

# Family-Based Whole-Exome Sequencing for Identifying Novel Variants in Consanguineous Families with Schizophrenia

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## Abstract

**Background:** Schizophrenia (SCZ) is a complex neuropsychiatric disorder characterized by pronounced genetic heterogeneity. Much of the genetic architecture of the disorder has not yet been clearly elucidated.

**Objectives:** In the present experimental genetic analysis study, we used the whole-exome sequencing (WES) approach to identify the SCZ-related genetic variants in consanguineous multi-affected families.

**Patients and Methods:** The current study was conducted between 2013 and 2015. The patients were recruited from two mental hospitals, including Razi hospital (Tehran, Iran) and Mirza Koochak Khan hospital (Rasht, Iran). All patients were diagnosed based on the DSM-IV-TR diagnostic criteria for SCZ. DNA samples from one proband for each of the three consanguineous Iranian families with twelve affected patients were subjected to WES. Then, a multi-step analysis strategy was employed to identify the genetic variants that may have potentially contributed to SCZ.

**Results:** After variant filtering, WES data revealed two previously known pathogenic mutations (rs450046 in *PRODH* and rs1800497 in *ANKK1* genes) and five novel variants in five genes (*NOS1*, *ANKK1*, *ARVCF*, *GRID1*, and *ANK3*), all of which were predicted to be causing damage by SIFT, Polyphen-2, and MutationTaster tools. Two of these novel variants (c.562C > T in *ANKK1* and c.7649G > T in *ANK3*) showed complete segregation in the families, which makes them good candidates for further case-control studies.

**Conclusions:** By applying WES, both novel and known SCZ pathogenic variants with complete or incomplete segregation in the families with multiple cases of schizophrenic patients were identified.

**Keywords:** Schizophrenia, High-Throughput Nucleotide Sequencing, Exome, Mutation, Family

## 1. Background

A large number of common disorders, including intellectual disabilities, schizophrenia (SCZ), and autism, previously thought to have arisen out of complex multifactorial inheritance, are now considered to be manifested as a result of a heterogeneous collection of rare mutations (1-4), the majority of which have yet to be identified. Identification of the genes responsible for these disorders enables molecular diagnosis of patients and facilitates carrier identification and prenatal diagnosis. Thus, a great deal of effort is now being directed toward identifying the genes responsible for these disorders. Recent advances in next generation sequencing (NGS) technologies, particularly whole-exome sequencing (WES), have significantly facilitated the process of gene identification of disorders.

SCZ is a severe neuropsychiatric disorder, and its wide range of clinical manifestations include positive symptoms such as hallucinations, paranoia, and delusions, as well as negative symptoms such as a flat affect or poverty of speech, lack of motivation, and some degree of cogni-

tive deficit (5). The lifetime prevalence of SCZ is approximately 4.0 per 1,000 worldwide (6). Classical twin, family, and adoption studies indicate a heritability of up to 80% for SCZ, reflecting a strong genetic influence (7, 8).

## 2. Objectives

In the present work, we applied WES as a mutation detection tool to identify SCZ-related genetic variants in a set of three consanguineous Iranian families with more than one schizophrenic member.

## 3. Patients and Methods

### 3.1. Study Population

The patients were comprised of subsets of larger Iranian families with multiple schizophrenic members based on their pedigree analysis (Figure 1). They were recruited from two mental hospitals, including Razi hospital (Tehran, Iran) and Mirza Koochak Khan hospital (Rasht,

Iran) between 2014 and 2015. This study was approved by the ethical committee of Tarbiat Modares University (approval code: 1088706, date 2014/01/28). All participants provided written informed consent prior to enrollment. Three families were considered eligible to enter the study based on the presence of parental consanguinity and a minimum of two children who were affected by SCZ. All patients were diagnosed based on the DSM-IV-TR criteria for SCZ. The families were excluded if the parents and at least two siblings of the patient were not available for blood sampling. The blood samples of the patients, their parents, and two siblings were collected. DNA samples of one patient from each family were subjected to WES, and the SCZ-related candidate variants were confirmed by Sanger sequencing. Samples from the family members were genotyped for the detection of the variants' mode of segregation for SCZ.

### 3.2. DNA Isolation

Peripheral blood samples were obtained and DNA extraction was performed using a QIAGEN DNA extraction kit. The ratio of the absorbance at 260/280 nm and 260/230 nm were measured by the NanoVuePluse (GE healthcare life science). High quality intact DNA with an optical density ratio of 260/280~1.8 and 260/230 > 1.5 were used for further analysis.

### 3.3. WES

For WES, 3 µg (in a volume of 100 µL) of extracted genomic DNA was sheared into fragments of about 150-200 bp. The fragmented DNA was ligated with adaptor sequences using the Illumina HiSeq2000. The agilent sureslect human exome kit (V4) was used for the capture and enrichment of the coding exons and the flanking of the intronic sequences. Paired-end sequencing was carried out using a HiSeq 2000 sequencer. More than 95% of the targeted coding exons were covered by at least 10 non-redundant sequencing reads.

### 3.4. SCZ Gene List

The literature was systematically reviewed to collate all genes associated with SCZ that had been identified in previous human and animal studies. This generated a list of 443 SCZ genes reported in candidate gene studies, case-control studies, genome-wide association studies, and methylation studies, among others.

### 3.5. Variant Filtering

After quality control of the Illumina reads, a burrows-wheeler aligner (BWA) V7.10 (9) was used to align the sequence reads with the human reference genome (UCSC version hg19), and the Unified Genotyper module of the

genome analysis toolkit (GATK) V. 3.3.0 (10, 11) was used for calling variants of both single nucleotide polymorphisms (SNPs) and insertions/deletions (INDELs). Variant annotation was performed by applying ANNOVAR (12). First, we searched for known pathogenic mutations in the SCZ candidate genes, and then the variants with a minor allele frequency (MAF) of more than 5% AF in the databases of the 1000 genomes project (<http://www.1000genomes.org>), dbSNP137, and the exome variant server (<http://evs.gs.washington.edu/EVS/v.0.0.14>) were excluded. An average of 4000 SNPs and INDELs were found after applying these filters. Variants were measured for the likelihood of having a damaging consequence on protein function with SIFT and PolyPhen-2, and then any benign or tolerated variant was excluded.

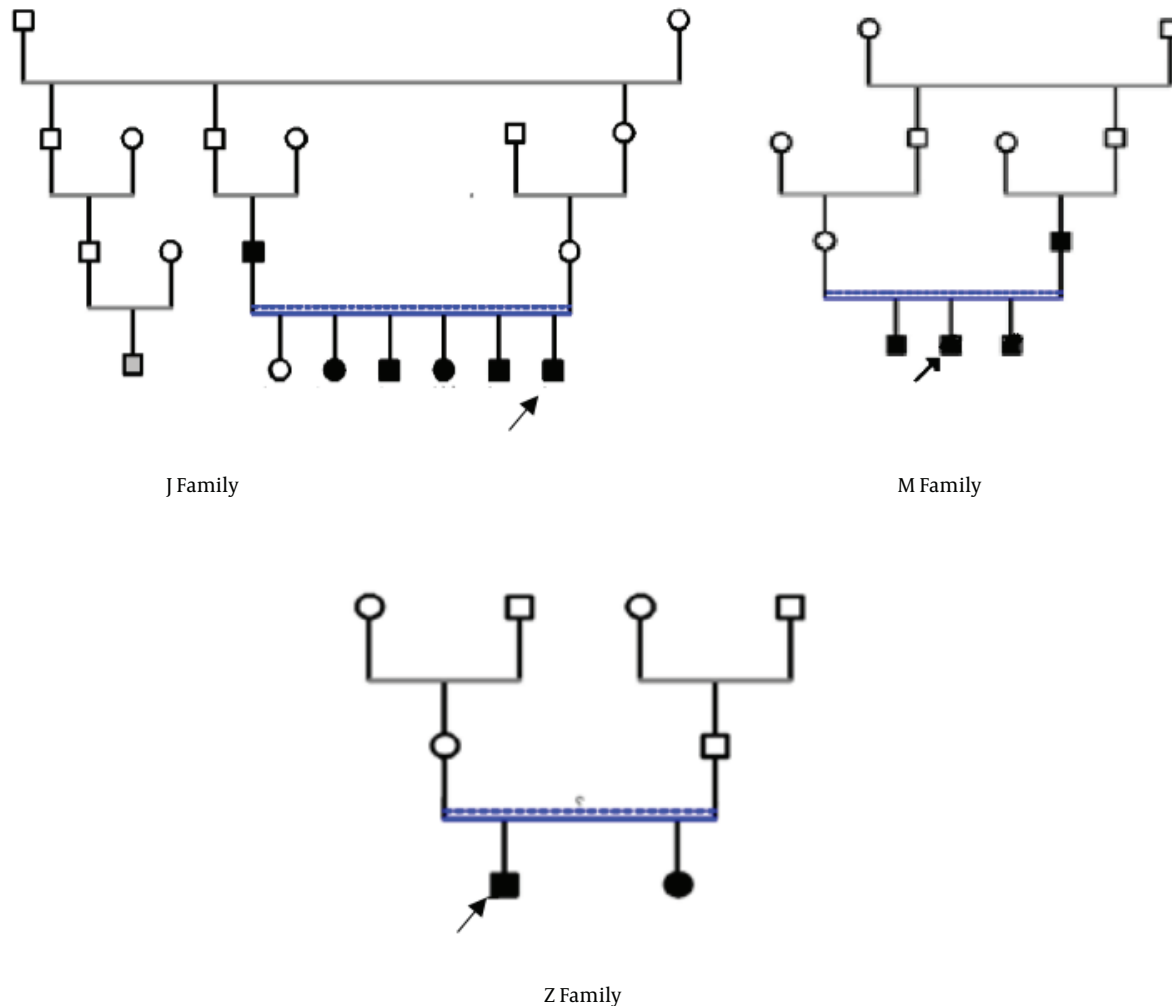
Since the phenotype of all affected subjects was considered to be similar, at first approach, our focus was on identifying the common variants among the affected subjects between families; however, no shared variant related to SCZ was identified. Consequently, we searched for variants within SCZ related genes in each proband separately (depicted in Figure 1 with arrows). The selected variants were further analyzed irrespective of being homozygous or heterozygous.

### 3.6. Mutation Confirmation and Segregation Analysis

For mutation confirmation and segregation analysis, we designed specific primers for the polymerase chain reaction (PCR) amplification of the prioritized variants. We used GeneRunner Primer software to design PCR primers (Table 1). PCR products were visualized on agarose gels and cycle sequenced.

**Table 1.** List of Primers for Sanger Sequencing of Candidate Variants

Gene	Primers	
	F	R
<i>NRCAM</i>	ACACGGCTAGTTCTCTGTGCTG	AGATCAGGCAGCTTGACACAAC
<i>ANKK1</i>	GCAAACATGGGTGCTCTTGG	CATCTCGGCTCCTGGCTTAG
	ACCTTGGAAACAGGCAGATAGC	GTTACACAGTCCCAGGCAAG
<i>PRODH</i>	GGCATCGTGCATTATTTTCTAC	GTAGGGCAGCACCTCCATC
<i>ANK3</i>	AGGAACAGGGTCAGCAAGAAG	ATTGGATGCCAGCAGTTCTG
<i>NOS1</i>	ATAGAAGAGGCCATGGGAGTG	TGTGACTTAATGGGTGGGAGG
<i>ARVCF</i>	TTAGAGGGTGAGGTACAGGGC	CACAGACTGACTGGCGTGAAG
<i>GRID1</i>	TTGGGCAGTGAATAGGGTG	CTGCTGCCTCCAGACAAGATG



**Figure 1.** Pedigrees of Family Trees for Those with Cases of SCZ. Affected Members for Whom WES Was Performed are Indicated with Arrows

#### 4. Results

In the present study, WES along with a multi-step analysis strategy were utilized to identify genetic variants with potential contribution to SCZ in three consanguineous families with multiple members affected by schizophrenia. Overall, we found eight mutations in seven genes, including neuronal cell adhesion molecule (*NRCAM*), proline dehydrogenase (*PRODH*), nitric oxide synthase 1 (*NOS1*), the armadillo repeat gene deleted in Velo-cardio-facial syndrome (*ARVCF*), glutamate receptor, ionotropic receptor, Delta1 (*GRID1*), ankyrin-3 (*ANK3*) and the ankyrin repeat and kinase domain containing 1 (*ANKK1*) (Table 2). The mutations were further subjected to segregation analysis and confirmatory Sanger sequencing (Figure 2).

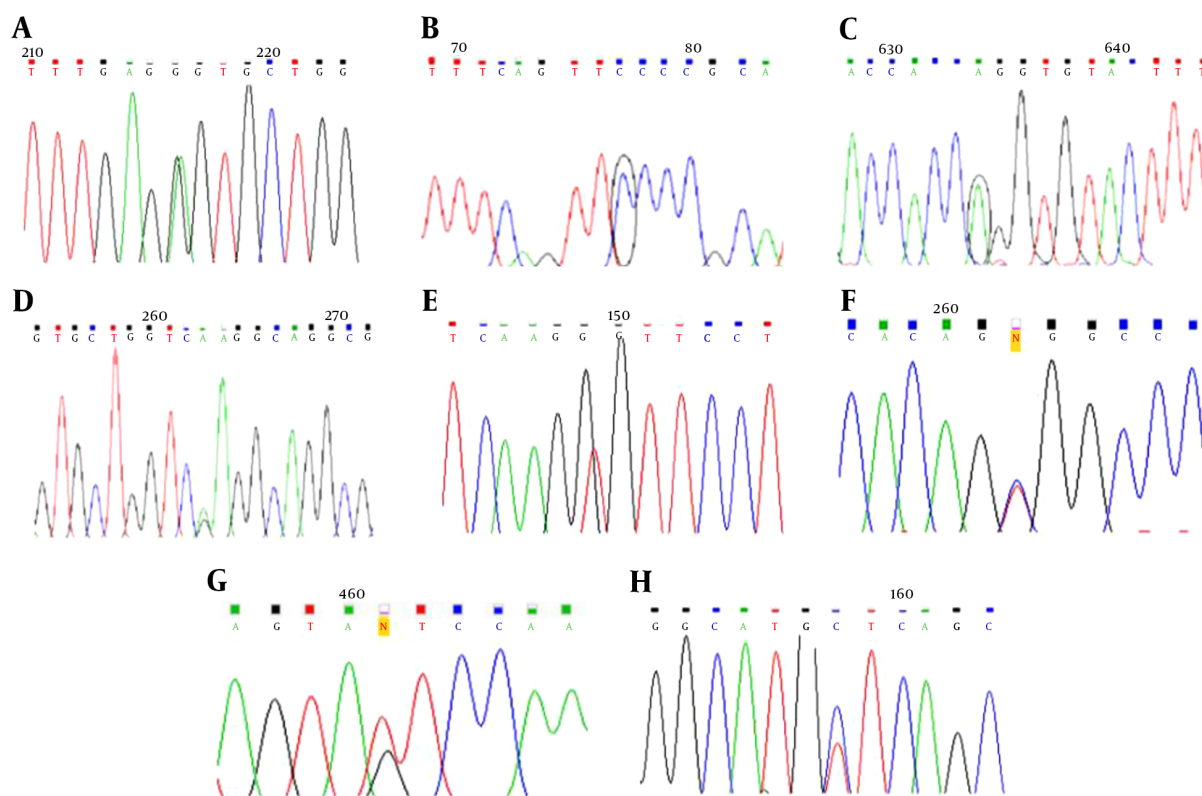
The results of WES revealed a homozygous missense (c.1562G > A) mutation in the *PRODH* gene (rs450046) in the affected subjects from all three families, which was confirmed by Sanger sequencing (Figure 2C). The results of the segregation analysis performed on the families showed incomplete segregation of the mutation, suggesting it is a variant with low or moderate penetrance.

Moreover, we identified a novel heterozygous missense mutation (c. 562C > T) in *ANKK1* in an affected patient of the J family, which was predicted to be damaging by SIFT, Polyphen-2 and MutationTaster tools (Figure 2H). This variant was confirmed by Sanger sequencing, and it showed complete segregation in the J family, making it a significant candidate for further population and case-control studies to order to pinpoint its role in SCZ pathogene-

**Table 2.** The Results of WES, Sanger Sequencing, and Segregation Analysis

Gene	Variant status	Variant in exome/sequencing	Variant in Sanger sequencing	Family	Segregation analysis
<i>PRODH</i>	Known pathogenic	rs450046/NM_016335.4: c.1562G > A: p.Arg521Gln	Confirmed	Z/J M	No
<i>ANKK1</i>	Known pathogenic	rs1800497/NM_178510.1: c.2137G > A: p.Glu713Lys	Confirmed	Z/J M	No
<i>ANKK1</i>	Novel	NM_178510.1: c.562C > T: p.Leu188Phe	Confirmed	J	Yes
<i>NRCAM</i>	Novel	NM_005010.4: c.55_55delC: p.Leu19 <sup>a</sup>	NM_005010.4: c.-104T > C	M	Yes
<i>ANK3</i>	Novel	NM_020987.3: c.7649G > T: p.Ser2550Ile	Confirmed	J	Yes
<i>NOS1</i>	Novel	NM_000620.4: c.2623A > G: p.Ser875Gly	Confirmed	Z	No
<i>ARVCF</i>	Novel	NM_001670.2: c.2774C > A: p.Pro925His	Confirmed	M	No
<i>GRID1</i>	Novel	NM_017551.2: c.2429G > A: p.Arg810His	Confirmed	M	No

<sup>a</sup>The variant was not confirmed by sanger sequencing.

**Figure 2.** Sanger Sequencing of Candidate Variants

A, variant c.2623A > G in the *NOS1* gene in heterozygous form; B, variant c.104T > C in the *NRCAM* gene in homozygous form; C, variant c.1562G > A in the *PRODH* gene in homozygous form; D, variant c.2137G > A in the *ANKK1* gene in heterozygous form; E, variant c.2774C > A in the *ARVCF* gene in heterozygous form; F, variant c.2429G > A in the *GRID1* gene in heterozygous form; G, variant c.7649G > T in the *ANK3* gene in heterozygous form; H, variant c.562C > T in the *ANKK1* gene in heterozygous form.

sis. In addition, a known heterozygote missense mutation (c.2137G > A) in the *ANKK1* gene (rs1800497) was detected in the affected members of all three families (Figure 2D). The mutation was further confirmed by Sanger sequenc-

ing. As segregation analysis within the families showed incomplete segregation of this mutation, this variant is suggested to have low or moderate penetrance for SCZ.

We also found a novel heterozygous missense muta-

tion (c.7649G > T) in the *ANK3* gene in the J family (Figure 2G), which was predicted to be damaging by SIFT, Polyphen-2, and MutationTaster tools. This variant was confirmed by Sanger sequencing and showed complete segregation in the family. This evidence suggests that this variant would also be a significant candidate for population and case-control studies in order to more effectively identify its precise role in SCZ pathogenesis.

A novel heterozygous missense mutation (c.2623A > G) in the *NOS1* gene in an affected subject from the Z family was identified as well. The mutation was further confirmed by Sanger sequencing (Figure 2A). Further bioinformatics analysis by SIFT, Polyphen-2, and MutationTaster tools showed that this variant is potentially damaging. The results of the segregation analysis performed on this family demonstrated incomplete segregation of the mutation, suggesting the mutation was a variant with low or moderate penetrance.

We also identified a novel heterozygous missense mutation (c.2774C > A) in the *ARVCF* gene in an affected subject from the M family, which was confirmed by Sanger sequencing (Figure 2E). Bioinformatics analysis by SIFT, Polyphen-2, and MutationTaster tools showed that the variant was potentially damaging. The results of the segregation analysis carried out on this family showed incomplete segregation of the mutation, suggesting it was a variant with low or moderate penetrance.

WES results further showed a novel heterozygous missense mutation (c.2429G > A) in the *GRID1* gene in an affected subject from the M family, which was confirmed by Sanger sequencing (Figure 2F). Analyzing the mutation with SIFT, Polyphen-2, and MutationTaster tools predicted that this variant was damaging. The results of the segregation analysis carried out on this family showed incomplete segregation of the mutation, suggesting that it is a variant with low or moderate penetrance.

A homozygous single-base deletion frame-shift mutation (c.55\_55delC p.Leu19X) in the *NRCAM* gene in an affected member from the M family was identified as well, but it was not confirmed by the Sanger sequencing results. However, Sanger sequencing revealed another homozygous T > C mutation in 5'UTR of this gene (rs1269634), functionally involved in signal peptide splicing (Figure 2B). This variant showed complete segregation with the disease through this pedigree as all homozygote-affected individuals and the normal heterozygote mother carried the mutation. However, this variant is regarded as polymorphic with MAF = 0.45 in the 1000 Genome Project and dbSNP. Thus, further case-control association and function analysis studies of this variant are required.

## 5. Discussion

Despite extensive investigations into SCZ, the details of its etiology remain largely unclear. Family, twin, and adoption studies have consistently suggested that, overall, genetic variation is the most important factor, which would be an indication of its high heritability. Up to now, mutations in over 100 genes have been found to be responsible for SCZ (2, 13). WES allows for the simultaneous screening of mutations in a large number of genes and is more cost effective compared to other genetic testing options when the number of genes is large. This is particularly true when the condition under study are extremely heterogeneous, as is the case with SCZ. In the present study, we investigated three consanguineous families with multiple cases of SCZ. The WES results showed eight mutations in seven genes, including *NRCAM*, *PRODH*, *NOS1*, *ARVCF*, *GRID1*, *ANK3*, and *ANKK1*.

*NRCAM* is located in the chromosome 7q region and encodes a neuronal cell adhesion molecule (CAM), with multiple immunoglobulin-like C2-type domains and fibronectin type-III domains. This ankyrin-binding protein is involved in neuron-neuron adhesion and promotes directional signaling during axonal cone growth. Of the candidate biological pathways for SCZ, the CAM pathways have repeatedly been linked to both psychosis and neurocognitive dysfunctions (14-16). In their study, Zhang et al. (17) investigated the association between CAM pathways and SCZ in the Chinese population. They reported that *NRCAM*, as one of the candidate molecules in this pathway, showed a modestly significant association with schizophrenia. The results of our study indicated a T > C mutation in 5'UTR of this gene, which revealed that it played a role in signal peptide alternative splicing. As alternative splicing generates multiple transcript variants of this gene, and different isoforms may functionally be involved in CAM pathways, suggesting that this mutation is a potentially important variant.

The c.1562G > A mutation in the *PRODH* gene (rs450046) is known as a pathogenic mutation for SCZ. *PRODH* encodes the proline dehydrogenase enzyme that catalyzes the first step in proline catabolism. The gene is located on chromosome 22 (22q11.2), a candidate region for several psychiatric disorders. It is noteworthy that one third of patients with 22q11 microdeletion syndrome (22q11DS), also known as DiGeorge or Velo-cardio-facial syndrome, develop SCZ or schizoaffective disorder (18, 19). Moreover, while the prevalence of the chromosome 22q11 microdeletions in the general population is one in 4,000 individuals, its frequency is estimated to be approximately 1% in adult patients with SCZ (20, 21). Therefore, as with other genes located in this region, *PRODH* is highly



implicated as a contributor to SCZ onset. Proline is a precursor of the neurotransmitter glutamate and has several characteristics suggesting that it functions as a CNS neuromodulator. Moreover, whereas high levels of plasma proline are found on a relatively common basis in patients with 22q11DS (22, 23), some evidence suggests that hyperprolinemia may lead to neurocognitive dysfunction (24) and may be involved in the cognitive and psychiatric features of 22q11DS [23]. In support of the functional significance of hyperprolinemia in SCZ, familial studies have indicated that *PRODH* deletion and *PRODH* missense mutations (25, 26) are functionally related to both moderately and severely decreased *PRODH* enzyme activity (25), which is also associated with hyperprolinemia in schizophrenic patients (26).

Another potentially important gene located in the region of chromosome 22q11 is the *ARVCF* gene (27). *ARVCF* plays a significant role in the cadherin-catenin complex and may modulate cadherin-mediated junction structures and cell-cell adhesion in various cell types during the development of different organs. It is conceivable that *ARVCF* influences the migration of neural crest cells by controlling the cell-cell adhesion. Thus, the lack of *ARVCF* might contribute to a migratory defect of neural crest cells. Given the role of *ARVCF*, it can be considered as a candidate gene for susceptibility to schizophrenia via alterations in neural development, which are linked with the neurodevelopmental hypothesis of schizophrenia. Previous family-based schizophrenia association studies showed that SNPs in the 3' region of *ARVCF* are linked to the risk of developing schizophrenia (27, 28). The bioinformatics analysis of reported *ARVCF* missense mutation in our study showed that this variant can alter *ARVCF* function, suggesting it may function as a potentially SCZ-related mutation.

The *ANKK1* gene is closely linked to dopamine receptor D2 (DRD2) on chromosome band 11q23.1. Deregulation of the dopaminergic neurotransmission system has been implicated in the pathogenesis of SCZ (29). Dopamine D2 receptor (DRD2), as a modulator of the dopamine neurotransmitter, plays a regulatory role in this system. The impairment of its function is known to be implicated in the development of SCZ. Currently, the most effective treatments for SCZ principally antagonize this receptor (30). DRD2 density has been shown to be increased in the brains of schizophrenic patients (31). The rs1800497 locus, which was previously thought to be located in DRD2, has been identified within exon 8 of *ANKK1* and likely modulates the function and expression of DRD2 due to its close proximity (32).

The *ANK3* gene encodes ankyrin-G with multiple isoforms in neurons, sharing three conserved domains including an N-terminal membrane association domain, a

spectrin-binding domain and a C-terminal tail (33). Numerous variants of this gene have been associated with bipolar disorder (BD) in genome-wide association studies (GWAS) and a large genome-wide study combining SCZ and BD (33, 34). A number of studies show that ankyrin-G exists in synapses. Additionally, its role in the axons at nodes of Ranvier is well characterized (35).

The first report of correlation between nitric oxide (NO) metabolism and SCZ disorder was published in the 1960s in Russia. No additional details regarding this connection were clarified until the beginning of the 1990s. In the brain and peripheral nervous system, NO displays many properties of a neurotransmitter. *NOS1*, an enzyme catalyzing the production of NO from L-arginine is functionally related to glutamate and dopaminergic systems. Recent GWA and WES studies defined the *NOS1* gene as a remarkable candidate gene in SCZ (36). In our study, the reported missense mutation in *NOS1* has the potential to influence the enzyme function, which provides supporting evidence that this mutation may lead to a biological susceptibility to schizophrenia.

The delta family of ionotropic glutamate receptors consists of glutamate D1 (GluD1) and glutamate D2 (GluD2) receptors. Although the role of GluD2 in the regulation of cerebellar physiology is well known, the function of GluD1 in the central nervous system, which is encoded by the *GRIN1* gene, remains elusive. Copy number variation studies showed that this gene is a potential candidate for autism spectrum disorder. Moreover, GWA studies have illustrated the role of GluD1 in neuron networks, which makes it an effective gene in SCZ, BD, and major depression (37).

To the best of our knowledge, this is the first work applying WES-based comprehensive molecular analysis in an investigation into Iranian families with SCZ. We identified both novel and known pathogenic variants with complete or incomplete segregation in the families. While, variants with incomplete segregation would be regarded as low to moderate penetrance effectors, complete segregation of two novel variants (in the *ANKK1* and *ANK3* genes) marks them as significant candidates for population or case-control studies. As Iran is a country with a high consanguineous marriage rate, these findings may provide supporting evidence for the efficiency of WES in the detection of causative or susceptibility variants in patients with SCZ.

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## Footnotes

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**Conflict of Interest:** The authors declare that they have no conflict of interest.

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