A pioneering study indicate role of GABRQ rs3810651 in ASD severity of Indo-Caucasoid female probands

Sharmistha Saha, Mahasweta Chatterjee, Swagata Sinha & Kanchan Mukhopadhyay

 Alteration in gamma aminobutyric acid (GABA), the principal inhibitory neurotransmitter, is speculated to be a potential risk factor for Autism Spectrum Disorder (ASD) due to an altered expression in the brain. Sensory, social, and emotional deficits of subjects with ASD were reported to be caused by an imbalance between excitatory and inhibitory neurotransmission as well as GABAergic dysfunction caused by inadequate receptor function. We for the first time studied association between ASD and a missense coding variant rs3810651 (I478F) in the GABRQ gene, encoding for one of the subunits of GABA<sub>A</sub> receptors. Stratified analysis on families with ASD probands (N = 251) and ethnically matched control subjects (N = 250) revealed marginally higher frequency of “A” allele and “AA” genotype in female ASD probands as compared to gender matched controls. Female probands demonstrated higher severity for Verbal communication ($\chi^2 = 5.75, P = 0.01$), Activity level ($\chi^2 = 7.26, P = 0.007$), as well as Level and consistency of intellectual response ($\chi^2 = 7.83, P = 0.005$) in presence of “A/AA” warranting further in-depth investigation on the role of rs3810651 in ASD.

Autism spectrum disorder (ASD) is a highly heritable complex disorder showing steep increase in prevalence throughout the world, including India. ASD is characterized by early childhood symptoms of (1) persistent deficits in social communication & interaction and (ii) restricted repetitive patterns of behaviors and interests, the extent of which vary from mild, moderate to severe. Number of boys affected with ASD is higher as compared to girls. Investigations have shown that genetic, epigenetic, and environmental factors influence ASD symptom severity. However, till date, diagnosis is exclusively based on medical history and presenting symptoms. Identification of novel genetic markers which can be used for recognizing ASD and its associated symptom severity thus becomes vital. Behavioral abnormalities of subjects with ASD are hypothesized to be due to an imbalance in the levels of excitatory/inhibitory neurotransmitters. Gamma aminobutyric acid (GABA), the principal inhibitory neurotransmitter, is crucial for maintaining neuronal plasticity as well as synchronized brain activity through regulation of other neurotransmitters. Accordingly, modulation of GABAergic signaling is now sought as an alternative approach for providing symptomatic remediation in subjects with ASD. GABA<sub>A</sub> receptor mediates fastest inhibitory synaptic transmission in the central nervous system. Sixteen different subunits determine the receptor’s agonist affinity, chance of opening, conductance, and other properties. Amongst these, significant expression of GABA<sub>δ</sub> receptor theta subunit in the brain and a combination of specific subunits was found to reduce affinity of the receptor to GABA<sub>δ</sub>. GABA<sub>δ</sub> receptor theta subunit is encoded by the GABRQ gene located at chromosome Xq28 and a truncating rare variant, c.306 G- & C, was detected in one female ASD patient. Another GABRQ coding variant, rs3810651, is a missense polymorphism in the last exon of GABRQ and was explored for association with neurological disorders like migraine, essential tremor and restless leg syndrome. However, to the best of our knowledge, till date this variant was not reported to have association with ASD.

We for the first time investigated association between rs3810651 and ASD to find out whether this SNP has any contribution in the etiology of the disorder. Further, due to the reported higher occurrence of ASD in boys and localization of GABRQ in the X chromosome, we have performed gender-based stratified analysis.

Manovikas Biomedical Research and Diagnostic Centre, Manovikas Kendra, 482 Madudah, Plot I-24, Sector J, EM Bypass, Kolkata, West Bengal 700107, India. Email: kanchanmvk@yahoo.com
Results

Comparative case–control genetic association analysis. Genotype of female participants followed the Hardy-Weinberg equilibrium. Population-based analysis failed to show any significant difference in frequency of rs3810651 alleles in all ASD probands, even after stratification based on gender (data not included for brevity). However, stratified analysis based on gender as well as disease severity exhibited marginally higher frequency of the “A” allele and “AA” genotype in the female probands with moderate severity as compared to gender matched controls (Tables 1 and 2; $\chi^2 = 4.56$ & $6.95$ respectively, $P = 0.03$).

Transmission disequilibrium test (TDT) analysis. Family based analysis revealed a trend for higher maternal transmission of the “A” allele to female probands, though the result was statistically insignificant (Table 3; $\chi^2 = 2.16$, $P = 0.14$).

Genotype–phenotype association analysis. ASD subjects exhibit varying level of behavioral characteristics which are deviant from neurotypicals. For assessing disease severity, we have used the Childhood Autism Rating Scale 2-Standard Test (CARS2- ST) covering 15 different phenotypes. Values for each phenotype assessed under CARS2-ST varies between 1 and 4 with 0.5 interval and subjects were categorized into mild to moderate (score 30.0–36.5) and severe (score 37.0–60.0) depending on the total score. Female ASD probands showed statistically significant association of the “A” allele and “AA” genotype with cumulative CARS score as is evident from positive Addvalue (Table 4, $P<0.03$; ST 1). Independent associations were also noticed in the female probands for all the 15 phenotypic domains included under CARS (Table 4, $P\leq 0.05$). On the other hand, no such statistically significant allelic association was observed for the male probands (Table 4; $P>0.05$). Female probands stratified based on disease severity demonstrated association of “A/AA” with higher severity for Verbal communication ($\chi^2 = 5.75$, $P=0.03$), Activity level ($\chi^2 = 7.26$, $P=0.007$), as well as Level and consistency of intellectual response ($\chi^2 = 7.83$, $P=0.005$).

Discussion

In silico analysis on X-linked synaptic genes predicted GABRQ A > T: Ile478Phe (i.e. rs3810651) to be non-pathogenic. Genomic DNA samples of ASD patients showed truncating mutations in GABRQ and GABRA3 in two different families with ASD probands though no association between rs3810651 and ASD was reported.

### Table 1. Population based comparative analysis on rs3810651 allelic frequency. N.B: Statistically significant difference is presented in bold.

| Allele | Control (N = 250) Moderate symptoms (N = 167) $\chi^2$ (P) | Female participants Moderate symptoms (N = 86) $\chi^2$ (P) | Male participants Moderate symptoms (N = 147) $\chi^2$ (P) | Severe symptoms (N = 58) $\chi^2$ (P) | Severe symptoms (N = 26) $\chi^2$ (P) |
|--------|----------------------------------------------------------|----------------------------------------------------------|----------------------------------------------------------|-----------------------------------|-----------------------------------|
| A      | 0.43 0.46 0.33 (0.56)                                   | 0.45 0.42 0.27 (0.69)                                    | 0.41 0.41 0.18 (0.67)                                    | 0.41 0.69 0.56 (0.68)             | 4.56 0.56 1.36 (0.68)             |
| T      | 0.57 0.54 0.44 (0.68)                                   | 0.55 0.58 0.59 (0.68)                                    | 0.59 0.59 0.44 (0.68)                                    | 0.59 0.31 0.44 (0.68)             | 0.44 0.24 1.36 (0.68)             |

| Allele/ genotype | Control | Probands | Probands with mild/ moderate symptoms | Probands with severe symptoms | $\chi^2$ (P) |
|------------------|---------|----------|--------------------------------------|-------------------------------|-------------|
| AA               | 0.17    | 0.24     | 0.38                                 | 0.45                          | 3.75 (0.15) |
| AT               | 0.48    | 0.39     | 0.62 (0.61)                          | 6.95 (0.03)                   |             |
| TT               | 0.35    | 0.37     | 0.69                                 | 0.22                          |             |

| Parents | Allele | T | NT | $\chi^2$ (P) |
|---------|--------|---|----|-------------|
| Both    | A      | 0.57 | 0.50 | 0.14 (0.70)  |
|         | T      | 0.43 | 0.50 |             |
| Father  | A      | 0.62 | 0.92 | 0.54 (0.46)  |
|         | T      | 0.38 | 0.08 |             |
| Mother  | A      | 0.62 | 0.36 | 2.16 (0.14)  |
|         | T      | 0.38 | 0.64 |             |

---

Scientific Reports | (2022) 11:7010 | https://doi.org/10.1038/s41598-021-86496-5 natureportfolio
On the other hand, rs3810651 was found to be a potential candidate for a common neurological disorder migraine and carriers of rs3810651 “AT” genotype showed increased risk of migraine, which was significant for female subjects. Frequency of the “A” allele was appreciably higher in the subgroup of female migraine patients with ≤ 15 years age of onset and subjects with “AA” genotype showed a lower mean age of onset of migraine attacks. rs3810651 “T” allele exhibited association with improvement in essential tremor. In Major depressive disorder patients, rs3810651 “A” allele showed significant positive association with antidepressant drug response. No significant association of the studied variant was noticed with restless leg syndrome.

Genotype of female participants recruited for the present study followed the Hardy–Weinberg equilibrium. Assumption taken into consideration while calculating Hardy–Weinberg equilibrium was (1) non-consanguineous marriage of parents, (2) same ethnicity, and (3) no genetic drift.

Our investigation for the first time revealed association of this variant with phenotypes of female ASD probands indicating a role of GABRQ rs3810651 A’ allele in the severity of ASD associated traits of female probands. In spite of having reasonable number of male probands, we failed to notice any significant association of GABRQ rs3810651 in this subgroup. An earlier study on a large dataset showed that female ASD probands with less than 70 IQ had overall more impairments than males with ASD in terms of social-communicative abilities, cognitive and adaptive abilities, and externalizing behaviors. Girls diagnosed with ASD were also reported to have a higher mean total problem score (hyperactivity, anxiety, and conduct, peer, and prosocial problems) and higher frequency of low IQ than boys with ASD. Comparative analysis on neurobehavioral profile exhibited poorer inhibition in females with ASD as compared to males. Females with ASD showed significantly increased grey matter volume in the right anterior cingulate cortex and right cerebellum, brain regions with neuronal activity typically mediated by GABA.

We infer from the present investigation that GABRQ rs3810651 may have a specific role in ASD severity which is dependent on gender. Our study has three major drawbacks; (1) limitation in number of female ASD probands. However, it has already been noticed that from human studies to model systems, limited availability of female

---

### Table 4. Association between rs3810651 alleles/genotypes and traits of ASD probands (N.B. Only statistically significant values are presented for female probands).

| CARS Domain               | Allele/Genotype | Male proband (N = 207) | Female proband (N = 44) |
|---------------------------|-----------------|------------------------|-------------------------|
|                           | AddVal | χ²(P) | AddVal | χ²(P) |
|                           |        |       |        |       |
| Cumulative score          | A      | +0.01 | 1.09(0.29) | +0.04 | 4.64(0.03) |
|                           | AA     | − −   | +0.20 | 6.27(0.01) |
| Relating to people        | A      | +0.06 | 0.11(0.74) | +0.51 | 4.42(0.03) |
|                           | AA     | − −   | +1.55 | 5.37(0.02) |
| Imitation                 | A      | +0.23 | 1.48(0.22) | +0.46 | 3.94(0.05) |
|                           | AA     | − −   | +1.43 | 6.00(0.006) |
| Emotional response        | A      | +0.32 | 2.21(0.14) | +0.53 | 4.85(0.03) |
|                           | AA     | − −   | +1.98 | 10.02(0.0001) |
| Body use                  | A      | +0.16 | 0.91(0.34) | +0.43 | 4.03(0.04) |
|                           | AA     | − −   | +1.74 | 5.15(0.02) |
| Adaption to change        | A      | +0.22 | 1.08(0.30) | +0.51 | 3.97(0.05) |
|                           | AA     | − −   | +1.52 | 6.13(0.01) |
| Visual response           | A      | +0.12 | 0.36(0.55) | +0.45 | 3.69(0.05) |
|                           | AA     | − −   | +1.92 | 8.99(0.002) |
| Listening response        | A      | +0.22 | 0.91(0.34) | +0.48 | 3.75(0.05) |
|                           | AA     | − −   | +1.24 | 6.20(0.008) |
| Taste, smell and touch    | A      | +0.31 | 1.25(0.26) | +0.77 | 5.77(0.02) |
|                           | AA     | − −   | +2.14 | 8.27(0.004) |
| Fear or Nervousness       | A      | +0.15 | 0.74(0.39) | +0.48 | 4.71(0.03) |
|                           | AA     | − −   | +1.64 | 8.56(0.003) |
| Verbal communication      | A      | +0.17 | 0.74(0.39) | +0.45 | 4.08(0.04) |
|                           | AA     | − −   | +1.35 | 7.25(0.007) |
| Activity level            | A      | +0.16 | 0.84(0.36) | +0.71 | 6.74(0.009) |
|                           | AA     | − −   | +1.49 | 8.32(0.003) |
| Level and consistency of Intellectual Response | A | +0.29 | 2.52(0.11) | +0.52 | 5.12(0.02) |
|                           | AA     | − −   | +1.67 | 7.43(0.006) |
| General impression        | A      | +0.19 | 1.16(0.28) | +0.42 | 3.74(0.05) |
|                           | AA     | − −   | +1.47 | 6.41(0.01) |
ASD samples and limited attention to gender as a biological variable have hindered our ability to describe as well as to understand the importance of sex difference in ASD pathology. Hence, further extensive research including larger female ASD samples is warranted to identify the importance of GABRQ gene in the etiology of ASD in sex balanced sample sets. (2) absence of high functioning (HF) ASD patients, i.e. with IQ > 80, in our study group. Our subjects were homogenous for age (i.e. 5.96 years ± 3.31), low IQ (< 80) and limited verbal ability. This could be one of the reasons for our observed higher trait scores in female probands, as low IQ have already been reported to be associated with poorer traits in female ASD patients. Having a comparative analysis on subjects with broader ASD phenotypes characterized by a more diverse sample in terms of high functioning vs. low-functioning would help to identify the precise association between GABRQ rs3810651 and ASD phenotype. (3) assessment of traits using CARS-2-ST. Analysis of association between GABRQ rs3810651 and ASD using diagnostic criteria mentioned in the DSM-5 and scores obtained through other ASD assessment tools like Autism Diagnostic Observation Schedule along with the Autism Diagnostic Interview-Revised may aid in strengthening our findings. Under the current scenario, we conclude that to identify the importance of GABRQ gene in the etiology of ASD further extensive research is warranted.

Materials and methods
Subject selection and sample collection. Indo-Caucasoid nuclear families with ASD probands (N = 251; 207 male & 44 female) and controls (N = 250; 147 male & 103 female) were recruited by child psychiatrists and clinical psychologists on the basis of DSM-IV and/or DSM-51,21 after obtaining informed written consent from participants and/or their legal guardians. Mean age of the probands was 5.96 years ± 3.31 (SD). No high functioning (HF) ASD patient, i.e. with IQ > 80, was available for our study. Hence, recruited ASD subjects were assessed by the Childhood Autism Rating Scale 2-Standard Test (CARS-2-ST)24 to measure symptom severity and subjects were categorized into mild-moderate and severe groups based on the cumulative CARS score with range of 30–36.5 and 37–60. The study protocol was approved by the Human Ethical Committee of Manovikas Kendra consisting of Scientists, Clinicians, Social worker, Advocate and Administrator (PR-006–14) which follow guidelines/regulations of the Committee. Genomic DNA was purified from peripheral blood of study participants following standard protocol25.

Genotyping and statistical analysis. Genotyping of coding region variant, rs3810651 of GABRQ was done by TaqMan-based allelic discrimination assay (Assay ID C__27492635_10, Thermo Fisher Scientific, Singapore). Population- and family-based analyses were performed using UNPHASED v 3.1.724. Quantitative Trait (QT) analysis, to identify association between rs3810651 and phenotypic domains incorporated under the CARS as well as cumulative CARS score, was also performed using UNPHASED (v.3.1.7) program24.

Received: 19 October 2020; Accepted: 16 March 2021
Published online: 26 March 2021

References
1. American Psychiatric Association. Diagnostic and Statistical Manual of Mental Disorders 5th edn. (Washington, 2013).
2. Wiśniowiecka-Kowalnik, B. & Nowakowska, B. A. Genetics and epigenetics of autism spectrum disorder—current evidence in the field. J Appl Genet 60, 37–47. https://doi.org/10.1007/s13353-018-00480-w (2019).
3. Saha, S., Saha, T., Sinha, S., Rajamma, U. & Mukhopadhyay, K. Autistic traits and components of the folate metabolic system: an explorative analysis in the eastern Indian ASD subjects. Nutri Neurosci 23(11), 860–867. https://doi.org/10.1080/1208415X.2019.154442 (2020).
4. Smidt, T. M., Milivojevic, B. & Kenner, C. Atypical excitation–inhibition balance in autism captured by the gamma response to contextual modulation. NeuroImage Clin 3, 65–72. https://doi.org/10.1016/j.nicl.2013.06.015 (2013).
5. Cellot, G. & Cherubini, E. GABAergic signaling as therapeutic target for Autism spectrum disorders. Front Pediatr 2, 70. https://doi.org/10.3389/fped.2014.00070 (2014).
6. Cossart, R., Bernard, C. & Ben-Ari, Y. Multiple facets of GABAergic neurons and synapses: multiple fates of GABA signaling in epilepsies. Trends Neurosci 28(2), 108–115. https://doi.org/10.1016/j.tins.2004.11.011 (2005).
7. Bonnert, T. P. et al. Theta, a novel gamma-amino-butyric acid type A receptor subunit. Proc Natl Acad Sci USA 96, 8981–8986. https://doi.org/10.1073/pnas.96.17.8981 (1999).
8. Piton, A. et al. Analysis of the effects of rare variants on splicing identifies alterations in GABA<sub>R</sub> receptor genes in autism spectrum disorder individuals. Eur J Hum Genet 21, 749–756. https://doi.org/10.1038/ejhg.2012.243 (2012).
9. Quintas, M. et al. Interaction between γ-amino-butyric acid receptor genes: new evidence in migraine susceptibility. PLoS ONE 8, 9. https://doi.org/10.1371/journal.pone.0074087 (2013).
10. García-Martín, E. et al. Gamma-amino-butyric acid (GABA) receptors: implications for clinical practice. J Neurol Neurosurg Psychiatry 82, 689–698. https://doi.org/10.1136/jnnp.2010.203937 (2011).
11. Jiménez-Jiménez, F. J. et al. Gamma-amino-butyric acid (GABA) receptors and risk for restless legs syndrome. Pharmacogenomics 11, 565–577. https://doi.org/10.2217/pgs.10.374 (2010).
12. García-Martín, E. et al. Gamma-amino-butyric acid (GABA) receptors and risk for restless legs syndrome. Mol Psychiatry 16(8), 867–880. https://doi.org/10.1038/mp.2010.54 (2011).
13. Pu, M. et al. Influence of genetic polymorphisms in the glutamatergic and GABAergic systems and their interactions with environmental stressors on antidepressant response. Pharmacogenomics 14(3), 277–288. https://doi.org/10.2217/pgs.13.1 (2013).
14. Frazier, T. W., Georgiades, S., Bishop, S. L. & Hardan, A. Y. Behavioral and cognitive characteristics of females and males with autism in the Simons Simplex Collection. J Am Acad Child Adolesc Psychiatry 53(3), 329–340. https://doi.org/10.1016/j.jaac.2013.12.004 (2014).
16. Dworzynski, K., Ronald, A., Bolton, P. & Happe, F. How different are girls and boys above and below the diagnostic threshold for autism spectrum disorders?. J. Am. Acad. Child Adolesc. Psychiatry 51(8), 788–797. https://doi.org/10.1016/j.jaac.2012.05.018 (2012).

17. Lemon, J. M., Gargaro, B., Enticott, P. G. & Rinehart, N. J. Executive functioning in autism spectrum disorders: a gender comparison of response inhibition. J. Autism. Dev. Disord 41(3), 352–356. https://doi.org/10.1007/s10803-010-1039-2 (2011).

18. Retico, A. et al. The effect of gender on the neuroanatomy of children with autism spectrum disorders: a support vector machine case-control study. Mol Autism 7, 5. https://doi.org/10.1186/s13229-015-0067-3 (2016).

19. Northoff, G. et al. GABA concentrations in the human anterior cingulate cortex predict negative BOLD responses in fMRI. Nat Neurosci 10, 1515–1517. https://doi.org/10.1038/nn2001 (2007).

20. Kissel, L. T. & Werling, D. M. Neural transcriptomic analysis of sex differences in autism spectrum disorders: current insights and future directions. Biol. Psychiat. https://doi.org/10.1016/biopsych.2020.11.023 (2021).

21. American Psychiatric Association. Diagnostic and Statistical Manual of Mental Disorders 4th edn. (Washington, 2000).

22. Schopler, E., Van Bourgondien, M. E., Wellman, G. J. & Love, S. R. Childhood Autism Rating Scale 2nd edn. (Western Psychological Services, 2010).

23. Miller, S. A., Dykes, D. D. & Polesky, H. F. A simple salting out procedure for extracting DNA from human nucleated cells. Nucleic Acids Res. 16, 1215. https://doi.org/10.1093/nar/16.3.1215 (1988).

24. Dudbridge, F. Pedigree disequilibrium tests for multilocus haplotypes. Genet Epidemiol. 25, 115–221. https://doi.org/10.1002/gepi.10252 (2003).

Acknowledgements
Authors are obliged to the volunteers for participation in the study.

Author contributions
S.S. conceptualized the work, performed genotyping, data analysis, drafted manuscript. M.C. performed genotyping and data analysis. S.S. helped in recruitment of A.S.D. patients and provided clinical input. K.M. guided study designing, execution, and edited the manuscript. All the authors approved the final manuscript.

Competing interests
The authors declare no competing interests.

Additional information
Supplementary Information The online version contains supplementary material available at https://doi.org/10.1038/s41598-021-86496-5.

Correspondence and requests for materials should be addressed to K.M.

Reprints and permissions information is available at www.nature.com/reprints.

Publisher’s note Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.

Open Access This article is licensed under a Creative Commons Attribution 4.0 International License, which permits use, sharing, adaptation, distribution and reproduction in any medium or format, as long as you give appropriate credit to the original author(s) and the source, provide a link to the Creative Commons licence, and indicate if changes were made. The images or other third party material in this article are included in the article’s Creative Commons licence, unless indicated otherwise in a credit line to the material. If material is not included in the article’s Creative Commons licence and your intended use is not permitted by statutory regulation or exceeds the permitted use, you will need to obtain permission directly from the copyright holder. To view a copy of this licence, visit http://creativecommons.org/licenses/by/4.0/.

© The Author(s) 2021