 %
Sex ratio		3.6	4.6
Age		33.76 ± 10.79	38.21 ± 10.83
Clinical subtypes	undifferentiated	–	48.9%
	paranoid	–	31.5%
	disorganized	–	18.3%
Treatment		–	385.2 ± 260.2 mg

Table 2
Primers of VEGF-A and KDR gene polymorphisms for PCR amplification.

SNPs	Primers (5′- 3′)	Annealing temperature	Amplicon sizes (bp)	Restriction enzymes
rs699947	**F:** GCACCTCCACCAAACCACAGCAACAT	55	*C/C*: 422	
(*-2578C/A*)	**R:** CAAGCCCCCTTTTCCTCCAACTCTCC		*A/A*: 264, 158 Bgl II	
rs833061	**F:** TGTGCGTGTGGGGTTGAGCG	55	*T/T*: 175	
(*-460 T/C*)	**R:** TACGTGCGGACAGGGCCTGA		*C/C*: 155, 20 BstUI	
rs3025039	**F:** AAGGAAGAGGAGACTCTGCGCAGAGC	60	*C/C*: 208	
(*+936C/T*)	**R:** TAAATGTATGTATGTGGGTGGGTGTGTCTACAGG		*T/T*: 122, 86 Nla III	
rs2305948	**F:** TGAGGTTAAAAGTTCTGGTGTCCCTGTT	58	*G/G*: 262	
(*+1192 G/A*)	**R:** AAATGTACAATCCTTGGTCACTCCGGGGTA		*A/A*: 232, 30 BstZ17I	
rs1870377	**F:** CCTCCTGTATCCTGAATGAATCT	58	*A/A*: 404	
(*+1719 A/T*)	**R:** GCCTCACATATTATTGTACCATCC		*T/T*: 213, 191 Alu I	

2.4. Statistical analysis

The Hardy-Weinberg equilibrium (HWE) was estimated for the studied SNPs in both groups using the Chi-square (χ^2) test. For statistical analysis purposes, SPSS software (version 23, Armonk, NY, USA) and SNPStats online software (https://www.snpstats.net/start.htm) were used.

SNPStats was used to assess the association between target SNPs and the risk of Scz under three inheritance models. The best-fit model was determined according to the lowest Akaike information criterion (AIC) value [35] (Table 3).

The strength of association between alleles or genotypes of control and case samples was evaluated with the odds ratio (OR) presented with 95% confidence intervals (CI) whenever χ^2 test was significant. Statistical significance was assigned at $p < 0.05$. Additionally, students' *t*-test was performed, using SPSS, to compare the symptom severity scale in patients with Scz according to *VEGF-A* genotypes before and after treatment. Regarding P-value adjustment (P_A), a binary logistic regression model was conducted with categorical and quantitative independent variables, adjusting for the effects of age and sex by analyzing the deviation of a sequential addition of each variable.

P_A and haplotype reconstruction were conducted with SNPStats. Linkage disequilibrium (LD) expressed in terms of the D′ and r' coefficients was estimated between paired SNPs using the same online software. We used also the Haploview (version 4.2) and a graphical view was generated. Moreover, LDlink (https://ldlink.nci.nih.gov) was performed to investigate haplotype frequencies, and patterns of linkage disequilibrium across European reference genome (Toscani in Italia).

3. Results

The distribution of *VEGF-A* and *KDR* genotypes were in keeping with those predicted by the Hardy-Weinberg equilibrium conditions in both healthy controls and patients (data not shown). The genotypic and allelic frequencies of investigated SNPs are depicted in Table 2. No statistical differences were observed between the polymorphism *rs699947* and predisposition to Scz (PA = 0.5). As for the second SNP *rs833061*, we note that the minor allele *rs833061*C* and the combined (*T/C + C/C*) genotype are more prevalent among cases (PA = 0.04 and PA = 0.04, respectively). With regard to *rs3025039* substitution, a strong association was seen for *T/T* genotype under the three inheritance models (Table 2). In terms of allele frequency, an increased risk of Scz was perceived (PA= <0.001). Concerning the *rs1870377* variant of the *KDR* gene, the obtained results evoked close values for both groups, indicating no significant association between the genotyped *KDR* mutation and Scz. Instead, the lower prevalence of the mutated *T/T* genotype (whatever the genetic mode of inheritance) as well as the minor allele *rs1870377*T* in healthy subjects suggests that the *rs1870377* polymorphism constitutes a risk for the development of this pathology. These positive correlations, reinforced by adjusted analysis for age and sex, remain highly significant with the lowest AIC conforming to the dominant model of inheritance for the *rs833061*, *rs3025039* polymorphisms and to the recessive one for the *rs1870377* polymorphism (Table 3).

According to data analysis of clinical subtypes of the disease, no significant association was found for polymorphisms at positions *rs699947*, *rs833061* and *rs2305948*. However, a higher distribution of *rs3025039* was found among undifferentiated and paranoid forms considering the dominant inheritance. The *rs1870377* substitution was correlated with paranoid and disorganized schizophrenia but not with the undifferentiated type of the disease conforming to the recessive mode of inheritance (Table 4).

When we performed a sex-stratified analysis, our results indicated that only undifferentiated males harboring *rs3025039*T* allele and paranoid males carrying *rs1870377*T* allele are more predisposed to Scz as expressed by the appropriate model of inheritance of each polymorphism (***PA***=<0.001; ***PA*** = 0.005 respectively). Similar results have been described for females without reaching the statistical significance; one should take into account the relatively small size of the designed cohort. However, allele differences were not significant for both sexes in terms of the other analyzed sites (Table 5).

In order to find out if there is an impact of the *rs699947* polymorphism on patients' symptomatology, we compared the severity of disease symptoms before and after treatment.

As shown in Table 6, cases with A/A genotype have more intense symptomatic scores with statistical significance for the SANS1 (p = 0.004) and SANS2 (p = 0.001) scales according to the recessive genetic model.

Similarly, heterozygous and homozygous carriers of VEGF-A *rs3025039* presented greater positive and negative symptoms score means. The difference is statistically significant after treatment as mentioned in the Table 7. For the *rs833061*, *rs1870377* and *rs2305948* SNPs we did not find any significant correlation with the scores of the psychometric parameters (BPRS, SANS and SAPS) whatever the genetic transmission mode.

We conducted haplotype analysis in order to estimate the combined effect of the *VEGF-A* and *KDR* polymorphisms. The *rs699947*C* ~ *rs833061*T* ~ *rs3025039*T* haplotype, with only one mutated allele *rs3025039*T*, conferred a high significant difference in patients (P = 0.016). Furthermore, this genetic combination exerts much important predisposing effect in the undifferentiated and paranoid forms (P = 0.03; P = 0.02 respectively). Among the last-mentioned subgroup, we noticed that *rs699947*A* ~ *rs833061*T* ~ *rs3025039*T* haplotype is overrepresented (P = 0.01) (Table 8).

Moreover, the *rs2305948*G* ~ *rs1870377*T* haplotype carrying the *KDR rs1870377*T* displayed significant increased frequencies in the whole group of patients and particularly among paranoid form (p = 0.013; p=<0.001 respectively) (Table 9).

Table 3
Different frequencies of VEGF-A and KDR polymorphisms between patients and controls according to the different inheritance models.

Inheritance Models	Genotypes	Controls n (%)	Patients n (%)	OR (95% CI)	P-value	AIC	PA
***rs699947* (*-2578C/A*)**							
Codominant	*C/C*	72 (36%)	72 (36%)				
	C/A	105 (52.5%)	97 (48.5%)	0.92 (0.6–1.4)			
	A/A	23 (11.5%)	31 (15.5%)	1.35 (0.7–2.5)	0.47	559	0.62
Dominant *C/C vs C/A + A/A*				1 (0.6–1.5)	1	558.5	0.65
Recessive *C/C + C/A vs A/A*				1.41 (0.7–2.5)	0.24	557.1	0.49
Alleles *rs699947*C*		249 (62.25%)	241 (60.25%)				
*rs699947*A*		151 (37.75%)	159 (39.75%)	1.08 (0.8–1.4)	0.56		0.50
***rs833061* (*-460 T/C*)**							
Codominant	*T/T*	137 (69.9%)	119 (59.5%)				
	T/C	56 (28.6%)	76 (38%)	1.56 (1.02–2.3)			
	C/C	3 (1.5%)	5 (2.5%)	1.92 (0.45–8.2)	0.09	550.2	0.13
Dominant *T/T vs T/C + C/C*				1.58 (1.04–2.4)	**0.03**	548.2	**0.04**
Recessive *T/T + T/C vs C/C*				1.65 (0.39–7)	0.49	552.5	0.49
Alleles *rs833061*T*		330 (84.2%)	314 (78.5%)				
*rs833061*C*		62 (15.8%)	86 (21.5%)	1.45 (1–2.09)	**0.04**		**0.04**
***rs3025039* (*+936C/T*)**							
Codominant	*C/C*	157 (78.5%)	122 (61%)				
	C/T	41 (20.5%)	69 (34.5%)	2.17 (1.3–3.4)			
	T/T	2 (1%)	9 (4.5%)	5.79 (1.2–27.2)	**<0.001**	544.1	**0.001**
Dominant *C/C vs C/T + T/T*				2.33 (1.5–3.6)	**<0.001**	543.8	**<0.001**
Recessive *C/C + C/T vs T/T*				4.66 (1–21.8)	**0.026**	553.6	0.062
Alleles *rs3025039*C*		355 (88.75%)	313 (78.25%)				
*rs3025039*T*		45 (11.25%)	87 (21.75%)	2.19 (1.4–3.2)	**<0.001**		**<0.001**
***rs2305948* (*+1192 G/A*)**							
Codominant	*G/G*	150 (75%)	149 (74.5%)				
	G/A	43 (21.5%)	47 (23.5%)	1.1 (0.6–1.7)			
	A/A	7 (3.5%)	4 (2%)	0.58 (0.1–2)	0.6	559.5	0.64
Dominant *G/G vs G/A + A/A*				1.03 (0.6–1.6)	0.91	558.5	0.61
Recessive *G/G + G/A vs A/A*				0.56 (0.1–1.9)	0.36	557.7	0.54
Alleles *rs2305948*G*		343 (85.75%)	345 (86.25%)				
*rs2305948*A*		57 (14.25%)	55 (13.75%)	0.95 (0.6–1.4)	0.83		0.84
***rs1870377* (*+1719 A/T*)**							
Codominant	*A/A*	114 (57%)	93 (46.5%)				
	A/T	74 (37%)	81 (40.5%)	1.3 (0.8–2)			
	T/T	12 (6%)	26 (13%)	2.6 (1.2–5.5)	**0.021**	552.8	**0.025**
Dominant *A/A vs A/T + T/T*				1.5 (1–2.2)	**0.035**	554.1	**0.023**
Recessive *A/A + A/T vs T/T*				2.3 (1.1–4.7)	**0.016**	552.7	**0.03**
Alleles *rs1870377*A*		302 (75.5%)	267 (66.75%)				
*rs1870377*T*		98 (24.5%)	133 (33.25%)	1.5 (1.1–2)	**0.006**		**0.005**

OR: odds ratio; **CI:** confidence interval; ***PA:*** Adjusted P-value for age & sex.
AIC (Akaike information criterion) provides a means for model selection;

In connection with the previous results, haplotype distribution profile of the *rs3025039* and *rs1870377* polymorphisms suggests that the observed predisposing effect may be driven by the allele *rs3025039*T*. However, among the paranoid subgroup the *rs3025039*T* ~ *rs1870377*T* haplotype carrying two mutated alleles conferred the highest significant difference as compared to the wild type combination. Estimated constructed haplotypes are shown in Table 10.

The D' and r values for controls and undifferentiated patients (considered as a whole group) are stated in Table 11. There was no evidence of LD between markers except for *rs833061* and *rs3025039* (p = 0,005).

Haploview analysis revealed moderate LD between *rs699947* and *rs833061* (D′ = 54) as compared to that conferred by *rs3025039* with *rs833061* (D′ = 16) and *rs699947* (D′ = 1) (Supplement 1).

LDlink (https://ldlink.nci.nih.gov) was used to investigate patterns of linkage disequilibrium across European reference genome (Toscani in Italia). Population-specific haplotype frequencies were calculated. LD Matrix was used to create interactive heatmap matrix of pairwise linkage disequilibrium statistics and LD pair was used to investigate correlated alleles for a pair of variants in high LD. We found that *rs3025039* was in linkage equilibrium with *rs699947* and *rs833061* mutation sites. Furthermore, *rs699947*A* allele is correlated with *rs833061*C* allele and *rs699947*C* allele is correlated with *rs833061*T* allele (Supplement 2, 3).

Regarding *KDR* receptor, we noted that the investigated polymorphisms, *rs2305948* and *rs1870377*, are in linkage disequilibrium (Supplement 4, 5).

4. Discussion

Several micro and macrovascular disorders, involving peripheral and cerebral vascularization have recently been identified as having direct effects on the pathophysiology of Scz. Researchers have focused on exploring serum or plasma *VEGF-A* levels in neuropsychiatric conditions like Major Depressive Disorder, First-Episode Psychosis or Multiple-Episode Schizophrenia [17,36]. We chose to analyze functional polymorphisms of the *VEGF-A* gene and its *KDR* receptor to identify the nature of the plausible link between Scz and angiogenesis from a vascular point of view. The *VEGF-A* gene, highly polymorphic, is localized on chromosome 6 at locus 6p12 [23].

In the promoter region, a *C/A* substitution is described at position −2578 *(rs699947)*. The allele *rs699947*C* has a binding site to the Hypoxia-Inducible Factor-1 (HIF-1) transcription factor which can be removed by the minor allele *rs699947*A* [24]. HIF-1

Table 4
Distribution of genotype and allele frequencies of *rs3025039* & *rs1870377* in control subjects and patients with different subtypes of Scz.

Genotypes n (%)						**Alleles n (%)**				
VEGF-A *rs3025039*	*C/C* **vs** (*C/T* + *T/T*)		**OR** (95% IC)	**P-value**	P_A	*rs3025039*C*	*rs3025039*T*	**OR** (95% IC)	**P-value**	P_A
Controls (n = 200)	157 (78.5%)	43 (21.5%)				355 (88.7%)	45 (11.3%)			
Undifferentiated (n = 103)	58 (56.3%)	45 (43.7%)	2.82 (1.6–4.7)	**<0.001**	**<0.001**	156 (75.7%)	50 (24.3%)	2.52 (1.6–3.9)	**<0.001**	**<0.001**
Paranoid (n = 57)	35 (61.4%)	22 (38.6%)	2.28 (1.2–4.3)	**0.01**	**0.03**	88 (77.2%)	26 (22.8%)	2.32 (1.3–3.9)	**0.002**	**0.01**
Disorganized (n = 37)	27 (73%)	10 (27%)	1.35 (0.5–2.9)	0.46	0.37	64 (86.5%)	10 (13.5%)	1.23 (0.5–2.5)	0.56	0.48
KDR *rs1870377*	(*A/A* + *A/T*) **vs** *T/T*					*rs1870377*A*	*rs1870377*T*			
Controls (n = 200)	188 (94%)	12 (6%)				302 (75.5%)	98 (24.5%)			
Undifferentiated (n = 97)	91 (93.8%)	6 (6.2%)	1.03 (0.3–2.8)	0.93	0.97	139 (71.6%)	55 (28.4%)	1.21 (0.8–1.7)	0.31	0.26
Paranoid (n = 69)	56 (81.1%)	13 (18.9%)	3.61 (1.5–8.5)	**0.003**	**0.002**	86 (62.3%)	52 (37.7%)	1.86 (1.2–2.8)	**0.003**	**0.002**
Disorganized (n = 32)	25 (78.1%)	7 (21.9%)	4.34 (1.4–12)	**0.008**	**0.017**	39 (60.9%)	25 (30.1%)	1.97 (1.1–3.4)	**0.01**	**0.03**

OR: odds ratio; **CI:** confidence interval; ***PA:*** Adjusted P-value for age & sex.
KDR: kinase insert domain receptor.

Table 5
Distribution of genotype and allele frequencies of *rs3025039* and *rs1870377* within undifferentiated and paranoid type according to sex.

	Males				**Females**			
	Controlsn (%)	Patientsn (%)	OR (95% IC)	P_A	Controlsn (%)	Patientsn (%)	OR (95% IC)	P_A
VEGF-A *rs3025039*								
Undifferentiated type								
Genotypes	(n = 157)	(n = 88)			(n = 43)	(n = 15)		
C/C	121 (77%)	47 (53.4%)			36 (83.7%)	11 (73.3%)		
C/T + *T/T*	36 (23%)	41 (46.6%)	2.9 (1.6–5.1)	**<0.001**	7 (16.3%)	4 (26.7%)	1.8 (0.3–9)	0.54
Alleles								
*rs3025039*C*	276 (87.9%)	130 (77.4%)			79 (91.9%)	26 (86.7%)		
*rs3025039*T*	83 (12.1%)	46 (22.6%)	2.5 (1.5–4.1)	**<0.001**	7 (8.1%)	4 (13.3%)	1.7 (0.3–7)	0.55
KDR *rs1870377*								
Paranoid type								
Genotypes	(n = 158)	(n = 45)			(n = 42)	(n = 24)		
A/A + *A/T*	149 (94.3%)	37 (82%)			39 (92.8%)	19 (79.1%)		
T/T	9 (5.7%)	8 (18%)	3.5 (1.2–10)	**0.017**	3 (7.2%)	5 (20.9%)	3.3 (0.5–23)	0.21
Alleles								
*rs1870377*A*	244 (77.2%)	57 (63.3%)			58 (69%)	29 (60.4%)		
*rs1870377*T*	72 (22.8%)	33 (36.7%)	1.9 (1.1–3.2)	**0.005**	26 (31%)	19 (39.6%)	1.4 (0.68–3)	0.37

OR: odds ratio; **CI:** confidence interval; ***PA:*** Adjusted P-value for age & sex.
KDR: kinase insert domain receptor.

is required to modulate the gene transcription in response to hypoxia via interaction with the Hypoxia Response Element (HRE) located in the 5′UTR region of the *VEGF-A* gene [37]. Moreover, the allele *rs699947*A* is linked to the insertion of 18 bp at position −2549 (rs144854329) [38] which is absent among individuals harboring the wild allele *rs699947*C* [39]. The homozygous *C/C* genotype increases the production of this growth factor in the Peripheral Blood Mononuclear Cell (PBMC) supernatant in culture from healthy subjects [40]. Furthermore, *rs699947* is involved in the development of some neurodegenerative disorders, such as Amyotrophic Lateral Sclerosis and Alzheimer's disease [41,42], as well as the severity of atherosclerosis [43] and psoriasis [44]. As regards mood disorders, this mutation is linked to treatment-resistant depression and not correlated to Scz in the Han Chinese population [45,46].

In our prospective study, the comparison of genotypic and allelic frequencies of the *rs699947* did not reveal any significant association (p>0.05) with the development of Scz in the Tunisian cohort. This result suggests that the predisposition to this psychosis is unrelated to the production of *VEGF-A* in hypoxic condition. On the other hand, carriers of *A/A* genotype display important SANS scale before (p = 0.004) and after (p = 0.001) treat-

Table 6
Comparison of symptom severity scale in patients with Scz according to the recessive model of *rs699947* before (1) and after (2) treatment (mean ± standard deviation).

	Genotypes		
	C/C + C/A (n)	*A/A* (n)	*p* (*C/C + C/A vs A/A*)
SAPS 1	32.4 ± 5.6 (125)	33.5 ± 7.2 (25)	0.4
SANS 1	33.2 ± 6.3 (123)	37.4 ± 6.1 (24)	**0.004**
BPRS 1	37.7 ± 3 (129)	37.9 ± 3.9 (27)	0.76
SAPS 2	25 ± 7.1 (134)	25.2 ± 8 (25)	0.9
SANS 2	26.3 ± 8.1 (132)	32.4 ± 7.6 (23)	**0.001**
BPRS 2	33.2 ± 3.8 (139)	33.5 ± 5.9 (27)	0.78

BPRS: Brief Psychiatric Rating Scale; **SANS:** Scale of the Assessment of Negative Symptoms;
SAPS: Scale of the Assessment of Positive Symptoms.

Table 7
Comparison of symptom severity scale in patients with Scz according to the dominant model of *rs3025039* before (1) and after (2) treatment (mean ± standard deviation).

	Genotypes		
	C/C (n)	*C/T + T/T* (n)	*p* (*C/C vs C/T + T/T*)
SAPS 1	32.1 ± 5.5 (90)	33.1 ± 6.6 (61)	0.3
SANS 1	33.2 ± 6.9(86)	34.6 ± 5.8(61)	0.2
BPRS 1	37.9 ± 3 (94)	37.9 ± 3.3 (63)	0.9
SAPS 2	24.1 ± 7.3 (96)	26.7 ± 7.5 (61)	**0.03**
SANS 2	25.5 ± 8.2 (92)	29.1 ± 7.9 (60)	**0.008**
BPRS 2	33.3 ± 3.5 (101)	33.8 ± 4.3 (63)	0.4

ment. It means that recessive allele *rs699947*A*, which down-regulate *VEGF-A* levels in hypoxic situations, confers the ability to have severe negative symptoms. According to a report on brain function, this genetic variation causes an attenuation of white and gray matter and arterial blood volume in healthy individuals [47]. Another work dealing with psychosis, including Scz, found that *rs699947* mutation is not associated with the intensity of positive symptoms and cognitive abilities of patients. Yet, they did not investigate relationships with negative symptoms [48].

Regarding *rs833061*, we noticed that the minor allele *rs833061*C* along with the combined *T/C + C/C* genotype are a little more frequent in patients with Scz compared to controls. This substitution, situated in the promoter region, enhances *VEGF-A* gene expression *in vitro* using the human breast cancer cell line MCF7 [25]. It is involved in the development of proliferative diabetic retinopathy, acute respiratory distress syndrome and endometriosis [49,50]. As for cancer susceptibility, results are often contradictory [51,52].

We also investigate, for the first time, the involvement of *rs3025039* polymorphism detected in the 3′UTR region that down regulates *VEGF-A* plasma concentrations among carriers of the allele *rs3025039*T* [26]. This mutation leads to the loss of a potential binding site for the transcription factor activator protein 4 (AP-4) [53].

In the current study, we showed a risk for the development of Scz with the allele *rs3025039*T* and the *T/T* genotype considering the three inheritance models. This predisposition remained valid for the paranoid form and more advanced in the undifferentiated subtype even after adjustment with covariates. This result is similar to that found for MCP-1 −362G/C among the same cohort [54]. When we compared the severity of disease symptoms, our findings demonstrate also greater symptomatic scores before and after treatment with statistical significance for the SAPS2 and SANS2 scales according to the dominant genetic model.

In order to evaluate the combined effect of the three targeted SNPs, we carried out a haplotypic analysis. The obtained results revealed that *rs699947*C ~ rs833061*T ~ rs3025039*T* haplotype, with only one risk allele *rs3025039*T*, present a statistical difference in the whole schizophrenic cohort ($p = 0.016$) and is also significant for the undifferentiated and paranoid group. We noted also that *rs699947*A ~ rs833061*T ~ rs3025039*T* is highly expressed among the last-mentioned clinical form.

As far as we know, this is the first report of these variants being examined for correlation with Scz. In agreement with our results showing that alleles associated with decreased production of *VEGF-A* predispose to the development or severity of Scz, *post-*

Table 8
VEGF-A haplotype frequencies in healthy controls & patients with Scz & according to paranoid and undifferentiated subtypes.

VEGF-A haplotypes	Percent haplotype frequencies		OR (95%CI)	P-value
	Controls	Scz patients		
rs699947 ~ rs833061 ~ rs3025039				
C ~ T ~ C	52.3	40.7		
A ~ T ~ C	23.3	20.3	1.07 (0.6–1.7)	0.8
A ~ C ~ C	10.2	11	1.4 (0.7–2.5)	0.24
C ~ T ~ T	5.5	12.6	2.5 (1.2–5.5)	**0.016**
C ~ C ~ C	2.8	6.1	2.2 (0.7–6.9)	0.16
VEGF-A haplotypes	Controls	Paranoid patients	OR (95%CI)	P-value
rs699947 ~ rs833061 ~ rs3025039				
C ~ T ~ C	52.3	39.7		
A ~ T ~ C	23.3	16.2	0.7 (0.3–1.8)	0.57
A ~ C ~ C	10.2	14.4	2 (0.9–4.5)	0.08
C ~ T ~ T	5.5	13.4	2.7 (1.1–6.5)	**0.02**
A ~ T ~ T	3.2	9.8	3.9 (1.3–11.6)	**0.01**
VEGF-A haplotypes	Controls	Undifferentiated patients	OR (95%CI)	P-value
rs699947 ~ rs833061 ~ rs3025039				
C ~ T ~ C	52.3	40.2		
A ~ T ~ C	23.3	22.6	1.1 (0.5–2.1)	0.75
A ~ C ~ C	10.2	7.5	1 (0.4–2.3)	0.99
C ~ T ~ T	5.5	13.8	2.5 (1–6)	**0.03**
C ~ C ~ C	2.8	5.8	2.1 (0.5–7.9)	0.25

OR: odds ratio; **CI:** confidence interval; **Scz:** schizophrenia.

Table 9
KDR haplotype frequencies in healthy controls & patients with Scz & according to paranoid subtype.

KDR haplotypes	Percent haplotype frequencies		OR (95%CI)	P-value
	Controls	Scz patients		
rs2305948 ~ rs1870377				
G ~ A	65	57		
G ~ T	20.6	29.3	1.5 (1.1–2.2)	**0.013**
A ~ A	10.4	9.7	1 (0.6–1.8)	0.81
A ~ T	3.8	3.8	1.1 (0.4–3)	0.75
KDR haplotypes	Controls	Paranoid patients	OR (95%CI)	P-value
rs2305948 ~ rs1870377				
G ~ A	65	48		
G ~ T	20.6	37.6	2.3 (1.4–3.7)	**<0.001**
A ~ A	10.4	14.3	1.9 (1–3.8)	0.05
A ~ T	3.8	0	0 (inf - inf)	1

OR: odds ratio; **CI:** confidence interval; **Scz:** schizophrenia; **KDR:** kinase insert domain receptor.

Table 10
Haplotype frequencies in healthy controls & patients with Scz & according to paranoid subtype.

KDR haplotypes	Percent haplotype frequencies		OR (95%CI)	P-value
	Controls	Scz patients		
rs3025039 ~ rs1870377				
C ~ A	67.8	53.3		
C ~ T	20.8	24.7	1.4 (0.9–2.1)	0.06
T ~ A	7.7	13.5	2.2 (1.2–4)	**0.01**
T ~ T	3.6	8.3	3 (1.2–7.5)	**0.01**
KDR haplotypes	Controls	Paranoid patients	OR (95%CI)	P-value
rs3025039 ~ rs1870377				
C ~ A	67.8	50.76		
C ~ T	20.8	25.74	1.5 (0.8–2.6)	0.13
T ~ A	7.7	11.69	1.8 (0.8–4)	0.16
T ~ T	3.6	11.81	4.9 (1.8–13.6)	**0.002**

OR: odds ratio; **CI:** confidence interval; **Scz:** schizophrenia; **KDR:** kinase insert domain receptor.

Table 11
Linkage disequilibrium between the three analyzed VEGF-A SNPs.

SNP	**D′ (r)**		**p-value**	
	rs833061	*rs3025039*	*rs833061*	*rs3025039*
rs699947	0.48 (0.29)	0.03 (-0.01)	0	0.81
rs833061	–	0.14 (0.13)	–	**0.005**

r: Pearson's correlation coefficient; **D':** Standardized linkage disequilibrium coefficient.

mortem studies have shown a decreased *VEGF-A* brain activity among cases. Moreover, levels of its mRNA in the prefrontal cortex (PFC) region as well as expression of its *KDR* receptor are significantly reduced [16,55]. *VEGF-A* acts as a neurotrophic factor in neurons and appears to play a critical role in adult neurogenesis [56,57]. The proliferation of neural stem cells in the hippocampus (first stage of adult neurogenesis) seems significantly restricted in this psychotic disorder [58]. *VEGF-A* can serve as a prognostic biomarker. In this context, high serum levels were linked to a better antipsychotic response, whereas low serum concentrations predicted resistance to drug therapy in acute-stage of Scz [59]. Also, elevated serum *VEGF-A* among cases with treatment-resistant Scz who had electroconvulsive therapy is positively associated with therapeutic effects [60].

Referring to several subsequent works, serum levels are elevated whereas brain *VEGF-A* expressions are decreased in individuals suffering from Scz. These results, looking contradictory, could be explained by mechanisms involving internalization and degradation of the VEGF/KDR complex that would inhibit cerebral *VEGF-A* signaling pathways [55]. Thus, the correlation proposed by our work between the two SNPs, generating elevated *VEGF-A* levels, and susceptibility to Scz would be consistent.

This study has additionally incorporated two other exonic polymorphisms at positions *rs2305948* (exon7) and *rs1870377* (exon 11). These SNPs, identified in the extracellular 3rd and 5th Ig-like domains of the *KDR* gene receptor, lead respectively to an amino acid change at residues 297 V > I and 472H > Q and influence *VEGF-A* binding efficiency. Indeed, the 3rd domain is critical for ligand fixation while the 5th domain is necessary for *VEGF-A* retention on *KDR* [28]. The genotype and allele distributions of *rs2305948* in patients are very close to that found in controls. This substitution did not appear to influence Scz as the difference is statistically insignificant ($p > 0.05$). Concerning the *rs1870377* polymorphism, *rs1870377*T* allele and *T/T* genotype (under the three genetic models) was related to the risk of developing this psychosis and more specifically to the paranoid subtype. Previously, our lab results highlighted genetic associations of TNFα/β [61] and IL-8 [62] particularly for the paranoid amongst the undifferentiated and disorganized clinical forms.

In connection with the above data, haplotypic analysis exhibited a higher prevalence of *rs2305948*G ~ rs1870377*T* haplotype in patients when compared to the wild type combination. Similar result with a stronger association is obtained for the paranoid group. Our data are consistent with the functional analysis of these two non-synonymous variants. Lowered mRNA levels generated by the minor allele *rs1870377*T* are plausibly caused by altering the splicing mechanism due to its proximity (3 bp) to the intron/exon boundary. Further, in HEK293 cells, *rs1870377* conveyed a 46% augmentation in *KDR* phosphorylation after VEGF-A165 stimulation ($p = 0.035$) while the other substitutions like *rs2305948* displayed no effect [63]. Moreover, when we compared the combined effect of the *VEGF-A rs3025039* and *KDR rs1870377* polymorphisms we noted that the observed predisposing effect may be driven by the allele *rs3025039*T*.

However, among the paranoid subgroup the *rs3025039*T ~ rs1870377*T* haplotype carrying two mutated alleles conferred the highest significant difference as compared to the wild type combination.

The attenuated *KDR* levels in the PFC are inversely associated with positive symptoms of Scz [55,64]. This receptor is expressed in neurons and blood vessels of the adult human brain [65]. Neurotrophic factors such as BDNF, neuroregulins and EGFs are involved in the pathogenesis of Scz [66,67] and reduced *KDR* expression in patients can provoke altered neurotrophic signaling in neuronal cells. Microcirculation anomalies are also linked to Scz [68]. Indeed, a diminished cerebral blood flow in frontal, parietal and occipital lobes, as well as in functional regions has been detected in patients [69]. Moving forward, *post-mortem* analysis revealed ultrastructural malformations, such as thickening of the capillary basement membrane and swelling of pericapillary astrocytic end-foot in the PFC and the visual cortex. As well, imaging studies exhibited larger retinal venules causing microvascular dysfunctions [70]. Thus, the impact of decreased *KDR* binding affinity in PFC can disseminate to nucleus accumbens and cause positive symptoms via glutamatergic neurons [64]. Interestingly, *KDR* inhibitors such as *sunitinib* and *sorafenib* generate acute and transient side effects similar to positive symptoms including visual and auditory hallucinations, paranoid delusions and aggressive behavior [71,72,73]. Other reports denoted that elevated *VEGF-A* serum levels are negatively correlated with total volume of the frontal lobe in subjects with Scz [74]. While stimulation of *KDR* by *VEGF-A* promotes its internalization and degradation [75], reduced *KDR* levels in Scz might reflect its constitutive activation [64].

To date, the link between *KDR* signaling and susceptibility to Scz remains to be precised. Selective invalidation experiments of, either the receptor or the Src family kinases, have demonstrated the involvement of the VEGF/KDR/Src kinase pathway in the guidance process.

During embryonic development, *KDR* allows guidance of subicular axons in a VEGF-independent manner. Moreover, *KDR* binds with Plexin D1/Nrp-1 complex and stimulates PI3K/Akt pathway in subicular neurons after fixation of Sema3E [76]. In migrating granule cells, *VEGF-A* modulates the association of *KDR* with NMDA receptor which facilitates calcium entry by the intermediary of Src kinases [77]. These different data highlight the complexity of *KDR* signaling that uses distinct pairs of receptors to interact with the nervous system independently of the vascular system.

To conclude, the present findings suggest that the studied SNPs correlated with the predisposition or the severity of Scz, were subsequently associated with a decreased *VEGF-A* levels or influence *KDR* binding affinity. These data need to be strengthened by further independent analyses. In spite of that, several limitations in this study should be considered such as the lack of gene expression or protein dosage assays to support the hypothesis of lower *VEGF-A* expression, as well as experiments to prove that polymorphisms affect receptor binding affinity.

Funding

All financial support has been provided by the Laboratory of Genetics, Biodiversity and Bioresource Valorization (GBVB) of Higher Institute of Biotechnology of Monastir (ISBM), University of Monastir, Monastir, Tunisia via the Ministry of Higher Education, Scientific Research of Tunisia.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgments

We deeply give thanks to all patients and healthy controls with whose cooperation this study was possible. We also gratefully acknowledge the crew of the Psychiatry Department of Monastir Hospital and the staff of the regional blood transfusion center of the same University Hospital for providing samples and clinical information.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.humimm.2022.04.003.

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