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TF and *TCF4* gene polymorphisms are linked to autism spectrum disorder: a case–control study

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Abstract

Objective: Although the prevalence of autism spectrum disorder (ASD) is increasing, appropriate diagnosis and prevention strategies are still lacking. This case–control study was designed to explore the association between ASD and the rs1867503 and rs9951150 polymorphisms of the *TF* and *TCF4* genes, respectively.

Methods: Ninety-six children with ASD and 118 healthy children were recruited and polymerase chain reaction–restriction fragment length polymorphism technique was applied for genotyping.

Results: The frequencies of the mutant allele G were 48% and 44% for the rs1867503 and rs9951150 polymorphisms, respectively. In our analysis, both *TF* and *TCF4* polymorphisms were associated with an increased risk of developing ASD. AG heterozygotes (OR = 3.18), GG mutant homozygotes (OR = 2.62), AG + GG combined genotypes (OR = 2.98), and G mutant alleles of

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TF rs1867503 (OR = 1.94) were associated with a significantly elevated risk of ASD. Likewise, AG heterozygotes (OR = 2.92), GG mutant homozygotes (OR = 2.36), AG + GG combined genotypes (OR = 2.72), and G minor alleles of *TCF4* rs9951150 (OR = 1.92) were associated with a significantly elevated risk of ASD.

Conclusions: Our results indicate that *TF* rs1867503 and *TCF4* rs9951150 polymorphisms may be strongly associated with the development of ASD in Bangladeshi children.

Keywords

Autism spectrum disorder, polymerase chain reaction, restriction fragment length polymorphism, genetic polymorphism, *TF* rs1867503, *TCF4* rs9951150, mutant allele G

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Introduction

Autism spectrum disorders (ASDs) are neurodegenerative disorders that are diagnosed primarily based on children's behaviors and characterized by insufficient development of normal social interaction with other people, impaired development of communicative ability, a lack of imaginative ability, and repetitive and stereotyped movements.[1–3] Anatomical and physiological changes such as frontal cortex overgrowth occur in the ADS brain during the prenatal period.[4,5] Underdeveloped cognitive areas affect decision-making, communication, and language.[5] A 2017 statistic suggests that the prevalence of ASD among children was 168, 161, 152, 100, 100, 69, 67, 49, 27, and 9.2 per 10,000 in the United States, Japan, Canada, the United Kingdom, Ireland, Denmark, Australia, China, Brazil, and Portugal, respectively.[6] The latest published data indicate that the prevalence of ASD is greater than 2%.[7] The diagnosis of ASD nevertheless currently lacks a unifying theory.[8] Early theories about the cause of ASD mainly focused on substandard parenting.[8] Newschaffer et al.[9] suggested that the causes of ASD mainly fall into three categories: genetic, environmental, and neurobiological. Factors including toxicity, teratogenic effects, trauma, and infections can also cause ASD.

Several independent studies and substantial evidence confirm that transferrin (*TF*; chromosomal location: 3q22.1) is one of the genes that confer susceptibility to ASD.[10,11] Transferrin is an iron-transporting plasma glycoprotein that controls iron levels in biological fluid.[10] The glycoprotein has two iron binding sites; these irons accumulate rapidly at the onset of myelination. A very recent study suggested that an elevated amount of oxalate in plasma plays a role in ASD by binding to the bilobal iron transport protein transferrin (hTF), thereby interfering with iron metabolism by inhibiting iron delivery to cells.[11] Therefore, the genetic modification of the transferrin gene may be linked to the development of ASD.[12] An investigation of the rs1867503 polymorphism of the *TF* gene reported that this genetic polymorphism plays a significant role in producing cognitive disorders such as ASD.[13]

Another gene related to ASD is transcription factor 4, 18q21.2 (*TCF4*; also known as *E2-2*, *SEF2,* or *ITF2*), a basic helix–loop–helix transcription factor that is frequently associated with cognitive dysfunction.[14–16] The autosomal dominant

mutation or deletion of *TCF4* results in Pitt–Hopkins syndrome , 18q deletion syndrome, and three rare ASDs (autistic disorder, Asperger syndrome, and pervasive developmental disorder).[17–19] A previous study indicated that in neurodevelopmental pathways *TCF4* target genes cluster mostly to schizophrenia, ASD, and ID risk genes.[20] Studies such as this have proven the association of these genes with ASD in some ethnic groups.

A study to validate the link between the *TCF4* rs9951150 and *TF* rs1867503 variants and ASD in Bangladeshi children has not yet been conducted. We performed this study because the diagnosis and treatment of ASD in Bangladeshi children are not adequately prioritized despite a prevalence nearly equal to that in other parts of the world. The study was conducted using polymerase chain reaction (PCR)-based amplification followed by a restriction fragment length polymorphism (RFLP) method to detect the associations of *TF* rs1867503 and *TCF4* rs9951150 with ASD. We anticipate that this study will help further elucidate ASD and improve procedures for its diagnosis and treatment.

Methods and Materials

Study design and sample and data collection

Two groups of children were selected for this case–control study. The first group consisted of 96 children with ASD (aged 3–15 years) who were recruited as cases from schools for children with ASD in Chittagong and Dhaka using the criteria of the Diagnostic and Statistical Manual of Mental Disorders, 4th Edition. We de-identified all patient details. A total of 118 healthy children (aged 3–15 years) were recruited as controls from areas in Dhaka and Chittagong, Bangladesh. All participants were randomly selected to investigate the risk of ASD due to the *TF* rs1867503 and *TCF4* rs9951150 polymorphisms.

Genotyping analysis was performed in the Laboratory of Pharmacogenomics and Molecular Biology, Department of Pharmacy, Faculty of Science, Noakhali Science and Technology University, Noakhali, Bangladesh. Ethical clearance was obtained from the ethical committee of the Noakhali Science and Technology University (ID-01/2018) and written consent was obtained from each participant before inclusion in the study. We collected consent from guardians for minors or those lacking the capacity to consent. Consent was obtained verbally and in writing (signature or fingerprints). The consent form was translated into the native language to ensure accurate understanding by participants.

The study was conducted in accordance with the International Conference of Harmonization for Good Clinical Practice and was in compliance with the Declaration of Helsinki and its further amendments.[21] Moreover, the study was performed following the Strengthening the Reporting of Observational Studies in Epidemiology guidelines, as described by von Elm et al.[22]

Selection of genes and single nucleotide polymorphisms

We selected additional ASD-susceptible genes and polymorphisms by analyzing their possible association with ASD. Transferrin factor is important for iron transportation[12] and lower iron levels are linked with ASD.[23,24] A genome-wide association study found a link between the rs1867503 polymorphism and ASD.[13] Another study reported the association of *TCF4* with autism.[20] Moreover, the frequency of the minor allele must be greater than 15% according to the 1000 Genomes database in the studied population.

DNA extraction and genotyping

Approximately 3 mL of blood was drawn from all patients and controls into a tube containing ethylenediaminetetraacetic acid disodium and stored at −80°C until the isolation of genomic DNA.[25,26] Genomic DNA was isolated from 96 children with ASD and 118 controls using the FavorPrep™ DNA isolation kit (Favorgen Biotech Corporation, Ping-Tung, Taiwan). The genotyping of the selected single nucleotide polymorphisms (SNPs) was performed using the PCR-RFLP method. The PCR conditions for rs1867503 consisted of an initial denaturation at 95°C for 3 minutes, 35 cycles of 95°C for 20 s, 55°C for 30 s, 72°C for 30 s, and a single-step final extension at 72°C for 5 minutes. The PCR conditions for the amplification of rs9951150 were similar except that the annealing temperature was 57°C instead of 55°C. After completion of PCR amplification, PCR products of 299 and 446 bp in size were obtained for rs1867503 and rs9951150, respectively, and were visualized in 1% (w/v) agarose gel. Targeted polymorphisms were identified by digesting with respective restriction enzymes under the conditions mentioned in Table 1.

Statistical Calculation

SPSS software package version 16.0 (SPSS Inc., Chicago, IL, USA) was used for statistical analysis. The deviation of variable allele frequencies in the control group from that of the patient group was assessed according to the Hardy–Weinberg equilibrium (HWE) and the chi-square test (χ^2). The genotype and allelic frequencies were reported as percentages. We evaluated the association of SNPs with ASD using genetic models including additive models and recessive and dominant models.[27,28] SPSS was further used to estimate odds ratios (ORs) and their 95% confidence intervals (CIs). For all analyses, a p-value of less than 0.05 was used to determine statistical significance. Statistical power was calculated using an online sample size estimator (OSSE; http://osse.bii.a-star.edu.sg/).

Results

All cases and controls were from the same ethnic group and were Bangladeshi by birth. The genotype frequencies of *TF* rs1867503 and *TCF4* rs9951150 were analyzed for 96 children with ASD and 118 healthy children. The distribution of demographic characteristics among study participants is summarized in Table 2. Among children with ASD, 70.83% were boys and 29.17% were girls, whereas 38.98% were boys and 61.02% were girls among controls. Average ages were 10.06 years and 10.81 years in the ASD and control groups, respectively.

Post-study statistical power calculation with OSSE (http://osse.bii.a-star.edu.sg/) using the minor allele frequencies of 48%

Table 1. Restriction enzyme digestion conditions and expected fragments.

Allele	Restriction Enzyme	Digestion Condition	Expected Fragments (bp)
rs1867503 *TF*	Fat*I*	Incubation at 55°C for more than 6 hours	AA: 76, 223 AG: 76, 223, 299 GG: 299
rs9951150 *TCF4*	Xba*I*	Incubation at 37°C for more than 6 hours	AA: 118, 328 AG:118, 328, 446 GG: 446

in cases and 32% in controls for rs1867503 to achieve 80% power showed that the 292 samples (146 cases + 146 controls) were required to achieve 80% statistical power. Given the sample size (96 cases and 118 controls), the statistical power of the study was 66.3%.

In the case of the rs1867503 SNP of the *TF* gene, 27.08% of children with ASD and 52.54% of controls carried the AA genotype. Fifty percent of children with ASD and 30.51% of controls carried the AG genotype, whereas 22.92% of children with ASD and 16.95% of controls carried the GG genotype. The frequencies of the G allele were 47.92% and 32.20% among children with ASD and controls, respectively. The chi-square values for the ASD and control groups were 0.0003 and 10.71, respectively. The frequency distribution of ASD cases and controls did not obey the HWE ($p < 0.05$; Table 3).

The G allele frequencies were 43.75% in patients and 28.81% in controls for the *TCF4* gene's rs9951150 SNP. The genotype frequencies of the rs9960767 variant were as follows: AA, 33.33%; AG, 45.83%; and GG, 20.83% in patients and AA, 57.63%; AG, 27.12%; and GG, 15.25% in controls. Only the genotype distribution data of ASD cases followed the HWE ($p > 0.05$: Table 4).

In the case of the rs1867503 SNP of the *TF* gene, children carrying the AG genotype were at 3.18 times higher risk (OR = 3.18, 95% CI = 1.69–5.97) of developing ASD compared with children carrying the AA genotype ($p < 0.05$). Children with the GG genotype were at 2.62 times higher risk (OR = 2.62, 95% CI = 1.22–5.60) of developing ASD compared with children

Table 2. Distribution of demographic variables of autism spectrum disorder children and controls.

Variables	ASD Children n = 96 (%)	Control Children n = 118 (%)
Sex, n (%)		
Male	68 (70.83)	46 (38.98)
Female	28 (29.17)	72 (61.02)
Age, years		
Mean (±SD)	10.06 (±7.03)	10.81 (±3.28)
Range	3–15	3–15
Weight, kg		
Mean weight (±SD)	34.20 (±18.12)	24.13 (±9.76)

Table 3. Allelic frequencies and Hardy–Weinberg equilibrium of rs1867503 genotypes among children with autism and healthy controls and their association with ASD.

	Autism n = 96 (%)	*p*- value	χ^2	Controls n = 118 (%)	*p*-value	χ^2	OR	*p*-value
TF rs1867503								
AA	26 (27.08)	0.986	0.0003	62 (52.54)	0.0011	10.71	1	–
AG	48 (50.00)			36 (30.51)			3.18 (1.69–5.97)	0.0003
GG	22 (22.92)			20 (16.95)			2.62 (1.22–5.60)	0.0128
Dominant model (AG + GG vs. GG)								
GG	22 (22.92)			20 (16.95)			1	–
AG + GG	70 (66.67)			56 (42.37)			2.98 (1.67–5.31)	0.0002
Recessive model (GG vs. AA+AG)								
AA + GG	74 (77.08)			98 (83.05)			1	–
GG	22 (22.92)			20 (16.95)			1.46 (0.74–2.87)	0.276
A allele	100 (52.08)			160 (67.80)			1	–
G allele	92 (47.92)			76 (32.20)			1.94 (1.31–2.87)	0.0010

$p < 0.05$ is considered statistically significant and $p > 0.05$ indicates consistency with Hardy–Weinberg equilibrium.

Table 4. Allelic frequencies and Hardy–Weinberg equilibrium values of rs9951150 genotypes among children with autism and healthy controls and their association with ASD.

	Autism (n = 96) (%)	*p*-value	χ^2	Controls (n = 118) (%)	*p*-value	χ^2	OR	*p*-value
TCF4 rs9951150								
AA	32 (33.33)	0.5004	0.454	68 (57.63)	0.0002	13.56	1	–
AG	44 (45.83)			32 (27.12)			2.92 (1.57–5.43)	0.0007
GG	20 (20.83)			18 (15.25)			2.36 (1.10–5.06)	0.0273
Dominant model (AG+GG vs. GG)								
GG	20 (20.83)			18 (15.25)			1	–
AG+GG	64 (66.67)			50 (42.37)			2.72 (1.55–4.76)	0.0005
Recessive model (GG vs. AA+AG)								
AA+AG	76 (79.17)			100 (84.75)			1	–
GG	20 (20.83)			18 (15.25)			1.46 (0.72–2.95)	0.290
A allele	108 (56.25)			168 (71.19)			1	–
G allele	84 (43.75)			68 (28.81)			1.92 (1.29–2.87)	0.0014

$p < 0.05$ is considered statistically significant and $p > 0.05$ indicates consistency with Hardy–Weinberg equilibrium.

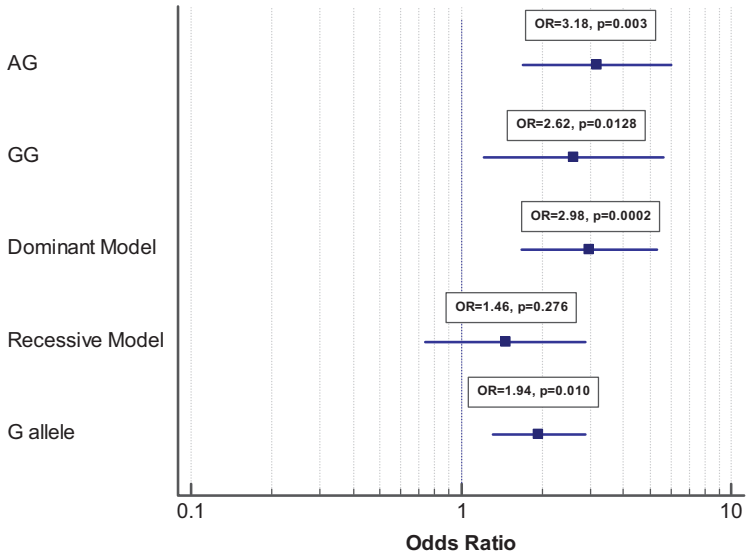

Figure 1. Forest plot of the *TF* gene's rs1867503 allele in the study population.

carrying the AA genotype ($p < 0.05$). Children carrying the combined AG + GG genotype (dominant model) were at 2.98 times higher risk (OR = 2.98, 95% CI = 1.67–5.31) for the development of ASD compared with children carrying the AA genotype ($p < 0.05$). However, children carrying the G allele were at 1.94 times higher risk (OR = 1.94, 95% CI = 1.31–2.87) of developing ASD compared with children carrying the A allele ($p < 0.05$; Table 3 and Figure 1).

Table 4 shows the allelic frequencies of *TCF4* rs9951150 genotypes among children

with ASD and healthy controls and their association with ASD. Children carrying the AG genotype were at 2.92 times higher risk (OR = 2.92, 95% Cl = 1.57–5.43) of developing ASD compared with children carrying the AA genotype ($p < 0.05$), whereas children carrying the GG genotype, were at 2.36 times greater risk (OR = 2.36, 95% Cl = 1.10–5.06) of developing ASD compared with children carrying the AA genotype ($p < 0.05$). Children with the combined AG + GG genotype (dominant model) were at 2.72 times greater risk (OR = 2.72, 95% Cl = 1.55–4.76) for the development of ASD compared with controls carrying the AA genotype ($p <$ the 0.05), whereas children carrying the G allele were at 1.92 times greater risk (OR = 1.92, 95% Cl = 1.29–2.87) of developing ASD compared with controls carrying the A allele ($p < 0.05$). No associations were observed between ASD and the recessive model (GG vs. AA + AG) of both SNPs in the population studied (Figure 2).

Finally, the genotype data for *TF* rs1867503 and *TCF4* rs9951150 for cases and controls were distributed according to sex. No significant difference was observed in the genotype distribution between males and females for both SNPs ($p > 0.05$; Table 5).

Discussion

Though the prevalence of ASD increases daily, appropriate diagnosis and prevention strategies are still lacking.[29] The heritability of ASD is 90%. However, identifying relevant genes responsible for the development of ASD remains challenging.[30] Multiple studies are underway to identify responsible genes. Hundreds of genes have already been positively linked to the development of ASD; these genes follow various biochemical pathways to perform their functions.[10–12] Ours was the first-ever attempt to investigate the association of *TF* and *TCF4* gene polymorphisms with ASD in Bangladesh and our initial findings are discussed.

Several studies have suggested the role of *TF* (rs1867503) and *TCF4* (rs9951150) in a variety of psychiatric symptoms and

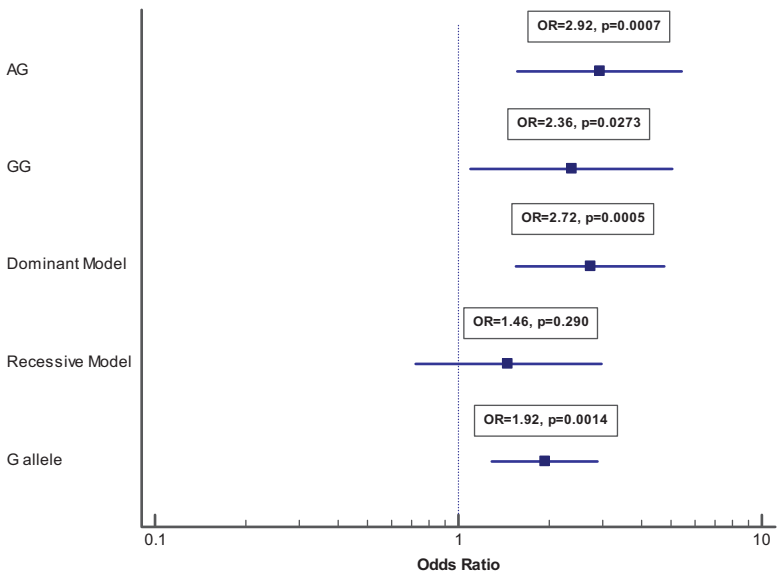

Figure 2. Forest plot of the *TCF4* gene's rs9951150 allele in the study population.

Table 5. Sex-based distribution of *TF* rs1867503 and *TCF4* rs9951150 genotype data.

	Autism (n = 96) (%)				Controls (n = 118) (%)			
	Male (68)	Female (28)	χ^2	*p*-value	Male (46)	Female (72)	χ^2	*p*-value
TF rs1867503								
AA	16	10	1.53	0.466	25	37	0.834	0.659
AG	36	12			15	21		
GG	16	6			6	14		
TCF4 rs9951150								
AA	21	11	1.24	0.539	24	44	2.50	0.287
AG	31	13			12	20		
GG	16	4			10	8		

diseases including phobic anxiety, obsessive-compulsive disorder, schizophrenia, and attention-deficit hyperactivity disorder. The *TF* polymorphism causes an increase or decrease of oxygen free radicals, which cause damage to neurons with the excess production of lipid peroxidation and are thus responsible for the oxidative stress associated with neurodegenerative disorders.[31] This polymorphism further causes the additional formation of ferrous, which stimulates hydroxyl formation and leads to brain cell damage.[32] The presence of a larger amount of the antioxidant superoxide dismutase—a marker of lipid peroxidation—was observed in a healthy group of Egyptian children than in an ASD group, indicating the polymorphism of transferrin.[33,34]

In this research, 118 healthy volunteers and 96 individuals with ASD were studied for the rs1867503 SNP of the *TF* gene. We observed a significant association between rs1867503 and ASD in Bangladeshi children. Children carrying the AG and GG genotypes were at 3.18 and 2.62 times greater risk, respectively, for the development of ASD compared with children carrying the AA genotype ($p < 0.05$). A statistically significant ($p < 0.05$) association with ASD was further observed in children carrying the combined AG + GG genotype (OR = 2.98, $p = 0.0002$), whereas children carrying the G allele were at 1.94 times greater risk for the development of ASD compared with controls carrying the A allele ($p < 0.05$). A comparative study conducted by Chauhan et al.[33] indicated an elevated level of lipid peroxidation in children with autism compared with their siblings without autism. The authors further detected increased oxidative stress, which is caused by reduced transferrin. A reduction in transferrin is also responsible for language difficulties in children with ASD. A study by Luck et al.[12] concluded that oxalate in plasma could play a role in ASD by interfering with iron transport by binding with transferrin (hTF) and that high levels of oxalate can cause iron deficiency anemia in children with ASD. Our SNP finding study also suggests such a relationship with ASD.

The *TCF4* polymorphism disrupts the columnar and laminar structure of the cortex, which is activity-dependent. The polymorphism further hampers calcium activity, which is responsible for neuronal excitability. These activities result in various autistic syndromes in children.[35] A study of *TCF4* regulation concluded that *TCF4* encodes a basic helix–loop–helix transcription

factor that merges with other factors to activate or suppress gene expression, causing two rare ASDs: Pitt–Hopkins syndrome and 18q deletion syndrome.[36] Our study validates some of the findings of previous researchers.[35,36]

In the case of the rs9951150 SNP of *TCF4*, children carrying the AG and GG genotypes were at 2.92 and 2.36 times greater risk of developing ASD compared with controls carrying the AA genotype ($p < 0.05$). Children carrying the combined AG + GG genotype were at 2.72 times greater risk for the development of ASD compared with controls carrying the AA genotype ($p < 0.05$), whereas children carrying the G allele were at 1.92 greater risk for the development of ASD compared with controls carrying the A allele ($p < 0.05$).

This study also reported a higher frequency of ASD in males (70.83%) compared with females (29.17%) in our study (male:female ratio = 2.43:1). Previous studies showed differences between male participant and female participants.[37] This difference may be attributed to the lack of accurate and early diagnosis; this assumption must be confirmed before drawing implications from the findings.[37] The minor allele frequencies in the Bangladeshi population were 32.20% and 28.8% for rs1867503 and rs9951150, respectively, in healthy controls and 47.92% and 43.75% for rs1867503 and rs9951150, respectively, in children with ASD. According to the 1000 Genomes database, the frequency we reported for rs1867503 is higher than that in the African population (21.9%) and close to that of the East Asian population (30.9%) for rs9951150. This study is significant considering the low number of autism-based genetic association studies in Bangladesh, where genetic studies on children with ASD are scarce because of the paucity of research funds. Our study population has a distinct genetic lineage that has been investigated earlier.[26] Moreover, a lack of proper healthcare support was found to improve the quality of life of children with ASD. We hope that our study will encourage healthcare providers and policymakers to appropriately diagnose children with ASD and provide them with the necessary genetic-based treatment.

Our finding supports the conclusion that the rs1867503 and rs9951150 SNPs are strongly associated with the development of ASD. We hope that our identification of the genetic basis of ASD in Bangladeshi children will help elucidate ASD etiology. Given that all cases and controls were from the same ethnic group and were Bangladeshi by birth, statistical biases due to ethnicity were unlikely. However, some study limitations should be noted. Only two known SNPs were selected from a public database; novel SNPs were not studied. Another limitation is that our study population was not large enough for the study to generate nationally generalizable results. Although we detected a strong association of SNPs with the development of ASD, a large-scale study may provide stronger evidence in the future.

Conclusion

This case–control study reveals that *TF* rs1867503 and *TCF4* rs9951150 polymorphisms may be significantly associated with ASD in Bangladeshi children. Ours is the first study of these polymorphisms in Bangladesh. The results are significant despite a limited number of cases and controls. We expect that the findings of this study will guide further large-scale studies.

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Author Contributions

MA, MSH, MAA: Blood sample collection; MAA, MB, NS, MAR: DNA extraction; MA, MSH, MAA, MB, NS, MAR, MS: PCR analysis and initial draft preparation; MA, MAB, MB, NS, MAR, MS: Data analysis, critical review, and interpretation of results; SSB and GMA: Conception and manuscript editing; MSI: Conception, supervision, institutional approval, editing, final check, and submission.

Availability of data and materials

All relevant data and study materials have been provided in the manuscript. Further information on data and study materials will be available from the corresponding author on reasonable request.

Declaration of conflicting interests

The authors declare no conflicting interests in preparing this article.

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