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Original Article

Relationship between Single Nucleotide Polymorphisms of *GRHL3* and Schizophrenia Susceptibility: A Preliminary Case-Control Study and Bioinformatics Analysis

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Grainyhead-like (GRHL) transcription factors were recently linked to the etiology of neural tube defects (NTDs). Overlapping patterns in the variation of schizophrenia (SCZ) incidence with that of NTDs suggests the presence of common etiological risk factors. This preliminary study was designed to examine the relationship between two missense variants of *GRHL3* gene (rs2486668C/G and rs545809A/T) and SCZ susceptibility among Iranians. Three hundred ninety subjects (192 patients confirmed with SCZ, and 198 healthy controls) were enrolled and genotyped. Statistical and bioinformatics analyzes were performed to determine the effects of the variants. *In silico* analyzes were performed to determine the effects of the variants on the secondary structure of GRHL3 protein and prediction of silencer motifs for each variation. Statistically significant differences were observed between the studied groups under codominant AA, dominant AT+AA, and recessive AA genetic contrast models for rs545809A/T. The presence of the A allele of rs545809A/T enhanced SCZ risk by 2.33 fold. In contrast, rs2486668C/G was not linked to SCZ susceptibility ($P > 0.05$). Bioinformatics analysis revealed that both missense SNPs caused substantial changes in the secondary structure of *GRHL3*-mRNA. Screening of the flanking sequences of rs545809A/T predicted silencer motifs for this SNP. Our results demonstrated that the rs545809A/T of *GRHL3* gene could affect the risk of SCZ in Iranian populations. Replication studies are warranted to confirm these results.

Key words: Bioinformatics, grainyhead-like 3, haplotype, polymorphism, schizophrenia

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Schizophrenia (SCZ) is mainly characterized by suspiciousness, isolation, distractibility, delusions, and hallucination. This mental disorder is affecting approximately about 0.07–0.43 per 1000 per year (1, 2). SCZ is highly heterogeneous, and is caused by environmental and genetic factors (3, 4). Systematic meta-analysis studies have already identified the relationship between functional single nucleotide polymorphisms (SNPs) of different candidate genes implicated in multiple cellular pathways (5). However, a few similarities were discovered in epidemiological investigations of SCZ and neural tube defects (NTDs) suggesting the existence of overlapping inheritance models and shared etiological risk factors (6).

Grainyhead-like (GRHL) transcription factors modulate proliferation and embryonic development (7). The three vertebrate orthologs of GRHL (GRHL1-3) have been contributed to the etiology and progression of squamous cell carcinoma and neural tube defects in the craniofacial regions (8). The highly conserved *GRHL3* gene (located in 1p36.11 region) has 19 exons encoding a transcription factor that mediates critical target genes during embryonic development, with mice lacking *Grhl3* exhibiting defective neural tube closure (9, 10). A few correlation studies genotyped SNPs located in *GRHL3* in different ethnicities, reaching no valid conclusion regarding the possible association of any allele, genotype or haplotype of these polymorphisms with the risk of disorders associated with the genetic backgrounds (11). Amongst potential gene variants with clinical implications, rs2486668C/G is located within the exon 2 of the *GRHL3*, with the minor allele frequency (MAF) of 0.29 according to the information from the 1000 genome project. This missense variant causes the exchange of aspartate to glutamate. Also, another variation in the exon 16 of this gene is rs545809A/T (MAF=0.15 according to the information from the 1000 genome project), which causes methionine to lysine amino acid exchange (12).

The manifestation of anxiety and neurodevelopmental disorders in SCZ affected patients has lately begun garnering attention (13, 14). To the best of our knowledge, no population-based variation detection study has reported the association between genetic variants spanning the *GRHL* genes. Therefore, this preliminary study was aimed to determine the functional variants in the *GRHL3* gene (rs2486668C/G and rs545809A/T) involved in the pathogenesis of SCZ in a population from Iran.

Materials and methods

Subjects

The present case-control study was performed between January and June 2019. The local Ethics committee of Zahedan University of Medical Sciences (Zahedan, Iran) verified the protocol of the study (Ethical code: IR.ZAUMS. REC. 1398.136), and informed consent was obtained from all the participants.

For determining the association between rs2486668C/G and rs545809A/T polymorphisms and SCZ, we examined 192 unrelated Iranian patients (113 men and 79 women, aged 18–76 years (mean 36.51) admitted to Baharan Hospital (Zahedan, Iran)) who met the criteria for SCZ based on American Psychiatric Association's Diagnostic and Statistical Manual of Mental Disorders (DSM-5) (15). Control group (displaying no sign of psychiatric and systematic disorders) consisted of 198 unrelated Iranian (119 males and 79 females, aged 20-79 years (mean 36.38)) from the same geographic area. These controls were checked for the exclusion criteria of DSM–V operational diagnostic criteria using a structured clinical interview. Additional exclusion criteria were a first-degree relative with psychosis, a history of head injuries, substance abuse or dependency, or any systemic illness and neurological abnormality.

DNA extraction and genotyping

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From each subject, a minimum of 5 ml blood sample was collected into ethylenediamine tetraacetic acid (EDTA)-contained tubes, and DNA isolation was carried out using the standard salting-out technique (16). Gel electrophoresis and NanoDrop spectrophotometry were used to assess the quality and quantity of extracted DNAs. For each SNP shown in Table 1, the specific primers for genotyping each SNP were designed using the PRIMER1 tool (available at http://primer1. soton. ac. uk/primer1.html), and were synthesized by Pishgaman Inc. (Tehran, Iran). The primer sequences specific for the alleles are shown in Table 2. The genomic sequence of two exons within *GRHL3* was amplified using allele-specific amplification refractory mutation system polymerase chain reaction (ARMS-PCR) method. For each PCR reaction, 8 µL of master mix (AmpliqonTaq 2x mastermix, Denmark), 4 µL sterile DNase free water, 1 µL of each primer (10 ng/mL), and 1 µL genomic DNA (~1 ng/µl) were mixed into a microtube to a final volume of 15 µL. The PCR proceeded under the following conditions: initial denaturation at 95 °C for 6 min, 32 cycles of 95 °C for 40 s, specific annealing temperature (according to Table 2 for each variant) for 40 s, and

Table 1. Characteristics of *GRHL3* gene variants.

SNP	Chromosome	Functional Consequenc	Chromosome position	Allele major/minor	Amino-acid Exchange	MAF
rs2486668G/C	1	Missense	24331573	C/G	D [Asp] ⇒ E [Glu]	0.268
rs545809A/T	1	Missense	24364274	T>A	M [Met] ⇒K [Lys]	0.292

SNP: single nucleotide polymorphism; MAF: minor allele frequency.

Table 2. Primers used for detection of rs2486668G/C, and rs545809A/T single-nucleotide polymorphisms in *GRHL3* gene.

Gene polymorphism	Primers	Sequence (5′ to 3′)	Annealing Temp.
rs2486668G/C	F (Common)	GATGAGGGGCTGGGGTGGTCATGG	62 °C
	R (Common)	CTAAATTTGACTCTCCTTACTTGC	
	F (G-allele)	GGAAGCTCAAGGCCGCAACACCG	60 °C
	F (C-allele)	GGAAGCTCAAGGCCGCAACACCC	
rs545809A/T	F (Common)	GAAACACCCAACACAACACAGTTC	62 °C
	R (Common)	GGTGGGTGTGTCATTCCGAGTTTAC	
	F (T-allele)	CCTGTGACTCAAGTGAGGAACGT	63 °C
	F (A-allele)	CCTGTGACTCAAGTGAGGAACGA	

F: forward; R: reverse.

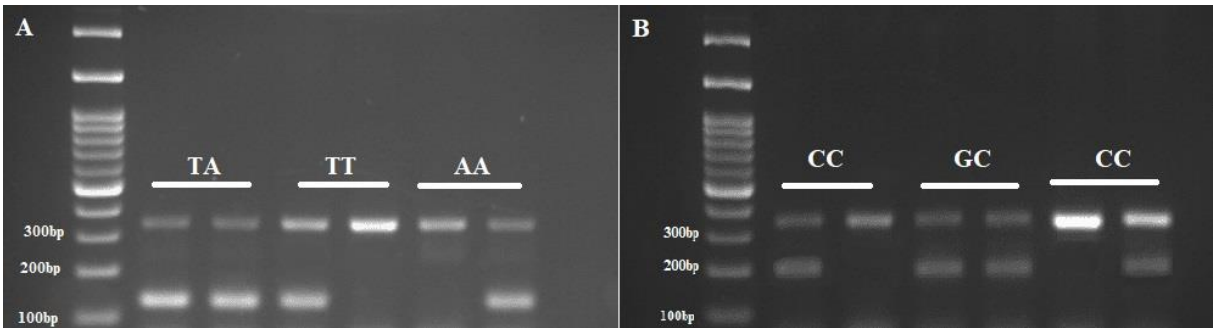

Fig. 1. Genotyping of *GRHL3* gene variants by allele-specific amplified refractory mutation system (ARMS)-PCR. The 130 bp amplicons represent either T or A allele-specific bands for rs545809A/T (A) while the 200 bp band represents either C or G allele-specific amplicons for rs2486668C/G (B). An outer 320 bp band confirmed the accuracy of PCR reactions.

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72 °C for 35 s. A final extension reaction was then performed at 72 °C for 8 min. The amplified fragments were then electrophoresed in 2% agarose gel, and stained with green viewer TM (Pars-Tous Biotechnology, Iran) for visualization under UV light. The amplicon size was 200 bp (for either C or G allele of rs2486668C/G), and 130 bp (for either T or A allele of rs545809A/T) displayed in agarose gel electrophoresis (Figure 1**)**. The accuracy of PCR reactions was confirmed by the presence of an outer 320 bp band amplified by specific common primers of both variants (Table 2 and Figure 1).

Bioinformatics analysis

Bioinformatics analysis was performed to determine the potential effects of rs545809A/T and rs2486668C/G variants located in the coding region of *GRHL3*, exons 2 and 16, respectively. The coding sequence of the *GRHL3* gene (accession number of NC_000001.11) carrying both minor and major alleles was translated via the ExPASy server for later applications. Chou–Fasman database was utilized for predicting the effect of SNPs in *GRHL3*-mRNAs on the secondary structures of the protein. The Chou-Fasman method is based on the local amino acid composition of single sequences in alpha helices, beta sheets, and turns (17). SpliceAid 2 database (a database for RNA target motifs and expression of human splicing factors) was applied to explore the effect of SNPs on the binding sites of transcriptional regulators in *GRHL3*-mRNA; in which an accurate graph represented each sequence. In the target sequences, a positive score was assigned to exonic splicing enhancer (ESE) and intronic splicing silencer (ISS) motifs and a negative score to exonic splicing silencer (ESS) and intronic splicing enhancer (ISE) motifs (18). WebLogo database was employed to illustrate the conservation of SNPs between different organisms (19).

Statistical analysis

Data were analyzed using SPSS v23 (SPSS Inc., IBM, USA). The genotypic and allelic distribution of the SNPs between groups were compared using the X^2 test. The logistic regression analyzes were used to measure the strength of association between variants and SCZ using 95% confidence intervals (CI) and odds ratio (OR). Using SNPAnalyzer 2.0, the analysis of haplotype and linkage disequilibrium (LD) was performed (20). The statistical significance was set at $P < 0.05$.

Results

Association studies

Age and sex were adjusted in SCZ patients and controls. No significant difference was observed regarding age (P = 0.80) and sex (P = 0.71) between studied groups. The mean age in SCZ patients and controls was 36.51±10.52 and 36.38±10.89, respectively. Genotypes and allele frequencies of *GRHL3* polymorphisms rs545809A/T and rs2486668C/G are presented in Table 3 and Table 4, respectively. Our findings indicated that A allele of rs545809A/T is a risk factor for SCZ, and its frequency was higher in SCZ subjects in comparison with healthy subjects (OR = 2.33 95% CI=1.74-3.13, P <0.0001). AA genotype of rs545809A/T in the codominant model was strongly associated with SCZ susceptibility (OR = 4.32 95%CI = 2.40-7.78, P < 0.0001). Moreover, the A allele of rs545809A/T in either dominant or recessive models significantly enhanced SCZ risk (OR = 2.17 95%CI = 1.43-3.31, P < 0.0001, and OR = 3.38 95%CI = 1.98-5.75, P < 0.0001, respectively). No significant difference was noticed between the studied groups regarding the allele frequency of rs2486668C/G (P = 0.08). We did not observe any significant link between patients diagnosed with SCZ and healthy controls under different contrasted genetic models for rs2486668C/G.

The interaction of *GRHL3* rs545809A/T and rs2486668C/G polymorphisms with the risk of SCZ is shown in Table 5. Some of the combined

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genotypes had a significantly higher frequency in SCZ subjects in comparison with the healthy group. In this regard, we found that AAGC, AAGG and ATGG genotypes were significantly associated with SCZ susceptibility (OR = 4.43 95%CI = 1.92-10.21, P <0.0001, OR = 6.29 95%CI = 2.12-18.67, P<0.0001, and OR= 2.24 95%CI= 1.12-4.46, P=0.02, respectively). The haplotype analysis indicated that the [T; G] haplotype of both missense variants has the highest frequency in healthy controls (Table 6). Furthermore, the frequency of [A; G] haplotype was significantly different when comparing the SCZ group and controls (OR = 2.60 95%CI = 1.83-3.67, P < 0.001). The amount of LD for rs545809A/T and rs2486668C/G polymorphisms was equal to 0.13 (P = 0.02), whereas LD > 0.8 represents a powerful linkage disequilibrium (Data not shown).

Table 3. Genotypes and allele frequencies of *GRHL3* rs545809A/T polymorphism in SCZ and control subjects.

rs545809A/T	SCZ, n (%)	Control, n (%)	OR (95%CI)	P-value
Codominant				
TT	54 (28)	91 (46)	1 [reference]	
AT	79 (41)	84 (42)	1.58 (1.00-2.50)	0.05
AA	59 (31)	23 (12)	4.32 (2.40-7.78)	< 0.0001
Allele				
T	187 (48.7)	266 (68.9)	1 [reference]	
A	197 (51.3)	120 (31.1)	2.33 (1.74-3.13)	< 0.0001
Dominant				
TT	54 (28)	91 (46)	1 [reference]	
AT+AA	138 (72)	107 (54)	2.17 (1.43-3.31)	< 0.0001
Recessive				
TT+AT	133 (69)	175 (88)	1 [reference]	
AA	59 (31)	23 (12)	3.38 (1.98-5.75)	< 0.0001
Over-dominant				
TT+AA	113 (59)	114 (58)	1 [reference]	
AT	79 (41)	84 (42)	0.95 (0.63-1.47)	0.80

SNP: single nucleotide polymorphism, SCZ: schizophrenia, *GRHL3*: Grainyhead-like 3, OR: odds ratio, CI: Confidence interval. The p-value <0.05 was regarded as statistically significant.

Table 4. Genotypes and allele frequencies of GRHL3 rs2486668C/G polymorphism in SCZ and control subjects.

rs2486668C/G	SCZ, n (%)	Control, n (%)	OR (95%CI)	P-value
Codominant				
GG	85 (44)	75 (38)	1 [reference]	
CG	74 (39)	77 (39)	0.85 (0.54-1.32)	0.47
CC	33 (17)	46 (23)	0.63 (0.37-1.09)	0.10
Allele				
G	244 (63)	227 (57)	1 [reference]	
C	140 (37)	169 (43)	0.77 (0.58-1.03)	0.08
Dominant				
GG	85 (44)	75 (38)	1 [reference]	
CC+CG	107 (56)	123 (62)	0.77 (0.51-1.15)	0.20
Recessive				
GG+CG	159 (83)	152 (77)	1 [reference]	
CC	33 (17)	46 (23)	0.69 (0.42-1.13)	0.14
Over-dominant				
CC+GG	118 (61)	121 (61)	1 [reference]	
CG	74 (39)	77 (39)	0.99 (0.66-1.48)	0.94

SNP: single nucleotide polymorphism; SCZ: schizophrenia; GRHL3: grainyhead-like 3; OR: odds ratio; CI: confidence interval. The p-value <0.05 was regarded as statistically significant.

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Bioinformatics analyses

In silico analysis revealed that both SNPs located within the *GRHL3* gene caused a methionine to lysine substitution (T→A substitution of rs545809 at position 16 and C→G substitution of rs2486668C/G at codon 2) (Figure 2). Predicting *GRHL3* rs545809A/T and rs2486668C/G effects on the secondary structure of GRHL3 protein revealed that both missense SNPs caused noticeable alterations on the secondary structure of mRNA (Figure 3). Screening of the flanking sequences of SNPs for enhancer and silencer motifs by SpliceAid2 tools predicted silencer motifs such as hnRNP H1, KSRP for T allele of rs545809A/T and silencer motifs such as hnRNP F for A allele of rs545809A/T (Figure 4). Moreover, the conservation of rs545809A/T, and rs2486668C /G SNPs was illustrated by the WebLogo tool, showing highly-conserved regions across multiple mammalian species (Figure 5).

Table 5. Interaction of *GRHL3* rs545809A/T and rs2486668C/G polymorphisms on SCZ risk.

rs545809A/T	rs2486668G/C	SCZ (%)	Control (%)	OR (95%CI)	P-value
AA	CC	6 (4.0%)	5 (3.2%)	1.83 (0.50-6.69)	0.36
AA	GC	26 (17.3%)	7 (4.5%)	5.64 (2.11-15.16)	<0.0001
AA	GG	14 (9.3%)	6 (3.9%)	3.54 (1.18-10.58)	0.02
AT	CC	10 (6.7%)	17 (11.0%)	0.89 (0.35-2.30)	0.82
AT	GC	23 (15.3%)	30 (19.4%)	1.17 (0.55-2.48)	0.69
AT	GG	29 (19.3%)	19 (12.3%)	2.31 (1.05-5.08)	0.02
TT	CC	10 (6.7%)	17 (9.0%)	0.89 (0.35-2.30)	0.82
TT	GC	9 (6.0%)	22 (14.2%)	0.61 (0.23-1.59)	0.31
TT	GG	23 (15.3%)	35 (22.6%)	1 [reference]	

SCZ: schizophrenia, OR: odds ratio, CI: confident interval. The p-value <0.05 was regarded as statistically significant.

Table 6. Haplotype analysis of *GRHL3* gene polymorphisms between SCZ patients and controls.

rs545809A/T	rs2486668G/C	SCZ	Control	OR (95%CI)	P-value
T	G	0.32	0.42	0.64 (0.48-0.86)	< 0.01
A	G	0.32	0.15	2.60 (1.83-3.67)	< 0.001
T	C	0.17	0.25	0.61 (0.43-0.86)	< 0.01
A	C	0.20	0.18	1.14 (0.79-1.63)	0.48

SCZ: schizophrenia; OR: odds ratio; CI: confidence interval. The p-value <0.05 was regarded as statistically significant.

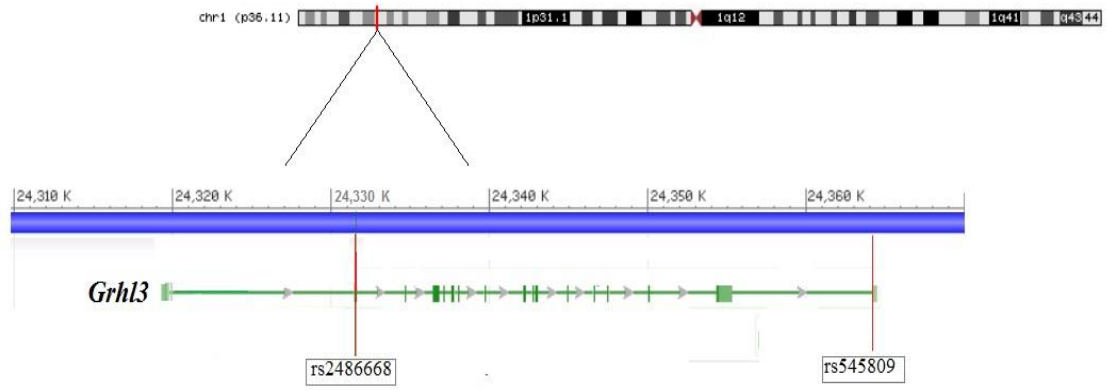

Fig. 2. Schematic diagram of the *GRHL3* gene demonstrating rs545809A/T and rs2486668C/G polymorphisms.

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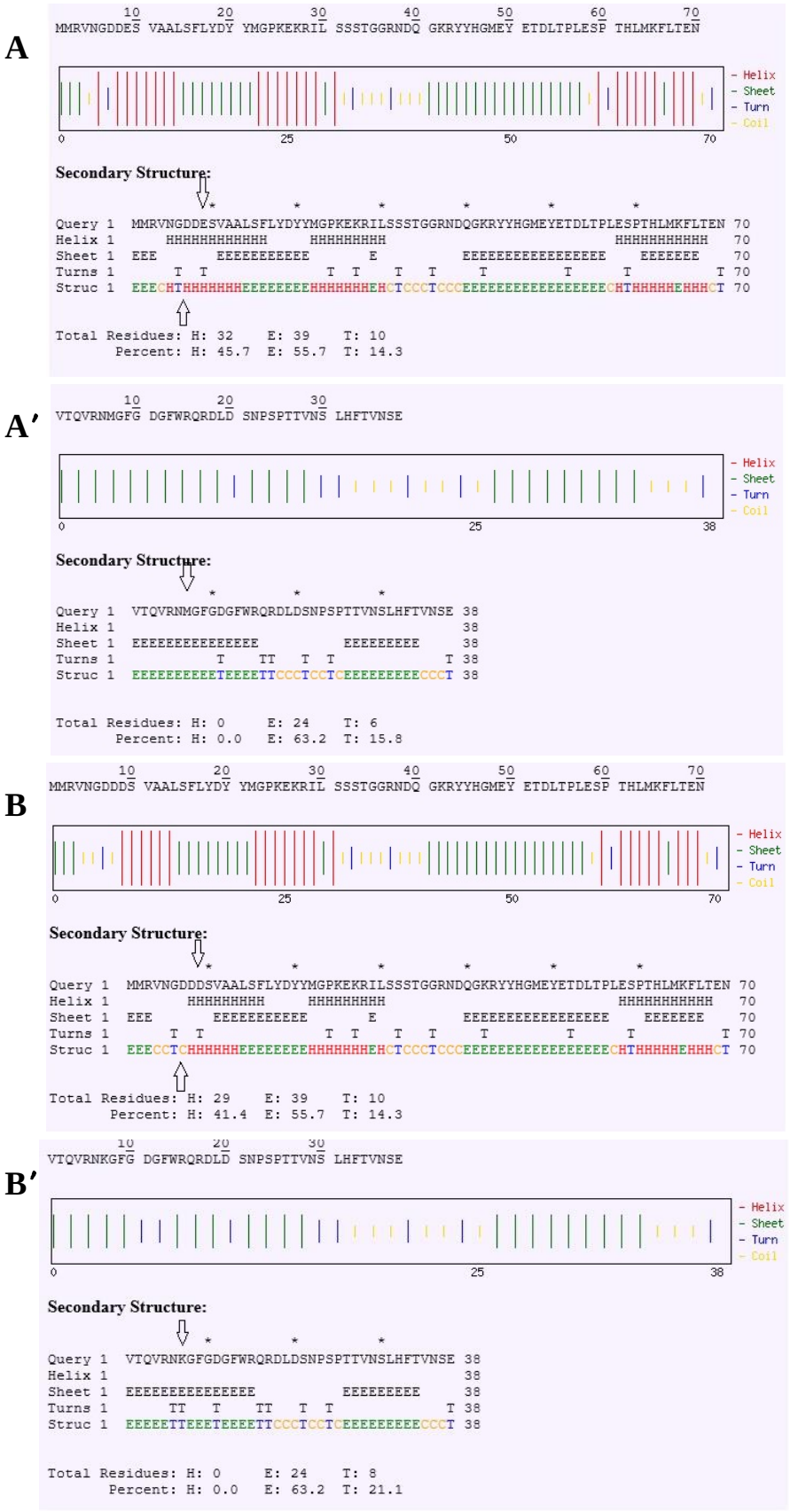

Fig. 3. Chou–Fasman's secondary structure predictions. Secondary structure predictions for (A&B) 9D and 9E, and (A′&B′) 7M and 7K phenotypes, respectively. The residues 9 and 7 are shown by arrowheads.

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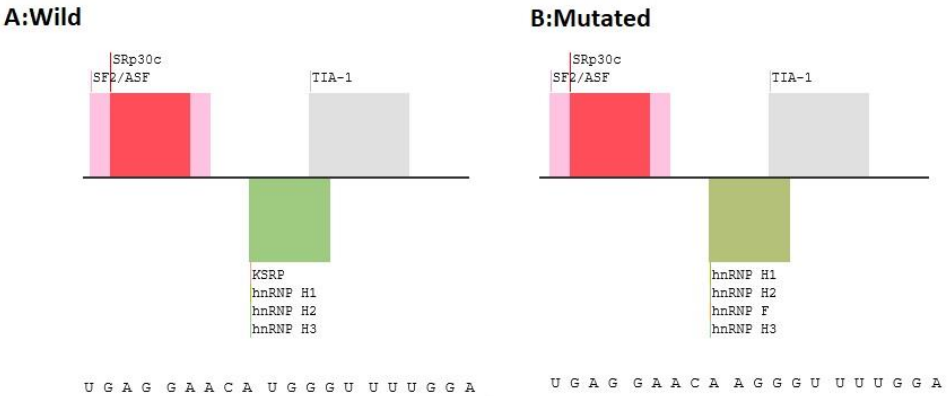

Fig. 4. Screening of the flanking sequences in the rs545809A/T polymorphism via SpliceAid 2 tool.Silencer motifs for (A) the T allele, and (B) the A allele are represented. hnRNP H1 and KSRP are silencer motifs for T allele; hnRNP F is the silencer motif for A allele.

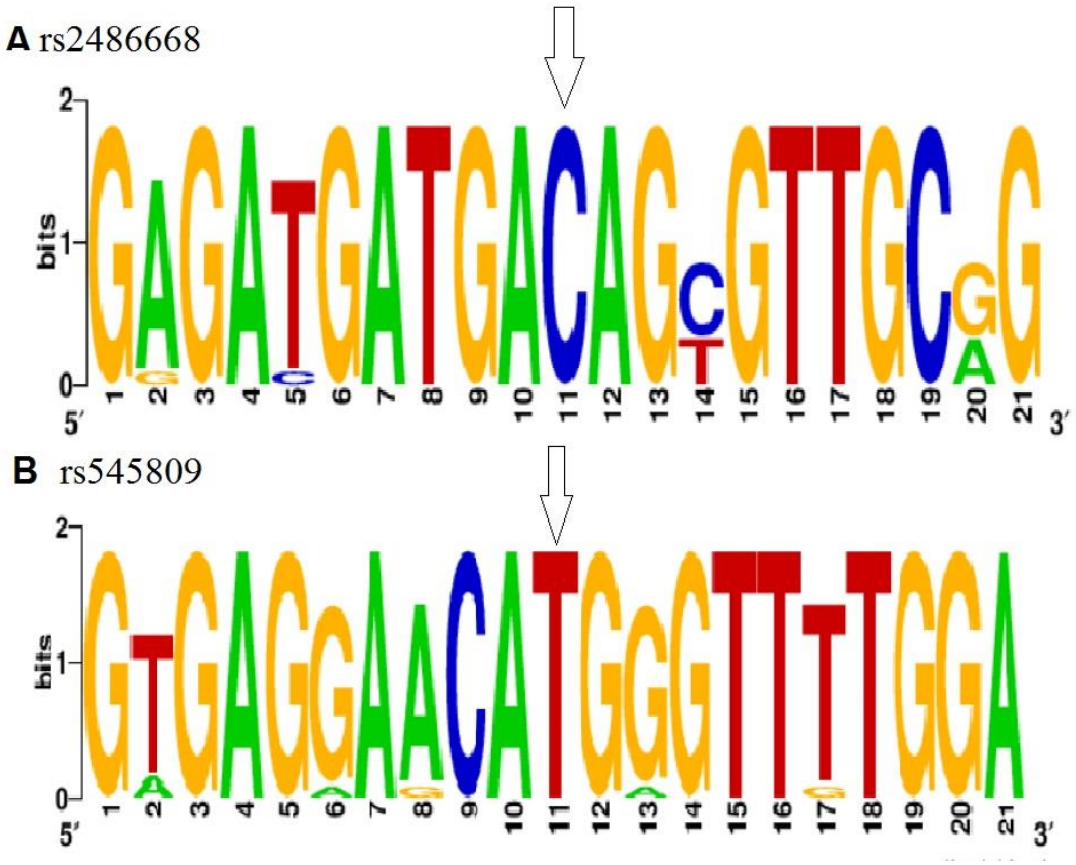

Fig. 5. The conservation of the DNA sequences across multiple mammalian species. A: rs545809A/T; B: rs2486668C/G. Sequences were analyzed using WebLogo server.

Discussion

In the current study, significant differences were detected in the genotype and allele frequencies of the rs545809A/T variant in *GRHL3* gene between the SCZ and healthy groups. The data showed the effect of the A allele of rs545809A/T on the increased risk of SCZ. Moreover, rs545809A/T polymorphism in either dominant or recessive models significantly enhanced SCZ susceptibility. In contrast, no significant association was observed

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between rs2486668C/G polymor-phism and SCZ risk under different genetic models. This represents a lack of association of the rs2486668C/G polymorphism with SCZ susceptibility in the Iranian population. The interaction of *GRHL3* rs545809A/T and rs2486668C/G polymorphisms showed that AA; GC, AA; GG, and AT; GG combined genotypes could increase the risk of SCZ. We have also found that [$A_{rs545809A/T}$; $G_{rs2486668C/G}$] haplotype conferred an increased risk of SCZ in our population. Bioinformatics analysis predicted that T→A substitution in the rs545809A/T variant could alter the mRNA secondary structure and silencer motifs.

The grainyhead-like proteins regulate many genes with different functions, including morphogenesis, tight junction, proliferation, cell adhesion, glutaminolysis, and epidermal phenotype (21, 22). *GRHL2* or *GRHL3* loss-of-function alleles, which have a high-level expression in the non-neural surface ectoderm, lead to NTDs (23). In mice with the *Grhl3* conditional knockout epidermis, downregulation of phosphatase and tensin homolog (Pten), a direct target of *Grhl3* regulation, was observed, which increases the activation of the PI3K/AKT/mTOR pathway (24). The roles of this pathway in the pathogenesis of psychiatric illnesses (25) and SCZ (26) were reported previously. A study by Rifat and colleagues has shown that deletions in the *GRHL3* gene have caused a distinct lower spinal closure defect associated with defective dorsolateral hinge points formation. Based on their observations, conditional loss of *GRHL3* gene within the mice brain affected locomotor activity and thus influenced hyperactivity related behaviors and anxiety (27).

Moreover, the role of maternal micronutrient deficiency in both NTDs and SCZ disorders have been reported previously(6). Craniofacial morphology (kind of minor physical anomalies) occurs in SCZ subjects and illustrates some subtle problems in embryonic growth. However, the involving mechanisms may not be explicitly relevant to those for NTDs (28). Because of the low incidence of both SCZ and NTDs, no studies have established a link between comorbidity of these two disorders (6). There are many studies which have shown the similar effect of gene polymorphisms on SCZ (29-31) and NTDs (32-34) risk.

Although the role of genetic polymorphisms on SCZ risk has been proposed in a large number of studies previously (35, 36), no study has reported the relationship between *GRHL3* gene polymorphisms and SCZ. However, a link between *GRHL3* polymorphisms and some diseases has been examined recently. Kikulska et al. observed that the polymorphisms in the *GRHL3* gene increased the risk of Non-melanoma skin cancer development (37). He and Bian examined the effect of *GRHL3* variants on non-syndromic orofacial clefts susceptibility in the Han Chinese cohort. In this study, no significant differences were observed in genotype and allele frequencies of *GRHL3*-rs2486668C/G and rs545809A/T polymorphisms between cases and controls (12). This study has some limitations as we carried out the research on a homogenous sample of Iranian ethnicity. Moreover, we did not perform sequencing to verify the genotyping results. However, the randomly repeated genotyping for about 20% of the samples showed no error in genotyping.

In conclusion, the current study demonstrated the association of rs545809A/T polymorphism in the *GRHL3* gene with SCZ susceptibility. However, no association was found between the other *GRHL3* missense variant (rs2486668C/G) and the risk of SCZ. Performing this experiment on larger sample sizes with different races may help to explain the role of these polymorphisms in SCZ development.

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Conflict of interest

Authors declare no conflict of interest.

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