Connection of *TF* and *TCF4* Gene Polymorphisms with ASD

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Abstract

Though the prevalence of autism spectrum disorder (ASD) is increasing day by day, there is still a lack of a proper way to diagnose or prevent ASD. There is no study carried out in the Bangladeshi children with ASD to evaluate the association of Transferrin (*TF*) and Transcription Factor 4 (*TCF4*) genetic polymorphisms. This genetic association study was designed to explore the association of rs1867503 polymorphism of *TF* and rs9951150 polymorphism of *TCF4* genes with ASD. We collected blood from 96 children with ASD and 118 healthy children of very similar age differences. Genotyping of these SNPs was performed by the PCR-RFLP method. SPSS (version 16) was used to estimate the odds ratio (OR) and their 95% confidence intervals (CI). The frequency of mutant allele G for rs1867503 and rs9951150 polymorphisms was found 48% and 44%, respectively. In our analysis, both *TF* and *TCF4* polymorphisms showed an increased risk for the development of ASD. AG heterozygote, GG mutant homozygote, AG+GG combined genotype, and G mutant allele of *TF* rs1867503 showed a significantly elevated risk of ASD development (OR=3.18, p=0.0003; OR=2.62, p=0.0128; OR=2.98, p=0.0002; and OR=1.94, p=0.001, respectively). Likewise, AG heterozygote, GG mutant homozygote, AG+GG combined genotype, and G minor allele of *TCF4* rs9951150 also showed a significantly elevated risk of ASD development (OR=2.92, p=0.0007; OR=2.36, p=0.0273; OR=2.72, p=0.0005; and OR=1.92; p=0.0014, respectively). Our results indicate that *TF* rs1867503 and *TCF4* rs9951150 polymorphisms are strongly associated with the development of ASD in Bangladeshi children.

Introduction

Autism spectrum disorders (ASD) are neurodegenerative disorders that are mainly diagnosed based on the behaviors of children, whose symptoms include deficit to develop normal social interaction with other people, impaired development of communicative ability, lack of imaginative ability, and repetitive, stereotyped movements (Casanova et al. 2002). Some changes occur in the anatomy and physiology of brain, such as overgrowth of the frontal cortex during the prenatal period in ASD (Casanova et al. 2002; Talkowski et al. 2012). On the other side, underdeveloped parts in cognitive areas affect decision making, communication and language (Talkowski et al. 2012). Abnormal growth of the hippocampus can affect the development of language syntax, semantics, and the capacity of creativity in language generation and a better understanding of words of a child. One in forty-two boys and one in 189 girls children have ASD worldwide (Autism Speaks, 2018) and the prevalence has increased 10 folds in the last 40 years (Hansen et al. 2015). A new statistic of 2017 shows that the prevalence of ASD among children in the selected countries was found 168, 161, 152, 100, 100, 69, 67, 49, 27, 9.2 per 10000 for USA, Japan, Canada, UK, Ireland, Denmark, Australia, China, Brazil, Portugal, respectively (Hansen et al. 2015). In 2013, a pilot study in Bangladesh found a prevalence of ASD was 0.15% (3% in Dhaka city and 0.07% in the rural area), and the ratio of boys and girls was 4:1 (Global autism movement and Bangladesh, 2014). It is still a case today that diagnosis of ASD lacks unifying theory (Mullegama et al. 2015). Early theories mainly focused on substandard parenting (Mullegama et al., 2015). Newschaffer et al. (2007) suggested that causes of ASD mainly fall into three categories, genetic, environmental and neurobiological. Some

other factors like toxicity, teratogenic effect, trauma, infections can also cause ASD (Newschaffer et al. 2007).

Transferrin (*TF*) (chromosomal location: 3q22.1) is one of the genes which has the most substantial evidence of ASD susceptibility with several independent studies (Davis et al. 2003; Konstantynowicz et al. 2012). TF is an iron transporting plasma glycoprotein that controls the iron level in the biological fluid (Davis et al. 2003). It has two iron binding sites, and these irons accumulate rapidly at the onset of myelination. A very recent study suggested that an elevated extent of oxalate in plasma might play a role in ASD by binding to the bilobal iron transport protein transferrin (hTF) and thereby interfering with iron metabolism by inhibiting iron delivery to cells (Konstantynowicz et al. 2012) So, genetic modification in the transferrin gene may manifest during the generation of ASD (Luck et al. 2013). An investigation was carried out on rs1867503 of transferrin gene and reported that genetic polymorphism of transferrin gene plays a significant role in generating cognitive disorders like ASD (Chaste et al. 2015).

Another particular gene related to ASD is Transcription Factor 4, 18q21.2, (*TCF4*; also known as *E2-2*, *SEF2* or *ITF2*) is a basic helix-loop-helix (bHLH) transcription factor (TCF) that is frequently associated with cognitive dysfunction (Sweatt, 2013; Forrest et al. 2014; Hill et al. 2014). Autosomal dominant mutation or deletion of *TCF4* results in Pitt Hopkins syndrome (PTHS) and 18q deletion syndrome, three rare ASD (Autistic disorder, Asperger syndrome, and Pervasive developmental disorder) (Brockschmidt et al. 2007; Amiel et al. 2007; Zweier et al. 2007). We found from a previous study *TCF4* target genes cluster in neurodevelopmental pathways mostly to schizophrenia, ASD, and ID risk genes (Forrest et al. 2018). These studies proved the association of these genes with ASD in some ethnic groups.

However, there is no study carried out in the Bangladeshi children with ASD to validate the association of rs9951150 variant of the *TCF4* gene and rs1867503 of the *TF* gene. Considering the current situation of ASD in Bangladeshi children, this study was performed with a polymerase chain reaction (PCR) based amplification followed by restriction fragment length polymorphism (RFLP) method to detect *TF* (rs1867503) and *TCF4* (rs9951150) association with ASD, and we hope it will help to understand ASD and to improve their diagnosis and treatment procedure.

Methods And Materials

Sample and Data Collection

Two groups of children were selected. One group consisted of 96 ASD children (aged 3-15 years) recruited as cases from the different schools for ASD children in Chittagong and Dhaka. Total 118 healthy children (aged 3-15 years) were recruited as controls from the different areas of Dhaka and Chittagong, Bangladesh. All of them were selected to investigate the risk of ASD due to polymorphisms of TF (rs1867503) and TCF4 (rs9951150). The 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. The study was directed as per the International

Conference of Harmonization (ICH) for Good Clinical Practice (GCP) and in compliance with the Declaration of Helsinki and its further amendments (World Medical Association Declaration of Helsinki, 2013).

DNA extraction and genotyping

About 3 ml of blood was drawn into a tube containing ethylenediaminetetraacetic acid disodium from all the patients and controls and stored at −80°C until the isolation of genomic DNA (Daly et al. 1998; Islam et al. 2013). Genomic DNA was isolated from 96 children with ASD and 118 controls by a kit method using a Favorprep DNA isolation kit. Genotyping of the selected SNPs was performed by a PCR-RFLP method. The PCR condition for rs1867503 consisted of an initial denaturation at 95^0C for 3 m, 35 cycles of 95^0C for 20 s, 55^0C for 30 s and 72^0 C for 30 s and a single step final extension at 72^0C for 5 m. The PCR condition for the amplification of rs9951150 was the same, except the annealing temperature was 57^0C instead of 55^0C. After completion of PCR amplification, two PCR products of 299 and 446 bp were obtained for rs1867503 and rs9951150, respectively, and these products were visualized in 1% (w/v) agarose gel. Targeted polymorphisms were identified by the digestion with the respective restriction enzymes and conditions mentioned in Table 1.

Table 1
Restriction enzyme digestion conditions and expected fragments

Allele	Restriction Enzyme (RE)	Digestion Condition	Expected Fragments (bp)
rs1867503 Transferrin	Fat*I*	Incubation at 55°C for more than 6hrs	AA: 76, 223 AG: 76, 223, 299 GG: 299
rs9951150 Transcription factor 4	Xba*I*	Incubation at 37°C for more than 6hrs	AA: 118, 328 AG:118, 328, 446 GG: 446

Statistical Calculation

SPSS software package, version 16.0 (SPSS, Inc., Chicago, IL), was used for statistical analysis. The deviation of variable allele frequencies in the control group from the patient group was assessed according to Hardy–Weinberg equilibrium (HWE) by chi-square test (χ^2). The genotype and allelic frequencies were reported as the percentage. SPSS was also used to estimate the odds ratio (OR) and their 95% confidence intervals (CI). For all analyses, the significant statistical value was considered at $p < 0.05$.

Result

Genotype frequencies of *TF* rs1867503 and *TCF4* rs9951150 were analyzed for 96 ASD children and 118 healthy children. The distributions of demographic characteristics among study subjects are summarized in Table 2. Among the ASD children, 70.83% were male and 29.17% were female, whereas 38.98% were male and 61.02% were female in controls. The average ages were 10.06 years in the ASD group and 10.81 years in the control group.

Table 2
Distribution of demographic variables of ASD children and controls

Variables	**ASD Children (n=96) (%)**	**Controls Children (n=118) (%)**
Gender, n (%)		
Male	68 (70.83)	46 (38.98)
Female	28 (29.17)	72 (61.02)
Age (years)		
Mean age, n (±SD)	10.06 (±7.03)	10.81 (±3.28)
Range	3-15	3-15
Weight (Kg),		
Mean weight, n (±SD)	34.20 (±18.12)	24.13 ((±9.76)

In the case of rs1867503 SNP of *TF* gene, 27.08% of ASD children and 52.54% of the controls carried AA genotype. 50.00% of ASD children and 30.51% of the controls carried AG genotype, whereas 22.92% of ASD children and 16.95% of the controls carried GG genotype. The frequency of G allele was 47.92% and 32.20% among the ASD children and controls, respectively. The chi-square values for the ASD and control groups were 0.0003 and 10.71, respectively. The ASD cases and controls frequency distribution do not obey ($p < 0.05$) the HWE (Table 3).

Table 3
Genotype and allelic frequency, HWE of rs1867503 allele of TF gene among autistic children and control healthy volunteers and their association with ASD

TF rs1867503	Autism (%) (n= 96)	p-value	χ2	Controls (%) (n=118)	p-value	χ2	OR	p-value
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**
$p < 0.05$ was considered as statistically significant and $p > 0.05$ indicates consistent with HWE								

For *TCF4* gene (rs9951150), G allele frequencies were 43.75% in the patients and 28.81% in the control subjects. The genotype frequencies of rs9960767 variant were as follows: AA, 33.33%, AG, 45.83%, and GG, 20.83% in the patients; AA, 57.63%; AG, 27.12% and GG, 15.25 % in the control subjects, while only cases genotype distribution data follows the in HWE ($p > 0.05$) as presented in Table 4.

Table 4
Genotype and allelic frequency, HWE values of rs9951150 Allele of TCF4 genotypes among autistic children and control volunteers and their association with ASD

TCF4 rs9951150	Autism (%) (n= 96)	p-value	χ2	Controls (%) (n=118)	p-value	χ2	OR	p-value
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 was considered as statistically significant, and p > 0.05 indicates consistent with HWE								

In case of rs1867503 of *TF* gene, children carrying AG genotype had 3.18 times (95% CI = 1.69-5.97) more risk in the development of ASD compared to children carrying AA genotype, which is statistically significant ($p < 0.05$). Children with GG genotype had 2.62 times (95% CI = 1.22-5.60) more risk for the development of ASD compared to the children carrying AA genotype, which is also statistically significant ($p < 0.05$). Children carrying combined genotype AG+GG (dominant model) had 2.98 times (95% CI = 1.67-5.31) more risk for the development of ASD compared to children carrying AA genotype, which is statistically significant ($p < 0.05$). On the other hand, children carrying G allele had shown 1.94 times (95% CI = 1.31-2.87) more risk for the development of ASD compared to the children carrying A allele which is also statistically significant ($p < 0.05$) (Table 3 and Figure 1).

Table 4 elicited rs9951150 allele of *TCF4* genotypes among ASD children and control volunteers and their association with ASD. Children carrying AG genotype had shown 2.92 times (95% Cl = 1.57-5.43) more risk in the development of ASD compared to children carrying AA genotype, and that is statistically significant ($p < 0.05$), whereas children carrying GG genotype had shown 2.36 times (95% Cl = 1.10-5.06) more risk for the development of ASD in compared to the children carrying AA genotype and that is statistically significant ($p < 0.05$). Children with combined genotype AG+GG (dominant model) have 2.72 times (95% Cl = 1.55-4.76) more risk for the development of ASD compared to the controls carrying AA genotype, and that is statistically significant ($p < 0.05$), whereas children carrying G allele, had shown 1.92 times (95% Cl = 1.29-2.87) more risk for the development of ASD in compared to the controls carrying A allele and that is statistically significant ($p < 0.05$). No association was found in the case of the recessive model (GG vs. AA+AG) for both SNPs with ASD in the studied population (Figure 2).

Discussion

Though the prevalence of ASD is increasing day by day, there is still a lack of a proper way to diagnose or prevent ASD. The heritability of ASD is 90%. However, it is challenging to identify relevant genes, which are liable for the development of ASD (Bailey et al. 1995). Multiple studies are going on to identify the responsible genes and already hundreds of genes are found positively accountable for the development of ASD, and these genes are following various biochemical pathways to show their functions (Davis et al. 2003; Konstantynowicz et al. 2012; Luck et al. 2013). It was the first-ever attempt in Bangladesh, and here, we reported our initial findings on the association of *TF* and *TCF4* genes polymorphisms with ASD from the perspective of Bangladesh.

Several studies considered *TF* (rs1867503) and *TCF4* (rs9951150) as role players in a variety of psychiatric symptoms and diseases, including phobic anxiety, obsessive-compulsive disorder, schizophrenia, and attention-deficit hyperactivity disorder. Polymorphism of *TF* causes an increase or decrease of oxygen free radicals, which are responsible for oxidative stress associated with the neurodegenerative disorder by causing damage of neurons with excess production of lipid peroxidation (Onyango et al. 2010). This polymorphism also causes more formation of ferrous, which stimulates hydroxyl formation and leads to brain cell damage (Bjørklund et al. 2020). A study with Egyptian children showed a large number of antioxidants, superoxide dismutase (SOD), which are markers for lipid peroxidation and showed the polymorphism of Transferrin (Chauhan et al. 2004; Meguid et al. 2011).

In our present research, for rs1867503 SNP of *TF* gene, 118 healthy volunteers and 96 individuals with ASD were studied. We have found a significant association between rs1867503 and ASD in Bangladeshi children. Children carrying AG and GG genotypes had 3.18 and 2.62 times more risk, respectively, in the development of ASD compared to controls carrying AA genotype, which is statistically significant ($p < 0.05$). Another statistically significant ($p < 0.05$) association was observed on children carrying combined genotype AG+GG have (OR = 2.98, $p = 0.0002$)), whereas children carrying G allele have shown 1.94 times more risk for the development of ASD in compared to the controls carrying A allele and that is statistically significant ($p < 0.05$). A comparative study was performed by Chauhan et al. (2004) in which they showed

an elevated level of lipid peroxidation in autistic children compared to their non-autistic siblings. They also found increased oxidative stress, which is caused by reduced transferrin. This reduced transferrin is also responsible for language difficulties in ASD children. Luck et al. (2013) conducted a study in which they described oxalate in plasma could play a role in ASD to interfere with iron transport by binding with transferrin (hTF), and this high oxalate can cause iron deficiency anemia (IDA) in children with ASD. Our SNP finding study has also suggested such kind of relation to ASD.

Polymorphism of *TCF4* disrupts the columnar and laminar structure of the cortex, which is activity-dependent. It also hampers calcium activity, which is responsible for neuronal excitability. These incidents result in different autistic syndrome in children (Page et al. 2018). A study about *TCF4* regulation by Blake et al. in which they investigated that *TCF4* encodes a basic helix-loop-helix transcription factor that merges with other factors to activate or suppress gene expression, which causes two rare ASDs, Pitt-Hopkins syndrome and 18q deletion syndrome (Meguid et al. 2011). Our present study has brought some positive outcomes to validate the findings generated by some previous researchers (Page et al. 2018; Blake et al. 2010).

In the case of rs9951150 SNP of *TCF4*, children carrying AG and GG genotypes have 2.92 and 2.36 times more risk in the development of ASD, respectively, compared to controls carrying AA genotype and that is statistically significant ($p < 0.05$). Children carrying combined genotype AG+GG have 2.72 times more risk for the development of ASD compared to the controls carrying AA genotype, and that is statistically significant ($p < 0.05$), whereas children carrying G allele have shown 1.92 times more risk for the development of ASD in compared to the controls carrying A allele and that is statistically significant ($p < 0.05$).

From this result, we can say that the rs1867503 and rs9951150 SNPs are strongly associated with the development of ASD. As we have identified the genetic basis of the Bangladeshi children with ASD, we hope it will be helpful to understand the etiology of ASD. However, some limitations of this study should be noted. Only two known SNPs were selected from a public database without novel SNP. Another limitation is that the study population we present here is not large enough to represent the actual scenario of the country. Though we have found a strong association, a large-scale study may provide stronger evidence.

Conclusion

This case-control study reveals that *TF* rs1867503 and *TCF4* rs9951150 polymorphisms are significantly associated with ASD in Bangladeshi children. However, it is the first study for these SNPs in Bangladesh with a limited number of cases and controls, and the results are significant. This study will be beneficial for further studies with a large-scale population.

Declarations

Ethics approval and consent to participate

The present study was directed in the Department of Pharmacy, Noakhali Science and Technology University, Noakhali-3814, Chittagong, Bangladesh. Ethical clearance was obtained from the ethical committee of the Noakhali Science and Technology University, and written consent from each patient was taken prior to their inclusion in the study. Consent was obtained verbally and in writing (signature or fingerprints). The consent form was translated into the native language for the understanding of participants.

Competing Interests

The authors report no conflicts of interest.

Consent for Publication

All the authors approved the submission of the manuscript for publication.

Availability of data and materials

The required data and materials were mentioned in the manuscript. Further information about data and materials will be available for the corresponding authors on a valid request.

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Authors Contributions

MA, MSH, M. Siddiul Islam, MAA, MMRM: Blood sample collection; MAA, MB, NS, MAR: DNA extraction; MA, MSH, MAA, MB, NS, MAR, MS: PCR analysis and initial draft preparation; MA, M. Siddiul Islam, MB, NS, MAR, MS: Data analysis, critically review, interpretation of results; GMA: Conception and edition of the manuscript; MSI: Conception, supervision, Institutional approval, edition, final check and

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Data availability

The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

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Figures

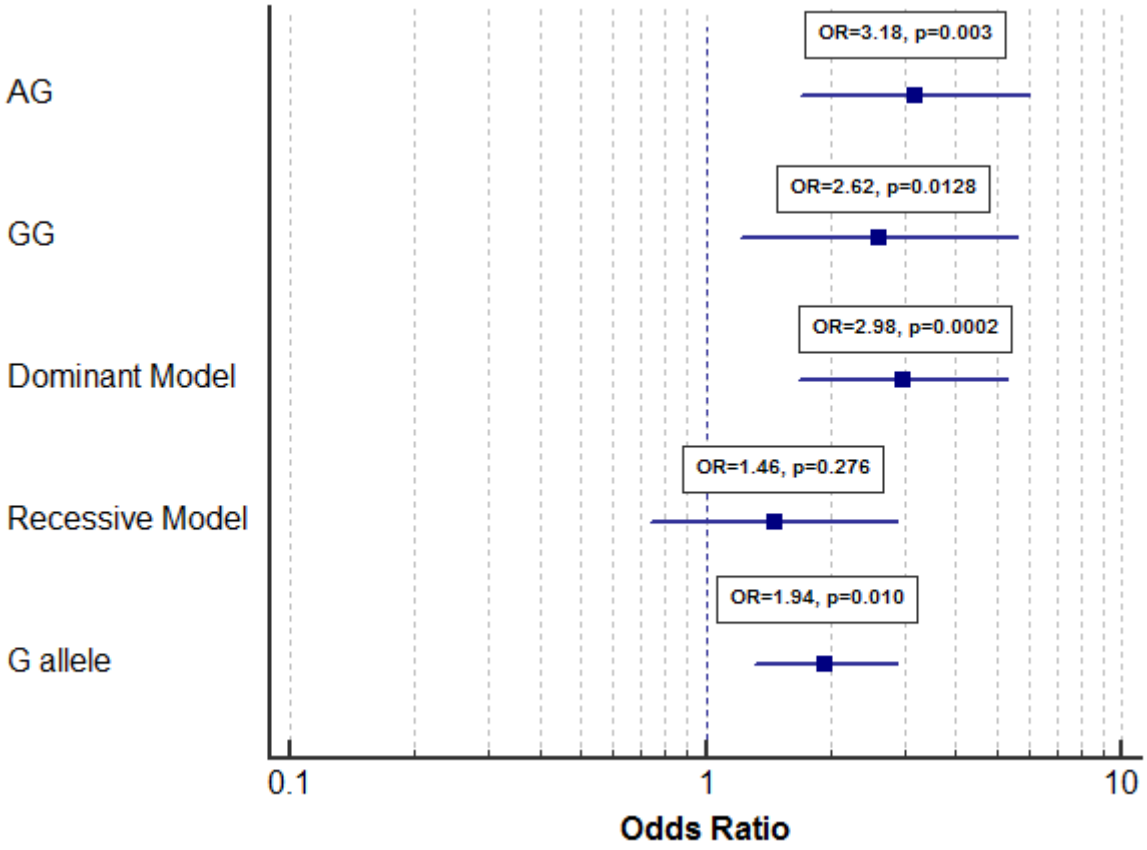

Figure 1

Forest plot of rs1867503 allele of TF gene in the study population

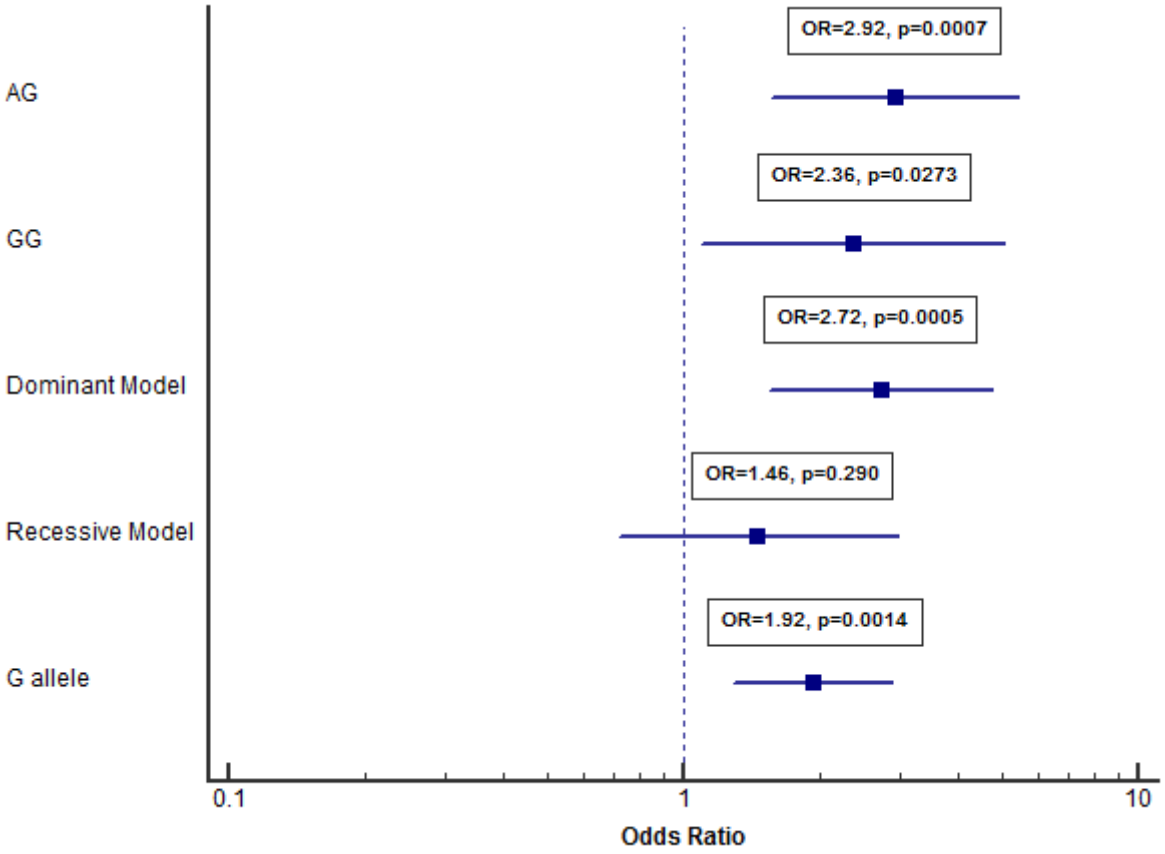

Figure 2

Forest plot of rs9951150 allele of TCF4 in the study population