



Original Article

Association Studies of Common Variants of TCF4 Gene Conferring Risk of Schizophrenia in Pakistani Patients

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ABSTRACT

Schizophrenia is a psychiatric disorder and it is strongly inherited disease with a heritability of 80% or more. Rare genetic mutations are more frequent in schizophrenia patients. These genetic variations interfere with brain development and include hundreds of distinct genes. Transcription factor 4 (TCF4) has been emphasized as major players for disruption of brain development as well as function and consequently, the onset of schizophrenia. The dysregulation of TCF4 gene expression in brain affects the process of pre pulse inhibition (PPI) and consequently profound reduction in sensor motor gating that may results in to the onset of schizophrenia. **Objective:** To find out the genetic association of common variants of TCF4 gene conferring risk of schizophrenia. **Methods:** It was a case control study in which statistically significant number of blood samples of confirmed diagnosed schizophrenic patients as well as age matched healthy control subjects were analyzed to screen out selected Single Nucleotide Polymorphisms (rs9960767, rs4309482, rs12966547, and rs2958182) of TCF4 gene for their association with schizophrenia. **Results:** Out of these four SNPs rs9960767 and rs4309482 were significantly associated with schizophrenia. p-values for SNPs rs12966547 and rs2958182 were greater than 0.05 in both healthy controls and in patients. **Conclusions:** The results of this study offer compelling evidence for the link between particular TCF4 gene polymorphisms and schizophrenia. Two SNPs, rs9960767 and rs4309482, were found to have a strong correlation with schizophrenia in the research population, according to the analysis.

INTRODUCTION

Schizophrenia is a psychiatric disorder and it is strongly inherited disease with a heritability of 80% or more. It is a devastating mental illness that weakens social and mental functioning and often leads to the development of comorbid disease. The prevalence of schizophrenia is 1% worldwide and is equally common in men and women [1]. The morbidity rate of schizophrenia patients is high in young adulthood. The onset of schizophrenia varies between men and women with respect to age as males tend to have a younger onset. The peak incidence for males and

females is in the decade 15–24 with an average reduction in lifespan of about 25 years [2]. There is a 10 percent lifetime chance of suicide in patients with schizophrenia. Both negative and positive symptoms have been observed in schizophrenic patients. Negative symptoms of schizophrenia allude to the lack of normal behaviors found in healthy individuals. Furthermore, Negative symptoms include poverty of speech, flat affect, loss of will or drive, loss of sense of pleasure and psychomotor retardation whereas positive symptoms include delusions,

hallucinations and florid thought disorders [1]. Schizophrenia may cause by duplications or deletions of DNA sequences in genes that play role in brain development or neuronal signalization. Recent genome-wide association studies highlight schizophrenia as highly polygenic disorder and abnormal connection between different genes network can also leads to schizophrenia [3]. One of the most well-known schizophrenia risk genes is transcription factor 4 (TCF4). The TCF4 gene (Entrez Gene ID 6925; ensemble ENSG00000196628) is a basic helix-turn-helix (bHLH) Ephrussi-box (also known as "E-box" or "E-") protein transcription factor that is found on chromosome 18q21.2. TCF4 also goes by the names E2-2, ITF2, and bHLHb19. It is 437 kb long, has 41 exons, and has a human protein length of between 511 and 773 amino acids [4]. TCF4 has been actively expressed in brain, especially in thalamic neurons and plays important role in differentiation of glial cells, especially the maturation of oligodendrocyte progenitors [5]. The microRNA mir137, a regulator of neuronal development targets TCF4 and disrupts the tissue specific tuning of its expression that may leads to Schizophrenia [4]. Furthermore, SNPs in miR-137 and its target genes have shown association with risk of schizophrenia, as result of dysregulated neurodevelopment [6]. The cognitive impairments and deficits in pre-pulse inhibition has been reported in mice with over expressing TCF4 in the forebrain [7]. The significant mRNA over expression of TCF4 has been reported in psychosis patients in comparison to controls as well as positive correlation with positive- and negative-symptom levels. Therefore, involvement of TCF4 variants in psychosis pathology especially abnormal neurodevelopment has been suggested in previous studies [8]. Furthermore, the increase in obstetric complications in children who later develop schizophrenia, cortical thickness reduction, enlargement of ventricles in the early phases of the disease, cognitive dysfunction, positive and negative symptoms support the neuro- developmental hypothesis for the onset of schizophrenia [8]. Most recent studies have found out the importance of common variants of TCF4 gene and their association with schizophrenia [9-11]. The significant association of SNP (rs9960767) of TCF4 gene has been observed with risk of schizophrenia in American and German populations [36]. Further evidence for the involvement of the same variant (rs9960767) for the risk of schizophrenia has been provided in British population [11]. Another SNP (rs2958182) of gene TCF4 appeared to be significantly associated with schizophrenia among Han Chinese population in recent studies [10]. The replication study of SNPs (rs4309482, rs12966547) showed their significant association with schizophrenia in Norwegian population [9]. The above-mentioned

association studies motivated for testing of selected Single Nucleotide Polymorphisms rs9960767, rs4309482, rs12966547 and rs2958182) of TCF4 gene for association with schizophrenia in Pakistani population. In this study, the genetic associations of common variants of TCF4 gene (rs9960767, rs4309482, rs12966547, and rs2958182) conferring risk of schizophrenia were carried out in case-control study pattern (case n=60, control n=60) in Pakistani population. The identification of disease susceptibility loci may lead to a better understanding of the biological mechanism of schizophrenia that will pave a way for the better diagnostic, prevention and treatment of schizophrenia.

METHODS

In k3 EDTA vials, blood samples from 60 Institute of Mental Health, Lahore confirmed diagnosed schizophrenia patients and 60 age-matched healthy control persons were taken. The preparation of these samples for molecular analysis. Following all ethical guidelines, blood samples were taken from patients, and clinical data were gathered from patient files. Blood samples were drawn by the knowledgeable paramedical staff. Using a DNA extraction by Thermofisher for purification of genomic DNA or by using the organic method of DNA isolation methodology outlined by *Maniatis et al.*, (1982), the DNA was isolated from the fresh blood samples [12]. Agarose gel electrophoresis was used to assess the DNA's quantity and quality. The quantity and purity of the DNA were evaluated using agarose gel electrophoresis. Using the Primer 3 program from the internet (<https://primer3.ut.ee/>), allele specific primers for allelic variations of TCF4 were created. For each marker, two universal primers and one allele-specific primer were created. Method of Hirotsu *et al.*, (2010) was followed for creating allele-specific primers, and the primer sequences and annealing temperatures are listed in Table 1 [13].

Table 1: Allele specific primer sequences for TCF4 alleles and their annealing temperatures.

TCF4 SNPs	Name of Primer	Sequences of primers	Annealing Temperature (°C)
rs9960767	T Reverse	5'-AGGGGTAATA ATTTGTGAAT-3'	51
	G Reverse	5'-AGGGGTAATAA TTTGTGAAG-3'	51
	Universal Forward	5'-CAAGAGATT CCATTGTATGC-3'	51
rs4309482	A Forward	5'-ATGCTAAGTG ACAGGAGTCA-3'	56
	G Forward	5'-ATGCTAAGT GACAGGAGTCG-3'	56
	Universal Reverse	5'-GATGTGTGT AGAGTTGGGCA-3'	56
	A Forward	5'-CCTATGTCC TTCCAAGAATA -3'	52

rs2958182	T Forward	5'-CCTATGTCC TTCCAAGAATT -3'	52
	Universal Reverse	5'- CAGCTCTTT CTAAACCCATA-3'	52
rs12966547	Universal Forward	5'- AGACTGAGA GAATTTAGAGT-3'	52
	T Reverse	5'-TCCACTCTG ACTTACTATT-3'	52
	C reverse	5'-TCCACTCTG ACTTACTATC -3'	52

Genomic DNA (10 ng), oligonucleotide primers (0.4 μ M each), dNTPs (200 μ M), 1X PCR Buffer, Taq Polymerase (1U) and MgCl₂ (2 mM) were all used in the PCR. The following PCR cycling circumstances were used: With varying annealing temperatures listed in table 1 for 30 seconds each, one cycle was performed at 95°C for 5 minutes, followed by 32 cycles at 95°C for 30 seconds, and one cycle was performed at 72°C for 5 minutes. Products from amplified SNPs were separated by gel electrophoresis and staining was performed with Ethidium Bromide (EtBr) and then seen under an Ultra Violet light source. The genotyping of the allelic variations of TCF4 was done using a gel-based approach. Sanger sequencing of purified PCR products from chosen samples was carried out to confirm the various allelic variants of TCF4 in order to validate the gel-based approach of SNP identification. BigDye Sequencing Kit was used to sequence the purified products using universal primers in accordance with the manufacturer's instructions (Applied Biosystems). Sanger sequencing was done at the business facilities. Chi-square, odds ratios (ORs), and 95% confidence intervals (CIs) were performed to estimate the association of genetic variations of TCF4 with schizophrenia. A 0.05 p-value was regarded as statistically significant. The haplotypes association with the risk of schizophrenia was assessed using Fisher's exact test. SHEsis, an online statistical analysis tool, was used for all statistical analysis.

RESULTS

This study comprised of Pakistani Schizophrenia patients (n=60) and age-matched normal healthy control subjects (n=60). DNA isolation was performed from fresh blood samples, and four variants of TCF4 gene containing (rs9960767, rs2958182, rs12966547 & rs4309482) were genotyped. The allele specific extension method was exploited to amplify the variant regions of TCF4 gene and amplified products were analyzed by agarose gel electrophoresis. The amplified PCR product for all four SNPs of TCF4 (two possible variants of each genetic marker) are shown in gel image (Figure 1).

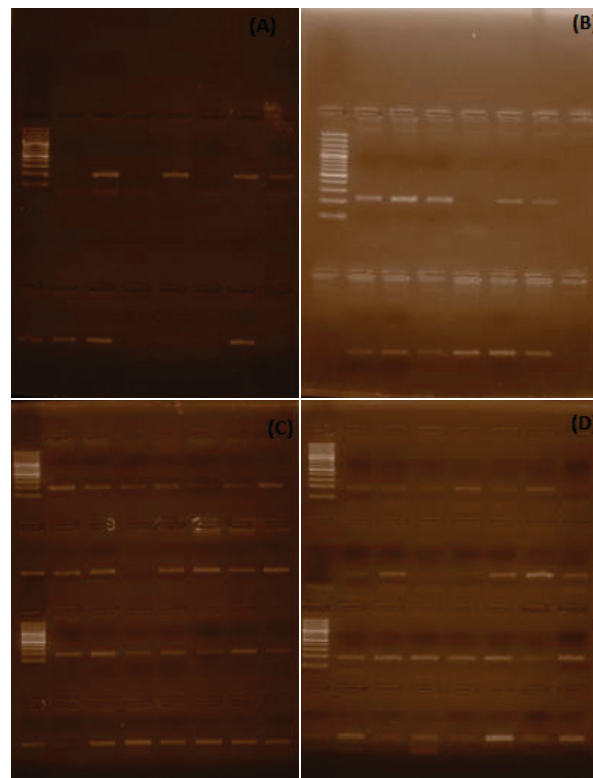


Figure 1: Gel images of four SNPs of TCF4 (A) rs2958182 (B) rs4309482 (C) rs9960767 (D) rs12966547

Four SNPs of TCF4 were genotyped in 60 Pakistani schizophrenic patients and in normal healthy individuals. Chi square analysis was performed for all four SNPs genotyped in schizophrenia patients and in controls. Table 2 shows p-values for each SNP and its association with schizophrenia risk in both case and control individuals. By considering p-values, two SNPs i.e. rs9960767 and rs4309482 of TCF4 showed significant association (<0.05) with risk of schizophrenia in Pakistani population ($p=7.47e-007$ and $p=0.000537$ respectively). However, other two SNPs rs2958182 and rs12966547 were not significantly associated with risk of schizophrenia in this population. The strongest association with the schizophrenia was observed for rs9960767 ($p=7.47e-007$) in Pakistani schizophrenia patients.

Table 2: The association of TCF4(rs9960767, rs4309482, rs2958182, rs12966547) with Schizophrenia in Pakistani patients

Gene	Marker ID	Odds Ratio	%95 CI	Fisher's p- value	Pearson's p- value
TCF4	rs9960767	7.666667	[4.131957~14.225168]	7.47e-007*	7.17e-007
	rs4309482	1.115533	[0.657053~1.893930]	0.000537 *	0.000537
	rs12966547	1.105820	[0.665594~1.837213]	0.925377	0.925376
	rs2958182	1.115533	[0.657053~1.893930]	0.245469	0.245373

TCF4=transcription factor4. *Marker showed significant association with Schizophrenia($p<0.05$)

The study further focused on haplotyping of all variants to check whether patients carrying more risk alleles are at greater risk of schizophrenia. Table 3 shows haplotypes of four SNPs of TCF4. In the four TCF4 SNPs, the haplotype analysis found 15 common haplotypes with frequencies greater than 0.03; 11 of these haplotypes were significantly associated with the risk of schizophrenia, demonstrating that patients carrying more risk alleles have a higher risk of developing schizophrenia.

Table 3: The association of TCF4 haplotypes with Schizophrenia

Gene	Marker	Haplotype	Case frequency	Control frequency	p-value	Odds Ratio [95%CI]
TCF4	rs99660767	G A C A*	7.57(0.063)	1.46(0.012)	0.037423	5.821 [0.884~38.316]
		G A T A*	8.34(0.069)	0.11(0.001)	0.00617	2.083 [0.820~5.294]
		G A T G*	6.76(0.056)	1.09(0.009)	0.03866	5.186 [1.458~18.455]
		G T C A*	7.16(0.060)	1.30(0.011)	0.03950	6.759 [0.977~46.751]
		G T C G	14.16(0.118)	7.30(0.061)	0.11660	5.821 [0.884~38.316]
		G T T A*	14.10(0.117)	3.03(0.025)	0.00523	2.083 [0.820~5.294]
	rs2958182	G T T G*	7.68(0.064)	1.21(0.010)	0.02639	5.186 [1.458~18.455]
		T A C A*	7.67(0.064)	17.89(0.149)	0.03368	6.759 [0.977~46.751]
	rs12966547	T A C G	6.58(0.055)	2.78(0.023)	0.20176	0.391 [0.161~0.952]
		T A T A*	0.00(0.000)	12.05(0.10)	0.00034	2.462 [0.592~10.243]
	rs4309482	T A T G	4.85(0.040)	4.12(0.034)	0.79831	1.191 [0.312~4.542]
		T T C A	10.68(0.08)	11.78(0.09)	0.81978	0.904 [0.378~2.159]
		T T C G*	7.94(0.066)	19.99(0.16)	0.01593	0.356 [0.150~0.847]
		T T T A*	6.49(0.054)	17.37(0.14)	0.01958	0.339 [0.132~0.869]
		T T T G*	6.79(0.057)	16.01(0.13)	0.04386	0.392 [0.153~1.000]

DISCUSSION

TCF4 is considered as a most susceptible gene that cause schizophrenia. This study comprised of Pakistani Schizophrenia patients (n=60) and age-matched normal healthy control subjects(n=60).

Four variants of TCF4 gene containing (rs9960767, rs2958182, rs12966547, rs4309482) were genotyped. In this study of patients of schizophrenia, significant association was seen between two SNPs of TCF4 [rs9960767, rs4309482 with $p=7.47e-007$ and $p=0.000537$ respectively] and Schizophrenia. These results are similar with the study of Stefansson et al., which showed that rs9960767, an SNP found in an intron of TCF4 (P.4.1_10_9), was one of seven single nucleotide polymorphisms (SNPs) that were reported as being related with schizophrenia at a genome-wide level, which included data from different GWAS [14]. This association was replicated in a study of Han Chinese patients using a different SNP (rs2958182, in high linkage disequilibrium (LD) with rs9960767). Another significant GWAS found rs4309482, which is intergenically located upstream of CCDC68 and downstream of TCF4 [15]. Finally, two novel TCF4 SNPs(rs17512836, which is located in intron 3 of TCF4, and rs12966547, which is in high LD with

rs4309482) were discovered and supported TCF4 as a disease gene for schizophrenia in recent meta-analyses. mRNA levels and TCF4 sequence variations are related to neurodevelopmental traits in psychotic illnesses. TCF4 and schizophrenia were first linked by the SNP rs9960767 in intron 3 of the gene (P. 4.1_10_9; OR 1.23 [1.15, 1.32]) [14]. Our findings also indicated a substantial link between the SNP rs4309482 and schizophrenia. These outcomes are consistent with those of several research. In a subsequent analysis (P147.8109; 1.09 [1.06, 1.12]), Steinberg et al., discovered that the mutation (rs4309482) downstream the TCF4 gene had achieved a genome-wide significance level [16]. In a GWAS mega study from the psychiatric GWAS collaboration, a second variation (rs12966547) was significantly linked to schizophrenia (P142.601010; OR 1.09 [1.06-1.12]). [17]. From the PGC schizophrenia analysis, a third variant, rs17512836, has also been linked to schizophrenia (P142.35108); upon follow-up, this resulted in a total p-value of 1.05106 (OR 1.23 [1.14-1.31])[17]. Physically (within 1.5 kb) in LD ($r^2=41$), rs12966547 and rs4309482 are practically identical markers. In the Caucasian population, rs4309482 and rs12966547 are in extremely weak LD with rs9960767 (D0140.05, $r^2=140$), indicating that they are

producing independent association signals with schizophrenia. Similar to rs4309482 and rs12966547, rs17512836 is in very low LD with both, but is in moderate LD with rs9960767 (D0141, r2140.52), indicating that the association signal there is not independent [18]. Another study by Wirgines et al., revealed a positive correlation between rs4309482 and SCZ. The two associated rs12966547 and rs4309482 are linked to less effective verbal fluency, which is a gauge of cognitive function, according to an analysis of known TCF4 risk variants for schizophrenia [19]. The identical risk variations, rs12966547 and rs4309482, were connected to a higher ventricular capacity even though this did not endure correction for multiple testing. One of the most common symptoms of schizophrenia is an increased ventricular volume[20].

CONCLUSIONS

In conclusion, the goal of this study was to determine how Pakistani patients' vulnerability to schizophrenia may be influenced by the TCF4 gene. The results of this study offer compelling evidence for the link between particular TCF4 gene polymorphisms and schizophrenia. Two SNPs, rs9960767 and rs4309482, were found to have a strong correlation with schizophrenia in the research population, according to the analysis. Overall, this research adds to the growing body of data that TCF4 is a schizophrenia susceptibility gene.

Authors Contribution

Conceptualization: FI, MAT

Methodology: NG

Formal analysis: FI, AM

Writing-review and editing: FI, MKAK, MAT

All authors have read and agreed to the published version of the manuscript.

Conflicts of Interest

The authors declare no conflict of interest.

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