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Article

Association of oxytocin receptor gene polymorphisms with autism spectrum disorder in Bengali of Bangladesh population

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Abstract: Autism spectrum disorder (ASD) is a group of sex-biased neurodevelopmental disorders characterized by core deficits in social interaction, communication and behaviors. Several lines of evidence indicate that oxytocin signaling through its receptor (OXTR), is vital in a wide range of social behaviors and role of *OXTR* polymorphism in ASD development has also been established in several populations. Therefore, an attempt was taken to determine whether genetic variations in the oxytocin signaling system contribute to ASD susceptibility in a part of Bangladeshi (BEB) population. We have investigated the role of *OXTR* polymorphisms (rs53576, rs2254298, rs2228485 and rs237911) in ASD development through PCR-RFLP method, based on case studies. A significant frequency ($p = 0.027$) for *OXTR* 'rs53576AA' risk genotype was found to be associated with ASD which is consistent with the previous study in Chinese but Caucasian and Japanese population. Besides, no significant association has been found for other *OXTR* variants (rs2254298, rs2228485 and rs237911) in this study. Understanding of these significant association with ASD development could be open a new clue aimed at clinical marker development for ASD diagnosis and treatment in future.

Keywords: autism spectrum disorder (ASD); *OXTR*; rs53576; polymorphism; BEB population

1. Introduction

Autism spectrum disorder (ASD) is a combination of neurodevelopmental disorders (Norbury *et al*., 2013) portrayed by developmental delays, impaired functioning in social and communicative skills and the presence of restricted, repetitive behavior (Volkmar *et al*., 2004) and there is neither any specific cause nor cure known for this condition because of heterogenic as well as complex nature of it (Harris, 2016). The establishment of social relationships and social communication is a fundamental developmental task of infancy. On 26th April 2018, the Central for Disease Control's (CDC) Autism and Developmental Disabilities Monitoring (ADDM) network updated the prevalence rate of ASD in United States as 1 in 59 children (1.7%) which is higher than the previous estimate of 1.5% or 1 in 68 children released in 2016 (CDC, 2018). ASD has already been identified as a burden of diseases in Bangladesh, and it has been assumed that the magnitude is high which makes it the 9th

top most countries of the world (Daily Prothom Alo, 2018).

Over the last half-century, varying conceptual models for etiologic factors in autism spectrum disorder (ASD) have been proposed, most emphasizing single cause hypotheses. But based on more than one decade of research; currently, we know that the etiology of ASD is complex, heterogeneous, and multifactorial (Betancur, 2011). Researchers have estimated the heritability of ASD at 55-80%, indicating that genetic influences are responsible for most of its etiology (Lichtenstein *et al*., 2010; Freitag, 2007). One of the prime deficits in ASD is the social deficit; it is not surprising that many researchers have attempted to find if there is any causative link between oxytocin and ASDs. Oxytocin (OXT) is a nine-amino-acid peptide hormone which exerts its effect by activating its specific G-protein-coupled oxytocin receptor (OXTR) to relays the messages to downstream effectors and plays a prominent role in social behavior across species (Hollander *et al*., 2007). Oxytocin hormone is known to play a vital role in the regulation of social recognition, affiliation, bonding, and attachment (Friedlander *et al*., 2019; Hollander *et al*., 2007). Animal studies have shown that oxytocin and vasopressin help regulate the social behavior of prairie voles, especially the formation of partner preference (Heinrichs *et al*., 2009). Notably, *OXTR* gene knockout mice models manifest impaired social memory while parturition was mostly unaffected (Takayanagi *et al*., 2005; Ferguson *et al*., 2000). Employing an intranasal administration paradigm revealed that OXT commonly stimulates all social cognitive functions, including the demonstration of a good emotional bond between parents and neonate (Ebstein *et al*., 2009). Numerous genetic studies in humans and animals have reported that associations between polymorphisms on the *OXTR* gene and phenotypes are related to social cognition (Tops *et al*., 2019; Ribeiro *et al*., 2018; Gong *et al*., 2017), affiliation, perspective taking and sociability in ASD (Reuter *et al*., 2017; Parker *et al*., 2014; Skuse *et al*., 2014). In the Chinese Han population, the *OXTR*-SNPs rs53576 and rs2254298 found to be significantly associated (Wu *et al*., 2005), but no association was found for rs2228485 and rs237911 in the studied ASD population. This *OXTR*-ASD association was also supported by a meta-analysis (LoParo and Waldman, 2015), and the role of the *OXTR* polymorphism in ASD development has also been established in several populations (Montag *et al*., 2017; Lakatosova *et al*., 2013; Campbell *et al*., 2011).

The availability of genetic testing on the basis of genetic risk factor, the number of potentially critical genetic findings would outstrip the capacity for reliable and valid interpretation and genetic counseling also can rapidly emerging insights into the neurobiology underlying autism pathophysiology (Newschaffer *et al*., 2007). As there is no available data on *OXTR*-ASD association in Bengali of Bangladesh (BEB) population, our research endeavored to assess the impacts of four *OXTR* polymorphisms (rs53576 and rs2254298: prominent and well-studied; rs2228485 and rs23791: less studied), in the susceptibility of ASD. To our knowledge, this is the first study in Bangladesh and therefore, in the present investigation we are focusing on the relationship between Oxytocin receptor (*OXTR*) gene polymorphism and ASD.

2. Materials and Methods

2.1. Ethical clearance

All procedures performed in this study involving human participants were under the ethical standard of Declaration of Helsinki, 1964. Ethical clearance for this study was taken from Chattagram Maa-O-Shishu General Hospital, Chittagong (ref: CMOSHMC/IRB/2018/6), Bangladesh. The written informed consent was obtained from the authority of respective institution and the legal guardian of all study participants, because maximum of the participants were children (<18 years).

2.2. Participants and diagnostic procedure

Individuals, who fulfill the ASD diagnostic criteria set by DSM-5 (American Psychiatric Association, 2019) are selected as subjects, and healthy individuals were selected as control. By taking face to face interview from respective parents and caregivers of both 103 ASD individuals and control groups, the data were collected. Thus efforts were put to diagnose all the enrollees using a structured questionnaire (data not shown), and then an experienced child psychiatrist evaluated each patient's data in terms of diagnosis. ASD diagnosis was made when a subject had all of the three symptoms in social communication and social interaction domain or at least had two manifestations of the restricted, repetitive patterns of behavior, interests, or activities domain. In addition to the ASD severity criteria in DSM-5, Childhood Autism Rating Scale (CARS) (Schopler *et al*., 1980, 1988) was also used in diagnosis and rating of autism with a 15-item scale consists of fourteen questions (each querying a discrete behavior and an additional question investigating the general impression about autism spectrum disorder) to determine the severity level of this disorder according to Rellini *et al*. (2004) and Ocakoğlu *et al*., (2018).

2.3. Sample collection

Considering the fact of less positive response from ASD individual's family in our study, blood samples of 50 ASD individuals and 50 healthy controls (age, sex matched) were collected to obtain deoxyribonucleic acid (DNA). Participants and their families were informed about the process and the complications that may develop before the blood sampling procedure. Then, 2.0 ml blood samples were taken in tubes containing tri-potassium ethylene diamine tetra acetic acid (K3EDTA) by expert phlebotomist experienced in working with ASD participants. Immediately after the sampling, obtained samples were delivered to the laboratory by maintaining cold chain where the genetic analysis was to be performed.

2.4. DNA extraction

Genomic DNA was extracted from collected blood samples by using PureLink®^{TM} Genomic DNA extraction kit (Invitrogen^{TM}, Thermofisher Scientific, MA, USA) according to the manufacturer's instructions.

2.5. Genotyping

Targeted four *OXTR* gene variants were amplified with polymerase chain reaction (PCR) using the primers listed in Table 1(a). Then the amplified products were genotyped by using PCR-RFLP method (Figure 1).

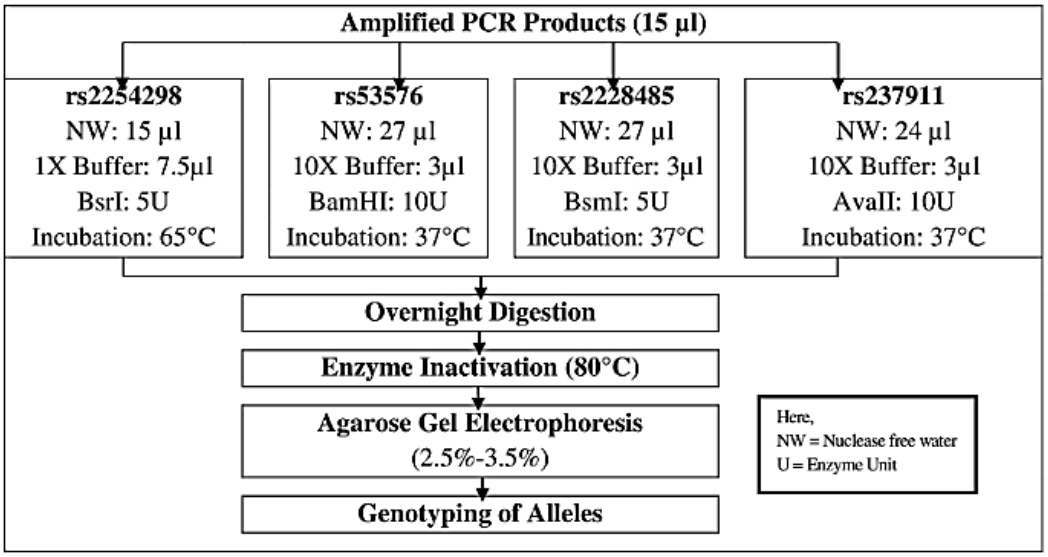

Figure 1. Flow chart of PCR-RFLP analysis.

The list of restriction enzyme was given in Table 1(b). Selected PCR products were sequenced (1st BASE Laboratories, Malaysia following Sanger method of DNA sequencing) and data was analyzed, where Single Nucleotide Polymorphisms (SNPs) was detected by SnapGene® Viewer tool (Version 4.2.2, from GSL Biotech; available at snapgene.com). The sequencing data was matched with the PCR-RFLP genotyping results.

Table 1(a). List of primers.

SNPs	Primer sequences (5′-3′)	Product length (bp)
rs53576	GCCCACCATGCTCTCCACATC	340
	GCTGGACTCAGGAGGAATAGGGAC	
rs2254298	TGAAAGCAGAGGTTGTGTGGACAGG	307
	AACGCCCACCCCAGTTTCTTC	
rs2228485	CCGTAAAGGGCTCGAAGG	394
	ACTTGACCAGGCGGCACA	
rs237911	CCCTTTACGGCTTGGCG	300
	CCGCTCATTTGCAGTGGCTCAG	

Table 1(b). List of enzymes.

SNPs	Product length (bp)	RFLP	Allele (bp)	
rs53576	340	BamHI	G 340	A 230/110
rs2254298	307	BsrI	A 135/164/8	G 101/34/164/8
rs2228485	394	BsmI	C 394	T 175/219
rs237911	300	AvaII	A 300	G 100/200

2.5.1. Allele and genotype frequencies calculation

Allele and Genotype frequencies of the study population were calculated by using Hardy-Weinberg equation (Hardy, 1908) with a web program (http://scienceprimer.com/hardy-weinberg-equilibrium-calculator) and tested with the Chi-square (χ^2) test for all of the Single Nucleotide Polymorphisms (SNPs) under consideration. After that, the calculated allelic frequencies were compared with the allelic frequency of BEB population (data were retrieved from dbSNP and 1000 genome projects) (Siva, 2008).

2.6. Statistical analysis

Data obtained from this study underwent descriptive and inferential statistical analysis. Noncontinuous data were expressed as percentages, and the comparison of the data was performed by Chi-square (χ^2) test. All the statistical parameters were calculated by SPSS software version 16.0 (SPSS Inc., Chicago, IL, USA). For all the statistical analysis the level of significance was set at $p < 0.05$.

3. Results

Interestingly, from the genotyping analysis, the frequency of rs53576 AA genotype was significantly (p= 0.027) higher (30%) in the ASD compared to the control (12%) group (Table 2). Consequently, our allelic frequency calculations (by using Hardy-weinberg equation) also showed that while the rs53576A allele acting as minor (A=0.410) in the control but major in the ASD (A=0.660, Table 3) which was also dissimilar from the calculated frequency of Bengali of Bangladesh (BEB) population (db SNP and 1000 genome projects). For the remaining SNPs (rs2254298, rs2228485, rs237911) the allelic frequency was quite similar with the calculated frequency for BEB population (Table 3). The summary of our findings is also listed on Table 2 and 3 and also in Figure 2, 3, 4, 5 and Figure 6 (a, b, c, d). To summarize, we have identified a particularly higher rs53576A allelic frequency in the studied ASD group.

Table 2. Summary of the genotyping results of four studied *OXTR* variants and comparison with controls.

***OXTR* variants (SNPs)**	**Genotype**	**ASD (n=50)**	**Controls (n=50)**	**χ^2 value**	**p value**
rs53576	GG	18% (9)	30% (15)	1.974	0.160
	AG	52% (26)	58% (29)	0.364	0.546
	AA	30% (15)	12% (6)	4.882	0.027*
rs2254298	GG	82% (41)	76% (38)	0.542	0.461
	AG	14% (7)	16% (8)	0.078	0.779
	AA	4% (2)	8% (4)	0.709	0.400
rs2228485	TT	78% (39)	70% (35)	0.832	0.361
	CT	18% (9)	20% (10)	0.065	0.798
	CC	4% (2)	10% (5)	1.382	0.239
rs237911	AA	62% (31)	66% (33)	0.174	0.677
	AG	38% (19)	34% (17)	0.174	0.676
	GG	0	0	-	-

*values were considered statistically significant when p<0.05.

Table 3. *OXTR* variants allelic frequency calculations.

SNPs		Allele	A freq.	A freq. of ref. population (BEB)	Genotype	G freq.
rs53576	ASD (n=50)	**G**	0.440	G: 0.5174 A: 0.4826	GG	0.194
					AG	0.580
		A	0.660		AA	0.435
	Control (n=50)	**G**	0.590		GG	0.348
					AG	0.484
		A	0.410		AA	0.168
rs2254298	ASD (n=50)	**G**	0.870	G: 0.7295 A: 0.2705	GG	0.756
					AG	0.226
		A	0.130		AA	0.016
	Control (n=50)	**G**	0.840		GG	0.706
					AG	0.260
		A	0.160		AA	0.026
rs2228485	ASD (n=50)	**T**	0.870	T: 0.9360 C: 0.0640	TT	0.756
					CT	0.260
		C	0.130		CC	0.017
	Control (n=50)	**T**	0.800		TT	0.640
					CT	0.320
		C	0.200		CC	0.040
rs237911	ASD (n=50)	**A**	0.810	A: 0.9128 G: 0.0872	AA	0.657
					AG	0.307
		G	0.190		GG	0.036
	Control (n=50)	**A**	0.830		AA	0.689
					AG	0.282
		G	0.170		GG	0.028

SNP=Single Nucleotide polymorphism; A freq.=allele frequency; G freq.=Genotype frequency; BEB=Bengali of Bangladesh.

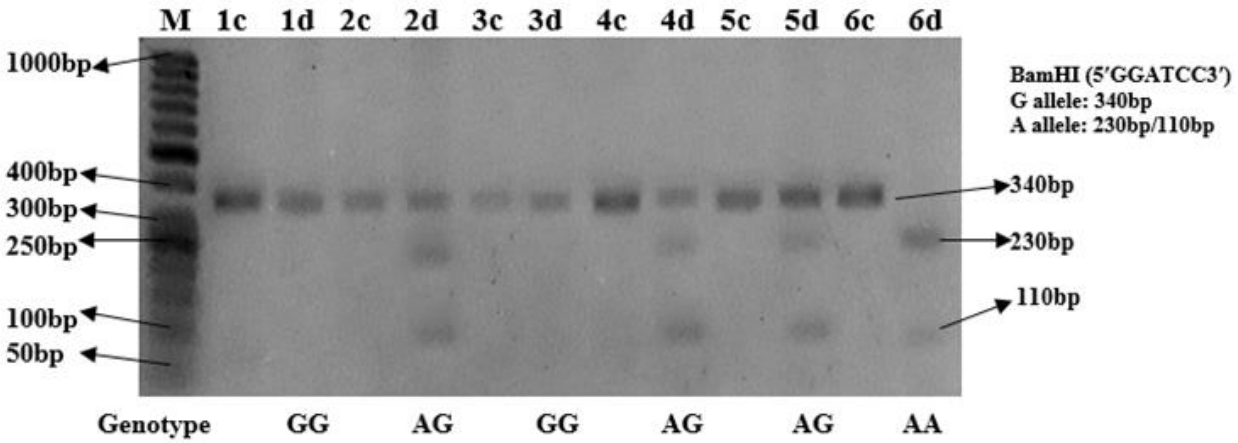

Figure 2. Genotyping of rs53576 by PCR-RFLP method. Here, M = 50bp DNA Ladder (Gene Ruler™, Thermofisher Scientific), c = Undigested PCR product, d = Digested PCR product. Lane 1d, 3d represents genotype GG; lane 2d, 4d represents genotype AG and lane 6d represents genotype AA. The digested products were visualized in 2.5% agarose gel.

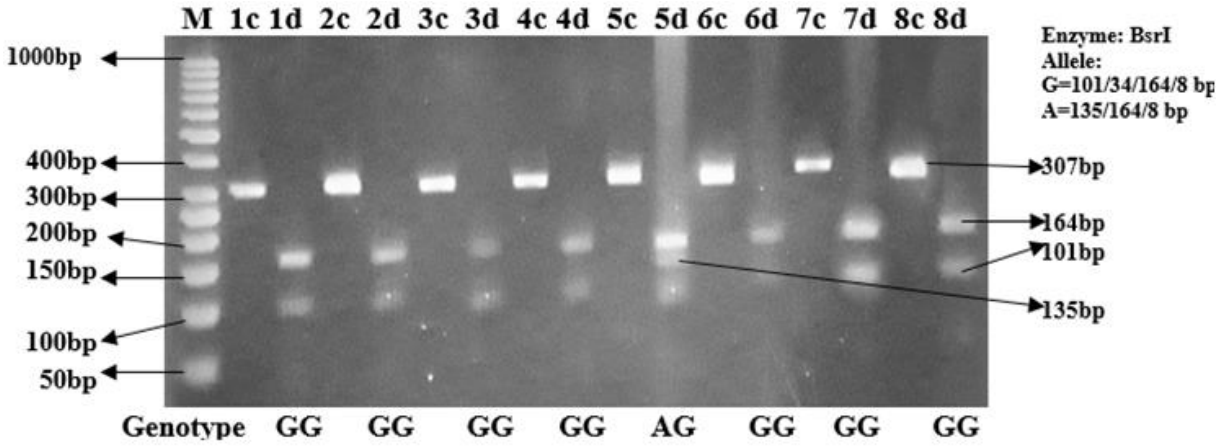

Figure 3. Genotyping of rs2254298 by PCR-RFLP method. Here, M = 50bp DNA Ladder, c = Undigested PCR product, d = Digested PCR product. Lane 1d-4d and 6d-8d represents Genotype GG. Lane 5d represents AG. The digested products were visualized in 3.5% agarose gel.

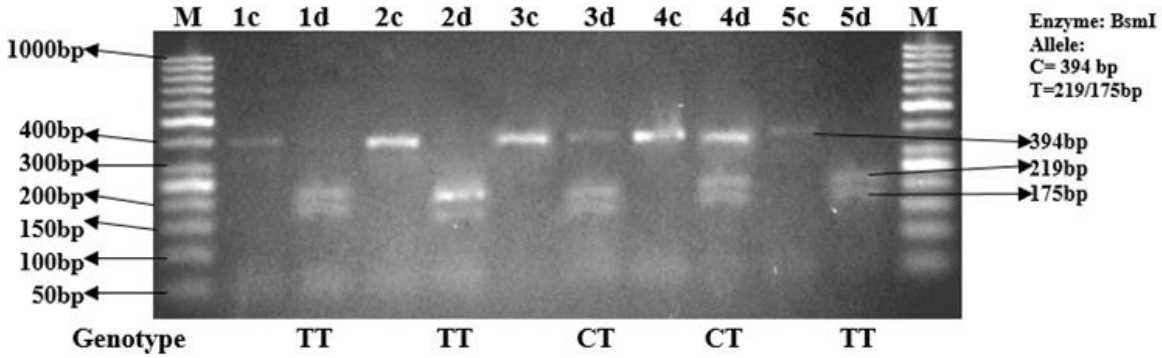

Figure 4. Genotyping of rs2228485 by PCR-RFLP method. Here, M = 50bp DNA Ladder, c = Undigested PCR product, d = Digested PCR Product. Lane 1d, 2d, 5d represent genotype TT; lane 3d, 4d represents genotype CT. The digested PCR products were visualized in 3.0% Agarose gel.

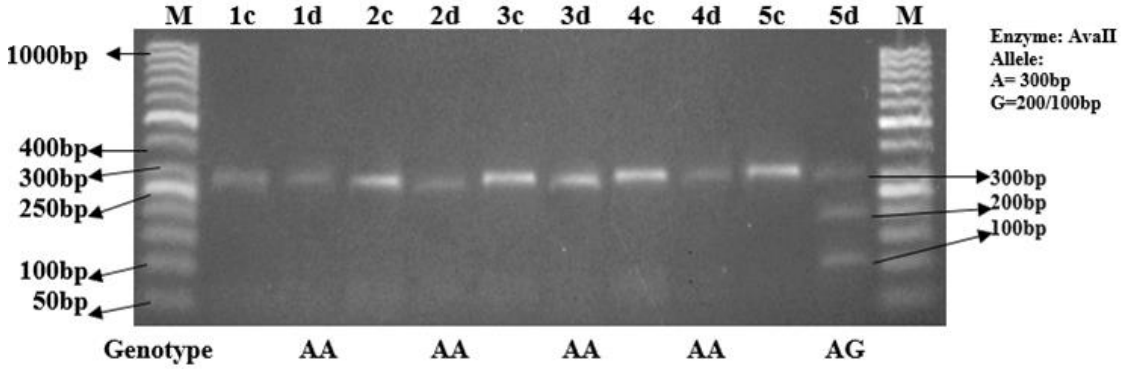

Figure 5. Genotyping of rs237911 by PCR-RFLP method. Here, M = 50bp DNA Ladder, c = Undigested PCR product, d = Digested PCR Product. Lane 1d to 4d represents Genotype AA. Lane 5d represents AG. The digested products were visualized in 3.0% agarose gel.

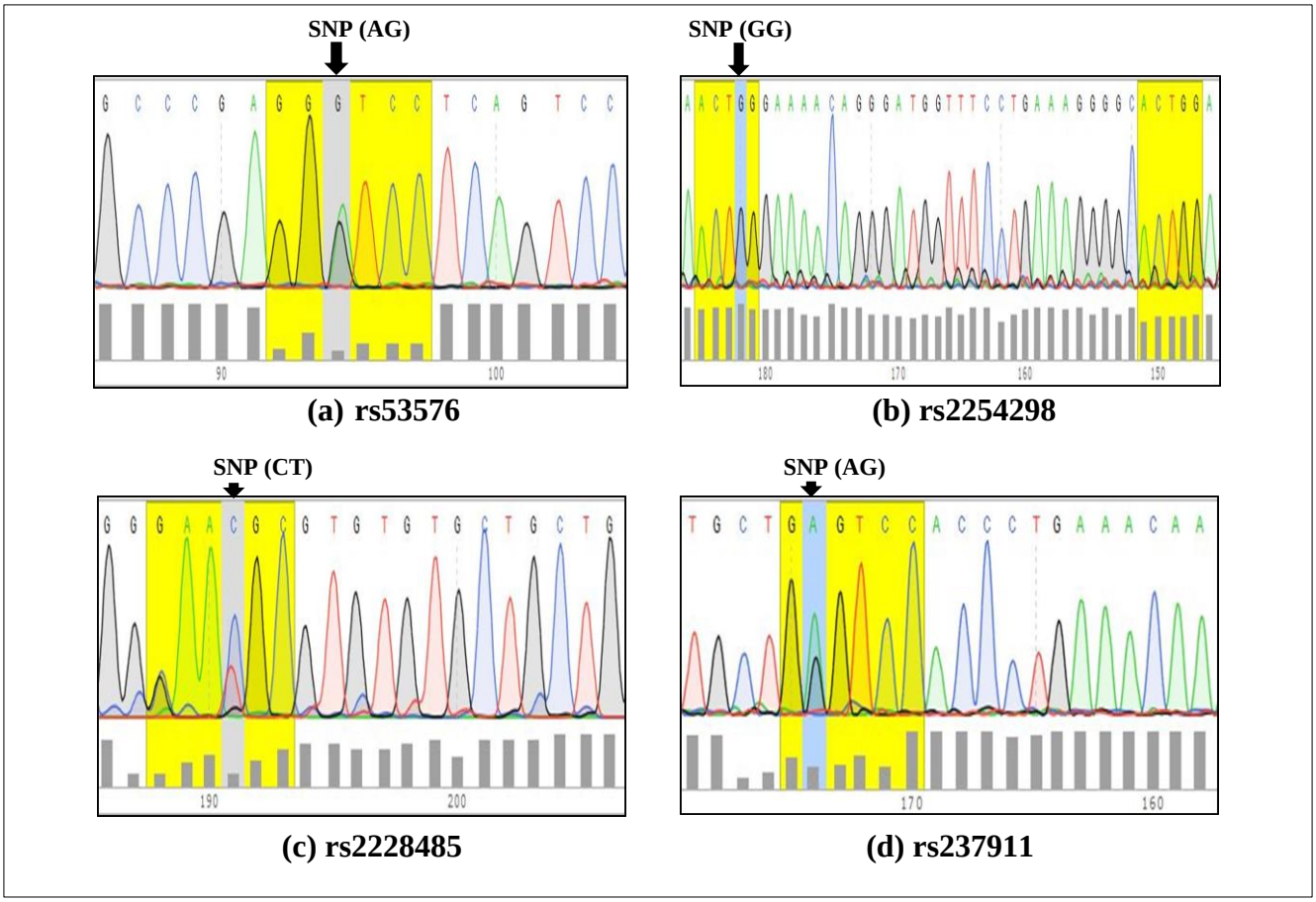

Figure 6 (a, b, c, d). Sequencing data.

4. Discussion

A group of conditions defined by qualitative abnormalities in reciprocal social interaction, distinctive patterns of communication and by a restricted, stereotyped, repetitive repertoire of interests and actions (WHO, 1992) was classified by ICD-10 as Autism Spectrum Disorder (ASD) under the Pervasive developmental disorders. Interest in the role of Oxytocin (OXT) hormone in the development of ASD has increased with the demonstration that this hormone plays an essential role in social attraction, affiliative behavior and bonding, as well as any disruption or change in this cascade, may lead to the development of social deficit (Greene *et al*., 2018; Busnelli and Chini, 2017; Hernandez, 2017; Olff *et al*., 2013). Failure of social adaptation on repeated exposure was seen in *OXT* knockout mice supports the function of oxytocin in integrating social olfactory information and facilitating the consolidation of social memory (Young *et al*., 2002). Moreover, several studies have demonstrated that ASD participants tend to have low plasma oxytocin levels (Green *et al*., 2001) and intranasally administered oxytocin results in improved eye contact, social memory and better use of social information among individuals with high functioning ASD (Bethlehem *et al*., 2013; Andari *et al*., 2010; Hollander *et al*., 2007; 2003).

There is an ongoing debate in the current literature about the relation between Single Nucleotide Polymorphisms (SNPs) in the Oxytocin receptor (*OXTR*) gene and manifestation of social behaviors. Many studies have performed analyses in which they genotyped multiple SNPs and/or haplotypes, but the findings are quite divergent with cultural differences (Loparo and Waldman, 2015). Interestingly rs53576 is one of the most studied SNPs which has shown to affect social behavior and homozygous AA has a significant contribution in the development of ASD in Chinese population (Wu *et al*., 2005), however no association of rs53576 was also found in Caucasian and Japanese population (Campbell *et al*., 2011; Liu *et al*., 2010). Furthermore, rs53576GG homozygotes had higher general sociality compared to A allele carriers (Li *et al*., 2015). In another study investigating the link between rs53576 and trust has shown that GG homozygous males were more trusting than AA males, but this pattern was not found for females (Nishina *et al*., 2015). A population-based study in Asian

and European population, it is reported that *OXTR* variant rs53576G allele is associated with better empathy characteristics in individuals than the A allele (Gong *et al*., 2017). It has been proved that rs53576 genotype GG and parental bonding history interact in influencing social development of infants (Truzzi *et al*., 2018). This SNP (A/G) is located in the third intronic region of the *OXTR* gene in chromosome 3, which is 6929bp downstream from the transcription start (TrxSt) site (Campbell *et al*., 2011). Introns in contemporary species fulfill a broad spectrum of functions and are involved in virtually every step of mRNA processing (Carmel and Chorev, 2012) but how rs53576A allele play a prominent role in social bonding by influencing the OXT-OXTR cascade is still unknown.

In molecular analysis of our study, the association of risk genotype rs53576 (AA) with ASD was statistically significant (30%, $p<0.05$) (Table 2). Though 'A' allele was minor (0.410) in our studied control population and reference BEB population, the frequency of this allele was major (0.66) in the studied ASD population (Table 3). The absence of this risk allele rs53576A in the other ASD participants explains that the presence of this risk allele rs53576A is not the only risk factor of ASD. There are several genetic and environmental conditions, which may lead to the development of ASD, either individually or combined (Di Napoli *et al*., 2014).

Consistent with the hypothesis that genetic variation at *OXTR* is possibly associated with differential susceptibility, carriers of the AG or AA genotype of the rs2254298 SNP are more vulnerable to developing a psychiatric condition, including autism (Brüne, 2012; Jacob *et al*., 2007) depression and anxiety disorders (Chen and Johnson, 2012; Thompson *et al*., 2011; Costa *et al*., 2009; Lucht *et al*., 2009). The first report about the positive association of rs2254298A allele with ASD was found in the Chinese population (Wu *et al*., 2005), and another study in Japanese population also showed consistency with this results (Liu *et al*., 2010). On the contrary, concerning autism as a disorder with a core deficit in social communication, over-transmission of the G allele in the intronic SNP rs2254298 found in Caucasian autistic subjects (Jacob *et al*., 2007). The G allele was hence linked to less friendly behavior, and thus, the A allele is suggested to be associated with prosocial behavior (Lerer *et al*., 2008). In our studied population, we found no significant association between rs2254298 polymorphism and ASD (Table 2). The major allele in our studied ASD population was rs2254298G (0.87, Table 3), which is consistent with the findings of Caucasian and Jewish population but with Chinese and Japanese population. So, the SNP rs2254298 may not play any role in the development of ASD in our studied population.

On the other hand, haplotypes involving *OXTR* rs2228485 showed an excess transmission from parents to affected offspring (Wu *et al*., 2005) with autism. In two separate studies, rs2228485 did not show any significant associations with ASD (Kelemenova *et al*., 2010; Chakrabarti *et al*., 2009). The rs2228485 is a synonymous coding SNP located in exon 3 of the *OXTR* gene (Algovik *et al*., 2010) which encodes amino acids of the oxytocin receptor (Gimpl and Fahrenholz, 2001). There is accumulating evidence that synonymous SNPs can affect splicing or messenger-ribonucleic-acid (mRNA) stability, thereby altering the gene products (Chamary *et al*., 2006). This line of arguing suggests a biological rationale of rs2228485. However, in our studied population, no significant association was found for any of the genotype (TT/CC/CT) (Table 2). The major allele was rs2228485T (Table 3) in both of the ASD and control population.

Being located in the promoter region rs237911 (A>G) polymorphism is a potential candidate but no positive association with ASD has been found in any population (Wu *et al*., 2005). In our studied population, rs237911A allele has been found as major allele in both ASD and control population (Table 3) and no significant association was found with ASD (Table 2).

In summary, the *OXTR* SNP 'rs53576A' allele may be the risk allele for BEB population, which is consistent with the previous findings in Chinese (Wu *et al*., 2005). Further studies of *OXTR* with larger sample size are recommended to analyze the real causal variants of the BEB population. Expected therapeutic resource for ASD can be oxytocin since this neuropeptide can modulate human social behavior cognition (Yamasue *et al*., 2016; Guastella *et al*., 2015), even though some controversies remains about its effectiveness, doses, treatment duration, administration route and timing of starting the treatment (Parker *et al*., 2017; Kosaka *et al*., 2016; Okamoto *et al*., 2016; Preti *et al*., 2014). We conclude that other untested *OXTR* polymorphisms may combinedly contribute to the development of ASD, thus further investigation on *OXTR* SNPs and clinical trials with oxytocin in young children should examine functional domain beyond those related to social behaviors.

5. Conclusions

This comprehensive study put light on association of oxytocin receptor (*OXTR*) gene variants with Autism Spectrum Disorder (ASD). To understand the genetic basis and the role of *OXTR* SNPs in ASD development, we have done the PCR-RFLP analysis and Sanger sequencing to genotype our studied BEB population; thus we found that *OXTR* SNP rs53576A allele significantly associated with ASD, which is consistent with previous

study in Chinese. Understanding how genetic variation may impact the ASD development is important for identifying novel biological treatments like intranasal oxytocin administration or etc.

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Conflict of interest
None to declare.

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