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RESEARCH ARTICLE

Sequencing of exon 1a promoter region and exon 8 of *GABRB3* gene for identification of SNPs in Iraqi autistic patients.

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Abstract

The γ-aminobutyric acid receptor β3 subunit (*GABRB3*) gene is located on chromosome 15q12 and it has been implicated in the neurobiology of ASDs. The aim of this study was to correlate between *GABRB3* and ASDs. This study included 40 autistic patients and 25 non-autistic controls (5 unaffected sibling and 20 healthynon-relatives). Genomic DNA was extracted from blood samples followed by PCR amplification of exon 1a promoter region and exon 8 of *GABRB3* for subsequent DNA sequencing using Sanger method. Identical bands related to the targeted regions were present in all samples. Samples of patients with similarities in autistic phenotypes were sent for direct sequencing by Macrogen Company, South Korea, along with control samples. BLAST results revealed that promoter region contained 16 SNPs (8 only in autistic patients, 2 only in controls, and 6 found in both). Three of them were common SNPs (rs7171660, rs4363842 and rs4906901) and were present in all (autistic and control samples) while the remaining promoter SNPs (13 SNP) are reported for the first time in this study. Exon 8 showed 100 % sequence identity in all samples as it had no SNPs, while the sequenced part of the following intron 8 appeared to have a SNP (rs3751582) in 3 autistic patients and 2 controls. SNPs found only in autistic patients are likely associated with ASDs and promoter SNPs could alter expression of the gene thus contributing to the pathogenicity of autism.

Introduction:-

Autism or (Autism Spectrum Disorders (ASDs) is a complex neurodevelopmental disorders characterized by severely impaired communication and a limited range of interests and behavior. It was first described by Kanner in 1943. A diagnosis of autism can typically be made by 3 years of age (Kanner, 1943;Limprasert, 2008). The prevalence of diagnosed ASDs was continuously increasing since it was first described so that by the year 2014, it was estimated to be ~1% with male to female ratio of approximately 4:1, suggesting a possible imprinting effect and involvement of the sex chromosomes (Ronemus*et al.*, 2014; Werling and Geschwind, 2013). The exact cause of autism is unknown but it is believed to be multifactorial with a strong genetic influence (Omar, 2012), so it is considered as a genetically influenced neurodevelopmental disorder, with abundant evidence pointing to dysfunction at the level of the synapse. There is extensive genetic heterogeneity, with perhaps hundreds of genetic variants involved (Carter and Scherer, 2013). Compelling evidence for a the genetic basis for autism has been provided by family studies, particularly twin studies, which demonstrate a significantly higher concordance rate for monozygotic versus dizygotic twins, with an overall heritability of 80%–90% (Folstein and Rosen-Sheidley, 2001). Autism spectrum disorders (ASDs) are mostly synaptic disorders due to the fact that dendritic spines have been found to be reduced in neurons of autistic patients; moreover, nearly all the genes associated with ASDs are involved in the formation, regulation, or normal function of the synapse (Zoghbi, 2003; Garber, 2007). Many studies have centered on genetic changes in ASD patients based on single-nucleotide polymorphisms (SNPs) analysis since they are considered genetic markers in studies of gene association with complex diseases (Jiao *et al.*, 2012). Genetic complexity of ASDs has been investigated using genetic approaches such as cytogenetic analysis, genome-wide

linkage and association scans, and candidate gene analysis (Chen *et al.,* 2014). *GABRB3*, mapped to chromosome 15q12, encodes the subunit β3 in the GABAA receptor (Gamma-aminobutyric acid receptor type A) where GABA (Gamma-aminobutyric acid) is the main inhibitory neurotransmitter in the brain (Sutcliffe *et al.,* 2003). Based on many family studies, chromosome 15q11-q13 which includes ASD-related genes, such as *GABRB3, GABRA5* has been considered to be an autism candidate region (Kim *et al.,* 2008).The human *GABRB3* gene has 10 exons and contains two promoter regions upstream of the alternative first exons, exon 1a and exon 1. Both alternative first exons are predicted to encode a signal peptide (Urak 2006).The aim of this study was to detect mutations in specific regions of*GABRB3*by direct sequencing in autistic patients and to identify association of detected mutations in ASDs.

Materials and Method:-

Forty patients (33 male and 7 female) with age range of (3-10) years old and diagnosed with ASDs were selected for this study and were from two local institutes specialized in caring of autistic children which are; Happy Family Specialist Center (Baghdad / Mansour), Rahman Specialist Centre (Baghdad/Yarmouk) and from Central Hospital of Pediatrics (Baghdad). All parents received a comprehensive description of the study, and gave written informed consent for their children's participation. Control group included twenty five non-autistic children ; five unaffected relatives (siblings) of patients and twenty unrelated children selected from Central Hospital of Pediatrics (Baghdad) in which they were attending for regular medical care. It was ensured that they had no neurological or psychological abnormalities as well as contagious diseases.

Three milliliters of blood were collected by vein puncture in EDTA anticoagulant tubes from all patients and control groups then stirred gently for few seconds to avoid blood's clotting. Total genomic DNA extraction from blood collected in EDTA tubes was applied using Wizard® Genomic DNA purification kit (Promega, USA)according to the manufacturer's protocol. After genomic DNA extraction, agarose gel electrophoresis was adopted to confirm the presence and integrity of the extracted DNA (Sambrook*et al*., 1989). PCR reaction was used to amplify the two targeted regions: exon 1a promoter using the specific primers(Forward:5'-GAACACAAAAACGAGCTTGATG-3' and Reverse: 3'-GGTCCAGGAGAGCCAGATG-5') and exon 8using the specific primers (Forward: 5'-TCCAGTCACCCCACAGTATTC-3' and Reverse: 3'-AAGGGTGTGTGGCTTGACAT-5')(Alpha DNA Company, Canada), these primers were designed by Chen *et al.* (Chen *et al.,* 2014).Components of PCR reaction and mixing amounts for both exon 1a promoter and exon 8 regions are shown in table 1.

Table (1): Components of reaction for amplification of **exon 1a promoter** region and **exon 8** of *GABRB3*.

Component	**Concentration**	**Component of one sample (µL)**	**Component of 10 sample (µL)**
Distilled water	---	12	120
Go Taq® green master mix	1 X	15	150
Forward primer	10 picomols/µL	0.5	5
Reverse primer	10 picomols/µL	0.5	5
DNA Sample	100 ng/µL	2	*
Total Volume	---	30	280

*DNA template of 2 µL for each sample

Optimization of PCR reaction was achieved after several trials to reach for the optimum conditions. The final Programs used are shown in Tables 2 and 3.

Table (2):PCR program **exon 1a promoter** of *GABRB3*.

No. of step		**Steps**	**Temperature (C°)**	**Time**	**No. Of cycles**
1		Initial denaturation	95	5 min	1
2	A	Denaturation	95	1 min	35
	B	Annealing	61	1 min	
	C	Extension	72	1 min	
3		Final extension	72	10 min	1

Table (3):PCR program for **exon 8** of *GABRB3*.

No.		Steps	Temperature (C°)	Time	No. Of cycles
1		Initial denaturation	95	5 min	1
2	A	Denaturation	95	1 min	35
	B	Annealing	60	1 min	
	C	Extension	72	1 min	
3		Final extension	72	10 min	1

The PCR products were separated by 2 % agarose gel electrophoresis, stained with ethidium bromide and visualized by ultraviolet light (302 nm) using (Gel Documentation System).

Sequencing and sequence alignment:-

Sequencing of exon 1a promoter region and exon 8 of *GABRB3* gene was performed by Macrogen company, South Korea. Samples of patients with related autistic phenotypes, along with control samples (9 samples for exon 1a promoter and 10 samples for exon 8) were selected for DNA sequencing. Homology search was conducted using Basic local alignment search tool (BLAST) program which is available at the National Center Biotechnology Information (NCBI) online at (http://www.ncbi.nlm.nih.gov). Translation of exons to polypeptide was achieved using EMBOSS Transeq tool which translates nucleic acid sequences to their corresponding peptide sequences available online at (http://www.ebi.ac.uk/Tools/st/emboss_transeq/) and the three dimensional structure was predicted using RaptorX tool which is a protein structure prediction server available online at (http://raptorx.uchicago.edu/) (Källberg*et al.*, 2012).

Statistical analysis:-

The statistical analysis is a very important final step in the research to analyses and evaluates the obtained results. Medical statistics of this study was conducted via computer based statistical program which was: Chi-square test was used to compare between percentages in this study and test the results for significant differences using Statistical Analysis System- SAS (2012) program. The statistical analysis tests used in this were as follows: P value < 0.05 is considered a significant correlation.

Results and Discussion:-

Description of study sample:-

The results presented in this study were based on analyses of data from a total of 65 cases: 40 autistic children, 5 non-autistic (siblings) and 20 non-relatives healthy children. The results of this study pointed to the higher risk of autism in males 33 (82.5 %) than in females 7 (17.5%) with a ratio of 4:1 table 4.

Table (4): Frequency rates for cases and controls according to gender groups

Case-control	Gender				Total
	Male	%	Female	%	
Autism	33	82.5	7	17.5	40
Non autistic (siblings)	0	0	5	100	5
Control (non-relatives)	10	50	10	50	20
Total	43	66.15	22	33.85	65

The ASDs prevalence being four times higher in males than in females could be due to the X-linked genes involved in brain development and cognition which are higher in proportion when compared with autosomal genes (Piton *et al.*, 2011). Werling and Geschwind observed that there is sex differences in autism spectrum disorders (Werling and Geschwind, 2013)

Polymerase chain Reaction (PCR) Results:-

Successful PCR amplification reaction for the targeted regions was confirmed by (2%) agarose gel electrophoresis when DNA bands of a molecular size related to the targeted regions were visualized under UV light (Gel

Documentation System) following 30 minutes staining with ethidium bromide. Molecular sizes for amplified exon 1a promoter and exon 8 were 769 bp and 495 bp respectively as shown in figure 1 and 2.

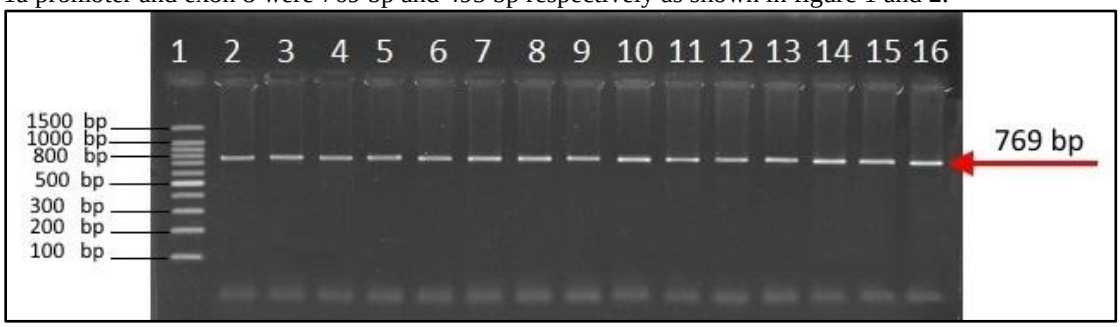

Figure.1: Agarose gel electrophoresis of PCR products of ***GABRB3* gene – exon 1a promoter** of the molecular size 769 bp indicated by red arrow. Bands were separated by electrophoresis on 2 % agarose gel at 90 voltages for one hour and visualized by gel documentation system after 30 minutes of staining with EtBr. Lane 1: DNA ladder (100-1500 bp). Lane (2-10): products for exon 1a promoter from autistic children. Lane (11-13): products for exon 1a promoter from relatives. Lane (14-16): products for exon 1a promoter from healthy children.

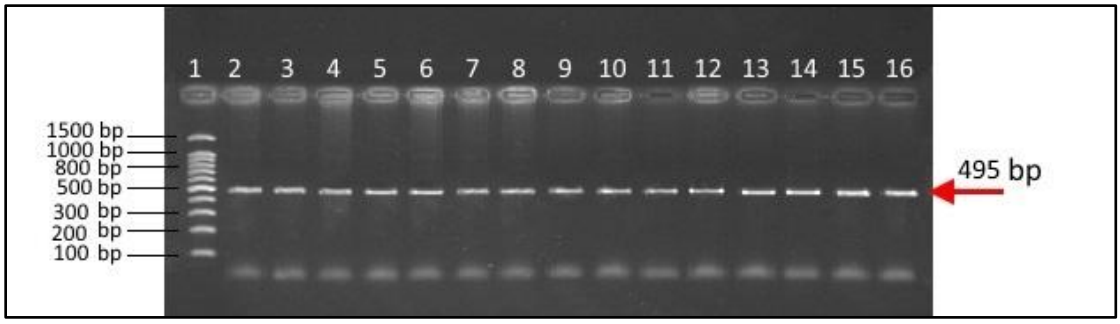

Figure.2: Agarose gel electrophoresis of PCR products of ***GABRB3* gene – exon 8** of the molecular size 495 bp indicated by red arrow. Bands were separated by electrophoresis on 2 % agarose gel at 90 voltages for one hour and visualized by gel documentation system after 30 minutes of staining with EtBr. Lane 1: DNA ladder (100-1500 bp). Lane (2-10): products for exon 8 from autistic children. Lane (11-13): products for exon 8 from relatives. Lane (14-16): products for exon 8 from healthy children.

Sequencing results of exon 1a promoter and exon 8 of *GABRB*:-

For exon 1a promoter region of *GABRB3*, BLAST alignment with the reference gene (GenBank: NG_012836.1) for all 9 samples of DNA sequence showed the presence of 3 common SNPs (rs7171660, rs4363842 and rs4906901) and 13 new ones located along this region. From all 16 SNPs detected in this region, six were mutual (found both in autistic and control samples), eight were found only in autistic patients while the remaining two were found only in controls. The three common SNPs were previously reported in other parts of the world. The SNPs rs4906901 (at -169 before exon 1) and rs7171660 (at -541 before exon 1) were reported by Urak and his colleagues in Austrian population (2006), while the SNP, rs4363842 was reported in Mexican and Honduras population (Tanaka *et al.*, 2012). As for exon 8, only one SNP (rs3751582) was found in three autistic patients and two controls while the remaining five DNA sequence samples showed 100 % identity when aligned using BLAST. Figure 3 shows the location of SNPs upstream of exon 1a of the gene and the SNP detected in exon 8. To our knowledge, this is the first time to report these SNPs in Iraq.

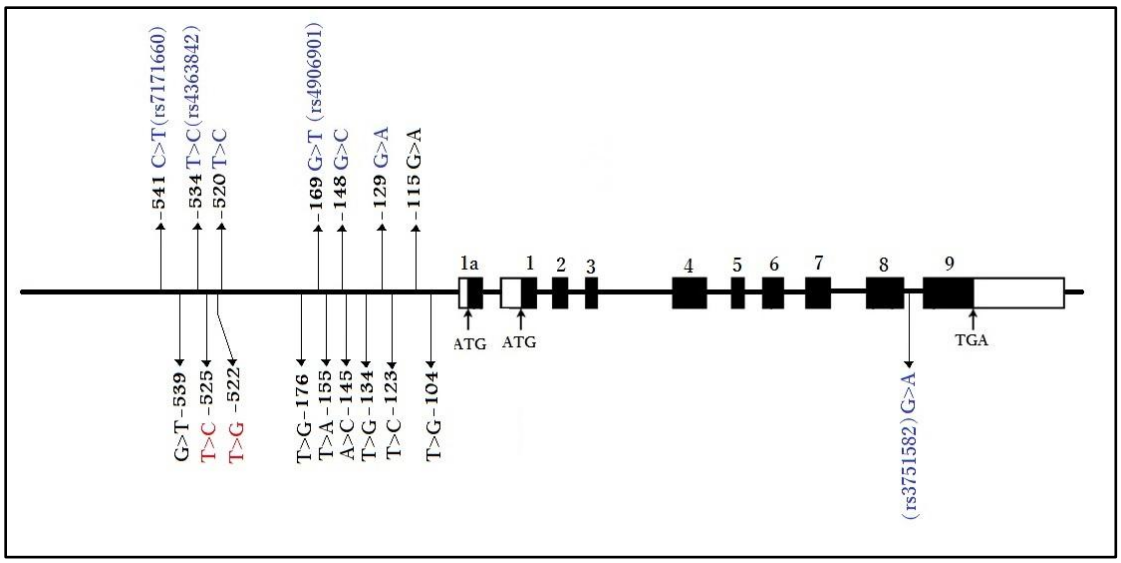

Figure.3: Distribution of SNPs identified in this study along *GABRB3* gene, black boxes are exons while the region upstream exon 1a is the promoter region.

Table 4 shows the detected SNPs with the number of samples they were detected in. SNPs in blue color were found in both autistic patients and controls; SNPs in red color were only found in controls while SNPs in black color were only found in Autistic patients

Table (4): Sample distribution of SNPs detected in this study.

Location	SNP	Autism	Control (healthy)	P-value
Exon 1a promoter	C>T at -541 (rs7171660)	6	3	0.028 *
	G>T at -539	1	0	0.155 NS
	T>C at -534 (rs4363842)	6	3	0.028 *
	T>C at -525	0	1	0.155 NS
	T>G at -522	0	1	0.155 NS
	T>C at -520	1	1	1.00 NS
	T>G at -176	1	0	0.155 NS
	G>T at -169 (rs4906901)	6	3	0.028 *
	T>A at -155	1	0	0.155 NS
	G>C at -148	1	1	1.00 NS
	A>C at -145	1	0	0.155 NS
	T>G at -134	1	0	0.155 NS
	G>A at -129	3	2	0.155 NS
	T>C at -123	1	0	0.155 NS
	G>A at -115	1	0	0.155 NS
	T>G at -104	1	0	0.155 NS
Intron 8	G>A (rs3751582)	3	2	0.155 NS

* ($P<0.05$), NS: Non-significant.

Statistical analysis showed that only the three common SNPs (rs7171660, rs4363842 and rs4906901) were significantly appearing in autistic patients, although furtherstudies are required with larger sample size to confirm the association of these SNPs with autism. The SNP rs3751582 is located in the beginning of intron 8 away from exon/intron boundaries; therefore, it is possibly not associated with splicing. Exon 8 encodes 81 amino acids that start from residue 124 to 169 within the polypeptide (NCBI). Translation of nucleotides to amino acids was achieved with EMBOSS Transeq tool. The obtained amino acid sequence was aligned using BLASTp which showed 100% compatibility with the reference peptide sequence for all the samples meaning that there was no change in amino acid sequence in all autistic patients and controls. The three dimensional structure was predicted using RaptorX tool as shown in figure 4.

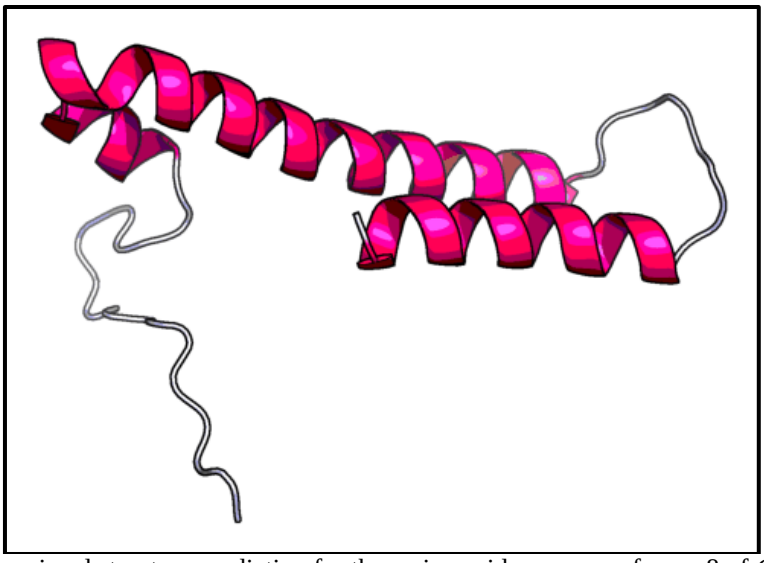

Figure 4: the three-dimensional structure prediction for the amino acid sequence of exon 8 of *GABRB3* gene, the sequence of all samples was identical with no change in amino acid sequence.

The results obtained in this study suggest the possible role of *GABRB3* promoter activity in the pathogenicity of autism. SNPs found only in autistic patients are likely associated with ASDs and promoter SNPs could alter expression of the gene thus contributing to the pathogenicity of autism. It is highly recommended for further studies to include the remaining regions of this gene in sequencing analysis as well as gene expression assays using real time PCR to confirm the association of this gene with autism.

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References:-

1. Carter, M. T. and Scherer, S. W. (2013). Autism spectrum disorder in the genetics clinic: a review. *Clinical genetics*, *83*(5): 399-407.
2. Chen, C.H.; Huang, C.C.; Cheng, M.C.; Chiu, Y.N.; Tsai, W.C.; Wu, Y.Y.; Liu, S.K. and Gau, S.S.F. (2014) Genetic analysis of *GABRB3* as a candidate gene of autism spectrum disorders. *Molecular autism*, *5*(1): p.1.
3. Folstein, S.E. and Rosen-Sheidley, B. (2001). Genetics of autism: complex aetiology for a heterogeneous disorder. *Nat. Rev*. Genet. 2(12):943-955.
4. Garber, K. (2007). Neuroscience. Autism's cause may reside in abnormalities at the synapse.*Science.* 317(5835):190-191.
5. Jiao, Y.; Chen, R.; Ke, X.; Cheng, L.; Chu, K.; Lu, Z. and Herskovits, E. H., (2012). Single nucleotide polymorphisms predict symptom severity of autism spectrum disorder. *Journal of autism and developmental disorders*, 42(6):971-983.
6. Kanner L. Autistic Disturbances of Affective Contact (1943). *Nervous Child*., 2:217-250
7. Kim, H.G.; Kishikawa, S.; Higgins, A.W.; Seong, I.S.; Donovan, D.J.; Shen, Y.; Lally, E.; Weiss, L.A.; Najm, J.;Kutsche, K. and Descartes, M. (2008). Disruption of neurexin 1 associated with autism spectrum disorder. *The American Journal of Human Genetics*, *82*(1):199-207.
8. Limpraert, P. (2008). Genetics of autism. *Siriraj Medical Journal*, 60(5): 305-309.
9. Källberg, M.; Wang, H.; Wang, S.; Peng, J.; Wang, Z.; Lu, H. and Xu, J. (2012). Template-based protein structure modeling using the RaptorX web server. *NatureProtocols*, 7:1511-1522.
10. Omar, S. K. (2012) Study of Prevasive developmental disorders among students in Mousil. *Ann. Coll. Med. Mosul* 2012; 38 (2): 52-61.
11. Piton, A.**;** Gauthier, J.; Hamdan, F.F.; Lafreniere, R.G.; Yang, Y.; Henrion, E.; Laurent, S.; Noreau, A.; Thibodeau, P.; Karemera, L.; Spiegelman, D.; Kuku, F.; Duguay, J.; Destroismaisons, L.; Jolivet, P.; Côté, M.; Lachapelle, K.; Diallo, O.; Raymond, A.; Marineau, C.; Champagne, N.; Xiong, L.; Gaspar, C.; Rivière, J.B.;

Tarabeux, J.; Cossette, P.; Krebs, M.O.; Rapoport, J.L.; Addington, A.; Delisi, L.E.; Mottron, L.; Joober, R.; Fombonne, E.; Drapeau, P. andRouleau, G.A. (2011). Systematic resequencing of X-chromosome synaptic genes in autism spectrum disorder and schizophrenia. Mol. Psychiatry.16(8):867-880.

12. Ronemus, M.; Iossifov, I.; Levy, D., and Wigler, M. (2014). The role of de novo mutations in the genetics of autism spectrum disorders. *Nature Reviews Genetics*, *15*(2): 133-141.
13. Sambrook, J.; Fritsch, E.F. and Maniatis, T. (1989). Molecular cloning: A laboratory manual. 2nd edition, Cold spring harbor laboratory press, New York.
14. Sutcliffe, J. S.;Nurmi, E. L. and Lombroso, P. J. (2003). Genetics of childhood disorders: XLVII. Autism, part 6: duplication and inherited susceptibility of chromosome 15q11-q13 genes in autism. *J Am Acad Child Adolesc Psychiatry*, 42:253–256.
15. Tanaka, M.; Bailey, J. N.; Bai, D.; Ishikawa-Brush, Y., Delgado-Escueta, A. V. and Olsen, R. W. (2012). Effects on promoter activity of common SNPs in 5′ region of *GABRB3* exon 1A. Epilepsia, 53(8): 1450-1456.
16. Urak, L.;Feucht, M.;Fathi, N.;Hornik, K. and Fuchs, K. (2006). A *GABRB3* promoter haplotype associated with childhood absence epilepsy impairs transcriptional activity. *Hum Mol Genet*, 15:2533–2541.
17. Wang, Z. and Moult, J (2001). SNPs, Protein Structure, and Disease. HUMAN MUTATION 17:263270.
18. Werling, D. M., and Geschwind, D. H. (2013). Sex differences in autism spectrum disorders. *Current opinion in neurology*, *26*(2): 146.
19. Zoghbi, H.Y. (2003).Postnatal neurodevelopmental disorders: meeting at the synapse.*Science*.302(5646):826-830.