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Article

A case-control study along with an epidemiological approach to *CNTNAP2* polymorphism among Bangladeshi ASD children

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Abstract: ASD (Autism Spectrum Disorder) is a neuropsychiatric disorder with a hereditary component, and its prevalence in South Asia was assessed 1, out of every 93 children. Moreover, recent studies suggested that the etiology of autism is thought to be linked to anomalies in the synapse, where mutation or deletion of synaptic gene *CNTNAP2* is responsible. Therefore, this research was aimed to find out specific signs and symptoms of ASD individuals as well as the distribution pattern of the *CNTNAP2* allelic variant (rs7794745) as a genetic risk factor in the Bangladeshi population. A case-control study including an epidemiological survey to investigate the association and pathophysiology of *CNTNAP2* (rs7794745) with ASD for the Bangladeshi population has been studied, where PCR-RFLP analysis and Sanger sequencing were used for 180 individuals (90 ASD samples and 90 healthy controls). Our retrieved data speculated a diverse clinical profile of ASD, in comparison to the control group (n=110); where 80.9% ($p \leq 0.001$) of ASD patients (n =100) had severe social interaction difficulties, 50% ($p \leq 0.001$) had language impairments, and 40.9% ($p \leq 0.001$) had behavioral abnormalities. Furthermore, findings from Pearson's chi-square test ($p = 0.001$) as well as logistic regression analysis of co-dominant ($p = 0.0083$), and recessive model ($p = 0.0075$) confirmed significant association between rs7794745 and in our studied sample. This research demonstrates the genetic variation of *CNTNAP2* found in our studied population could open a new clue to identifying a reliable biomarker for early diagnosis of ASD though it is recommended that more study is needed with a larger group population.

Keywords: autism spectrum disorder; *CNTNAP2*; epidemiological study; PCR-RFLP

1. Introduction

ASD is a term used to characterize a group of early-onset social communication deficits and repetitive behaviors that are linked to a strong genetic component, varying levels of intellectual disability starting in childhood, and other factors (Lord *et al*., 2020). In addition to these main symptoms, individuals with autism are more likely to have co-occurring psychological or neurological conditions, the most common of which are hyperactivity and

attention disorders such as ADHD, anxiety, bipolar disorder, depression, tourette syndrome, and epilepsy (Lord *et al*., 2020). In children with ASD, the intensity of core symptoms and comorbidities varies widely, but gastrointestinal issues, sleep disorders, and poor diet are common comorbidities (Yang *et al*., 2021). According to the American Academy of Pediatrics (AAP) guidelines, accurate diagnosis, early detection, familial and social support, intelligence (e.g., IQ), and linguistic skills are all important factors in the rehabilitation of children with ASD (Hyman *et al*., 2020). No epidemiological studies in Bangladesh have been undertaken to assess the etiology of ASD in infants, their age at diagnosis and treatment, their features and clinical manifestations, or the effects of familial and social support on their clinical symptoms, using specific clinical subgroups. Environmental risk factors (e.g., prenatal and perinatal factors, as well as maternal nutritional and lifestyle factors) that play an essential role in the advancement of ASD have been described in several systematic reviews. However, none of the environmental risk factors or susceptible genes appear to be carried by all people with ASD (Lord *et al*., 2020), and this heterogeneity has been a major roadblock to effective therapy development. As a result, identifying clinical subgroups and developing useful biomarkers are critical. On the other hand, several complex processes control the number, size, shape, and intensity of neuronal synapses across development and throughout life. Synaptic changes arise as a result of variations in the molecular structure of synapses and chemical modifications to synaptic proteins (Monteiro and Feng, 2017). The *CNTNAP2* (Contactin-associated protein-like 2) gene, encoding the neuronal cell adhesion transmembrane glycoprotein Caspr2, has been proposed to be one of the major susceptibility genes for neurodevelopmental disorders, including autism spectrum disorder (ASD) (Canali *et al*., 2018). The *CNTNAP2* gene spans 2.3 Mb on chromosome 7q35-36. Endophenotype analysis revealed that *CNTNAP2* has a major role in ‘language development in ASD and other language-related disorders (Peñagarikano and Geschwind, 2012). *CNTNAP2* is known as the very first ASD-predisposition gene that has been broadly replicated, with the clearest indication of autism susceptibility from multiple independent studies (Beiranvandi *et al*., 2020), (Zare *et al*, 2017). One of the non-coding variants in *CNTNAP2* is rs7794745 that located in intron 2 (Arking *et al*., 2008). (Koeda *et al*., 2015) suggested that A/T in rs7794745 more severely affects the reduction of cerebral response to human voice perception. Researchers found that despite the absence of behavioral abnormalities homozygotes for the risk allele (T) showed significant cerebral morphological variation, including reductions in grey and white matter volume in several regions (cerebellum, fusiform gyrus, and occipital and frontal cortices) that have already been implicated in ASD, suggesting that this polymorphism could disrupt frontal-occipital connections (Peñagarikano and Geschwind, 2012). In the Pakistani population, it was shown that A/T in rs7794745 of *CNTNAP2* is a risk genotype for ASD (Khalid *et al*., 2020). According to (Nascimento *et al*., 2016), a research finding on the Brazilian population reported this SNP is a susceptible factor for the Brazilian population while (Werling *et al*., 2016) found no association with the population of Zurich. This SNP was also found as a significant risk factor for the Chinese Han and Thi-Qar populations (Fang *et al*., 2021), (Issa *et al*., 2018). The available literature on the prevalence of these polymorphisms in various populations and their links to autism is somewhat ambiguous. As a consequence, the biological relevance of the *CNTNAP2* gene in ASD is still being researched.

Since ASD includes a variety of pathological conditions, a global biomarker of all subgroups may not exist; and studies have failed to divide children with ASD into meaningful subgroups due to small sample sizes and methodological limitations (Yang *et al*., 2021). As a result, identifying the exact biomarkers for each subgroup has been difficult. Hence, research into ASD in preschool children in Bangladesh is needed, as well as their ages at diagnosis and treatment, prominent comorbidities, and the influence of family and societal support on their symptoms. Therefore, this research aimed to find out specific signs and symptoms of ASD individuals and their parent's demographic profiles through the appropriate questionnaire. Moreover, the distribution pattern of the *CNTNAP2* allelic variant (rs7794745) as a genetic risk factor in the susceptibility of ASD also investigated.

2. Materials and Methods

2.1. Ethical clearance

'Ethical Clearance' for this research was taken from The Ma Shishu O General Hospital in Chattogram, (ref: CMOSHMC/IRB/2018/6) Bangladesh. All participants' legal guardians and the administration of their respective institutions provided written informed permission because the maximum number of participants were children.

2.2. Socio-demographic study and clinical profiling of ASD

Demographic data of 210 (100 ASD individuals and 110 control) populations (age-sex matched) has been collected to study different co-morbidities through a questionnaire. The core dimension of diagnostic symptoms and clinical profile with co-morbidities of ASD were studied. The co-occurrence of two or more disorders in the

same person is known as comorbidity. The majority of people with autism have one or more comorbid psychiatric illnesses. Sleep disorders, anxiety, epilepsy, depression, and Attention Deficit Hyperactivity Disorder (ADHD) are the most common co-morbid diagnoses, although eating disorders, Obsessive Compulsive Disorder, bipolar disorder, hetero-aggression, and self-harm are also common in autistic patients (Kong, 2018). The socio-economic impact of this disorder in the region of Chattogram and the mental health condition of the subject's parents due to stress, hormonal imbalance, antibiotic treatment, etc. were also studied. Besides, age, blood group, and situations throughout the pregnancy period of the subject's mother have been also considered.

2.3. ASD Severity Level Calculation

The ASD severity level (criteria determined by DSM-5; American Psychiatric Association, 2013) was calculated by using CARS scoring for the 90 ASD patients who enrolled for the molecular analysis of this study (Supplementary Table 1).

Supplementary Table 1. Summary of CARS scoring.

CARS Scoring	ASD Data	Control Data	*p*-value
CARS Range	30-60	-	-
CARS Mean ±SD	39.83±5.36	-	<0.001
Mild to Moderate <37	35 (36.5%)	-	<0.001
Severe ≥37	61 (63.5%)	-	<0.001

2.4. Inclusion and exclusion criteria

Data and samples were collected from all of Chattogram City's autistic schools and centers throughout this research. During the Covid-19 outbreak, samples have been collected from the patient's residence due to lockdown. Pre-diagnosed patients who meet the DSM-5 ASD diagnostic criteria (American Psychiatric Association, 2013) are chosen as subjects, while healthy individuals who are 'Non-ASD' are chosen as a control group. There is no medical test (such as a blood test) for autism spectrum disorder (ASD), and diagnosing this disorder can be challenging. Due to the complexity, severity, and overlap of ASD symptoms with those of other psychiatric conditions, it is critical to appropriately identify ASD. The Diagnostic and Statistical Manual of Mental Disorders (DSM) is a diagnostic tool produced by the American Psychiatric Association (APA) that serves as a common language for clinicians, researchers, and public health officials. The DSM-5 includes the most up-to-date criteria for diagnosing mental disorders, as well as extensive descriptive text, establishing a common language for clinicians to communicate about their patients and establishing consistent and reliable diagnoses that can be used in mental disorder research (Regier *et al*., 2013). For people with autism spectrum disorder, the DSM-5 provides a degree of support ratings for social communication (SC) and restrictive and repetitive behaviors (RRB). In both SC and RRB, the specifiers help physicians to utilize the clinical judgment to distinguish between three classifications: Level 1 ("Requiring support"), Level 2 ("Requiring significant assistance"), and Level 3 ("Requiring very substantial support") (Gardner *et al*., 2018). Inclusion criteria for ASD subjects considered in data and sample collection are "the presence of the three core difficulties of social interaction, behavior, and language", and "previously diagnosed by a pediatrician or psychologist". On the other hand, the exclusion criterion includes "Patients who didn't agree to give the blood sample", "Patients who do not fulfill the DSM-5 criteria of ASD", and "Patients' guardians who didn't agree to give the information about their children".

2.5. Sample Collection

Following patient's counseling, blood samples (2.0 ml) were withdrawn by an expert phlebotomist in accordance with WHO blood withdrawal guidelines (World Health Organization, 2010) from 90 ASD patients and 90 control groups and were kept in sterile, labeled anticoagulant (K3EDTA) containing vacutainer tubes and stored them into -20℃ refrigerator for further experiment.

2.6. DNA Extraction

The genomic DNA was extracted from blood samples using the, as directed by the manufacturer. Thermo Scientific NanoDrop 2000 spectrophotometer was used to quantify the extracted genomic DNA. Nuclease-free water was utilized as a standard, and all measurements were carried out according to protocol.

2.7. Genotyping

All extracted DNA samples were subjected to PCR amplification. Then the amplified products were genotyped by using the PCR-RFLP method. Used primer and the list of restriction enzymes were given in (Supplementary Table 2). Selected PCR reactions were sequenced using the Sanger method of DNA sequencing and data was examined, with the SnapGene® Viewer tool to detect Single Nucleotide Polymorphisms (SNPs) (Version 5.2.4).

Supplementary Table 2. Primer and enzyme were used in this experiment.

SNP	Primer sequence (5'-3')	Product Length	Restriction Enzyme	Allele (bp)	
(CNTNAP2)	**F:** AATACGGACCAAGATACCAAC	315bp	TasI	**A**	**T**
rs7794745	**R:** TTCAGACCAACAGTGCCTT			(315)	(95/220)

2.8 Statistical analysis

For all of the Single Nucleotide Polymorphisms (SNPs) under consideration, the study population's allele and genotype frequencies were calculated using the Hardy-Weinberg equation (Hardy, 1908) with a web program (http://scienceprimer.com/hardy-weinberg-equilibrium-calculator) and tested with the Chi-square test. The computed allelic frequencies were then compared to the BEB population's allelic frequencies (data were retrieved from dbSNP and 1000 genome projects). MedCalc was used to calculate the odds ratio (OR) and 95% confidence interval (CI) to determine the relative risk conferred by a certain allele and genotype. In addition, Binary logistic regression was used to undertake a series of tests for the SNP using dominant, receive, and over-dominant genetic models. Other statistical analyses were performed in the SPSS software package, version 17.0 (SPSS, Inc., Chicago, IL).

3. Results

This research aimed to find the current situation and association of *CNTNAP2* (rs7794745) among the ASD population in the southern part of Bangladesh through a demographic study, clinical profiling, and molecular analysis (Table 1, 2, and 3).

Table 1. Demographic profile of the studied population.

Criteria	ASD (n=100)	Controls (n=110)	Chi-square value	*p*-value
Age	10±5.041	11.83±5.05	31.879	-
BMI	20.52± 7.3	20.29±4.42	345.679[a]	-
Male	86% (86)	53.6% (59)	-	-
Female	14% (14)	46.4% (51)	-	-
Male: Female	6.14:1	1.15:1	28.025[a]	≤0.001**
Age of recognition of symptoms of ASD				
24 months	58.5% (38)	-	-	-
25-48 months	36.9% (24)	-	-	-
After 48 months	4.6% (3)	-	-	-
Age of diagnosis of ASD				
24-36 months	63.1% (41)	-	-	-
37-60 months	33.8% (22)	-	-	-
After 60 months	3.1% (2)	-	-	-
Presence of three core diagnostic criteria				
Social Interaction difficulties	80.9% (89)	5.5% (6)	159.582[a]	<0.001**
Behavioral problem	40.9% (88)	7.3% (8)	144.338[a]	<0.001**
Language Impairment	50%(87)	9.1% (10)	136.776[a]	<0.001**

Note. Here, ** values are considered statistically highly significant when $p \leq 0.001$. Data are shown as mean ± standard deviation (SD) for Age and BMI.

Table 2. Summary of the co-morbidities of ASD.

Criteria	ASD (n=100)	Control (n= 110)	Chi-square value	*p*-value
Intellectual disability	96.9% (94)	5.5% (6)	166.561[a]	<0.001**
Sensory problem	80.6% (75)	4.5%(5)	100.999[a]	<0.001**
Sleep disturbance	59% (59)	6.4% (7)	67.341[a]	<0.001**
Generalized anxiety	4% (4)	7.9% (5)	0.038[a]	0.845
Inappropriate mood swing	41% (41)	3.2% (2)	50.449[a]	<0.001**
ADHD	86% (86)	2.9% (3)	147.824[a]	<0.001**
Epilepsy	30% (30)	6.4% (7)	20.162[a]	<0.001**
Bipolar disorder	35% (35)	0	-	<0.001**
Dyslexia	8% (8)	9.1% (10)	0.080[a]	0.778
Obsessive-compulsive disorder (OCD)	5% (5)	0	-	0.157
Depression	2% (2)	7.4% (8)	3.211[a]	0.073

Note. Here, ** values are considered statistically highly significant when $p \leq 0.001$ and *considered statistically significant when $p \leq 0.05$. Fisher's exact test was done instead of the chi-square test whenever a cell frequency was with a null value and in that case, instead of the chi-square value, fisher's exact value was taken.

Table 3. Summary of the symptoms present in ASD.

Criteria	ASD	Control (n=110)	Chi-square value	*p*-value
Poor eye contact	35.5% (39)	5.5% (6)	45.640[a]	<0.001**
Poorly integrated verbal & non-verbal	58.2% (64)	0.9% (1)	124.838[a]	<0.001**
Do not like to play with children of the same age	66.4% (73)	1.8% (2)	122.361[a]	<0.001**
Ignores when called	53.6% (60)	0.9% (1)	90.396[a]	<0.001**
Deficits in maintaining& understanding relationship	61.8% (68)	8.2% (9)	107.289[a]	<0.001**
Excessive fear of noise, cover ears frequently	24.5% (27)	2.7% (3)	37.575[a]	<0.001**
Aloofness	37.3% (41)	5.5% (6)	54.981[a]	<0.001**
Lack of curiosity about the environment	63.6% (70)	1.8% (2)	113.303[a]	<0.001**
The facial expression doesn't fit the situation	35.5% (39)	0	-	<0.001**
Inappropriate crying/laughing	56.4% (62)	2.7% (3)	96.147[a]	<0.001**
Ignoring pain & temper tantrums	31.8% (35)	0.9% (1)	43.147[a]	<0.001**
Hates crowded places	43.6% (48)	3.6% (4)	54.534[a]	<0.001**
Repetitive behaviors	40.9% (45)	3.6% (4)	61.955[a]	<0.001**
Resistance to change	43.6% (48)	2.7% (3)	77.962[a]	<0.001**
Restlessness	67.3% (74)	21.8% (24)	83.696[a]	<0.001**
Stereotypical movements	52.7% (58)	3.6% (4)	95.202[a]	<0.001**
Presence of activity to hurt others	41.8% (46)	10% (11)	45.993[a]	<0.001**
Hyper/ hypo reactivity	51.8% (57)	11.8% (13)	66.158[a]	<0.001**
Playing with toys in an unimaginative way	51.8% (57)	0.9% (1)	83.336[a]	<0.001**
Preferring to maintain a strict routine	57.3% (63)	0.9% (1)	102.732[a]	<0.001**
Having a strong like/dislike of certain food	63.6% (70)	7.3% (8)	98.743[a]	<0.001**
Presence of self-hurting activity	37.3% (41)	7.3% (8)	45.951[a]	<0.001**
Preferring to avoid using spoken language	70.9% (87)	0	-	<0.001**
Speaking in a pre-learned phrase	50% (55)	0.9% (1)	99.644[a]	<0.001**
Speech sound as monotonous	50% (55)	0	-	<0.001**
Seeming to talk at people, rather than sharing a two-way conversation	52.7% (58)	0	-	<0.001**
Coordinated motor movements	85% (85)	75.5% (83)	10.965[a]	<0.001**
Uncoordinated motor movement	19% (19)	0	-	<0.001**

Note. Here, **values are considered statistically highly significant when $p \leq 0.001$ Fisher's exact test was done instead of the chi-square test whenever a cell frequency was with a null value, and in that case, instead of the chi-square value fisher's exact value was taken.

3.1. Epidemiological study

3.1.1. Age groups of ASD parents

Six groups were considered to categorize ASD parents into different age ranges. Mothers between the ages of 26-30 (32.78%) and fathers between the ages of 36-40 (32.78%) are accountable here for having children with ASD (Figure 1 and 2).

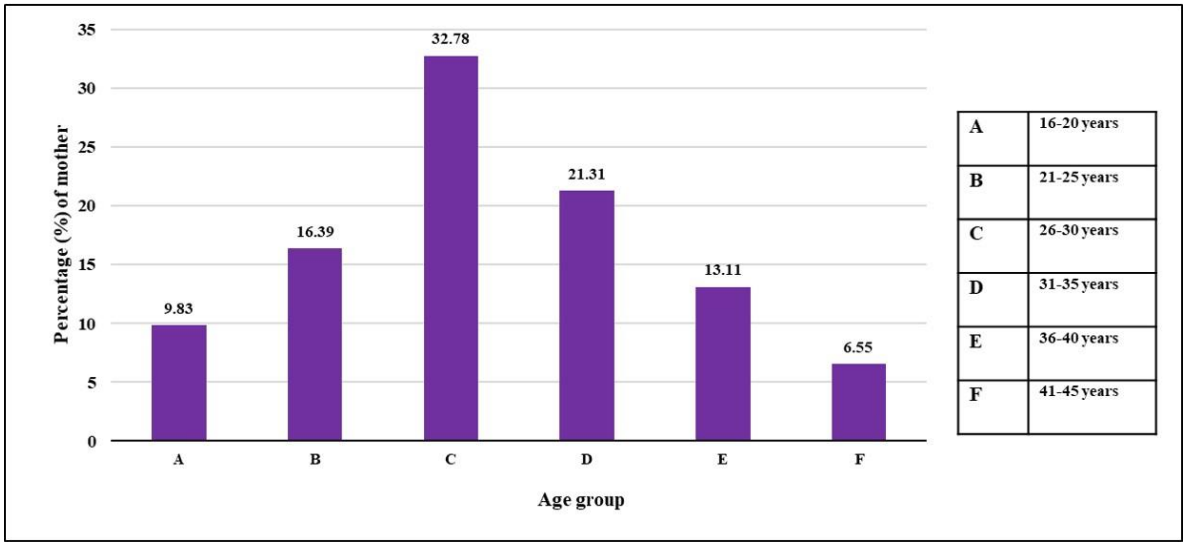

Figure 1. The age range of ASD mothers.

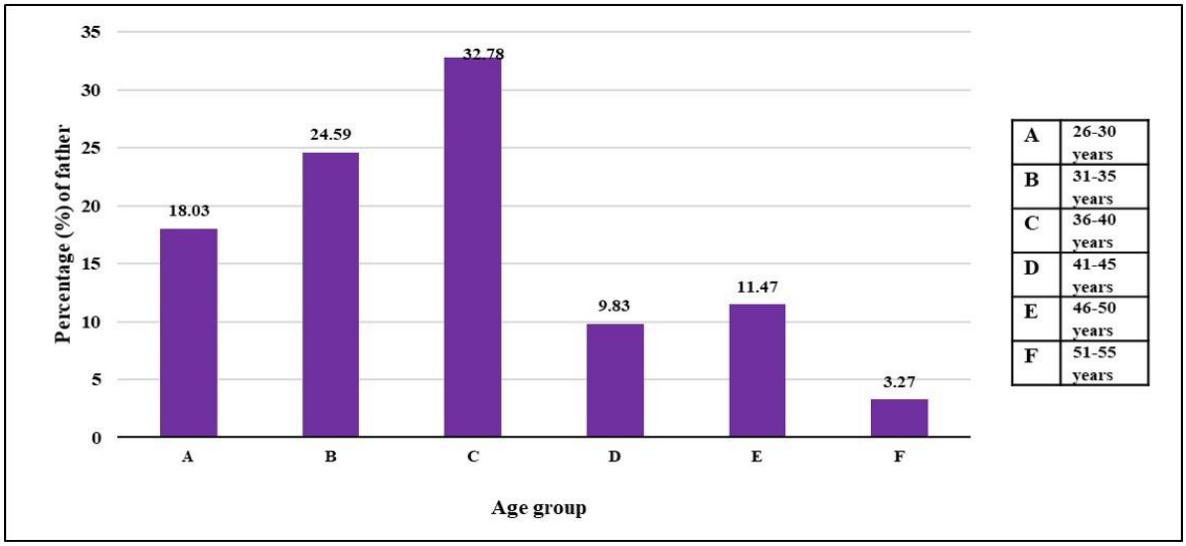

Figure 2. The age range of ASD fathers.

3.1.2. Blood groups of ASD parents

The blood groups of the majority of ASD parents; mothers (23.8%) and fathers (31.7%) were B+ than other blood groups (Figure 3).

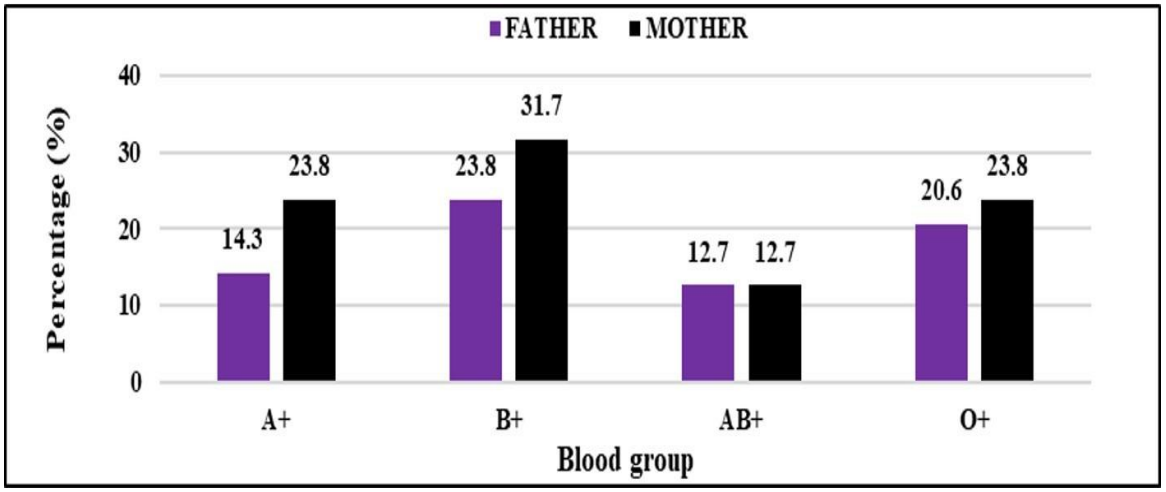

Figure 3. Frequency of blood groups of ASD parents.

3.1.3. Other features of ASD mothers

It is noteworthy that ASD mothers suffer greatly throughout pregnancy, with 61.9% reporting stress over their child's condition, as well as anxiety, depression, and mental trauma, and 28.6% reported difficulties during birth. Few mothers (7.9%) received dental amalgam therapy and antibiotic treatment. 46% of ASD mothers reported being harassed by society for having a special child with ASD. 81% of mothers said that maintaining ASD children through schooling, training, therapy, counseling, and other means is costly and that the available resources are insufficient to help their child progress (Figure 4).

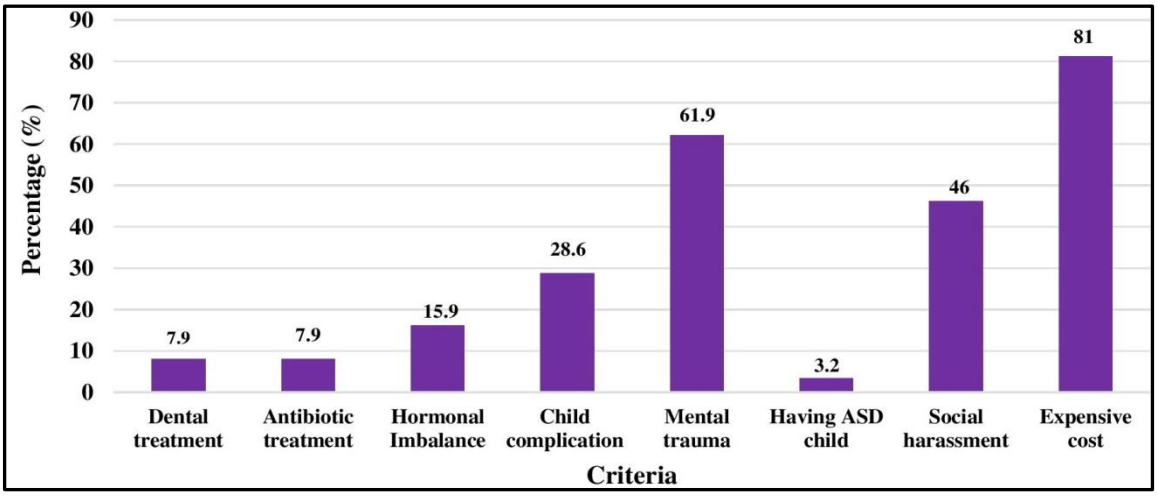

Figure 4. Difficulties faced by ASD mothers.

3.2. Molecular analysis

Interestingly, the frequency of the rs7794745TT genotype was considerably (p = 0.001) greater in the ASD group compared to the control group whereas risk genotype TT (15.56%), wild type AA (38.89%), and AT (45.56%) were present in the ASD, and TT (1.11%), AA (45.56%), and AT were present (53.33%) in the control group (Table 4, Figure 5). Sanger sequencing of amplified PCR products also confirmed the presence of homozygous TT polymorphism (Figure 6). Thus using the Chi-square test, a significant association between ASD and a healthy population was observed in the genotype distributions of the *CNTNAP2* gene (rs7794745) polymorphism (TT, p = 0.001). Moreover, in the binary logistic regression analysis, the co-dominant (OR = 16.4, 95% CI = 2.05-131.05, p = 0.0083), and recessive model (OR = 16.395, 95% CI = 2.11-127.58, p = 0.0075) (Table 4) also showed significant association for ASD and Bangladeshi population. We calculated genotype frequencies for all the alleles and genotypes by applying the Hardy Weinberg Equation while rs7794745T acts as minor, both in the ASD population and reference population (BEB) which is listed in Table 5.

Table 4. Summary of CNTNAP2 (rs7794745) genotyping result (by Chi-square test) and (by Binary logistic regression model).

rs7794745 SNP	ASD (%) (n=90)	Control (%) (n=90)	χ^2 – test	*p*-value	Genetic models	Odd ratio (OR)	95% Confidence Interval (CI)	*p*-value
AA	38.89% (35)	45.56% (41)	0.820	0.451	Co-dominant model			
AT	45.56% (41)	53.33% (48)	1.089	0.371	AA	1	-	-
TT	15.56% (14)	1.11% (1)	12.291	0.001**	AT	1.0006	(0.54-1.85)	0.9985
					TT	16.4	(2.05-131.05)	0.0083*
					Dominant model (AT+TT vs AA)			
					AA	1	-	-
					AT+TT	1.315	(0.73-2.38)	0.366
					Recessive model (TT vs AA+AT)			
					AA+AT	1	-	-
					TT	16.395	(2.11-127.58)	0.0075*

Note. Here, *values are considered statistically significant when $p \leq 0.05$ and **values are considered statistically highly significant when $p \leq 0.001$. Logistic regression analysis revealed that rs7794745 was associated with ASD both in co-dominant and recessive model and (OR = 16.4), (95% CI = 2.05-131.05), p = (0.0083) and (OR=16.395), (95% CI= 2.11-127.58), p = (0.0075).

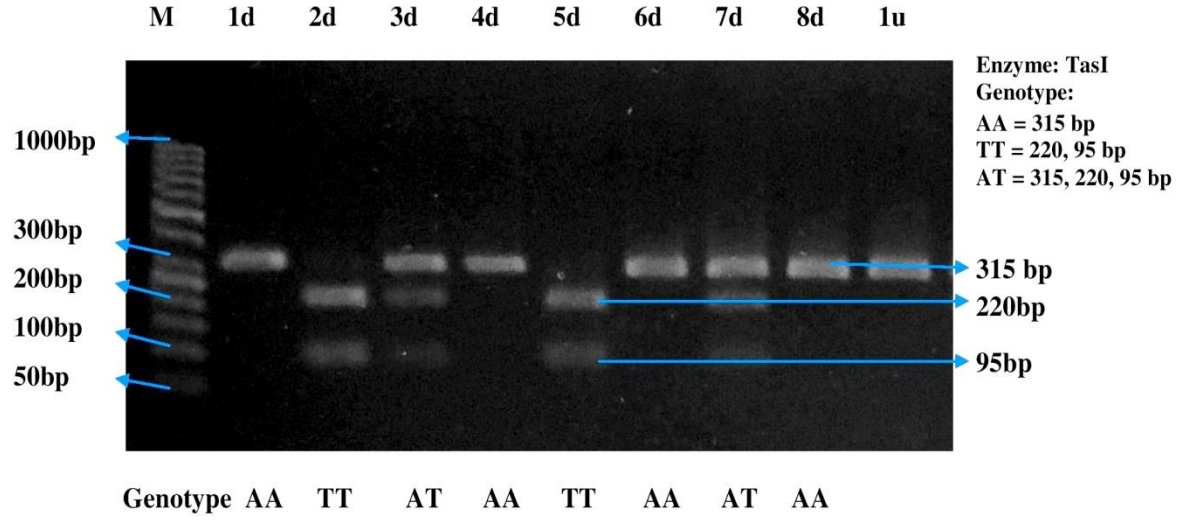

Figure 5. Genotyping of rs7794745 by PCR-RFLP method and visualized in 2% agarose gel.

Here, M = 50bp DNA Ladder (Gene Ruler, Thermo Fisher Scientific Baltics UAB, Lithuania) and 1u = undigested PCR product. In a digested PCR product lane 1d, 4d, 6d, 8d represents the genotype AA; Lane 2d, 5d represents the genotype TT, and lane 3d, 7d represents the genotype AT.

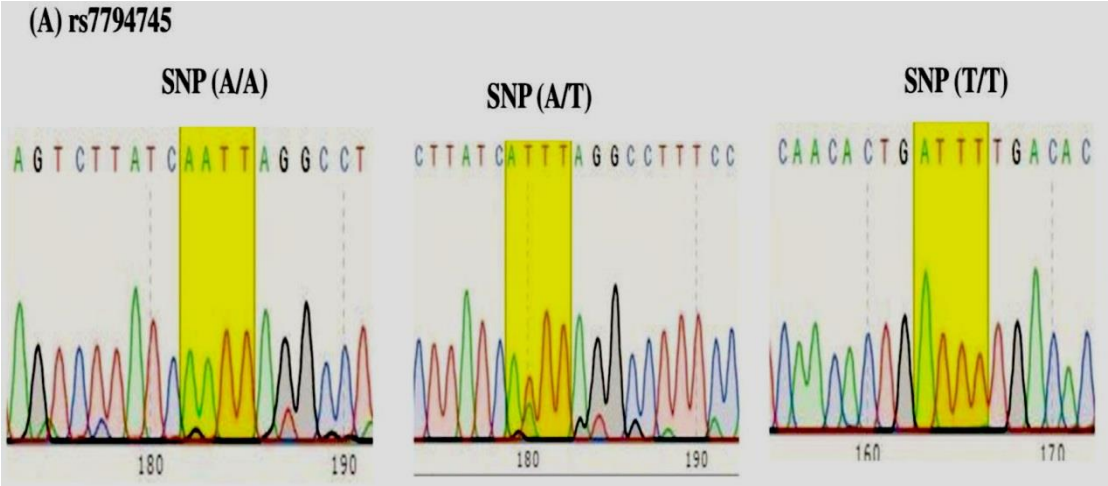

Figure 6. Shows the presence of (A/A) homozygous alleles, (A/T) heterozygous alleles, and (T/T) homozygous alleles in chromatogram data.

Table 5. Summary of Allele and Genotype frequency calculation.

Gene Variants (SNP)	Allele	Allelic Frequency	Allele freq of ref. population	Genotype	Genotypic Frequency	Observed Cases
rs7794745 (CNTNAP2)	A	0.617	A: 0.6279 T: 0.3721	AA	0.381	35
	T	0.383		AT	0.473	41
	(ASD n=90)			TT	0.147	14
	A	0.722		AA	0.521	41
	T	0.278		AT	0.401	48
	(Control n= 90)			TT	0.077	1

4. Discussion

Depending on the current situation, the World Health Organization (WHO) reports that 0.76% of children have ASD worldwide; however, this only accounts for around 16% of the world's children (Hodges *et al*., 2020), and globally, the number of people with ASD is growing every day. Even while ASD symptoms are neurological, they manifest as behavioral characteristics that vary in severity with age (Hyman *et al*., 2020). In our research, the mean age of ASD participants was 10±5.041, where the highest number of individuals were from the aged group of 6-8 years and the second-highest number of aged groups were 3-5 years. As we performed our demographic analysis around most of the schools of Chattogram city, so it is satisfactorily implied that the frequency of age group 3-8 years was highest than others as being standard age group of getting schooled. The mean BMI of ASD children (age group 3 to 20 years) resulted in 20.52±7.3, which is almost equal BMI shown in the control group 20.29±4.42, which indicates ASD children's BMI is not much different from typically developed (TD) children.

During gender ratio analysis for ASD studies, notable and consistent ASD features were diagnosed more frequently in boys compared to girls (Akhter *et al*., 2018). The sample population included 86 male and 14 female ASD subjects, resulting in a 6.14:1 affected male-female ratio; where the control group ratio was 1.15:1 (Table 1). Males are more likely to have ASD, a recent meta-analysis found that the true male-to-female ratio is lower than the 3.5:1 trend is similarly found in previously 4:1 (Demily *et al*., 2017) (Loomes *et al*., 2017) (Tartaglia *et al*., 2017) and according to the DSM-5, males are diagnosed with autism spectrum disorder four times more frequently than females (Loomes *et al*., 2017). Even though we couldn't include the overall population of Bangladesh, our result represents the gender trend for ASD prevalence in Chattogram province.

Studies found, that ASD develops at the age of three and lasts the rest of a person's life, however, symptoms may be flagrant as they grow older (CDC centers for disease control and prevention, 2021). Within the first few months of life, some children with ASD display signs of potential issues. On the other hand, some children with ASD continue to grow normally until they are 18 to 24 months old, at which point they avoid acquiring new

skills or losing the ones they already have. According to research, one-third to half of the parents of children with ASD find a problem before their child's first birthday, and about 80%–90% noted problems by the age of around 24 months (CDC, 2021). Our data also showed that most of the parents (58.5%) recognize ASD symptoms at the age of 24 months and the less frequent age of recognition (4.6%) was after 48 months (Table 1). In our study, most of the cases (63.1%) were re-diagnosed by a special psychologist within the age of 24-36 months and less frequent cases (3.1%) were diagnosed after the 60 months.

We used CARS to measure the severity level of autistic individuals which can make the difference between children with autism and those who have other developmental delays. CARS consists of 15 elements that cover various ASD symptoms and allow for an accurate comparison of an affected child's behaviors and activities to a healthy child's predicted developmental growth (Sharma *et al*, 2018b). Moreover, the DSM-5 divides core symptoms into two categories: confined, repeated patterns of behavior, and social communication/interaction. We found core ASD symptoms as social interaction difficulties in most of the subjects 80.9% ($p<0.001$), and behavioral problems in 40.9% ($p\leq0.001$) significantly. Another most important hallmark feature of autism is communication difficulties which were found at 50% ($p\leq0.001$) (Table 1) among our studied people. Each ASD people communicates in a specific way, sometimes they have a strong grasp of the language while others are unable to speak at all or only speak a few words (CDC- centers for disease control and prevention 2021). The signs and symptoms of ASD differ widely, and the majority of people have a unique combination of social, behavioral, and language issues (Johnson *et al*., 2007).

We found some co-morbidities had the highest degree of significant association with ASD in our studied population as intellectual disability 96.9% ($p \leq0.001$), sensory problems 80.6% ($p \leq0.001$), sleep disorder 59% ($p \leq0.001$), inappropriate mood swing 41% ($p \leq0.001$), epilepsy 30% ($p \leq0.001$), bipolar disorder 35% ($p \leq0.001$), and ADHD 86% ($p \leq0.001$) (Table 2). The most common comorbidity in people with ASD is ADHD, who have average intelligence or intellectual disability (Lord *et al*., 2020). Both disorders have a genetic predisposition; they are more common in boys than in girls.

In ASD patients, bipolar disorder (BPD) is normal and frequently presents during adolescence (Vannucchi *et al*., 2014). An accurate diagnosis of dual ASD/BPD will lead to more tailored treatments and an increase in the patients' quality of life. Some other co-morbidities such as anxiety, depression, OCD, dyslexia, etc (Sharma *et al*., 2018b) have a strong association with ASD, though we found a poor connection of these disorders in our population such as only 4% ($p \leq0.845$) for anxiety disorder, 5% ($p \leq0.157$) for OCD, and 2% ($p \leq0.073$) for depressive disorder.

The quality and quantity of reciprocal social interaction in children with ASD are significantly reduced. According to research, children with ASD initiate fewer social interactions and receive fewer social overtures from other children (Corbett *et al*., 2014). From our study, a maximum of the individuals had poor eye contact (35.5%, $p \leq0.001$), struggled in maintaining and understanding relations (61.8%, $p \leq0.001$), do not like to play with other children (66.4%, $p \leq0.001$), ignores when called (53.6%, $p \leq0.001$), having excessive fear of noises (24.5%, $p \leq0.001$), having lack of curiosity about the environment (63.6%, $p \leq0.001$) and unusual expression of emotions such as unexpected crying or laughing (56.4%, $p \leq0.001$) (Table 3), etc. this means that social interaction difficulties were substantially higher in our studied ASD population than in the control group.

High rates of substantial financial costs for people, communities, and facilities are also linked with ASD (Buescher *et al*., 2014). In children with ASD, emotional and behavioral issues are common (Matson and Cervantes, 2014), and rates of social anxiety, intellectual disabilities, attention deficit hyperactivity disorder, and oppositional disorder are higher than in the general population (Brereton *et al*., 2006), (Emerson and Hatton, 2007), (Totsika *et al*., 2011). Sleeping, eating, and toilet issues have now become more problematic. Individuals with ASD may spend considerable time flapping their arms or rocking from side to side (CDC, 2021). In our findings, 40.9% ($p \leq0.001$) of the population was having repetitive behaviors, 43.6% ($p \leq0.001$) was resistant to any kind of change, and 67.3% ($p \leq0.001$) had impatience. Hyper/hypo-reactivity is a notable behavioral problem seen in the ASD population which we found 51.8% ($p \leq0.001$) in our study. The percentage of the ASD population who maintain a strict daily routine is 57.3% ($p \leq0.001$). Self-injurious activity is especially common in people with autism. Head-banging, self-biting, skin scratching, hair pulling, and hitting oneself against hard objects are all examples of self-injurious actions (Steenfeldt-Kristensen *et al*., 2020), which we found 37.3% ($p\leq0.001$) in our research. Besides, we observed that 41.8% ($p \leq0.001$) of ASD children engage in behavior that harms others. Another significant behavioral problem of autism is having a like/dislike of certain food (CDC, 2021) which we found 63.6% ($p \leq0.001$) in this survey.

Language impairments have been related to neuroanatomical and functional abnormalities is a hallmark feature of ASD (Herringshaw *et al*., 2016). This impairment can be summarized by some symptoms that had been found in our research such as avoiding using spoken language (70.9%, $p \leq0.001$), speaking in a pre-learned

phrase (50%, $p \leq 0.001$), and seeming to talk at people rather than sharing a two-way conversation (52.7%, $p \leq 0.001$) (Table 3). Language deficiency in ASD may be caused by polygenic interaction impairment during early developmental stages, but the exact underlying mechanisms are still uncertain (Newbury *et al*., 2010).

We may infer from the evaluation of ASD symptoms that the autism spectrum disorder has a general pattern of impairments in social interaction, speech, and behavioral disturbances. The time between first noticing symptoms and receiving a diagnosis is critical because it can open up a new window for treatments that lead to improvements in essential areas. So, knowledge of early signs and symptoms is necessary for ASD parents.

The relationship between ASD risk and parental age, especially maternal age, and increased parental age is one of the most consistently reported perinatal risk factors for ASD (Guinchat *et al*., 2012), (Idring *et al*., 2014), (Sandin *et al*., 2012). In our survey, the mean age of mothers is 29.79±6.47 and the father's mean age is 36.23±5.79, which indicates parental age is not a risk factor for our population. There is also evidence that risk varies depending on the age of the parents (Shelton *et al*., 2010). The epigenetic alteration, confounding by genetic responsibility or social determinants of reproductive age, and mediation by age-related pregnancy risks are all probable mechanisms that could underpin these associations (Lee and McGrath, 2015).

It seemed believed that ABO blood type was linked to ASDs in some way as parents' blood types are passed on to their children (Wu *et al*., 2012). We surveyed families with ASD to see if they have a particular ABO blood type where it has been shown that ASD mothers had 23.8% A+, 31.7% B+, 12.7% AB+, 23.8% O+ blood types while fathers had 14.3% A+, 23.8% B+, 12.7% AB+ and 20.6% O+ blood group. In this result, we can say that the B+ blood group of parents probably had a trend of risk for having an ASD child. In another study, It is also postulated that some unknown genetic mechanisms of a parent's blood type can contribute to the risk of autism, disrupting fetal neurodevelopment (Wu *et al*., 2012).

However, through antibody transfer and the impact of immune markers on the developing nervous system, maternal immune-mediated conditions and autoimmune reactions can influence the risk of ASD (Kohane *et al*., 2012), (Zerbo *et al*., 2015). Some other maternal factors could be a risk for autism; a significant percentage in our research 7.9% of mothers had antibiotic treatment during pregnancy, 15.9% of ASD mother’s experienced hormonal imbalance during their pregnancy, while 61.9% of mothers suffered depression, anxiety, and various sorts of mental stress (Figure 4).

Bullying of children with Autism Spectrum Disorder (ASD) is a serious concern in today's society. According to studies, children with Autism Spectrum Disorder (ASD) face social isolation and prejudice solely because of their physical disabilities. A child with ASD and their parents were exposed to a variety of social and behavioral situations. Parents of autistic children have voiced similar feelings since witnessing and enduring such social mistreatment over time (Rahman *et al*., 2019). We inquired about their experiences, and 46% of parents said that having an ASD child causes them to be socially harassed. In light of the above special conditions and social care, parents attempted to overcome the obstacles and establish a normal relationship with either the autistic child or the social agents.

Researchers discovered many genes that are required for synapses to develop and function properly which are *CNTNAP2*, *SHANK3*, *NLGN3*, and *NLGN4X*, etc. (Zare *et al*., 2017). In our age-sex-matched case-control study, we focused on genetic variants of rs7794745 of *CNTNAP2* (contactin-associated protein-like2), to determine their allelic and genotypic frequencies and to investigate their positive or negative association with ASD development in the Bengali of Bangladesh (BEB) population living in Chattogram, Bangladesh. To the best of our knowledge, this is the first study in the Chattogram area of Bangladesh to combine demographic profiling of ASD patients with their allelic distribution pattern which investigation does not reflect the fashion of the overall BEB population; rather, it found the trend of ASD pathogenesis in the studied population.

CNTNAP2 is located in the central and peripheral nervous systems, where it is strongly distributed in the frontal and temporal lobes, striatum, dorsal thalamus, and specific layers of the cortex, among other places (Rodenas-Cuadrado *et al*., 2014), (Scott *et al*., 2019). As a result of its effect on neural connectivity, neural migration, synapse growth, and synaptic communication, *CNTNAP2* plays a critical role in the formation of neural circuits. According to knock-out and knockdown studies; its absence results in decreased dendritic arborization and also decreased inhibitory interneurons, excitatory synapses, and inhibitory synapses (Anderson *et al*., 2012), (Gdalyahu *et al*., 2015), (Peñagarikano *et al*., 2011). Seizures, epilepsy, hyperactivity, and other behavioral abnormalities all are found in *CNTNAP2* knock-out mice, which are related to ASD (Peñagarikano *et al*., 2011). It is clear that the *CNTNAP2* gene plays an important role in neural development, and its dysfunction significantly raises the risk of neurological dysfunction. Another study demonstrated that the *CNTNAP2* gene is a transcriptional factor whose expression is regulated by the forkhead box P2 (FOXP2) gene, which is involved in language processing (Koeda *et al*., 2015). rs7794745 is a non-coding variant in *CNTNAP2* that is found in an intron (Arking *et al*., 2008). Introns in contemporary species fulfill a broad spectrum of functions are involved

in virtually every step of mRNA processing (Carmel and Chorev, 2012). There is an ongoing debate in the current research about the relation between rs7794745 SNP and ASD. The rs7794745 SNP has been described as a significant risk factor for the ASD population in several countries including the Iranian population (Zare *et al*., 2017), (Fang *et al*., 2021). Investigating the relationship between rs7794745 and ASD has shown that the TT genotype (rs7794745) of the *CNTNAP2* gene may be linked to an increased risk of autism in the Brazilian population (Nascimento *et al*., 2016). Some negative associations were also found from meta-analysis and family base study in the Wuerzburg and Zurich population (Werling *et al*., 2016). In molecular analysis of our study, the risk genotype rs7794745 (TT) association with ASD was found statistically highly significant (15.56%, $p \leq 0.001$) both in the chi-square tests and binary logistic regression models. The absence of this risk allele rs7794745TT in other ASD populations suggests that this risk allele is not the only cause of ASD. There are a variety of genetic and environmental factors that can contribute to the development of ASD, either separately or in combination (Di Napoli *et al*., 2015).

Since establishing specific treatments for each type of ASD appears very challenging, therefore our findings on synaptic genes, together with epidemiological data, may be useful in stratifying individuals and determining how and when to treat them.

5. Conclusions

In summary, this study revealed that the *CNTNAP2* polymorphism plays a crucial role in the development of ASD in the southern part of the Bengali of Bangladesh (BEB) population. For further confirmation of this risk gene in the (BEB) population, larger studies of *CNTNAP2* with a larger sample size may be suggested. Our research went through significant demographic studies which highlight the prevalence of the disease that appears male-biasness among ASD individuals. Our study also focused on finding out the mental effect on mothers of ASD patients which suggests it as a significant factor for our population as well as will also help for further study with other recognizable factors of ASD parents.

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Data availability

All relevant data are within the manuscript.

Conflict of interest

None to declare.

Authors' contribution

Conceptualization and Methodology: [Lolo Wal Marzan, Hafsa Siddiqua]; Formal analysis and investigation: [Hafsa Siddiqua]; Epidemiological Study: [Hafsa Siddiqua, Mst. Sharika Ahmed, Md. Arzo Mia]; Writing - original draft preparation: [Hafsa Siddiqua]; Writing - review and editing: [Lolo Wal Marzan, Yasmin Akter, Md. Arzo Mia]; Resources: [Lolo Wal Marzan, Yasmin akter, Mahmood Ahmed Chowdhury], Supervision: [Lolo Wal Marzan, Yasmin Akter]; Approval of final manuscript [All Authors].

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