Molecular Evaluation of exons 8 and 22 of the *SHANK3* gene in Autism Spectrum Disorders

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Abstract

Autism spectrum disorders are a group of neurodevelopmental disorders with a complex and heterogeneous etiology. Studies have shown that genetic factors play an important role in the aetiology of these diseases. Recently, de novo mutations, frameshifts and deletions have been described in the *SHANK3* gene, also known as *ProSAP2* gene, which encodes a synaptic scaffolding protein. All the participants of this study had normal karyotypes and underwent screening for Fragile-X syndrome. Subsequently, they were analyzed by direct sequencing of different points of exons 8 and 22 of the *SHANK3* gene. None of the study participants presented with changes in these regions. These findings may be due to the fact that mutations, deletions and duplications of the *SHANK3* gene are rare.

Key-words: Autism Spectrum Disorders, 22q13.3, *SHANK3*

Introduction

Autism Spectrum Disorders (ASDs) are a clinically complex group of childhood disorders that have firm evidence of an underlying genetic etiology.[1] The group of ASDs includes autism, as well as Pervasive Developmental Disorder not otherwise specified (PDD-NOS) and Asperger's syndrome.[2] The diagnosis of ASDs is based on impairments in reciprocal social interaction and communication, and restricted and stereotyped patterns of interests and activities with abnormal development apparent within the first 3 years of life.[3] The prevalence of ASDs is estimated at 1 per 150 children.[4] Cases are isolated or associated and a recognized cause is identified in ~10% of individuals, most commonly fragile-X syndrome, tuberous sclerosis and cytogenetically detectable chromosome abnormalities.[5,6,7] Standard karyotype analyses

show chromosomal rearrangements in 3%-6% of cases, the most common being deletions and duplications on chromosomes 2q, 7q, 15q and 22q.[8,9]

The *SHANK3* gene, mapped on chromosome 22q13.3, has been extensively studied in this population. This gene encodes the postsynaptic density protein (PSD) specialized at excitatory synapses, where it may function as a master scaffolder forming large sheets that may well represent the platform for the construction of the PSD complex, where it binds directly to neuroligins.[10,11] The PSD is usually located at the tip of dendritic protrusions of about 1-2 μm in length, termed dendritic spines, and separated from the presynaptic transmitter-containing terminal by the synaptic cleft. Thus shank proteins are not considered to provide a direct scaffolding function for transmitter receptors, but rather work indirectly by connecting different types of scaffold/receptor complexes, leading to the concept of a "master" or "higher order" scaffold. This view is strongly supported by different protein interactions in which Shank proteins are involved.[12] In addition to their function of assembling the PSD during synaptogenesis, the *SHANK3* protein may play a role in synaptic plasticity and in the regulation of dendritic spine morphology.[13]

SHANK3 mutations, deletions and duplications are rare, occurring in only 1.1% of patients with ASD. However, the genotype-phenotype correlation suggests that individuals with severe language and social impairment might be good candidates for *SHANK3* mutation screening.[14]

Two recent studies reported possible correlations between mutations or small cytogenetic rearrangements affecting the *SHANK3* gene and an ASD phenotype chiefly characterized by severe verbal and social deficits. The first study found a deletion of 142 Kb in intron 8, one frameshift mutation (E409X) in exon 21, a deletion of 800 kb, and R12C and R300C mutations in exons 1 and 8, respectively. [8] The second work reported a missense mutation (Q321R) in exon 8, a 15-nt deletion in exon 21 and deletions of 277 Kb, 3.2 Mb and 4.36 Mb in 22q13. [2]

The lack of one functional copy of the gene would thus lead to severe neurological deficits because individual neurons cannot provide enough Shank proteins for synapse development and maintenance. In addition, it makes sense that the amount of Shank protein in dendrites is tightly controlled, most likely by local synthesis derived from dendritically transported mRNAs, and activity-dependent degradation through the ubiquitin proteasome system.[12]

This current pilot study evaluated exons 8 and 22 of the *SHANK3* gene by direct sequencing.

Subjects and Methods

This study was approved by the Research Ethics Committee (CEP) of the Medicine School in São José do Rio Preto and CONEP (process 25000.015469/2007-59). After obtaining written informed consent from parents, a total of 30 (22 male and 8 female) unrelated individuals with ASDs were studied. These individuals, of mixed ethnicity and representative of the Brazilian population, were aged from 5 to 30 years old (mean = 14.16 and standard deviation = 6.30 years). Fifteen (50%) were diagnosed as autistic,

11 (36.67%) with PDD-NOS and 4 (13.33%) with Asperger's syndrome. The patients were from two specialized autism schools and all individuals were conclusively diagnosed by psychiatrists using different methods. Additionally, all were evaluated by an interdisciplinary team composed of a psychiatrist, neurologist, geneticist, psychologist, speech therapist and nurse before participating in this study. Besides the clinical evaluation, the diagnosis of ASD was made if the patient met the DSM-IV criteria. Patients were submitted to clinical and karyotypic examinations and molecular investigations of the *FMR1* gene. Only those individuals with normal results for these tests were included in the study. We also excluded patients with evidence of any other psychiatric or neurological conditions and those with other genetic syndromes. Genomic DNA was isolated from the leukocytes of peripheral blood[15] and the primer sequences were designed using the Primer3 program.

For all patients, the entire exon 8 and parts of the coding region of exon 22 of the *SHANK3* gene were screened.

Exon 8 was targeted using the forward and reverse primers (5´-CAGCTGTGATTCCCTCTTCC-3´ and 5´-GGGAAGAACCAAGGTTCAGA-3´) flanking the positions 8671 and 8870 of DNA producing an amplification of 200 bp. Exon 22 was targeted using two pairs of primers; the first flanked the positions 46748 and 46934 of DNA, producing an amplification of 186 pb (forward primer 5´-GAAGTCACCCGAGGACAAGA-3´ and reverse primer 5´-CACAGCCGCTGACTGCAT-3´) and the second flanked the positions 47435 and 47617 of DNA, producing an amplification of 183 pb (forward primer 5´-CAAGCCCAAGCTCAAGTCC-3´ and reverse primer 5´-GGGAAGAACCAAGGTTCAGA -3´).

Amplification was performed in a reaction volume of 25µL containing 1x PCR Buffer, 50mM of $MgCl_2$, 1.25mM dNTPs (GE Healthcare), 50ng genomic DNA, 10µM of each primer, and 5U Taq DNA polymerase (Invitrogen). The PCR reaction was carried out in a GeneAmp® PCR System 9700 (Applied Biosystems, CA) with 4 min of denaturation at 94ºC, followed by 35 cycles of 94ºC for 45s, 45s at an annealing temperature of 62ºC, and for exon 8, an extension for 1 min at 72ºC, with 5 min denaturation at 95ºC, followed by 30 cycles of 95ºC for 30s, 30s at the annealing temperature at 57ºC, and for each primers of exon 22, extension for 1 min at 72ºC.

PCR products were purified with ethanol. [16] Sequencing reactions were carried out with the Applied Biosystems Dye-Terminator v3.1 Kit (Applied Biosystems, USA) and analyzed on a 3730 DNA analyzer (Applied Biosystems, CA). Analysis of sequences was performed using the DS Gene 2.0 program (Accelrys, USA) and a comparison with existing sequences was made using GenBank (www.ncbi.nlm.nih.gov/nuccore/24137474).

To date 30 individuals have been investigated. Karyotyping was performed when possible on probands resulting in 27 individuals and 9 subjects for molecular genetic testing of the Fragile-X syndrome with one affected individual in each family. Direct sequencing of exon 8 and two exon 22 regions of the *SHANK3* gene was performed for all subjects.

Results

All subjects had wild sequences according to Genbank. Thus, all participants had normal sequences from 8700 to 8870, the position of exon 8 in the *SHANK3* gene. Furthermore, they were normal considering the regions from 46748 to 46934 and from 47435 to 47617 positions of DNA of exon 22 of the *SHANK3* gene when analyzed by direct sequencing.

Discussion

SHANK3 is located on chromosome 22q13.3, spans approximately 57kb and contains 23 exons. Seven of the exons are alternatively spliced, which may influence the spectrum of SHANK-interacting proteins. *SHANK3* expression in the brain seems to be confined to the cortex, hippocampus and cerebellum.[17] Owing to its emerging role in neuropsychiatric disorders, *SHANK3* was first implicated in the field of neuro-psychiatric disorders when a patient with 22q13.3 deletion syndrome was found to have a *de novo* reciprocal translocation that disrupted the gene. Subsequent studies have identified *de novo* deletions and mutations of *SHANK3* in individuals with autism, thus corroborating with previous reports about the role of the gene within the autistic phenotype.[18]

Exon 8 of the *SHANK3* gene has 78 pb and exon 21 has 2254 pb. A *de novo* mutation has been described in exon 8, characterized by the exchange of A962G mRNA, which resulted in a heterozygous Q321R substitution.[2] In this study the entire exon 8 was screened in all participants but no changes were identified.

A frameshift mutation (E409X) and a 15-nt deletion have also been found in exon 21.[2,8] We initially chose to study this site (exon 21) as it had previously been reported as disrupted[2,8], but the sequences of primers described by the authors correspond to exon 22 of the human *SHANK3* gene according to GenBank data. In personal correspondence with Christian R. Marshall, one of authors, it was noted that the sequence described was correct, but that it corresponded to exon 22 instead of 21. Thus, we chose to study parts of exon 22 and all the subjects were normal.

A correlation between mutations or small cytogenetic rearrangements affecting different exons of *SHANK3* and ASD is possible.[2,14,19] Based on diagnostic tests, karyotyping and fragile-X testing must be requested for all patients with ASD. In the presence of dysmorphic features and evident neurological symptoms, it is reasonable to suspect the chromosomal rearrangements exist even if the karyotype appears normal. Depending upon availability and cost, BAC or CGH analysis is strongly advised in these cases. As these technologies will become progressively more available, it will be important not to restrict them to patients with dysmorphia, as microdeletions and microduplications are

also common among patients with idiopathic, non-dysmorphic ASD.[14] However genetic screening is a powerful tool when dealing with monogenic Mendelian disorders that are characterized by direct genotype-phenotype correlations. In the case of complex disorders, such as ASD, widespread genetic testing would not only be expensive and time-consuming, but also generally inappropriate due to the etiological complexity.[20]

Our findings were normal possibly due to the fact that mutations, deletions and duplications in *SHANK3* are rare events, only occurring in approximately 1.1% of individuals with ASD [14,18,21] and in this pilot study we investigated a small number of Brazilian subjects. Sykes et al. (2009)[21] not found SNP and copy number variants (CNVs) in *SHANK3* but so rare mutations potencially could have been missed within the gene, cause sequense analysis was not conducted, but we did and not found too.

There are numerous and variable changes in ASD, involving different chromosomal regions and many genes have been proposed as candidates including the one investigated here. Maybe genetic screening programs for the *SHANK3* gene could be implemented but not for all patients with ASD, only for those patients with severe verbal and social deficits bearing in mind that some *SHANK3* mutations have also been found to be transmitted to unaffected siblings of ASD probands.[2]

The etiological complexity results in misinformation about what deserves to be initially included in the protocols of laboratory investigations of affected individuals.

Conflict of Interest Statement. The authors declare that they have no competing interests.

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References

1. Kumar RA, Christian SL: Genetics of Autism Spectrum Disorders. Curr Neurol Neurosci Rep 2009; **9**:188-97.
2. Moessner R, Marshall CR, Sutcliffe JS, Skaug J, Pinto D, Vincent J *et al*: Contribution of *SHANK3* mutations to autism spectrum disorder. Am J Hum Genet. 2007; **81**: 1289-1297.
3. Lord C, Cook EH, Leventhal BL, Amaral DG: Autism spectrum disorders. Neuron 2000; **28**: 355-363.

4. Herbert MR: Contributions of the environment and environmentally vulnerable physiology to autism spectrum disorders. Curr Opin Neurol. 2010; Jan 16. Epub ahead of print 16 January 2010;
5. Folstein SE, Rosen-Sheidley B: Genetics of autism: complex aetiology for a heterogeneous disorder. Nat Rev Genet 2001; **2**: 943-955.
6. Xu J, Zwaigenbaum L, Szatmari P, Scherer SW: Molecular cytogentics of autism. Curr Genomics 2000; **5**: 347-364.
7. Veenstra-Vanderweele J, Christian SL, Cook EH Jr: Autism as a paradigmatic complex genetic disorder. Annu Rev Genomics Hum Genet 2004; **5**: 379-405.
8. Durand CM, Betancur C, Boeckers TM, Bockmann J, Chaste P, Fauchereau F *et al*: Mutations in the gene encoding the synaptic scaffolding protein SHANK3 are associated with autism spectrum disorders. Nat Genet. 2007; **39**: 25-27
9. Newbury DF, Warburton PC, Wilson N, Bacchelli E, Carone S; International Molecular Genetic Study of Autism Consortium *et al*: Mapping of partially overlapping de novo deletions across an autism susceptibility region (AUTS5) in two unrelated individuals affected by developmental delays with communication impairment. Am J Med Genet A. 2009; **149A**: 588-597.
10. Baron MK, Boeckers TM, Vaida B, Faham S, Gingery M, Sawaya MR *et al*: An architectural framework that may lie at the core of the post-synaptic density. Science 2006; **311**: 531-535.
11. Meyer G, Varoqueaux F, Neeb A, Oschlies M, Brose N: The complexity of PDZ domain-mediated interactions at glutamatergic synapses: a case study on neuroligin. Neuropharmacology 2004; **47**: 724-733.
12. Kreienkamp HJ: Scaffolding proteins at the postsynaptic density: shank as the architectural framework. Handb Exp Pharmacol. 2008; **186**: 365-80.

13. Bonaglia MC, Giorda R, Mani E, Aceti G, Anderlid BM, Baroncini A *et al*: Identification of a recurrent breakpoint within the SHANK3 gene in the 22q13.3 deletion syndrome. J Med Genet. 2006; **43**:822-828.

14. Lintas C, Persico AM: Autistic phenotypes and genetic testing: state-of-the-art for the clinical geneticist. J Med Genet. 2008; **46**:1-8.

15. Miller SA, Dykes DD, Polesky HF: A simple salting out procedure for extracting DNA from human nucleated cells. Nucleic Acids Res. 1998; **16**: 1215.

16. Sambrook J, Russel DW: Molecular Cloning a Laboratory Manual. Cold spring harbor laboratory press: New York, USA, 2001.

17. Streng M, Kim E: The shank family of scaffold proteins. J Cell Sci. 2000; 113:1851-1856.

18. Gauthier J, Spiegelman D, Piton A, Lafrenière RG, Laurent S, St-Onge J *et al*: Novel de novo SHANK3 mutation in autistic patients. Am J Med Genet B Neuropsychiatr Genet. 2009; **150B**:421-424.

19. Marshall CR, Noor A, Vincent JB, Lionel AC, Feuk L, Skaug J *et al*: Structural Variation of Chromosomes in Autism Spectrum Disorder. Am J Hum Genet. 2008; **82**: 477-488.

20. Caglayan AO: Genetic causes of syndromic and non-syndromic autism. Dev Med Child Neurol. 2010; Jan 5. Epub ahead of print 5 January 2010.

21. Sykes NH, Toma C, Wilson N, Volpi EV, Sousa I, Pagnamenta AT *et al*: Copy Number Variation and Association Analysis of SHANK3 as a Candidate gene for Autism in the IMGSAC Collection. Eur J Hum Genet. 2009; **17**: 1347-1353.