Bangladesh Med Res Counc Bull 2021; 47: 250-259 DOI: https://doi.org/10.3329/bmrcb.v47i3.59237

RESEARCH PAPER

Role of Non-Coding Variants of *NLGN-3* and *4x* Genes along with Non-Genetic Factors in Autism Spectrum Disorder (ASD) in Selected Population in Bangladesh

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Abstract

Background: Autism Spectrum Disorder (ASD) is a group of neurodevelopmental disorder which is now a hotbed worldwide. Being located on chromosome X and playing a vital role in synaptic transmission, NLGN-3 and 4X have been drawn the attention of many researchers as the most essential and functional candidate genes for ASD pathogenesis. However, there are many contradictory results considering their role in developing ASD in different populations.

Objective: This study was aimed to investigate the association of NLGN-3 and 4X genes along with non-genetic factors to develop ASD in Bangladeshi population.

Methods: In this study, we analysed rs4844285 and rs11795613 of NLGN-3 gene and rs3810686 and rs6638575 of NLGN-4X gene for both family-based association analysis and case-control based association analysis using polymerase chain reaction (PCR) and DNA sequencing. Along with this, demographic data were also analysed. A total of 60 members of 15 families, including ASD subjects and another 60 ASD subjects and 60 healthy people were included in this study.

Results: Allele A and genotype AA of NLGN-3 rs4844285 were found to be a probable risk factor for developing ASD in our studied population (p=0.031 for allele A, S χ2= 2.707, OR=1.833 for genotype AA). We also found different birth complications in this studied population which can be considered to intensify the risk of ASD along with the abnormalities of genes.

Conclusion: From this study, it is clear that not only genetic abnormalities but also some birth anomalies play a vital role for the risk of developing ASD.

Keywords: Autism spectrum disorder, NLGN gene, Genetic abnormalities, Birth complications

Introduction

Autism Spectrum Disorder (ASD) is a condition that appears very early in childhood development, varies in severity, and is characterised by a deficiency in acculturation, defect in lingual and gestural communications, and tiresome pattern of behavior.[1,2] People with ASD may also experience sleeping problems and irritability. Sometimes they fell anxiety, over-responsivity, and gastrointestinal problems.[3-5] Epilepsy, dental issues, and other mental problems are also found to be shared in some ASD patients.[6]

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Both genetic and environmental aspects are thought to prompt in advancing the ASD risk, but the actual reason is still unknown.

The prevalence of ASD is about 1% worldwide, and in the larger picture, 1 in 59 children are affected with ASD.[7,8] Two different population-based studies have found this disorder to be extremely heritable at a rate of about 40-80%.[9,10] Study has revealed that monozygotic twins are at higher risk of developing ASD than dizygotic twins.[11]

More than 20% of the world's population resides in the South Asia region, but the frequency of ASD occurrence is still mainly in the dark in this region.[12] In Bangladesh, the reported prevalence was 0.2%, 0.84%, and 0.15%.[13-15] According to the research findings by the Ministry of Social Welfare, Bangladesh

2016, 19% of total neurological disabilities was recorded as the rate of autism.[16] Moreover, almost 3,00,000 children are suffering from ASD where 1 in every 150 girls and 1 in every 94 boys having ASD in Bangladesh according to Autistic Children's Welfare Foundation, 2020.[17]

ASD susceptible genes have been identified by genome-wide linkage studies and genetic association studies, where most of which are involved in the development of the brain.[18,19] As ASD is prevalent in male than female, the role of the X chromosome in the etiology of ASD has been under consideration, and thus, both *NLGN-3* and *NLGN-4X* genes are thought as good positional and functional candidate genes in ASD susceptibility.[20]

Neuroligin a cell surface adhesion protein that is homologous to acetylcholinesterase and other esterases, binds to β-neurexin, a cell surface protein to form functional synapses.[21,22] Neuroligins family includes five members of type 1 transmembrane protein of which NLGN3, NLGN4X, and NLGN4Y are located on sex chromosomes.[23] Proteins encoded by *NLGN* genes are mostly expressed in the brain and interact with presynaptic NRXNs in a Calcium-dependent manner.[24] The X-linked *NLGN3* and *NLGN4X* are the first discovered ASD-associated genes in this pathway.[25]

Neuroligin 3 (*NLGN3*) gene, located on Chr: Xq13 and composed of eight exons with a start codon in exon 2.[26] Two brothers with autism and Asperger syndrome respectively were first reported to have a point mutation on *NLGN3* (R451C).[25] *NLGN4*, the fourth member of the neuroligin family gene, is located on Chr: Xp22.3 with six exons.[27] Study has found that 1bp insertion in the *NLGN4* gene causes a frameshift mutation and thus premature termination (D396X), while another study revealed 2bp deletion causes nonspecific X-linked mental retardation in an individual with or without autism or PDD-NOS.[25,28]

In Bangladesh, number of cases of ASD is about 10.5 lakhs, however, unfortunately, this disorder is one of the least understood ones in our country.[29] Comparing with the statistical data, research on a molecular level to assess the genetic basis of ASD is at the nascent stage in our country. This study thus aimed to identify the actual cause of ASD in this population and to quest if there is any connection between ASD and single nucleotide polymorphisms of *NLGN-3* and *NLGN-4X* genes.

Materials and Methods

Study subjects and collection of samples: A total of 60 members of 15 families, including ASD children, were analysed in family-based association analysis. Another 60 ASD children and 60 healthy people without any previous history of neurological disorder were recruited for case-control based association analysis. The ASD patients were diagnosed with the diagnostic and statistical manual of mental disorder-IV criteria autistic disorder. The age of the ASD children was between 4-30 years. Three milliliter of peripheral blood was collected from each of the subjects. Questionnaire was provided to all the patients to obtain demographic data. Ethical clearance was taken from the Ethical Review Board (ERB) of Chittagong Medical College and Hospital, Chittagong. Each family was informed about this study and written informed consent was obtained from them.

Categorisation of patients: Based on the ASD Assessment Scale/Screening Questionnaire, the patients were divided into four categories, i.e. 0-49=No ASD, 50-100=Mild ASD, 101-150=Moderate ASD, and >150=Severe ASD.

Selection of SNPs: Four SNPs of *NLGN-3* and *NLGN-4X* genes were selected based on the literature study.[30, 31] The selected SNPs and their position in the chromosome were also recorded in (table I).

PCR amplification and sequencing: Genomic DNA was extracted from the whole blood sample using the standard phenol-chloroform method. The entire coding regions of *NLGN-3* and *4X* gene for all the subjects were analyzed by PCR and direct sequencing using four pairs of primers (table II). The cycling conditions consisted of 35 cycles with an initial denaturation at 95ºC for 5 minutes following a denaturation event of 30 seconds at 95ºC, annealing at 57ºC for 30 seconds, and finally an elongation period of 30 seconds at 72ºC.

Statistical analysis: All the statistical analyses in this study were performed with the help of SPSS V.26. Both allelic and genotypic distribution for case and control samples besides with Odds ratio (OR) were set on using Fisher's exact test. The two-sided test results were considered to be appropriate. *p*-value <0.05 was considered to be significant.

Results

ASD severity analysis: Based on ASD Assessment Scale/Screening, we found 33 patients at severe ASD stage and 15 and 12 patients at moderate and mild ASD states respectively (figure 1).

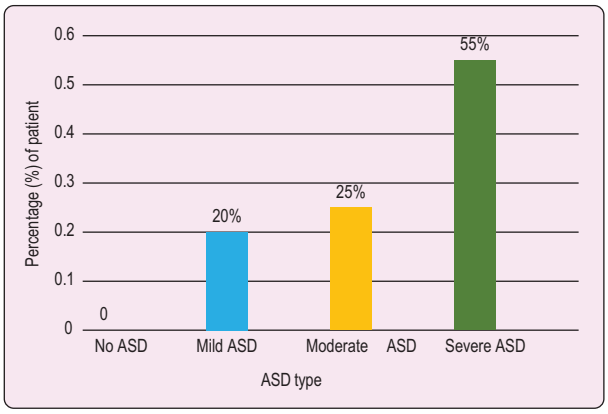

Figure 1: Categorisation of selected patients according to ASD Assessment Scale/Screening Questionnaire in percentage scale

Family-based association analysis: Evaluation of the sequencing data disclosed that all the 15 ASD patients have genotype AA (100%) for *NLGN-3* rs4844285 while most of the parents and siblings possessed GG genotype (90% and 80% respectively). That means this genotype was not inherited from either parent. On the other hand, no significant association was found for rs11795613 of *NLGN-3* gene with ASD in our studied population (table III).

For *NLGN-4X* rs6638575, it was observed that the GG genotype was frequent in patients (100%) as well as in their parents (70%) and siblings (60%). On the contrary, CC genotype of rs3810686 of *NLGN-4X* gene was dominant in siblings (100%) while most of the patients and their parents have TT genotype (60% and 40% accordingly) (table III).

Table I: List of analysed SNPs and their position in the chromosome

Gene	Gene Product	SNPs	Length (bp)	Position of SNPs	Alleles
NLGN-3	Neuroligin- 3	rs11795613	124	chrX:7114748	T>C
		rs4844285	86	chrX:71150394	G>A
NLGN-4X	Neuroligin- 4x	rs6638575	191	chrX5894600	A>G
		rs3810686	186	chrX:5892533	C>A/C>G/C>T

Table II: List of primers used for DNA sequencing

SNPs	Primers	
	Forward Primer	Reverse Primer
NLGN-3 rs11795613	5'-CCTGGGCATGTGAAACCT-3'	5'-CCTGTGGAAAGAGGGAAGTA-3'
NLGN-3 rs4844285	5'-TCTGAGGTTGGTAGGGTACAGT-3'	5'-CCTGCAGGTTTAAGAGACCTT-3'
NLGN-4X rs6638575	5'-TTTTGAAGGGGAAGTGTTATC-3'	5'-AGTCAAGGGCTGTTACAGAT-3'
NLGN-4X rs3810686	5'-ACAAGATCAACTTCTGACCCT-3'	5'-TATCAAGTGTCCTTGGCTGAG-3'

Table III: Genotype frequency distribution of selected rs of *NLGN*-3 and *NLGN*-4X genes in patients and family members

Gene	Genotype	Number (%) of Patients(n=15)	Number (%) of Parents(n=30)	Number (%) of Siblings(n=15)
NLGN-3rs4844285	AA	15(100%)	3(10%)	3(20%)
	AG	0	0	0
	GG	0	27(90%)	12(80%)
NLGN-3rs11795613	GG	9(60%)	12(40%)	3(20%)
	AG	0	6(20%)	3(20%)
	AA	6(40%)	12(40%)	9(60%)
NLGN-4Xrs6638575	AA	0	0	1(20%)
	AG	0	9(30%)	3(20%)
	GG	15(100%)	21(70%)	9(60%)
NLGN-4Xrs3810686	CC	3(20%)	12(40%)	15(100%)
	CT	3(20%)	6(20%)	0
	TT	9(60%)	12(40%)	0

Table IV: Allele frequency distribution in 60 ASD patients and 60 healthy individuals

GeneSNPs	MAF (Case)	MAF (Control)	*p* Value	χ^2	OR95% CI
NLGN-3rs4844285(G>A)	0.60	0.45	0.028	5.414	1.833(1.098-3.061)
NLGN-3rs11795613(A>G)	0.35	0.40	0.088	3.380	0.615(0.366-1.034)
NLGN-4Xrs6638575(A>G)	0.30	0.225	0.240	1.743	1.476(0.827-2.636)
NLGN-4Xrs3810686 (C>A/C>G/C>T)	0.425	0.475	0.517	0.606	10.817(0.491-1.359)

Abbreviations: CI-confidence interval; MAF-minor allele frequency; *NLGN*-neuroligin; OR-odds ratio. Fisher's Exact Test. Significance level: $p>0.05$, $\chi^2>1$, OR>1.

Case-control based association analysis: A significant genetic association between NLGN-3 rs4844285 and ASD was observed from the case-control based analysis, with A allele to be considered as one of the risk factors of ASD ($p=0.028$, $\chi2=5.414$, OR=1.833, 95% CI=1.098-3.061). Both case and control samples displayed a similar type of genotypic and allelic pattern for the rest of the rs of the NLGN-3 gene and the two rs of NLGN-4X gene (table IV, table IA).

Analysis of demographic data: Interpretation of demographic data on patients disclosed that all the selected patients had experienced some sort of birth complications during pregnancy period and after birth. C-section delivery was pervasive among all the patients. Besides, most of the patients suffered from anemia, pneumonia, and hypoxia immediately after birth or the first few weeks of birth. Some patients also underwent neonatal exposure to viruses like CMV, Rotavirus, etc. After analysing the demographic data, it was emanated that asthma, gout, allergy, thyroid problems, and autoimmune disorders were recurrent in most of the mother and diabetics, high blood pressure and cardiac issues were found to be frequent in most father. All the demographic data were also recorded (table IIA).

Table IA: Distribution of gene SNP and genotypes

GeneSNP	Genotype	No. in cases	No. in controls
NLGN-3rs4844285	GG	24	33
	AA	36	27
NLGN-3rs11795613	GG	21	28
	AA	39	32
NLGN-4Xrs6638575	GG	42	45
	AG	0	3
	AA	18	12
NLGN-4Xrs3810686	TT	24	27
	CC	33	30
	TC	3	3

Table II : Demographic data obtained from the parents and the level of ASD of all patients

Patient No.	Complications of the mother during pregnancy and delivery	Complications of father	Complications of the patient immediately after delivery	Level of ASD of patient
1	Asthma, c-section delivery	High bp		Severe
2	Exposer to CMV, c-section delivery, hypoxia	High bp, cardiac issues	Pneumonia	Severe
3	Gout, c-section delivery			Moderate
4	Allergy, Premature delivery	High bp		Moderate
5	Exposer to Rotavirus, c-section delivery	Allergy	Diarrhea	Severe
6	Post-mature delivery			Mild
7	Asthma, Premature delivery, hypoxia	High bp		Moderate
8	Gout, Exposer to CMV, c-section delivery	Diabetics	Pneumonia	Severe
9	Exposer to Rotavirus, c-section delivery	High bp	Diarrhea	Severe
10		Cardiac issues		Moderate
11	Gout, Post-mature delivery			Severe
12	Thyroid problems, Premature delivery			Mild
13	Exposer to CMV, c-section delivery, hypoxia		Pneumonia	Severe
14	Autoimmune disorders, Asthma, c-section delivery	High bp, diabetics		Severe
15	Exposer to CMV, Post-mature delivery	High bp	Pneumonia	Moderate
16				Mild
17	Autoimmune disorders, Exposer to Rotavirus, c-section delivery		Diarrhea	Severe
18	Exposer to CMV, c-section delivery	Cardiac issues	Pneumonia	Severe
19	Asthma, Premature delivery			Mild
20	Exposer to Rotavirus, c-section delivery	High bp	Diarrhea	Severe
21	Exposer to Rotavirus, c-section delivery	Allergy	Diarrhea	Severe
22	Post-mature delivery			Mild
23	Asthma, Premature delivery, hypoxia	High bp		Moderate
24	Exposer to CMV, c-section delivery	Cardiac issues	Pneumonia	Severe
25	Exposer to CMV, c-section delivery, hypoxia		Pneumonia	Severe
26	Exposer to Rotavirus, c-section delivery	Allergy	Diarrhea	Severe
27				Mild
28	Exposer to CMV, c-section delivery, hypoxia		Pneumonia	Severe
29	Exposer to Rotavirus, c-section delivery	High bp	Diarrhea	Severe
30	Thyroid problems, Premature delivery			Mild
31	Exposer to CMV, c-section delivery, hypoxia		Pneumonia	Severe
32		Cardiac issues		Moderate

Table II : Continued

Patient No.	Complications of the mother during pregnancy and delivery	Complications of father	Complications of the patient immediately after delivery	Level of ASD of patient
33	Gout, Post-mature delivery			Severe
34				Severe
35	Post-mature delivery			Mild
36	Gout, c-section delivery			Moderate
37				Severe
38	Asthma, Premature delivery, hypoxia	High bp		Moderate
39	Premature delivery			Severe
40				Mild
41	Exposer to Rotavirus, c-section delivery	Allergy	Diarrhea	Severe
42	Exposer to CMV, c-section delivery	Cardiac issues	Pneumonia	Severe
43	Asthma, Premature delivery, hypoxia	High bp		Moderate
44	Gout, c-section delivery			Moderate
45	Asthma, c-section delivery	High bp		Severe
46	Autoimmune disorders, Exposer to Rotavirus,		Diarrhea	Severe
47				Mild
48	Exposer to Rotavirus, c-section delivery	Allergy	Diarrhea	Severe
49	Exposer to Rotavirus, post mature delivery	High bp	Diarrhea	Severe
50				Moderate
51	Asthma			Severe
52	Premature delivery			Mild
53				Severe
54		High bp		Severe
55	Thyroid problems			Moderate
56				Severe
57		Cardiac issues		Moderate
58	Gout, Post-mature delivery			Severe
59				Mild
60	Gout, c-section delivery			Moderate

Abbreviations: CMV-Cytomegalovirus; bp-Blood pressure.

Table V: Interrelationship between the pattern of genotype and ASD category

Genotype of *NLGN*-3 with ASD score			Number of Patients	Genotype of *NLGN*-4X with ASD score			Number of Patients
rs4844285	rs11795613	ASDCategory	(%)	rs6638575	rs3810686	ASDCategory	(%)
AA	GG	Severe	12(20%)	AA	CC	Severe	3(5%)
AA	AA	Severe	15(25%)	AA	TT	Severe	3(5%)
GG	AA	Severe	9(15%)	GG	TT	Severe	15(25%)
AA	GG	Moderate	6(10%)	GG	CC	Severe	9(15%)
GG	AA	Moderate	12(20%)	GG	CT	Severe	3(5%)
AA	GG	Mild	3(5%)	AA	CC	Moderate	9(15%)
GG	AA	Mild	3(5%)	GG	CC	Moderate	6(10%)
				GG	TT	Moderate	6(10%)
				AA	CC	Mild	3(5%)
				GG	CC	Mild	3(5%)

Association of total ASD score with NLGN-3 and NLGN-4X genes: Genotype pattern AA-AA of the selected rs of *NLGN-3* and GG-TT of the selected rs of *NLGN-4X* gene were found to be associated with causing severe ASD in most of the cases. In a few cases, GG-TT pattern of NLGN-4x gene was also found to be associated with a moderate level of ASD. An interesting fact was observed from both family-based analysis and case-control based analysis that these patterns of genotype was also present in parents, healthy siblings, and also in healthy controls (table V).

Discussion

The case-control based study generated a significant association between SNPs in *NLGN-3* and ASD in our studied population, but no notable result was found for *NLGN-4X* polymorphisms. In addition to this, the family-based association analysis did not provide any strong association. In the case-control study, one SNP of *NLGN-3* (rs4844285) was significantly associated with ASD at a 5% level of significance. The A allele of *NLGN-3* rs4844285 was found as the risk allele of ASD in our studied sample. These results may suggest a potential role of *NLGN-3* in ASD, which is relevant to the previous studies.[21,30]

In contrast to the case-control study, no significant evidence was provided by the family-based study up to the limit of appreciation. This could be due to the limited number of families with insufficient information. For *NLGN-4X* rs6638575 G allele and GG genotype and for rs3810686 T allele and TT genotype were observed to be preferentially transmitted to the affected individuals and thus, undoubtedly point towards the inheritance postulation.

Based on the role of the X chromosome to develop ASD, previous studies observed some controversial results.[20, 23, 32-34] A study on the Chinese population also found no involvement of these SNPs of *NLGN-3* and 4*X* in the development of ASD but other SNPs (rs3747333 and rs3747334) of *NLGN-4X* gene were discovered to be correlated with ASD in the Chinese Han population.[20,23] On the other hand, missense mutations in the neuroligin-4 gene were found to be associated with autism susceptibility in the Bulgarian population and four novel synonymous substitutions were found in the Japanese ASD population.[32,33] So, this can be assumed that the effect of polymorphisms of the neuroligin gene varies depending on sample size and ethnicity as well as ASD heterogeneity.

One of the most important parts of this study is the analysis of demographic data of patients. From the data of the patient's history and the disease history of family members, we have found that obstacles that occurred during pregnancy and at the time of birth have resulted in severe ASD stage in most of the cases and also found to be related with some moderate to a mild level of ASD. These complications like misposition of the baby, c-section delivery, premature or post-mature delivery, hypoxia, etc. directly or indirectly in some points, affect different regions of the brain and thus may be hypothesized to be responsible for ASD development. We also found that the rate of patients

affected with pneumonia immediately after birth or after a few months of birth was also frequent in our sample. Diarrhoea and anaemia were also spotted to be persistent in mother and also in patients which may be pointed as a major cause of the defect in the brain. Thus, the birth complications and prenatal exposure to different viruses may also be anticipated as a causal factor of ASD like the previous studies where birth complications and environmental exposure were found to be related with ASD.[35,36]

From the history of diseases of the family members, we noticed that patients with a mother or father who have high blood pressure, cardiac issues, diabetics, and allergy, scored moderate to severe levels of ASD in our study population. Therefore, parental disorders may also be predicted to associate with ASD risk in our studied population along with genotypic dysfunction.

Most of the people of this region neglect necessary precautions during pregnancy which results in different complications at the time of labour and after the birth of the child. At this point, we may hypothesise that these complications are also becoming a risk factor for developing ASD in our population. Therefore, we can draw an end line by pointing on the fact that, these anomalies with the partial effect of a defect in selected genes may take to the court for intensifying the risk of inaugurating ASD in the current study population.

Conclusion

Polymorphisms in the *NLGN-3* rs4844285 gene along with complications during pregnancy period and labour conditions are associated with developing ASD risk in our studied population. We did not find any noteworthy results for other SNPs for both family-based and case-control based study. This is because of the small sample size in our study. So, further study with larger sample size and more information about family members and patients may be recommended for exploring the association more accurately.

Acknowledgements

This study was supported by the grant of Bangladesh Medical Research Council (BMRC) and the grant of Advanced Research in Education (GARE), Ministry of Education, Bangladesh. The authors gratefully acknowledge the Nishpap Autism Foundation, Chittagong, and the Prerona Autism School, Chittagong for their committed support and encouragement in the management of patients and parents. The authors also like to extend their gratitude to Mrs Rawshan Jahan (Teacher, Nishpap Autism Foundation) for her helping hand in sample collection.

Conflict of interest: None

Funding : Bangladesh Medical Research Council (BMRC) and Advanced Research in Education (GARE), Ministry of Education, Bangladesh

Ethical approval: Bangladesh Medical Research Council (BMRC)

Submitted: 21 December 2020

Final revision received: 07 October 2021

Accepted: 17 October 2021

Published: 01 December 2021

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