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Association Between *GSK3β* Gene Polymorphisms (rs334558 and rs3755557) with Schizophrenia Risk

Maryam Montazeri Dehbarez [1], Dor Mohammad Kordi Tamandani[1], Nasim Naeimi[1]*, Shahram Vaziri[2] and Mohammad Shekari[3]

[1] Department of Biology, Faculty of Science, University of Sistan and Baluchestan, Zahedan, Iran
[2] Department of Clinical Psychology, Faculty of Psychology and Social Sciences, Islamic Azad University of Rodehen Branch, Tehran, Iran
[3] Center for Research of Molecular Medicine, Hormozgan University of Medical Sciences, Bandar-Abbas, Iran

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Corresponding authors:
✉ N. Naeimi
naeimi@science.usb.ac.ir

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ABSTRACT

Numerous studies have recognized *GSK3ß* as a gene associated with susceptibility to schizophrenia (SZ), highlighting its essential function in neurodevelopment and its potential as a target for antipsychotic medications. In this study, we examined the connection between two polymorphisms of the GSK3ß gene, rs334558 and rs3755557, and their potential link to the risk of schizophrenia. We performed a case-control analysis to investigate this relationship within a population from southern Iran. DNA samples were isolated from 100 cases with schizophrenia and 100 normal controls. Then, we used a polymerase chain reaction (PCR) followed by the restriction fragment length polymorphism method (PCR-RFLP) to examine the *GSK3ß* SNPs (rs334558 and rs3755557) patterns. The frequency of genotypes, alleles, and the Hardy-Weinberg equilibrium were evaluated. Research has shown that individuals with the TA genotype and those with the combined TA+AA genotype at the rs3755557 (T/A) locus have a significantly higher risk of developing schizophrenia (OR= 2.94; 95% CI: 1.54-5.61, P= 0.0001) and (OR= 3.78; 95% CI: 2-7.13, P< 0.0001), respectively. Similarly, the GA genotype and the combination of GA+AA genotypes at the rs334558 (G/A) locus also notably increase the risk of schizophrenia, (OR= 6.62; 95% CI: 3.4-13; P< 0.001) and OR = 7.63; 95%CI: 3.95-14.7; P< 0.001), respectively. In this investigation, our findings have elucidated a substantial correlation between polymorphisms in the *GSK3ß* gene and the susceptibility to schizophrenia. The analysis indicated that variations in genotype frequencies between the control and the patient group could serve as a diagnostic criterion for schizophrenia. It is recommended that subsequent research involving larger sample sizes across diverse genetic populations be conducted to corroborate the current findings.

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Introduction

Psychosis or schizophrenia (SZ) is a condition characterized by disruptions in typical emotional reactions and cognitive functions. It manifests through delusions and hallucinations, disorganized thoughts, and a range of symptoms affecting mood, movement, behavior perception, and memory (Costantini *et al.*, 2020; Sargazi *et al.*, 2022). This condition predominantly affects individuals between the ages of 25 and 35 with an estimated prevalence rate of approximately 1% (Brannock *et al.*, 2020). Genetic and environmental factors can effectively treat this

disorder (Nia *et al.*, 2021). The causes of schizophrenia are variable, and 80% of genetic factors are known as risk factors. Suspicious genes or specific clinical phenotypes in psychiatric patients were suggested through genome-wide association studies (GWAS) (Ripke *et al.*, 2013). About 287 genomic loci associated with SZ were identified in 2020 (Trubetskoy *et al.*, 2022).

Cognitive impairment which is a symbol of schizophrenia is associated with various factors. These factors include changes in synaptic plasticity and neural networks, growth disorder or disruption in signaling pathways, structural organization that affects brain flexibility, and ultimately neurodegeneration (Mould *et al*., 2021; Kharawala *et al*., 2022). Glycogen synthase kinase 3β (*GSK3β*) is located on human chromosome 3q13.33 and consists of 12 exons. It is expressed in the hippocampus with 286 kb length (Hur & Zhou 2010; Latapy *et al*., 2012). This gene contains two serine-threonine kinases, is involved in metabolism and immunity, and is also a factor in psychiatric diseases (Kaidanovich-Beilin and Woodgett, 2011).

GSK3β is critical in signaling pathways, *WNT*, and *PI3K/AKT* as it is involved in neurodevelopmental abnormalities of the nervous system, and some pathological cellular processes and intracellular cascades are carried out through *AK*. This pathway is required for the proper development of entering the fetal forebrain, and these brain regions are damaged in disorders such as bipolar disorders and SZ (Wexler & Geschwind 2011; Pan *et al*., 2015).

GSK3B plays multiple roles in the development of the nervous system, including modulation of synaptic plasticity, intracellular trafficking, apoptosis, and regulation of gene transcription. *GSK3B* is involved in various critical processes during neural network development and regulation (Chen *et al*., 2020). Emamian *et al*. (2004) showed a decrease in *GSK3β* protein and *GSK3β* mRNA levels in the frontal cortex of SZ subjects. Different results have been recorded in the study of the relationship between the *GSK3β* gene and schizophrenia. Accordingly, this issue needs further investigation in other populations.

Some single nucleotide polymorphisms (SNPs) within the *GSK3B* gene locus have been linked to a heightened risk of developing bipolar disorder and SZ. This association is thought to be due to their impact on brain structures and associated with mental health conditions (Rampino *et al*., 2021).

Studies conducted by Li *et al*. (2011) indicate that SNPs in the *GSK3β* gene can influence both transcription and splicing processes, and enhance the activity of the *GSK3β* promoter. This gene is related to various neurodegenerative diseases. Rs33455 was associated with the risk of Parkinson's disease (Kwok *et al*., 2005). Tang *et al*. (2013) showed that in schizophrenic and bipolar patients, *GSK3B* polymorphism (inactivating mutation 334558) may be used for diagnosis.

In the investigation of SNPs (rs334555, rs119258668, and rs11927974) of the *GSK-3β* gene, it was observed that it may be associated with internal bipolar depression (Saus *et al*., 2010). The variant rs334558 of the *GSK-3β* gene has been linked to conditions such as unipolar depression, bipolar disorder, and dementia (Terao *et al*., 2020).

Considering the importance of identifying and treating schizophrenia, investigating polymorphisms related to effective genes can provide a clearer perspective to achieve these goals. The current study examined the potential interaction effects of the *GSK3β* gene polymorphisms rs334558 (G/A) and 3755557(T/A) about their association with various neurological diseases, particularly the gene's involvement in the development of schizophrenia.

Materials and Methods

Subjects and data collection

We performed an association analysis using a case-control design with samples obtained from the population in southern Iran, specifically at Shahid Mohammadi Hospital in Bandar-Abbas.

According to the following conditions, we selected 100 cases and 100 controls. Based on the Diagnostic and Statistical Manual of Mental Disorders, Fourth Edition (DSM-IV), patients with schizophrenia were diagnosed.

Patients underwent a comprehensive evaluation conducted by a specialized psychiatrist. This assessment was supplemented by a thorough review of medical records and interviews with

family members to gather clinical insights. All pertinent information, including personal history, hospital records, and family background, was meticulously documented. Detailed accounts regarding the onset and progression of mental health disorders were collected.

The selection criteria of the participants in this study were defined as follows: People were included only if they had no history of mental health disorders. In contrast, the exclusion criteria specified that individuals with a family history of psychiatric disorders, a history of alcoholism, epilepsy, neurological diseases, substance abuse, or other symptomatic psychoses, as well as individuals suffering from physical problems, were not eligible to participate. All participants, including both schizophrenia patients and healthy controls, were sourced from Hormozgan province in Iran. Informed consent was obtained from all participants.

We identified two risk SNPs, rs3755557 and rs334558, which are located in the *GSK3β* promoter region (-1727A/T, -50C/T respectively). This study aimed to elaborate further on the important role of the *GSK3β* gene polymorphism in the genetic susceptibility to SZ and determine the association between *GSK3β* SNPs in patients with SZ. The genomic structure of the *GSK3β* gene, along with the positions of the evaluated SNPs, is illustrated in Figure 1.

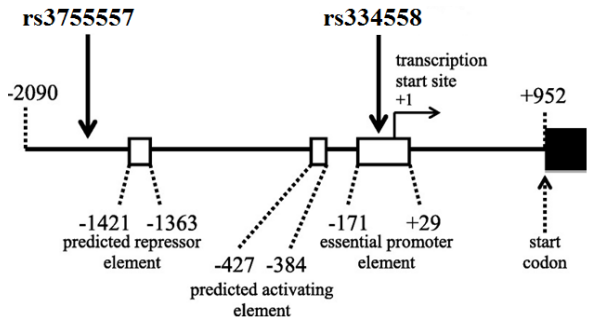

Fig. 1. SNPs in the promoter region of the *GSK-3β* gene regulatory cis-elements for transcription are indicated according to the report of Lau *et al*. (1999).

SNP genotyping

In the next stage of the study, 5 ml of blood was drawn from both the case and control groups and transferred into EDTA tubes. These samples were subsequently frozen at -20°C. Genomic DNA was extracted using the salting-out technique. The specific polymorphisms were selected based on prior research and data from various databases SNP (http://snpper.chip.org/). SNPs, specifically rs334558 and rs3755557, were genotyped using PCR-based RFLP analysis for all participants. Details regarding the primers and restriction enzymes employed are provided in Table 1. The PCR procedures for both SNPs were identical and conducted in a total volume of 25 µl. This included 1 µl of 100 ng genomic DNA, 2.5 µl of 10X, PCR-buffer, 0.75 µl of 50mM, $MgCl_2$, 0.5 µl of 10mM, dNTPs, 0.5 µl each of 10ppm, forward and reverse primers, and 0.25 µl of 5U, super *Taq* polymerase. PCR for the rs3755557 variant was performed in the phases of initial denaturation (95°C for 5 minutes), 40 cycles [denaturation (95°C for 45 s), annealing (58°C for 45s), and extension (72°C for 45s)], final extension (72°C for 7 minutes). In contrast, PCR conditions for rs334558 were performed at annealing (61°C for 45 s) and 35 cycles. Subsequently, a 15-µl aliquot of the PCR product was fully digested using 0.5 U of a restriction enzyme and then analyzed using agarose gel electrophoresis, with gels prepared at 3% for rs3755557 and 1.5% for rs334558.

Statistical analysis

The relationship between polymorphisms at the *GSK3ß* gene, specifically rs334558 and rs3755557, and also the risk factor of schizophrenia was evaluated by calculating OR and 95% CI. To examine the association between discrete variables, Fisher's exact test was employed, while the chi-square tests were utilized for other categorical data assessments. The SPSS software (version 21, Inc USA) was used to verify the genotyping distribution of single nucleotide polymorphisms for Hardy-Weinberg equilibrium. A significant difference was defined as a P-value was calculated less than 0.05.

Results

Specifications of samples

In two groups, 100 SZ patients and 100 healthy controls according to reviews. In the control group, there were 85 (85%) males and 15 (15%) females. There were 58(58%) and 42(42%) males and females in the case group,

respectively. The average age of the control group was 31.48± 9.70 years, while that of the patient group was 40.2± 10.06 years (P> 0.05). Both groups had ages ranging from 20 to 49 years. The study also examined the genotypic frequencies and their association with the risk of developing SZ.

Table 1. Details of the using primers for GSK3β rs334558 (G/A) and 3755557(T/A).

SNPs	Primer sequence (5'→3')	Tm (ºC)	Product length (bp)
rs334558 (G/A)	5'- TTTATAGACGCCCTCCCTTCGCTT-3' 5'- TTCCTTCCTTCCTTTGTCACTTGGC-3'	58	722bp
rs3755557 (T/A)	5′-GCCGCCATCCTGATTGTAATCCAGTGG-3′ 5′-GCTTACTTTGTTCTGTCCCAAGTCC-3′	61	120 bp

Genotype frequency analysis

The frequency of AA, AG, and GG genotypes for rs334558 (G/A) and rs3755557 (T/A) polymorphisms in the GSK3β gene was evaluated using the chi-square method and a significant difference was observed between the two groups of cases and controls with P <0.05 (Table 2). 100 SZ individuals and 100 controls were successfully genotyped for the two *GSK3β* polymorphisms. The frequencies of genotypes for the rs334558 (G/A) and rs3755557(T/A) polymorphism locus of *GSK3β* were assessed as a significant relationship.

In the current study, the genotypes rs334558 (G/A) were observed in both control and schizophrenia samples. GG genotype was found in 61% of controls and 17% of SZ samples, GA genotype appeared in 39% of controls and 72% of SZ samples, while the AA genotype was not present in SZ samples (0%) but accounted for 11% in controls. The occurrence of homozygous and heterozygous genotypes in the SZ and healthy groups showed a significant difference (P < 0.05). We observed a significant association between patients and the GA genotype of *GSK3β*, rs334558 (OR= 6.62; 95% CI: 3.4-12.86; P <0.0001). The combined genotypes GA+AA genotypes at rs334558 increased the risk of schizophrenia disorder (OR= 7.6; 95%CI: 3.95-14.75; P< 0.0001). The GA genotype and GA+AA combined genotype were more in the SZ group (Table 2).

The control samples exhibited genotypes at rs3755557 with frequencies of TT (37%), TA (52%), and AA (11%). In contrast, the schizophrenia group showed different frequencies: TT (13%), TA (70.2%), and AA (17%). A notable distinction was observed between the two groups (P< 0.05). The TA genotype at the rs3755557 SNP was linked with a significantly increased risk for schizophrenia (OR= 2.94; 95%CI: 1.54-5.61; P< 0.001). The combined genotypes TA+AA at the rs3755557 also increased the risk of schizophrenia (OR= 3.78; 95% CI: 2-7.13; P< 0.001).

Discussion

Neurological diseases are important causes of disability and mortality and affect the lives of millions globally. Investigating the association of polymorphisms with the risk of SZ is important, helps to understand the mechanisms, and provides an index for prognosis.

Schizophrenia is mostly controlled by a polygenic mechanism. The role of heredity has been investigated in many studies in the development of schizophrenia and it shows that genetic predispositions play an important role in its occurrence (van de Leemput *et al.*, 2016).

Polymorphisms in genes can significantly influence the clinical manifestations of diseases, leading to the observation of varying symptoms among patients who possess different genotypes at the same genetic locus.

Our analysis of the rs334558 (G/A) polymorphism revealed that individuals with the GA genotype were approximately 1.84 times more prevalent in the SZ group compared to the control group (P< 0.0001) and the increase is specifically related to the rs334558 (G/A). This suggests that the GA genotype may be an important genetic risk factor for schizophrenia in the Iranian population. Moreover, in rs3755557 (T/A), the frequency of homozygous AA in control and SZ was shown to have a significant difference (1.54 times; P<0.001). TA+AA genotype is about 1.33 times higher in the SZ group. A significant relationship between polymorphisms and the risk of SZ was observed,

and it indicates that these two polymorphisms are related to SZ susceptibility.

The odds ratio shows risk indicators and predicts the onset of the disease (Paccalet *et al.*, 2016).

In our study, there were two GSK3β polymorphisms according to Table 2. The results showed that rs334558 (G/A) with GA genotype (OR= 6.62; 95%CI: 3.4-12.86; P<0.0001) and rs3755557 (T/A) with TA genotype (OR= 2.94; 95%CI: 1.54-5.61; P< 0.001) could be a risk for schizophrenia because these relationships were significant (Table 2).

Table 2. The comparison of genotype frequencies in rs 334558 (G/A) and rs 3755557 (T/A) in the *GSK3β* gene in SZ and control groups

Genotypes/Alleles	SZ (n=100)	Control (n=100)	P- Value	OR	95% CI
rs334558 (G>A)					
GG	17 (17%)	61 (61%)	Ref= 1	-	-
GA	72 (72%)	39 (39%)	**0.0001***	6.62	3.4- 12.8
AA	11 (11%)	0 (0%)	**0.0001***	5.33	2.8- 13.84
GA+AA	83 (83%)	39 (39%)	**0.0001***	7.6	3.95- 14.75
Allels					
G	122 (61%)	165 (82.5%)	Ref= 1	-	-
A	78 (39%)	35 (17.5%)	**0.0001***	4.02	1.05- 3.8
rs3755557(T>A)					
TT	13 (13%)	37 (37%)	Ref=1	-	-
TA	70 (70%)	52 (52%)	**0.001***	2.94	1.54- 5.61
AA	17 (17%)	11 (11%)	**0.001***	3.1	2.5- 8.32
TA+AA	87 (87%)	63 (63%)	**0.001***	3.78	2- 7.13
Allels					
T	141 (70.5%)	156 (78%)	Ref=1	-	-
A	59 (29.5%)	44 (22%)	**0.001***	2	1.51- 3.8

SNPs= single-nucleotide polymorphisms, OR= Odds ratios; 95%CI= % Confidence interval, schizophrenia (SZ). *= P < 0.05.

The identified genetic abnormality is associated with *GSK-3*-related mental illnesses. Lithium serves as a therapy for Alzheimer's disease by inhibiting the *GSK-3* activity. In comparison to a placebo, lithium has been found to significantly reduce symptoms and complications, demonstrating SD= -0.41, 95%CI: -0.81 to -0.02, and P= 0.04 (Matsunaga *et al.*, 2015).

Numerous studies have indicated that the *GSK3β* gene is crucial for neurodevelopment, However, its genetic risk variants have not been studied (Szczepankiewicz *et al*., 2006). *GSK3β* gene SNPs of rs496250 and rs12630592 are associated with SZ, and these polymorphisms also affect the response to antipsychotic drugs (Del'Guidice *et al*., 2015). Also, regarding this gene, Li *et al*. (2011) conducted a study on 2550 samples in the Chinese population and reported that rs334558 (G/A) could affect the expression of GSK3β, which wasn't consistent with the results in Koreans.

In another research on depressed patients and healthy subjects, in 4 SNPs, including rs334558 (G/A), a notable correlation was found between this SNP and the administration of the antidepressant fluoxetine and citalopram (Tsai *et al*., 2008). In the 2023 study, the results showed that SNPs rs6438552, rs12630592, and rs3732361 were correlated with an increased risk of schizophrenia among individuals in the southern Fujian region of China (Xu *et al*., 2023). Chen *et al*. (2023) showed that three rs16901943, rs7733427, rs2168878 in Catenin Delta 2 gene (CTNND2) were correlated with SZ (Chi2 = 7.484, 11.576, and 5.391 and, df= 1, P= 0.006, 0.00067and 0.02 respectively). In a comparison of patients and control group, rs35753505 SNP of Neuregulin 1 gene (*NRG1*) with C and T alleles and CT, CC, and TT schizophrenia genotypes were analyzed and psychopathology scores in psychopathology symptoms were significantly increased in SZ patients (Moradkhani *et al.*, 2023).

The study by Ambrozová *et al*. showed that in women, the risk of developing schizophrenia with AG and GG genotypes of the insulin-degrading enzyme (IDE) gene is increased by more than 1.55 times and has a potential genetic predisposition with P <0.05 (Ambrozová *et al*., 2023). Our study showed that the frequency of AA and GA genotypes of rs334558 (G/A), and AA and TA genotypes of rs3755557 (T/A) in the

SZ group was higher than the control group and increased the susceptibility of schizophrenia, and was considered an effective factor. The significant difference confirms this relationship in the population of southern Iran.

In a study, Keshri *et al*. (2022) found that rs6489630 genotypes of the NT-3 neurotrophin-3 gene increase susceptibility to schizophrenia (CT and TT genotypes). Furthermore, rs6332 is associated with patients showing a significant reduction in memory scores (AG and AA genotypes). GSK3β signaling pathways were studied concerning schizophrenia. Increased GSK3β activity and disruption of PI3K/AKT/GSK3β and Wnt/GSK3β/β-catenin processes potentially lead to GSK3β overactivation and may influence schizophrenia risk (Mizuki *et al*., 2021). High levels of GSK-3β signaling have been shown in neurodegenerative diseases, so inhibition of this pathway could play a role in therapy (Rippin and Eldar-Finkelman, 2021). In transgenic mice, increased expression or SNP alteration in the GSK3β is associated with reduced brain volume. This decrease reflects the brain changes observed in people with schizophrenia (Steen *et al.*, 2006).

In patients with schizophrenia, a significant reduction of approximately 40% in the levels of *GSK3* protein, mRNA, and kinase activity was noted in the anterior cortex. Yang *et al*. (2020) identified that rs 6438552 and rs12630592 in the *GSK3β* gene may play a role in the development of this patient (Yang *et al*., 2020). However, contrasting findings by Meng *et al*. (2008) indicated that rs12630592 was not linked to schizophrenia susceptibility within the Chinese population. A summary of these findings indicated that increased *GSK3β* activity could be associated with SZ potential. A low number of samples used can be considered a limitation of the present study because using more samples reduces the probability of errors and leads us to a more reliable one. In addition, certain factors such as history, environment, diet, as well as culture can also be involved in the observed differences .The data presented in this study is limited and we examined rs334558 and rs3755557 in 200 samples. Our data show that there is a significant association between SNPs (rs334558, rs3755557) and susceptibility to schizophrenia, and these SNPs are associated with an increased risk of schizophrenia. In completing the results, it is important to examine multicenter studies and the effect of environmental interactions on genetic factors.

Conclusion

The prognostic value of rs334558 and rs3755557 polymorphisms of the *GSK3β* gene were investigated in predicting the risk of schizophrenia. Our findings showed that rs334558 (G/A) with GA, AA genotypes, and rs3755557 (T/A), with TA, AA showed a significant correlation with SZ. A key aspect of this study is that the polymorphisms rs334558 and rs3755557 significantly contribute to the development of schizophrenia in the southern Iranian population. Nevertheless, further investigation is required to identify genetic markers linked to other clinical manifestations of SZ. Future research should prioritize the expression of the GSK3β gene in larger, more diverse populations to clarify its role in schizophrenia. In addition, the GSK3β gene has several polymorphic loci, and according to the GWAS findings, we suggest a complete study of its SNPs to identify the relationship between this gene and schizophrenia.

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Author contribution

M.MD: Conceptualization, Data curation, Formal analysis, Investigation, Writing- original draft; DM. KT: Review and editing, Research conception, Protocol/project development, Conceptualization, Supervision, Project administration, Supervision; N.N: Manuscript editing, Research conception, Design, Review and Editing management, Methodology, Validation, Investigation; M.SH.: Performing the

experiments, Data acquisition, Statistical analysis, Writing Data Software: analysis and interpretation, Data collection, Review and editing; SH.V: Writing-conceptualization, Data curation, Formal analysis, Investigation, Data collection, Writing-original draft, Review and Editing.

Conflict of interests

The authors declare no conflict of interest.

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