Investigation the Relationship of Autism Spectrum Disorder and FOXP2, GRIN2B, KATNAL2, GABRA4 Genes

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INTRODUCTION

Autism spectrum disorder (ASD) affects approximately 1/100 of the population and genetic factors are known to play an important role in the etiology. ASD is a complex neurological disorder characterized by behavioral and psychological problems and repetitive behavioral patterns in children. Patients experience problems in social relations, language development and communication skills. Symptoms are present from early childhood and affect daily activities. Children with ASD diagnosis have higher rates of language problems, intellectual disability, and epilepsy compared to the general population. For ASD, a wide spectrum of behavioral disorders can be categorized under three headings: 1) Social interaction disorder, 2) Language and communication disorder, 3) Areas of interest and activity diversity (1). Intellectual failure, epilepsy, and dysmorphic features may be seen in patients. Children who are followed up with a diagnosis of autism spectrum disorder may develop their speaking and communication skills later on and socialize with their peers at different levels at school age. However, most of these patients require lifelong special education (2).

In studies for the etiology of autism, chromosomal anomalies and molecular pathologies are reported. Some chromosomal anomalies that can be determined by conventional cytogenetic analysis, and various copy number changes that can be observed by molecular methods are associated with autism. Autism clinical findings may also be caused by single gene mutations as in most of the genetic syndromes. ASD has become an important health problem in terms of increasing diagnosis and morbidity rates. Although increasing awareness in medical and social settings is a positive development, the desired levels have not been reached yet. It is important to diagnose autism in the early period, to start education programs early, to increase and enrich the existing skills and to gain new skills, as a result, permanent and significant improvements in the quality of life of patients. Clarifying the etiopathogenesis is of great importance in planning the appropriate treatment, providing genetic counselling related to the course of the disease (3, 4).

10% of ASD cases have single gene defects as seen in other such diseases. It has been reported in many studies that idiopathic autism is of genetic origin. Epidemiological studies report the frequency of autism spectrum disorders with a frequency of 1–2/100 and a male to female ratio of 4–5:1 (5).
Many studies were conducted with the next generation sequencing technology that has greatly contributed to the understanding of the genetic etiology of autism. These studies reported three important clues about the genetic etiology of autism: Rare de novo mutations are important in autism genetics, mutant genes encode proteins found in excitatory synapses, and the same mutation in the same gene can lead to different phenotypes (6).

The siblings of the ASD cases have 2–8% increased risk of having ASD compared to the general population. Monozygotic twin studies implicate 60% concordance. Different genetic approaches can be used for ASD diagnosis including whole genome analysis, linkage analysis (in the presence of more than one affected individual in the family), and screening of known causative genes (7–9).

We have decided to screen the FOXP2, GRIN2B, KATNAL2 and GABRA4 genes in this study, based on their functions in the central nervous system and according to the results of studies reporting a significant relationship with ASD in the literature. To our knowledge, these four genes have been evaluated together in the same ASD study for the first time. Chromosome analysis, array-CGH and Fragile X mutation analysis are routinely performed in patients with ASD in accordance with an algorithm. The genetic etiology could not be clarified when these tests give a negative result. These patients are directed for whole exome or whole genome sequencing analysis which are more expensive and the results can be harder to evaluate. In this study, we aimed to determine the genes that may have a role in the etiology of ASD and the genes which are amenable to screening before exome and genome analysis so that cost-effective and labor friendly genetic tests could be applied. In the light of the results of the whole genome analysis performed in many families, it has been suggested that different genes may play a role in the etiology of ASD. Referring patients with ASD pre-diagnosis from different disciplines (Child and Adolescent Mental Health, Pediatric Neurology, General Child Health and Diseases, Family Medicine, etc.) to the medical genetics clinic will provide important contributions to support and confirm the clinical diagnosis. The genetic tests will contribute to the elucidation of etiopathogenesis and genetic counselling. In our study, we aimed to investigate the relationship between FOXP2, GRIN2B, KATNAL2 and GABRA4 genes and ASD.

METHOD

Ninety six patients diagnosed with ASD according to DSM-V criteria in the Department of Child and Adolescent Mental Health, Trakya University Faculty of Medicine, were included in our study. Written informed consent forms were obtained from the legal guardians of all cases. For our study, approval of the ethics committee was obtained from Trakya University Faculty of Medicine Scientific Research Ethics Committee with the decision number of 06/09.

The symptoms of autism were assessed by The Childhood Autism Rating Scale (CARS). Patients had CARS scores above 30 (cut-off for diagnosis of childhood autism). CARS is a 15-item behavior-rating scale designed to detect and quantify symptoms of autism as well as to distinguish them from other developmental disabilities. Each item on the CARS is scored on a Likert scale, from 1 (no signs of autism) to 4 (severe symptoms). The maximum CARS score is 60, and the cut-off for a diagnosis of autism is 30. To assess their attention deficit hyperactivity disorder (ADHD) findings Conners Parent Rating Scale-Revised Short (CPRS-RS) was used.

In our cohort, the results of chromosome analysis, array-CGH analysis and Fragile X analysis showed no pathology. Array CGH was performed using Agilent 4x180K ISCA CGH + SNP Array, testing 170,359 copy number changes located with an average interval of 25.3 kb. Genomic DNA was isolated from 2 ml of peripheral blood sample taken into an EDTA tube using EZ1 DNA Blood 200 µl Kit (Qiagen, Hilden, Germany) and EZ1 Advanced XL (Qiagen, Hilden, Germany) nucleic acid isolation device were used for this purpose. The concentration and purity of the DNA samples were analyzed in NanoDrop (NanoDrop 2000C, Thermo Scientific, USA) and the DNA samples were stored at -20°C.

Specific primers for FOXP2, GRIN2B, KATNAL2 and GABRA4 genes were designed using NCBI primary blast. Nextera XT Library Preparation Kit was used to generate DNA libraries required for next generation sequencing. The amplicons obtained by polymerase chain reaction were sequenced in Illumina MiSeq (Illumina) after barcoding according to Nextera XT Library Preparation Kit (Illumina) instructions. Variants were determined and analyzed using the Genomize Seq Software (Genomize, Turkey) program from Fastq data obtained using MiSeq Reporter Software. IGV 2.4.8 (http://software. broadinstitute. org/software/igv/) was used for visual analysis of variants. Pathogenicity of variants was evaluated using databases (HGMD, NCBI dbSNP Database, PubMed) and in silico analysis methods such as MutationTaster, PolyPhen, SIFT, in line with ACMG-2015 guidelines.

RESULTS

Of the 96 ASD cases, 21.87% (21) were female and 78.12% (75) were male. The mean age of the patients was 10.17 and the age range was between 1–17. With a high range, 87 (90.6%) of 96 cases had a comorbidity. Thirty-six patients had a comorbidity with attention deficit hyperactivity disorder, thirty patients had intellectual disability, sixteen patients had epilepsy and five patients had anxiety disorder with ASD diagnosis.

No pathogenic or likely pathogenic variant was detected in FOXP2, GRIN2B, KATNAL2 and GABRA4 genes, however, in 50 (52%) cases, a total of 69 intronic variants of unknown clinical significance in these four genes were detected (Table 1). Twenty six of these variants were in GABRA4, 22 in FOXP2, 13 in KATNAL2, and 8 in GRIN2B gene. 23 variants were novel which were not previously reported in the literature, and 46 variants were defined in the dbSNP database. In-silico analyzes performed to evaluate the pathogenicity of 23 novel variants, and the minor allele frequency was accepted as <0.01.

DISCUSSION

All exons and exon-intron junctions of FOXP2, GRIN2B, KATNAL2 and GABRA4 genes were screened in 96 ASD patients by targeted sequencing. Pathogenic and/or likely pathogenic variants could not be detected in these genes in our cohort. Various intronic variants with unknown clinical significance were detected in 50 cases, 23 of them were novel.

In recent years, advancements in technology have become an important tool enabling the generation of information about genetic/epigenetic regulatory networks, chromatin structure, nuclear structuring and genome variations. In this study, sequencing analyzes of all exon, exon-intron junctions were performed with the next generation sequencing method in FOXP2, GRIN2B, KATNAL2 and GABRA4 genes, and the analysis of the four genes are completed in a short time.

Technological advancements have enabled the identification of a large number of genes that constitute a comprehensive framework to better understand the complexity and heterogeneity of ASD (10). To date, hundreds of ASD genes have been identified with different pathogenic roles in the development of autism. In addition to dominant, recessive and gene-environment mechanisms, polygenic mechanisms in patients with ASD have been investigated more in recent years (11, 12, 13). In studies using targeted gene analysis, several synaptic cell adhesion molecules and other molecules, such as NLGN3, NLGN4 (14), NRXN1
| Case | GABRA4 gene variations | FOXP2 gene variations | GRIN2B gene variations | KATNAL2 gene variations | dbSNP |
|------|------------------------|-----------------------|-----------------------|-------------------------|-------|
| 1    | ENST00000264318.3:c.494+96C>T |                      |                      |                         |       |
| 2    | ENST00000408937.3:c.1341+95G>A |                      |                      |                         |       |
| 3    | ENST00000356157.7:c.726+298A>G |                      |                      |                         |       |
| 4    | ENST00000264318.3:c.494+98C>T |                      |                      |                         |       |
| 5    | ENST00000356157.7:c.722-114ST>G |                      |                      |                         |       |
| 6    | ENST00000609686.1:c.412-149dupA |                      |                      |                         |       |
| 7    | ENST00000408937.3:c.721+1048T>C |                      |                      |                         |       |
| 8    | ENST00000356157.7:c.332+110_332+114delCTGCAinsTGCG |                      |                      |                         |       |
| 9    | ENST00000264318.3:c.721+1273C>T |                      |                      |                         |       |
| 10   | ENST00000408937.3:c.672-298T>C |                      |                      |                         |       |
| 11   | ENST00000408937.3:c.1257+499A>G |                      |                      |                         |       |
| 12   | ENST00000408937.3:c.1844+182T>G |                      |                      |                         |       |
| 13   | ENST00000408937.3:c.1258-404C>A |                      |                      |                         |       |
| 14   | ENST00000408937.3:c.258+100A>G |                      |                      |                         |       |
| 15   | ENST00000408937.3:c.334-612_334-609delACAC |                      |                      |                         |       |
| 16   | ENST00000408937.3:c.687_695dupGCAGCAGCA (p.Gln232_Gln234dup) |                      |                      |                         |       |
| 17   | ENST00000408937.3:c.258-100A>G |                      |                      |                         |       |
| 18   | ENST00000264318.3:c.875-86T>A |                      |                      |                         |       |
| 19   | ENST00000408937.3:c.1135-284G>A |                      |                      |                         |       |
| 20   | ENST00000356157.7:c.1010+101G>A |                      |                      |                         |       |
| 21   | ENST00000408937.3:c.1135-284G>A |                      |                      |                         |       |
| 22   | ENST00000264318.3:c.875-86T>A |                      |                      |                         |       |
| 23   | ENST00000264318.3:c.875-86T>A |                      |                      |                         |       |
| 24   | ENST00000408937.3:c.1135-284G>A |                      |                      |                         |       |
| 25   | ENST00000356157.7:c.1135-284G>A |                      |                      |                         |       |
| 26   | ENST00000408937.3:c.1135-284G>A |                      |                      |                         |       |
| 27   | ENST00000356157.7:c.1135-284G>A |                      |                      |                         |       |
| 28   | ENST00000408937.3:c.1135-284G>A |                      |                      |                         |       |
| 29   | ENST00000408937.3:c.1135-284G>A |                      |                      |                         |       |
| 30   | ENST00000408937.3:c.1135-284G>A |                      |                      |                         |       |
| 31   | ENST00000408937.3:c.1135-284G>A |                      |                      |                         |       |
| 32   | ENST00000408937.3:c.1135-284G>A |                      |                      |                         |       |
| 33   | ENST00000408937.3:c.1135-284G>A |                      |                      |                         |       |
| 34   | ENST00000408937.3:c.1135-284G>A |                      |                      |                         |       |
| 35   | ENST00000408937.3:c.1135-284G>A |                      |                      |                         |       |
| 36   | ENST00000408937.3:c.1135-284G>A |                      |                      |                         |       |
The gender ratios in our study were consistent with the literature; the male: female ratio was 3.57 (75/21) in our cohort and in literature it is reported that the number of boys diagnosed with ASD is higher than girls (28).

The rapid development of genomic testing technology, bioinformatics approaches and artificial intelligence will facilitate genetic testing results and interpretation as they gain more experience in testing patients applying for diagnosis. Due to the clinical heterogeneity and diagnostic uncertainty in ASD, many studies are required to gain more experience in genetic tests and treatment approaches.

There were several limitations of our study in which we used the candidate gene approach to investigate genetic risk factors in ASD. First of all, the candidate gene approach enabled us to analyse only a limited number of genes. Secondly, we could only traced de-novo inheritance since all cases were isolated. Thirdly, our cohort had a relatively small sample size. Increasing awareness for the genetic etiology in ASD and related neurobehavioral conditions is a necessity for providing treatment services. Correct diagnosis, correct orientation of the family with correct genetic counselling will increase the quality of life of ASD cases and increase the usefulness of genetic tests for ASD.

CONCLUSION

In 50 of 96 cases included in the study, intronic variants of unknown clinical significance were classified according to ACMG-2015 criteria. Considering that there may be differences in the classification of these variants with unknown clinical significance over time, the variants will be re-evaluated. In our study, the relationship between ASD and the FOX2, GRIN2B, KATNAL2 and GABRA4 genes could not be established since we could not detect pathogenic or likely pathogenic variants. In order to elucidate the genetic etiopathogenesis associated with ASD, comprehensive molecular genetic studies such as whole exome or whole genome sequencing studies are required in different populations with higher number of cases.

**Ethics Committee Approval:** For our study, approval of the ethics committee was obtained from Trakya University Faculty of Medicine Scientific Research Ethics Committee with the decision number of 06/09.

**Informed Consent:** Written informed consent forms were obtained from the legal guardians of all cases.

**Peer-review:** Externally peer-reviewed.
REFERENCES

1. Woodbury-Smith M, Paterson AD, O’Connor I, Zarrei M, Yuen RK, Howe JL, Thompson A, Parlier M, Fernández B, Piven J, Scherer SW, Vieland V, Sztatmari P. A genome-wide ideogram study of autism spectrum disorder and the broad autism phenotype in extended pedigrees. J Neurodev Disord 2018;10:20. [Crossref]

2. Kalsner L, Twachtman-Bassett J, Tokarski K, Stanley C, Dumont-Mathieu T, Cotney J, Chamberlain S. Genetic testing including targeted gene panel in a diverse clinical population of children with autism spectrum disorder: Findings and implications. Mol Genet Genomic Med 2018;6:171–185. [Crossref]

3. Ayhan F, Konopka G. Genomics of autism spectrum disorder: approach to therapy. F1000Res 2018;7:627. [Crossref]

4. Varghese M, Keshav N, Jacot-Descombes S, Warda T, Wicinski B, Dickstein DL, Harony-Nicolos H, De Rubeis S, Drapeau E, Buxbaum JD. Hof PR. Autism spectrum disorder: neuropathology and animal models. Acta Neuropathol 2017;134:537–566. [Crossref]

5. Muhle RA, Reed HE, Stratigos KA, Veenstra-VanderWeele J. The Emerging Autism Spectrum Disorders Unit with the project number of 2018/340. This study was funded by Trakya University Scientific Research Projects Unit with the project number of 2018/340. [Crossref]

6. Turner LM, Stone WL, Pozdol SL, Coonrod EE. Follow-up of children with autism spectrum disorders from age 2 to age 9. Autism 2006;10:243–265. [Crossref]

7. Narita A, Nagai M, Misono S, Ogishima S, Tamiya G, Ueki M, Saruk K, Makino S, Obara T, Ishikuro M, Yamakana C, Matsuoka H, Kuniyoshi Y, Murakami K, Ueno F, Noda A, Kobayashi T, Kobayashi M, Usuazi K, Ohtsuri H, Hohaza A, Kikuya M, Metoki H, Kuriyama S. Clustering by phenotype and genome-wide association study in autism. Transl Psychiatry 2020;10:290. [Crossref]

8. Yin J, Chun CA, Zavadenko NN, Pechatnikova NL, Naunoya YY, Doddapaneni P, Muzny DM, Schaaf CP, Grigorenko EL. Next Generation Sequencing of 134 Children with Autism Spectrum Disorder and Regression. Genes (Basel) 2020;11:E853. [Crossref]

9. Imamura A, Morimoto Y, Ono S, Kurotaki N, Kanegae S, Yamamoto N, Kinosita H, Tsujita T, Okazaki Y, Ozawa H. Genetic and environmental factors of schizophrenia and autism spectrum disorder: insights from twin studies. J Neural Transm (Vienna) 2020;127:1501–1515. [Crossref]

10. de la Torre-Ubieta L, Won H, Stein JL, Geschwind DH. Advancing the understanding of autism disease mechanisms through genetics. Nat Med 2016;22:345–361. [Crossref]

11. Chahrour M, O’Roak BJ, Santini E, Samaco RC, Kleiman RJ, Manzini MC. Mutations in the gene encoding the synaptic scaffolding protein SHANK3 are associated with autism spectrum disorders. Nat Genet 2008;40:159–169. [Crossref]

12. Tordjman S, Cohen D, Anderson GM, Botbol M, Canitano R, Coulon N, Roubertoux PL. Repint of “Reframing autism as a behavioral syndrome and not a specific mental disorder: Implications of genetic and phenotypic heterogeneity”. Neurosci Biobehav Rev 2018;89:132–150. [Crossref]

13. Butler MG, Rafi SK, Manzano AM. High-resolution chromosome ideogram representation of currently recognized genes for autism spectrum disorders. Int J Mol Sci 2015;16:6464–6495. [Crossref]

14. Jamain S, Quach H, Betancur C, Rastam M, Colineaux C, Gillberg IC, Soderstrom H, Giros B, Leboyer M, Gillberg C, Bourgeron T. Mutations of the X-linked genes encoding neuregulins LGN3 and LGN4 are associated with autism. Nat Genet 2003;34:27–29. [Crossref]

15. Kim HG, Kishikawa S, Higgins AW, Seong IS, Donovan DJ, Shen Y, Lally E, Weiss LA, Najm J, Kutsche K, Kutsche K, Descartes M, Holt L, Braddock S, Troxell R, Kaplan L, Volkmar F, Klin A, Tsatskinis K, Harris DJ, Noens I, Pauls DL, Daly MJ, MacDonald ME, Morton CC, Quade BJ, Gussela JF. Disruption of neurexin 1 associated with autism spectrum disorder. Am J Hum Genet 2002;88:199–207. [Crossref]

16. Arlazarov M, Abrahams BS, Stone JL, Duval JA, Perederiy JV, Bomar JM, Sebat J, Wigler M, Martin CL, Ledbetter DH, Nelson SF, Cantor RM, Geschwind DH. Linkage, association, and gene-expression analyses identify CNNTAP2 as an autism-susceptibility gene. Am J Hum Genet 2002;82:150–159. [Crossref]

17. Berkel S, Marshall CR, Weiss B, Howe J, Roeth R, Moog U, Ennis V, Roberts W, Sztamari P, Dino B, Bonin M, Riess A, Engels H, Sprengel R, Scherer SW, Rappold GA. Mutations in the SHANK2 synaptic scaffolding gene in autism spectrum disorder and mental retardation. Nat Genet 2010;42:489–491. [Crossref]

18. Durand CM, Betancur C, Boeckmann TM, Bockmann J, Chaste P, Fauchereau F, Nguyen R, Rastam M, Gillberg IC, Ancaksarater H, Sponheim E, Bougran-Botros H, Delorme R, Chabane N, Mournen-Simeoni MC, de Mas P, Bieth M, Rogé B, Héron D, Burglen L, Gillberg C, Leboyer M, Bourgeron T. Mutations in the gene encoding the synaptic scaffolding protein SHANK3 are associated with autism spectrum disorders. Nat Genet 2007;39:25–27. [Crossref]

19. Sebat J, Lakshmi B, Malhotra D, Troge J, Lese-Martin C, Walsh T, Yarmoh B, Yoon S, Kratsinitz A, Kendall J, Leotta A, Pai D, Zhang R, Lee YH, Hicks J, Spence S, Lee AT, Puura K, Lehtimaki T, Ledbetter D, Gregersen PK, Bregman JU, Sutcliffe JS, Jobanputra V, Chung W, Warburton D, King MC, Skuse D, Geschwind DH, Gilliam TC, Ye K, Wigler M. Strong association of de novo copy number mutations with autism. Science 2007;316:445–449. [Crossref]

20. Sanders SJ, Mruthu MT, Gupta AR, Murdoch JD, Raubeson MJ, Willsey AJ, Ercan-Sencicek AG, DiLullo NM, Parashar KN, Stein JL, Walker MF, Ober GT, Teran NA, Song Y, Fishawy PE, Murtha RC, Choi M, Overton JD, Bjornsdon RM, Carriero NJ, Meyer KA, Bilguvar K, Mane SM, Sestan N, Litton RP, Guenel M, Roeder K, Geschwind DH, Devlin B, State M. New de novo mutations revealed by whole-exome sequencing are strongly associated with autism. Nature 2012;485:237–241. [Crossref]

21. Tiwary BK. The cognitive and speech genes are jointly shaped by both positive and relaxed selection in the human lineage. Genomics 2020;112:2922–2927. [Crossref]

22. Vargha-Khadem F, Gadian DG, Copp A, Mishkin M. FOXP2 and the neuroanatomy of speech and language. Nat Rev Neurosci 2005;6:131–138. [Crossref]

23. Carlson SL, Bokshaj JP, Morrow AL. Ethanol Regulation of Synaptic GABA A Receptors Is Prevented by Protein Kinase A Activation. J Pharmacol Exp Ther 2016;357:10–16. [Crossref]

24. Taylor MJ, Rosenquist MA, Larsson H, Gillberg C, D’Onofrio BM, Lichtenstein P, Lundstrom S. Etiology of Autism Spectrum Disorders and Autistic Traits Over Time. JAMA Psychiatry 2020;77:936. [Crossref]

25. Monyer H, Burnath N, Laurie DJ, Sakmann B, Seeburg PH. Developmental and regional expression in the rat brain and functional properties of four NMDA receptors. Nature 1994;42:529–540. [Crossref]

26. Dunleavy JEM, Okuda H, O’Connor AE, Merriner DJ, O’Donnell L, Jamsai D, Bergmann M, O’Byran MK. Katanin-like 2 (KATNAL2) functions in multiple aspects of haploid male germ cell development in the mouse. PLoS Genet 2017;13:e1007078. [Crossref]

27. Merikangas AK, Almasy L. Using the tools of genetic epidemiology to understand sex differences in neuropsychiatric disorders. Genes Brain Behav 2020;19:e12660. [Crossref]