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Duplication-mediated down regulation of *SAP97* in a family with ASD and Tourette syndrome

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Abstract

Autism spectrum disorder (ASD) and Gilles de la Tourette syndrome (GTS) display broad clinical overlap with strong male bias and shared synaptic susceptibility genes. In this study we report ASD, GTS, ADHD, OCD, anxiety and depression in a 3 generation family with a 1.6Mb duplication of chromosome 3q29. We tested for expression of the gene thought most responsible for psychiatric and behavioural deficits at this locus *synapse-associated protein 97 (SAP97).* Comparative QPCR analysis indicated *SAP97* levels did not increase but rather decreased up to 2.5 fold in all family members tested with the duplication. Females displayed incomplete gender-limited phenotypic penetrance in direct correspondence with their decreased doses of *SAP97*. Conversely, there was a suggestion of *SAP97* dose-sensitive male bias with respect to ASD and GTS. These findings are consistent with ASD's prior association with the reciprocal 1.6Mb deletion at 3q29 and *SAP97's* prior familial linkage to GTS. SAP97 is a post-synaptic component of the neurexin trans-synaptic connexus (NTSC) - a mutation hotspot for ASD and GTS – that intersects with the DISC1 signalling pathway.

Keywords: SAP97, DISC1, Autism, Tourette syndrome, Depression, Anxiety, ADHD, OCD

Abbreviations: *SAP97*: *Synapse-Associated Protein 97* gene; NTSC: Neurexin Trans-Synaptic Connexus; PCR: Polymerase Chain Reaction; GTS: Gilles de la Tourette syndrome; ASD: Autism Spectrum Disorder; OCD, Obsessive Compulsive Disorder; ADHD: Attention Deficit Hyperactivity Disorder; DISC1: Disrupted in Schizophrenia 1

Introduction

ASD (MIM 209850) and GTS (MIM 137580) are both complex neurodevelopmental disorders with strong familial association [1-3]. Epidemiological, phenomenological and genetic evidence demonstrate broad overlap between ASD and GTS [4,5] with both exhibiting high incidence in first-degree relatives, high monozygotic to dizygotic concordance [6], with both conditions beginning during childhood with a high male preponderance with compulsive behaviours, obsessions, involuntary movements (tics in GTS and stereotypies in ASD), poor speech control and echolalia common in both conditions [7]. Attention deficit hyperactivity disorder (ADHD) is also present in both ASD and GTS [7,8]. GTS is over represented in ASD, with 5% having GTS and up to 40% experiencing tics [7]. Similarly, the rate of autism in GTS exceeds that expected by chance, with reports of autistic disorder in around 5%, subclinical autistic symptoms occurring in a third and a further two-thirds showing social deficits relating to the autism spectrum [9]. ASD and GTS are both heterogenous polygenic psychiatric disorders that exhibit strong molecular overlap with numerous shared synaptic susceptibility genes [4,5,10]. Neurexin trans-synaptic connexus (NTSC) gene families have been recurrently associated with, and represent a mutation hot spot for ASD and GTS [4,11]. Here we report a 3 generation family with a complex psychiatric phenotype segregating with a 1.6Mb duplication of 3q29 with reduced rather than increased levels of *SAP97*. This study expands the number of shared NTSC gene families recurrently associated with both ASD and GTS [4].

Materials and Methods

CGH micro-array analysis: Genomic DNA samples were isolated from peripheral blood samples from all participants, using standard phenol-chloroform extraction. Copy number variation (CNV) analysis of family DNAs was performed using ISCA 60k genome-wide CGH version 2 and analysed using BlueFuse-Multi software. Genome wide resolution was set to ~400kb and any change smaller than this was not reported.

Comparative QPCR of *SAP97*: Total RNA was extracted from peripheral blood samples from all participants using the Qiagen RNeasy kit (Qiagen) and treated with DNase I according to manufacturers' instructions. For each reaction, 1 µg of total RNA was reverse transcribed to generate cDNA using oligo-dT primer and Superscript II reverse transcriptase (Life Technologies). Quantitative real-time polymerase chain reaction (PCR) experiments were performed in duplicate using cDNA derived from 50 ng total RNA, 1.75 µM forward and reverse primer and Power SYBR-Green PCR Master Mix (Life Technologies) in a Rotorgene QPCR machine under the following amplification conditions: 95°C for 10 minutes, followed by 40 cycles of 95°C for 15 seconds and 60°C for 1 minute.

Primers:

SAP97-F (5'- CCAACAGAAGCTGTTCTTCCCTCTCCT-3');

SAP97-R (5'-TCATAATCTGCATCTGTGCCATTAACGTA-3');

18srRNA-F (AACCCGTTGAACCCCATT);

18srRNA-R (CGCTACTACCGATTGGATGG).

The correct amplicon sizes were confirmed using agarose gel electrophoresis against a low molecular weight DNA ladder (New England Biolabs). Comparable PCR efficiency of all primer pairs was validated by serial cDNA dilution: Comparative quantification of target gene expression was calculated using the threshold cycle (Ct) values of the target gene normalized against that of the endogenous reference 18srRNA and expressed as relative levels of gene expression derived from two different experiments performed in duplicate and annotated as mean fold difference compared to normal controls **(Table 1).** Expression levels of other genes within the duplication were not tested.

Clinical Findings

The proband **(Figure 1)** is a Caucasian male aged 15 years who after an uneventful birth showed developmental delay and learning difficulties at school. The assessment of intelligence using Weschler Intelligence Scale for Children (WISC) at the age of 8 years found him to be functioning at a moderately low level. He had spelling and writing difficulties and received a diagnosis of ADHD and later ASD at age 13. In addition to these features, he exhibited a number of motor and vocal tics from very early childhood. Motor tics over time included blinking, eye staring, facial twitch, nasal flare, facial grimacing, head nod forward, shoulder shrug, hand and arm tic, kicking, smelling, licking, kissing others, jaw protrusion, grinding of teeth, clicking the neck as well as a number of vocal tics including, grunting, throat clearing, barking, coughing and raspberries and high pitched shriek/scream sounds. He also reported obsessive compulsive behaviours (OCB) such as an urge to touch, forced touching, arithmomania (counting obsessions), arranging and ordering things in a particular way, as well as self-injurious behaviours, echopraxia, echolalia, and non-obscene socially inappropriate behaviours. The tics took a waxing and waning course with one type of tic being replaced by another, with the ability to voluntarily suppress the tics for brief periods of time although at the expense of mounting inner tension and a rebound effect, all of which are characteristic of GTS [12]. The mother (II-2) and maternal grandmother (I-1) displayed no remarkable facial features or behavioural deficit. The proband's older sister (III-2) aged 18 years presented with OCD, depression, anxiety, cleft palate and hearing loss - the latter 2 presentations being shared with her half-brother (III-1) from the maternal side who did not inherit the duplication or present with any behavioural deficit.

Results

CGH Analysis: A duplication was detected at chromosome 3q29 in the proband, his sister, mother and maternal grandmother **(Figure 1).** The coordinates of this duplication (Chr3: 197224783-198801470 (NCBI build 36) approximate those reported for the recurrent 1.6Mb duplication reported in 3q29 micro-duplication disorder and the reciprocal 1.6 Mb deletion most commonly reported in 3q29 micro-deletion syndrome

Table 1. *Family genotypes & psychiatric phenotypes.*

Maternal Relation	Pedigree#	Duplication	SAP97*	Phenotype
Grandfather	I-1	No	Normal	Normal
Grandmother	I-2	Yes	-1.7	Normal
Mother	I-2	Yes	-2.0	Normal
Male Proband	III-2	Yes	-1.5	ASD, GTS, ADHD, ID, OCD
Sister	III-3	Yes	-2.5	OCD, Depression, Anxiety
Half brother	III-3	Yes	Normal	Cleft Palate & Hearing Loss
Normal controls			± 1.25	

Legend: *Comparative RT-PCR of *SAP97* expression in familial blood samples annotated as mean fold difference relative to the mean for normal controls. Tourette syndrome (GTS), Autism Spectrum Disorder (ASD), Attention Deficit Hyperactivity Disorder (ADHD), Intellectual Disability (LD), Obsessive Compulsive Disorder (OCD).

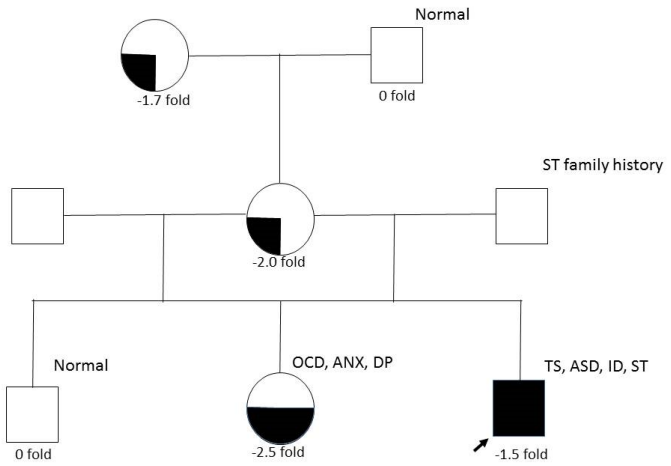

Figure 1. *Family Pedigree with Male Proband arrowed. Legend: ~3q29 duplication & reduced level of SAP97; ~ OCD/OCB; ~Autism Spectrum Disorder (ASD); ~Tourette syndrome (GTS); Anxiety (ANX); Depression (DP); Stickler syndrome (ST).*

[13-15]. The 1.6 Mb micro-duplication spaned at least 20 genes in order *TFRC, ZDHHC19, SLC51A, PCYT1A, TCTEX1D2, TM4SF19-AS1, TM4SF19, UBXN7, RNF168, C3orf43, WDR53, FBX045, NRR05, CEP10, PIGX, PAK2, SENP5, NCBP2, PIGZ, RP3AP30, MF12, MF12-AS1, SAP97/DLG1, DLG1-AS1* and *BDH1* where *SAP97/DLG1* overlaps the *DLG1-AS1* gene transcribed in the opposite direction. The 1.6Mb micro-duplication of 3q29 has not previously been associated with behavioural or psychiatric disorders [13-15]. By comparison, the reciprocal 1.6Mb deletion of 3q29 has been associated with learning problems, ASD, anxiety, depression, OCB and schizophrenia (SCZ) [13,15]. Given these inconsistencies in association we repeated the CNV analysis and confirmed the coordinates of the duplication. In addition, we tested expression levels of the gene thought most responsible for the behavioural and psychiatric deficits at this locus *synapse-associated protein 97 (SAP97)*.

SAP97 QPCR: Comparative QPCR analysis indicated that *SAP97* levels did not increase but rather decreased up to 2.5 fold in all family members tested with the duplication **(Table 1).** Family members without the duplication recorded *SAP97* levels indistinguishable from normal control levels. The proband had a 1.5 fold reduction in *SAP97* compared with normal control individuals and those unaffected male members of the family without the duplication **(Table 1).** The 3 female members of the family with the duplication had levels of *SAP97* that were lower than that of the proband **(Table 1).** The probands' sister had the lowest level of *SAP97* (-2.5 fold) as well as being the only female member of the family with a behavioural deficit and a psychiatric profile including depression, anxiety, obsessive compulsive disorder (OCD) and cleft palate **(Figure 1)**.

Discussion

This is the first report of ASD, GTS, OCD or ADHD associated with the recurrent 1.6Mb duplication of 3q29 [13-15]. Albeit, ASD has shown prior association with the reciprocal 1.6Mb deletion of 3q29 [13,16] which is wholly consistent with the finding in this study that the level of *SAP97* was reduced, not increased, in all members of the family with the duplication. Furthermore, *SAP97* showed prior linkage with GTS in a large Dutch pedigree using SNP D3S1311 localised within the *SAP97* gene [16,17]. In the Dutch pedigree both males and females presented with GTS with multiple motor tics, vocal tics, ADHD and obsessive compulsive symptoms and other family members with incomplete penetrance of either motor tics or vocal tics [17]. Tics are also common in ASD (~40%), moreover, GTS commonly associates with ADHD (~60%) and OCD (~50%) [4,10,12].

In the present study the probands' sister (Female III-2 in Figure 1) was the only female in the family to present with a psychiatric and behavioural profile including OCD, depression and anxiety but not ASD or GTS indicating incomplete penetrance. The probands' sister also had the lowest level of *SAP97* in the family. This correlation between *SAP97* dose and the severity of the phenotype in females thus suggested incomplete gender-limited SAP97 dose-dependent phenotypic penetrance **(Table 1)** [4,18-20]. Conversely, the proband had the smallest reduction in *SAP97* and the most severe phenotype with ASD, GTS and OCB suggesting *SAP97* dose-sensitive male bias with respect to ASD, GTS and OCB **(Figure 1).** These *SAP97* gender associated dose-deficiency correlations with psychiatric phenotypes are of particular interest given the high male preponderance of ASD and GTS and the recent finding that SAP97 levels within the synapse are maintained by DISC1-mediated stabilisation [21]. Moreover, *DISC1* is hemizygous deleted in ASD and GTS [22-25]. It is therefore possible that the *DISC1-LOH* pathway to ASD and GTS may be mediated, at least in part, through a reduction in *SAP97* [21]. However, notwithstanding these compelling dose associations between *SAP97*, ASD and GTS it is probable that genes other than *SAP97* either within the duplicated region of 3q29 and/or elsewhere in the genome moderate or even dictate the final clinical phenotype.

SAP97 is a post-synaptic signalling scaffold that binds trans-synaptically with NRXN4/CNTNAP2 and is thus a component of the NTSC - a recognised mutation hot spot for ASD and GTS [4]. In this context, the present study expands the number and nature of NTSC genes recurrently associated with both ASD and GTS to include *SAP97* [4,10] thus expanding the NTSC mutation hotspot in ASD and GTS to overlap the DISC1 pathway. SAP97 forms post-synaptic complexes with AMPA and NMDA-type glutamate receptors; negatively regulates the surface expression and activity of potassium channel KCNA1/Kv1.1; and positively regulates the surface expression and activity of the major glutamate transport channel EAAT2β [26-30]. Indeed, knock-down of *SAP97* increases extracellular glutamate concentrations [27] consistent with an increased excitatory signalling model for ASD and GTS [30,31]. Knock-down of SAP97 also increases the activity of the master kinase GSK3β [21] which has more known targets than any other

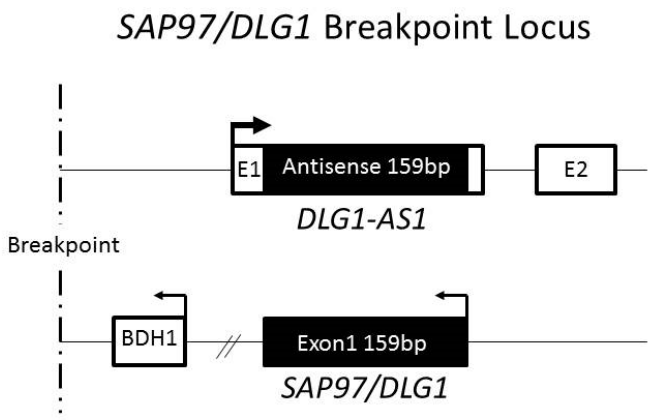

Figure 2. *Overlapping configuration of the SAP97/DLG1 gene with its antisense transcription unit DLG1-AS1 located at the penultimate position in the 1.6Mb 3q29 duplicated segment.*

kinase including the phospho-mediated down-regulation of creatine transporter SLC6A8 which facilitates ATP recycling wholly consistent with the recurrent LOH of SLC6A8 reported in ASD [32,33]. GSK3β also regulates mitochondrial motility and is actively translocated into the mitochondria where it regulates mitochondrial structure and function /ATP production.

This study demonstrates the merit of interrogating CNVs for candidate gene expression and more particularly duplications where the molecular consequences are more difficult to predict. However, the molecular basis of the ~1.9 mean fold reduction in *SAP97* in the affected family **(Table 1)** remains unresolved. Silencing of duplicated genes and transgenes is not novel but we are not aware of any similar report of a 'gene block duplication' silencing one of the genes located wholly within both the parent and duplicated block of genes. One possible explanation for this novel occurrence is that the duplication may have triggered a relative increase in the transcription of the overlapping *SAP97*-antisense transcription unit *SAP97-AS1 (DLG1-AS1)* located at the penultimate position within the duplication **(Figure 2).** An increase in *SAP97-AS1* transcription could potentially knock-down *SAP97* expression from the cis, trans and duplicated alleles. Questions also arise regarding whether *SAP97* dose deficiencies are sufficient in and of themselves to differentially regulate for one or the other gender-restricted psychiatric disorders associated with *SAP97* [22-25, 34]. Variants within one of the pathways downstream of SAP97 and/or DISC1 may determine how the primary genetic anomaly presents clinically. Indeed, some functions of SAP97 appear redundant to the function of other closely related SAP family members PSD95/SAP90/DLG4, PSD93 and SAP102 [35] where variations in their level of compensation may be a factor in the presentation of the final family phenotype.

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