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Identification of a 7q33-q35 microdeletion disrupting the *CNTNAP2* gene in a Brazilian Stuttering case

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Abstract

Speech and language disorders are some of the most common referral reasons to child development centers accounting for approximately 40% of cases. Stuttering is a disorder in which involuntary repetition, prolongation or cessation of the sound precludes the flow of speech. About 5% of individuals in the general population have a stuttering problem, and about 80% of the affected children recover naturally. The causal factors of stuttering remain uncertain in most cases; studies suggest that genetic factors are responsible for 70% of the variance in liability for stuttering, whereas the remaining 30% is due to environmental effects supporting a complex cause of the disorder. The use of high-resolution genome wide array comparative genomic hybridization (array-CGH) has proven to be a powerful strategy to narrow down candidate regions for complex disorders. We report on a case with a complex set of speech and language difficulties including stuttering who presented with a 10 Mb deletion of chromosome region 7q33-35 causing the deletion of several genes and the disruption of *CNTNAP2* by deleting the first 3 exons of the gene. *CNTNAP2* is known to be involved in the cause of language and speech disorders and Autism Spectrum Disorder (ASD) and is in the same pathway as *FOXP2*, another important language gene, which makes it a candidate gene for causal studies speech and language disorders such as stuttering.

Keywords

microdeletion; *CNTNAP2*; stuttering

Introduction

Fluent speech production is a process that involves three major steps: conceptualization, formulation and articulation [Levelt 1999] and disturbances of this balanced cascade can lead to speech disorders such as stuttering. Stuttering is a common worldwide communication disorder characterized by syllable repetitions and/or prolongations, and disturbances in the ability to produce smooth flow of speech [Conture and Kelly 1991;

Wingate 1964]. The disorder is first noted during early childhood; approximately 80% of affected children improve naturally within 1 to 4 years of onset [Andrews 1964; Yairi and Ambrose 1999]. Several studies reported females recover more often than males, resulting in a male-female ratio of 4:1 in older children and adults [Buchel and Sommer 2004; Felsenfeld 2002; Yairi and Ambrose 1999].

The primary cause of stuttering remains unknown in the majority of cases. Andrews et al. [1991] and Felsenfeld et al. [2000] reported that genetic effects contribute to approximately 70% of the variance in liability for stuttering whereas the remaining 30% are due to environmental effects, supporting a complex cause of the disorder. Familial aggregation of stuttering has been studied extensively, with an increase of approximately 15% in first-degree relatives of probandus, compared to 5% in the general population [Ambrose et al., 1993].

Speech disorders have a broad clinical phenotype usually of unknown cause and pathogenesis; one of the biggest challenges is to define homogeneous subgroups for causal studies. Stuttering can occur as an isolated event or as part of a more complex phenotype or syndrome. The identification of important genetic factors will increase the knowledge of the causes of stuttering, along with diagnosis and treatment. Recently, several genome wide studies have reported susceptibility loci for stuttering at 18p11 [Shugart et al., 2004]; 12q23 [Riaz et al., 2005]; on chromosomes 2, 7, 9 and 15 [Suresh et al., 2006]; chromosome 3, 13 and 15 [Raza et al., 2010; Wittke-Thompson et al., 2007] but the results remain controversial and, to date, no strong candidate genes have been reported. A study with Pakistani families and unrelated individuals from Pakistan and North America identified mutations in *GNPTAB*, *GNPTG* and *NAGPA* genes that were associated with stuttering [Kang et al., 2010].

Different strategies can be used for gene identification, among those, the recent use of DNA microarrays (array-CGH) has helped to identify a new form of submicroscopic genomic imbalance named copy number variants (CNV) that are the result of common gains or losses of genomic regions and contribute substantially to human genetic diversity; they may cover important genes responsible for human diseases and their identification will contribute to increased knowledge of genetic factors in complex conditions such as stuttering.

We report one case with a complex set of speech, language and learning difficulties with notable problems in speech fluency characterized by stuttering and cluttering, developmental delay, and some minor phenotypic signs such as broad nasal root, mild thoracic asymmetry, broad halluces, and distal toe phalanges with a 10 Mb deletion involving chromosome region 7q33-35 identified by array-CGH. The region is a candidate for extended analysis and contains at least one strong candidate gene, *CNTNAP2*, which is known to be involved in language and speech problems and Autism Spectrum Disorder (ASD) [Alarcon et al., 2008; Poot et al., 2009; Rossi et al., 2008; Stromswold 2008; Vernes et al., 2006].

Material and Methods

The proband, male, born in 1988, is the second child of a normal 23-year-old mother and her normal, non-consanguineous 25-year-old husband. Pregnancy was normal; no toxic, infectious, or traumatic incidents or X-ray exposure was reported. Delivery was normal. Birth weight was 3,150 g (>25th centile), length was 49 cm (25th centile). Neonatal development was normal. Neuropsychological development and language acquisition were delayed (first words at 3 years age) and stuttering was noticed early when phrasal constructions began. Examination at age 19 showed a weight of 59 kg (<25th centile), height of 168 cm (75th centile), OFC of 57.5 cm (<50th centile). He presented with severe

fluency disorder characterized by stuttering and cluttering, minor phenotypic signs such as broad nasal root, mild thoracic asymmetry, and broad halluces and distal toe phalanges. Brain MRI showed mild cerebral and cerebellar atrophy and a large callosal splenium. High resolution GTG banding (850 bands) and Fluorescent in Situ Hybridization (FISH): WCP 7-Spectrum Green-VYSISR) were performed.

An audiological evaluation was performed by tonal audiometry, tympanometry, acoustic immittance and ABR. Video, audio and recording of speech were evaluated by ABFW Test. Cognitive assessments were performed using Wechsler Intelligence Scale for Adults, Wisconsin Cards Sorting Test, Token Test, and the Illinois Test of Psycholinguistic Abilities.

The study was approved by the Ethical Committee at the Hospital de Reabilitação de Anomalias Craniofaciais - USP - Bauru, SP, Brazil and signed consents were obtained prior to sample collection.

DNA Microarray Analysis

Genomic DNA was isolated from a peripheral blood sample from the proband and parents and the concentration was measured by a NanoDrop spectrophotometer (ND-1000 V.3.1.2). The microdeletion was detected by a genome wide copy number scan using the Affymetrix Genome Wide Human SNP Array 6.0 (Affymetrix, CA, USA). These arrays offer genome wide coverage with almost 2 million SNPs and CNV probes and measure DNA copy number differences between a reference genome and the sample genome. The results were analyzed using three different software packages: Affymetrix Genotyping Console, Partek Genomics Suite and Nexus Copy Number.

Quantitative Real Time PCR

The quantitative real time PCR was performed with DNA from the proband, father, mother and a normal control using DNA-binding dye SYBR Green I. This method enables both detection and quantification as absolute number of copies of a specific sequence in a DNA sample. We designed primers for the first 5 exons of the *CNTNAP2* gene; all the samples were analyzed in duplicates.

The fold changes per sample were calculated based on methods previously described [Weksberg et al., 2005]. We compared the target sample (proband) with normal reference controls to obtain the fold changes.

$$\Delta KC_t = KC_{t/control} - KC_{t/affected}$$

$KC_{t/control}$ = 'Corrected C_t ' of the test primer for the control samples.
 $KC_{t/affected}$ = 'Corrected C_t ' of the test primer for the affected samples.

Where ΔKC_t – represents copy number gain or loss per sample (fold changes).

ΔKC_t values of 0 (± 0.35) indicate an equal ratio of the test and reference, which corresponds to no loss and therefore no genetic abnormality. A ΔKC_t value of -1 (± 0.35) indicates loss of one copy (microdeletion), for the affected samples while a value of +1, (± 0.35) would indicate copy gain consistent with microduplication [Weksberg et al., 2005].

Results

High-resolution GTG banding (at 850 bands) showed a 7q33-q34 deletion. Fluorescent in situ hybridization (FISH): WCP 7- Spectrum Green-VYSISR) ruled out translocation of the deleted region. Parents had normal chromosomes (data not shown). Audiological evaluation assessed by tonal audiometry, tympanometry, acoustic immittance, and ABR showed normal parameters. Video and audio recordings of speech samples were evaluated through the ABFW Test and showed high frequency of breakdowns of speech that included stutter disruptions such as repetitions and prolongation of sounds and syllables, involuntary pauses, reiteration, and associated movements (blinking). Cognitive assessment using Wechsler Intelligence Scale for Adults, Wisconsin Cards Sorting Test, Token Test, and the Illinois Test of Psycholinguistic Abilities revealed the patient's mental performance has normal intellectual level VIQ (86), PIQ (96), FSIQ (90), working memory index (87) and speed processing index (103). The Wisconsin test also showed a perseverative response indicating a difficulty in flexibility strategies. Speech and language evaluation showed moderate difficulty in resolution of complex verbal commands mainly for comprehension of syntactic structures or poor verbal communication. Receptive oral language was better than expressive language. His global academic performance in arithmetic, reading, and writing evaluated through TDE showed low scores in comparison with the normal range.

The genome wide copy number scan analysis using microarray showed a 10 Mb deletion encompassing the region 7q33-q35 that includes several genes and disrupts the gene *CNTNAP2* by deleting the 3 first exons. Fig. 1 shows details of the deleted region.

In order to confirm the breakpoints of the deletion and the disruption of the gene, we performed a quantitative PCR using Sybr Green I dye. We designed primers for the first 5 exons of the gene and the results show a pattern compatible with deletion of one copy for the exons one to three and normal values for exons 4 and 5, confirming the microarray results. The quantitative PCR plots and calculations are shown in fig. 2 and Table I.

The deletion of the first three exons of the *CNTNAP2* gene also results in deletion of *FOXP2* binding sites present in intron one of *CNTNAP2*. Since this patient is hemizygous for the *FOXP2* protein binding sites in the *CNTNAP2* gene there may be a disruption of the molecular mechanism between free and bound *FOXP2* protein.

Discussion

Stuttering is a complex disorder that involves genetic and environmental causes. It is a common and sometimes severe disorder that is distributed worldwide and has variable phenotypic severity, it can be syndromic or occur as an isolated event. Several studies have reported important evidence of genetic factors that may be involved in its cause [Ambrose et al., 1993; Andrews et al., 1991; Felsenfeld et al., 2000; Riaz et al., 2005; Suresh et al., 2006] although the wide phenotypic spectrum of language and speech disorders makes it difficult to identify homogenous subgroups for causal studies that could lead to the identification of major, causative genomic variants.

The use of high-resolution microarrays has enhanced discovery of loci and genes for human diseases, especially complex diseases. The current generation of DNA microarrays such as Affymetrix array SNP 6.0 offers excellent genome-wide coverage with almost 2 million SNPs and CNV probes and measures DNA copy number differences between a reference genome and the sample genome. The use of ultrahigh resolution makes it possible to find with precision the microdeletion boundaries.

For a long time, it was thought that stuttering arose from abnormalities of the tongue, larynx, peripheral muscles of the speech, and psychological response to the environment. However, recent studies suggest that stuttering dysfunction resides in the brain [Canhetti-Oliveira and Richieri-Costa 2006].

Our study reports a Brazilian case with complex neurodevelopmental phenotype including stuttering that presents with a 10 Mb deletion at 7q33-q35 involving several genes. Some of the deleted genes are of particular interest due to expression in the brain including: *CNTNAP2*, *CHRM2*, *HIPK2*, *DGKI*, *EPHB6*, *PTN* and *SVOPL*. Here we summarize the importance of some of the deleted genes in this case.

CHRM2 is one of 5 human genes encoding muscarinic acetylcholine receptors and is involved in neuronal excitability, synaptic plasticity and feedback regulation of acetylcholine release [Gosso et al., 2007]. Muscarinic receptors are involved in many processes in the brain, including attention, learning, memory and cognition [Baxter and Chiba 1999]. *CHRM2* is a functionally important membrane-bound protein that is negatively coupled to adenylyl cyclase and is widely expressed in a range of tissues including brain, cardiac, prejunctional cholinergic nerve endings, and smooth muscle [Fenech et al., 2004]. In 2004 Wang et al. reported an association between SNPs in *CHRM2* and alcoholism and depressive syndrome [Wang et al., 2004]. Behavioral and pharmacological animal studies involving muscarinic receptors have shown their importance for acquisition and retrieval of several learning tasks [Carey et al., 2001; Gautam et al., 2006; Orsetti et al., 1996; Seeger et al., 2004] but there are no data about speech disorders and *CHRM2*.

Other deleted genes show association with disease, including *HIPK2* with leukemia, *DGKI* with schizophrenia and bipolar disorder, *EPHB6* with neuroblastoma, and *CNTNAP2* with ASD susceptibility [Alarcon et al., 2008; Poot et al., 2009; Rossi et al., 2008] and Gilles de la Tourette syndrome which includes complex involuntary motor and vocal tics [Belloso et al., 2007].

Among all these *CNTNAP2* is probably one of the most promising candidates for the speech problem based on previous published data although the hypothesis needs confirmatory studies. This gene was previously related to language disorders [Abrahams et al., 2007; Alarcon et al., 2008; Belloso et al., 2007; Poot et al., 2009; Rossi et al., 2008; Verkerk et al., 2003; Vernes et al., 2008], is highly expressed in the cerebral cortex in areas involved with language function [Abrahams et al., 2007; Vernes et al., 2006] and is in the same pathway of *FOXP2*, another important language gene. A recent study reports the disruption of *CNTNAP2* in a case with ASD and speech delay and compares different rearrangements of *CNTNAP2* with different phenotypes [Poot et al., 2010].

The *CNTNAP2* gene belongs to the superfamily of the neurexins, a group of transmembrane proteins that mediate cell-cell interactions in the nervous system. A recent expression study showed that *CNTNAP2* is consistently expressed in circuits involved in higher cortical functions, including language [Abrahams et al., 2007; Bellen et al., 1998; Peles et al., 1997; Poliak et al., 1999]. Another recent study showed that the transcription factor *FOXP2* regulates the expression of *CNTNAP2* by binding to a regulatory sequence in intron 1 of the gene; they also showed complimentary patterns of expression of *FOXP2* and *CNTNAP2* in cortical lamination at 18 to 22 weeks of gestation and concluded that these 2 genes form a shared neurogenetic pathway that is involved in language and speech disorders [Vernes et al., 2008].

Vertek et al. [2003] showed that *CNTNAP2* is disrupted in a family with Gilles de la Tourette syndrome (GTS) involving complex involuntary motor and vocal tics; they discuss that disruption or decreased expression of *CNTNAP2* could lead to a disturbed distribution

of the K^+ channels in the nervous system, thereby influencing conduction and/or repolarization of action potentials, causing unwanted actions or movements in GTS [Verkerk et al., 2003]. Lu et al. [2009] showed that stuttering cases have an altered connectivity in the basal ganglia compared with non-stuttering controls that may be involved in the difficulties related to speech control [Lu et al., 2009a; Lu et al., 2009b]. As well as the unwanted actions seen in GTS patients, stuttering is a motor problem that impairs normal speech ability. The deletion found in our case supports the causal role of *CNTNAP2* in patients with stuttering and similar motor speech problems.

Our study shows evidence that one or more genes in the 7q33-q35 region may play a causal role in speech motor problems as stuttering with a special focus on a partial deletion of *CNTNAP2* including a deletion of the binding site for the transcription factor *FOXP2*.

Acknowledgments

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Figure 1.
Details of the deleted region – Top: heat map showing the deletion at 7q33-35; Bottom: a
expanded view of the deleted region based on UCSC Genome Browser with *CNTNAP2*
highlighted by a gray circle

Figure 2.

Confirmation of the microarrays results and detection of the deletion of the first 3 exons of *CNTNAP2* by quantitative PCR using 2 sets of primers for each of exons 1 to 5. The plots show the pattern of amplification in parents, control and from proband samples. The parents and control samples amplify normally while the patient sample presents a delay of amplification due to the deletion.

Table 1

Copy Number calculations based on qPCR with SybrGreen

Sample	Primer	Ct	KCt	ΔKCt	
PROPOSITUS	primer 1_1	25.4	25.6	-1.2	Loss of 1 copy
		25.8			
	primer 1_2	24.3	24.2	-1.0	
		24.0			
	primer 2_1	24.5	24.7	-1.3	
		24.8			No copy number loss
	primer2_2	25.6	25.3	-0.7	
		25.0			
	primer 3_1	25.1	24.9	-1.2	
		24.7			
	primer 3_2	24.6	24.5	-1.0	
		24.3			No copy number loss
	primer 4_1	23.6	23.6	0.7	
		23.5			
	primer 4_2	25.0	25.2	0.5	
		25.5			
	primer 5_1	24.4	24.5	0.3	
		24.7			
	primer 5_2	24.2	24.1	0.1	
		23.9			

Copy number calculation where: Ct represents the point at which the fluorescence crosses the threshold; KCt is the corrected Ct value and ΔKCt represents copy number gain or loss per sample (fold changes). The analysis show ΔKCt values consistent with loss of 1 copy of exons 1, 2 and 3 and normal values for exons 4 and 5 in the propositus.