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# Copy number variation in pediatric multiple sclerosis

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## Abstract

**Background—**Pediatric onset multiple sclerosis (MS) accounts for 2–4% of all MS. It is unknown whether the disease shares the same underlying pathophysiology found in adult patients or an extreme early onset phenotype triggered by distinct biological mechanisms. It has been hypothesized that copy number variations (CNVs) may result in extreme early onset diseases because CNVs can have major effects on many genes in large genomic regions.

**Objectives and methods—**The objective of the current research was to identify CNVs, with a specific focus on de novo CNVs, potentially causing early onset MS by competitively hybridizing 30 white non-Hispanic pediatric MS patients with each of their parents via comparative genomic hybridization (CGH) analysis on the Agilent 1M CGH array.

**Results and discussion—**We identified 10 CNVs not overlapping with any CNV regions currently reported in the Database of Genomic Variants (DGV). Fifty-five putatively de novo CNVs were also identified: all but one common in the DGV. We found the single rare CNV was a private variation harboring the SACS gene. SACS mutations cause autosomal-recessive spastic ataxia of Charlevoix-Saguenay (ARSACS) disease. Additional clinical review revealed that the patient with the SACS gene CNV shared some features of both MS and ARSACS.

**Conclusions—**This is the first reported study analyzing pediatric MS CNVs. While not yielding causal variation in our initial pediatric dataset, our approach confirmed diagnosis of an ARSACS-like disease in addition to MS in the affected individual, which led to a more complete understanding of the patient's disease course and prognosis.

### Keywords

Multiple sclerosis; copy number variation; pediatric



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**Conflicts of interest**

The authors declare that there are no conflicts of interest.

## Introduction

Multiple sclerosis (MS) is a chronic central nervous system disease characterized by dysregulated inflammation, myelin loss, and axonal pathology. The modest, albeit well documented heritability of MS suggests complex gene-environment interactions contributing to risk.[1–3] In Europeans and their descendants, MS is a common cause of neurological disability in young adults but onset before the age of 18 years accounts for only a modest 2–4% of all MS patients.[4] This relatively rare pediatric form of the disease typically progresses more slowly than in adults, although it often causes permanent disability at a younger age. The earlier the age of onset, the more evenly distributed the disease is between males and females, with the disease becoming more prevalent in females as age of onset increases.[5] Additionally, the ethnic distribution of prevalence in pediatric MS appears to be different to that of adult onset MS: that is, there is a higher relative frequency of non-white individuals with pediatric MS compared to adult onset MS.[4] Although it is not known for certain if the disease shares the same underlying pathophysiology as adult MS, pediatric MS may represent an extreme early onset phenotype of the disease.

Multiple genome-wide association screens in MS have been completed in the past five years, lengthening the list of risk loci and supporting the long-held view that MS susceptibility results from multiple common allelic variants.[6] However, copy number variations (CNVs)[7] associated with MS have not been reported. These variations can be inherited or occur de novo. CNVs can have an effect on gene dosage, allele dosage, and conformation of genomic regions. CNV associations have been identified for several complex diseases,[8] and large-scale CNV alterations of the genome can also result in highly penetrant early onset disease phenotypes.[9] Our objective was to identify CNVs, with a specific focus on de novo CNVs, potentially causing early onset MS.

## Materials and methods

### Subjects

Genomic DNA from 30 non-Hispanic white pediatric MS patients (age of onset≤14 years) and their parents were assayed with the Agilent SurePrint G3 Human 1M comparative genomic hybridization (CGH) array (Agilent Technologies Inc.). Each patient was competitively hybridized to an array with each parent separately, resulting in 60 total assays. Subjects for this study were recruited at the specialized pediatric MS centers of Stony Brook University Medical Center and the University of California San Francisco (UCSF) Medical Center. Patients met the International pediatric MS diagnostic criteria.[10] Although the intention was to select only sporadic pediatric MS patients, a single female parent in this dataset was later identified to be diagnosed with MS, and another female parent was diagnosed with clinically isolated syndrome. All participants gave written informed consent. The study was approved by the Stony Brook and UCSF institutional review boards.

### Assay

Before hybridization to arrays, samples of genomic DNA were labeled with the Agilent Enzymatic labeling kit, hybridized to the Agilent SurePrint G3 Human 1M CGH, and

scanned using the Agilent Microarray Scanner, all according to manufacturer's instructions. Array images were analyzed using Feature Extraction v10.5 (Agilent Technologies, Inc.), and the resulting files were imported into Nexus software (Biodiscovery, Inc.) for analysis.

Nine samples were rerun on the array due to a poor initial sample quality/amount. Also included in the rerun were samples to measure the quality and consistency of the assays. We chose to rerun the highest quality array from the original dataset to test repeatability of CNV calls. Moreover, we ran an additional control array by self-hybridizing a sample to get an estimate of false positive rates under these assay conditions and this particular design. Examination of this control array, under the same CNV calling parameters, revealed no called CNVs. These results indicated that the CNV calling settings were stringent enough to minimize our false positive rate. The replicated best quality sample control resulted in 14 total called CNVs. Two of these CNVs were only called in one of the replicates (one in each replicate). However, both of these discordant CNVs (both deletions) appear to have been a result of false negatives in the sample without the call, rather than false positives in the sample with the CNV called (See Supplementary Material, Figure 1 online). That is, in both instances the log R ratios of the sample without the deletion was trending toward a deletion.

Based on these two arrays, the settings to identify CNVs in all arrays were: the rank segmentation algorithm,[11,12] significance threshold for identifying different segments=$5.0\times10^{-9}$, maximum contiguous probe spacing=1000 Kbp, minimum number of probes per segment=8, high gain=1.1, gain=0.4, loss=−0.4, big loss=−1.1, 3:1 sex chromosome gain 1.2, 4:1 sex chromosome gain 1.7, and percent outliers to remove=3.

## Analysis

Nexus Copy Number (Biodiscovery, Inc.) was used for the identification of CNVs. The quality of each array was assessed by two methods. First, arrays with a quality score greater than 0.2 (as per suggestion of the Nexus Copy Number User Manual: $n$=6) were removed. Additionally, arrays with an unusually high number of CNV calls ($n$=3) were removed. These nine arrays were performed again, and all reruns met the two quality standards.

Two criteria were used to determine de novo copy number events. First, a detected CNV in an individual had to be identified when compared to both parents (i.e. each child-parent array had to give evidence of a duplication or deletion in the same location). However, both parents could be heterozygous for common CNVs and a homozygous child would then appear to be de novo copy number variant when compared to both parents even though the CNV is inherited. As a secondary criterion, these CNVs had to be absent or rare in the Database of Genomic Variants (DGV).[13] The second criterion insures that the CNV is rare in the general population, therefore making it unlikely that both parents are heterozygous for the CNV. All genetic positions reported herein correspond to the Human National Center for Biotechnology Information (NCBI) Build 36.1 genomic map.

It should be noted that all discussion of the classes of these CNVs (duplication or deletion) is with reference to the parental genomes to which the individual is compared. For example, an apparent duplication can occur when a child has three copies and is compared to a parent with two copies, or a child has two copies and is compared to a parent with a deletion. All

descriptions of CNVs herein describe the CNV state of the child as compared to one or both parents, not necessarily describing the absolute copy number.

### Conventional cytogenetic analysis

Metaphase chromosomes from peripheral blood cultures were prepared and banded (G-banding) following standard protocols.[14] The chromosomes were analyzed under a microscope and karyotyped using the CytoVision system (Applied Imaging).

### Fluorescence in situ hybridization (FISH)

Bacterial Artificial Chromosome (BAC) probes RP11-88F2 (chr13:22,746,386–22,936,999), RP11-357N10 (chr13: 22,335,638–22,522,444) and RP11-768O2 (chr13: 23,626,965–23,808,779) that are located in the duplicated region were selected using University of California, Santa Cruz (UCSC) genome browser (http://genome.ucsc.edu) and were purchased from EmpireGenomics. The probes RP11-88F2 and RP11-357N10 were labeled with fluorescein (green) and RP11-768O2 was labeled with ROX (red). FISH slides were pretreated with 2×SSC for two minutes at 73°C, 0.5 mg/ml pepsin (Sigma) in 0.01N HCl for five minutes at 37°C, and 10% phosphate buffered formalin (FisherScientific) for five minutes at room temperature (RT), followed by sequential dehydration in 70%, 85%, and 100% ethanol for one minute each at RT. The labeled probes were added on the FISH slides and co-denatured with the slides for five minutes at 75°C, followed by overnight hybridization at 37°C. After hybridization, the slides were washed in 0.4×SSC/0.3% NP40 for two minutes at 73°C and 2×SSC/0.1% NP40 for one minute at RT. The slides were then counterstained with DAPI II (Abbott Molecular) and the signals were analyzed using the CytoVision system (Applied Imaging).

## Results

Thirty pediatric MS patients were analyzed for de novo CNVs that could be associated with the disease. Twenty-one of the patients were female, and the average age of onset was 11.13 years. Twenty-four individuals had a relapsing–remitting disease course, and the disease course was unclear for six individuals. Using the single-nucleotide polymorphism (SNP) rs3135388 as a proxy for being HLA-DRB1*15:01 (human leukocyte antigen) positive, 13/28 affected individuals (46.42%) were found to be carriers of DRB1*15:01. The MS genetic burden (MSGB) has been proposed as a useful tool to summarize genetic load.[15] MSGB values were available for 27 out of the 30 pediatric patients. The MSGB values for the pediatric patients were compared to reference healthy adult white controls ($n$=566), adult MS patients with no family history of MS ($n$=1225), adult MS patients with family history of MS ($n$=791), and MS patients with no family history and an age of onset greater than two standard deviations above the adult onset mean ($n$=29; Supplementary Material, Figure 1 online). Based on a non-parametric two-tailed Wilcoxon rank-sum test, the pediatric patients had a significantly ($p$<0.05) higher MSGB values than controls but were not significantly different from the three groups of MS patients, indicating that the study dataset is genetically indistinguishable from other well-characterized adult MS datasets.

Across the 60 arrays (each pediatric patient hybridized with his/her parents separately), a total of 807 copy number variations were identified (Figure 1). The total number of CNVs per array ranged from 7–20 (Figure 2(A)). Minimum detected CNV length was 22.5 kb, and the maximum CNV length was 2 Mb (Figure 2(B)). Ten total CNVs were identified that did not overlap with any CNV regions in the DGV (Table 1). Two of these CNVs were deletions, eight were duplications, and the length of these CNVs ranged from 22.5 kb to 131 kb. Genes in these regions include RAB19, MKRN1, PSAT1, MGAT5, LOC151162, TMEM163, USP31, GMDS, FAM150A, MCC, GPHN, and FAM155A. None of these CNVs were identified in more than one individual (i.e. they were found to be private) or in an individual vs both parents (i.e. not de novo).

### Putative de novo CNVs

A total of 55 CNVs were identified that differed between a patient and both parents and were the same type of event (duplication or deletion), totaling 21 unique putatively de novo CNV regions (Supplementary Material, Table 2 online). All but one of these regions had a relatively high frequency in the DGV, suggesting that they may actually have been inherited rather than de novo (i.e. under our assay conditions, both parents were likely heterozygous for these CNVs). Parents were more likely to both be heterozygous for a CNV that is found in high frequency in the general population, and the patient can appear to be similarly different in copy number from both (duplication or deletion vs both parents) if both parents are heterozygous for the CNV. A 1.4 Mb duplication on chromosome 13 in a single affected individual overlaps a region in the DGV with very infrequent CNV calls (four CNVs out of 2137 individuals studied: Figure 3), which may be compatible with a de novo event. This region harbors nine genes: SGCG, SACS, TNFRSF19, MIPEP, PCOTH, C1QTNF9B, MIR2276, SPATA13, and C1QTNF9.

### Validation of de novo chromosome 13 duplication

Cytogenetic analysis of this individual revealed an apparently normal female karyotype and the duplication could not be detected by conventional chromosome banding analysis due to limited resolution (Supplementary Material, Figure 3 online). To validate the findings, we performed interphase FISH on the patient, both parents, and five normal controls. Two hundred cells were examined for each individual in the FISH study. Gain of a signal in the duplicated region was detected in 72% of the cells of the patient. However, this gain was not seen in both parents and the controls (patient and parents: Figure 4). These results clearly confirmed the de novo duplication in the patient. With two probes located at each end of the duplicated region, it was further demonstrated that the duplicated region appears to be a direct duplication (Figure 5).

### Case clinical features

As one individual harbored a CNV that includes the SACS gene for autosomal recessive spastic ataxia of Charlevoix-Saguenay (ARSACS), her clinical history and diagnostic studies were further evaluated for ARSACS (OMIM 270550). This patient was heterozygous for DRB1*15:01 (extrapolated from rs3135388 SNP genotype), and was risk allele positive for 13 of the 17 well established MS risk SNPs.[15]

The patient had gradual onset of right hand tremor, clumsiness, and mild balance problems from the age of 13 years onward. The past history of the patient was notable for mild learning difficulty and dyslexia. At 15 years she developed a transient sensory relapse which improved following intravenous steroids. Magnetic resonance imaging (MRI) showed lesions that met the 2001 International Panel Criteria for MS[10] in that the lesions exclusively involved the white matter and had a distribution that included the periventricular, infratentorial, juxtacortical, and subcortical locations. At the time of her MRI, neurological examination showed mild cerebellar signs and areflexia. Electromyography and nerve conduction studies were normal. Initially, there was progression on the MRI but in the past three years on disease-modifying therapy she has been radiologically and clinically stable. Taken together, the patient had some features of ARSACS (a history of progressive symptoms and neuropathy) and clear features of MS (history of a relapse and MRI and cerebrospinal fluid (CSF) findings). The conclusion is that the patient has both disorders.

## Discussion

Given the fact that a single CNV can influence many genes and disrupt the whole genomic region through structural changes, it is possible that a de novo CNV could result in an extreme phenotype such as early onset of a given disease. Following this hypothesis, the current study searched for de novo CNVs in 30 pediatric MS patients. This study is the first reported CNV analysis in pediatric MS patients. A single de novo CNV on chromosome 13 was identified and validated. The chromosome 13 duplicated region harbors nine genes. Although a role for several of the genes in MS susceptibility could be hypothesized, the SACS gene was of most interest because of its role in another neurodegenerative disease. The SACS gene encodes the sacsin protein, and mutations in this gene cause ARSACS disease.

Patients with ARSACS disease may present with progressive ataxia, dysarthria, spasticity, distal muscle wasting, neuropathy, and nystagmus. Most of these symptoms are also common in MS. However, a progressive course is extremely rare in pediatric MS[5] and areflexia is uncharacteristic of MS. Based on the SACS CNV, and additional testing, it was determined that this patient, who had previously been diagnosed only with MS, suffers from both MS and an ARSACS-like disease.

Many other CNVs of putative interest to MS susceptibility were identified. These regions fall into two classes: CNVs not reported in the DGV and putative de novo CNVs (i.e. CNVs that were identified in an individual when compared to both parents). In addition to the SACS gene region, 20 other putative de novo CNV regions were identified. Many of these regions contain paralogous genes, which is not surprising since gene families tend overlap with segmental duplications,[16] and segmental duplications predispose the regions to duplication or deletion.[17] Since CNVs in these regions were at a relatively high frequency in the DGV, it is likely that most of these were inherited from heterozygous parents rather than de novo: however, it is possible that some of these regions harbor genes affecting MS susceptibility. A putatively de novo deletion was observed in two patients on chromosome 17 that harbors the N-ethylmaleimide-sensitive factor (NSF) gene. NSF is present in neuronal intranuclear inclusion bodies in neuronal intranuclear inclusion disease,[18] a type of

progressive ataxia, and NSF is involved in the regulation of $GABA_B$ receptor signaling.[19] Additionally, a deletion was observed in the HLA region of a patient when compared to both parents, which included the HCP5P10, HLA-H, HCG2P7, HCG4P6, and HLA-A genes. This CNV is in the major histocompatibility complex (MHC) class I region, and variants in this region have previously been shown to be negatively associated with MS.[20–24]

While only a single validated de novo CNV was identified, several inherited CNVs were identified that did not overlap with those in the DGV. Although none of these regions were de novo (only identified vs a single parent), they may be interesting in regards to disease because of their rarity in the general population. Makorin ring finger protein 1 gene (MKRN1), which was in a duplicated region, is highly expressed during embryonic brain development in the mouse,[25] and it is therefore plausible that the gene could be involved in brain development abnormalities that lead to MS susceptibility. Mutations in phosphoserine aminotransferase 1 (PSAT1; single copy deletion in a patient) have been associated with abnormally low levels of PSAT resulting in several neurological symptoms including seizures, hypertonia, and psychomotor retardation.[26] The gephyrin gene (GPHN), which is located in a novel duplicated region, is a neuronal organizational protein that has been associated with hyperekplexia (OMIM 149400), a neurological disorder characterized by stiffness immediately after birth and an excessive startle reflex.[27] Finally, the mannosyl (alpha-1,6-)-glycoprotein beta-1,6-N-acetyl-glucosaminyltransferase gene (MGAT5) which lies in a duplicated region in a patient, has previously been associated with MS severity.[28–31] All of these putative loci warrant follow-up screening in an adequately powered dataset.

In summary, the objective of the present study was to identify de novo CNVs associated with pediatric onset MS. Several CNVs were identified that were not present in the DGV: however, all of these CNVs appeared to be inherited. Additionally, several putative de novo CNVs were identified. CNVs in all but one of these regions were reported with relatively high frequency in the DGV, making it unlikely that variants in these regions could be causative for MS. Finally, a de novo duplication was identified and validated in a single patient. Part of this CNV covered a region that was reported as variant with a very low frequency in the DGV. The SACS gene (with mutations which are responsible for ARSACS disease) is in this region, and the patient had clinical features consistent with both MS and an ARSACS-like disease. Although no definitive MS causative CNVs were identified, the present study resulted in the diagnosis of a disease, thus facilitating a better understanding of the patient's prognosis.

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were seen at the National Pediatric MS Center at Stony Brook University Hospital or the UCSF Regional Pediatric MS Center.

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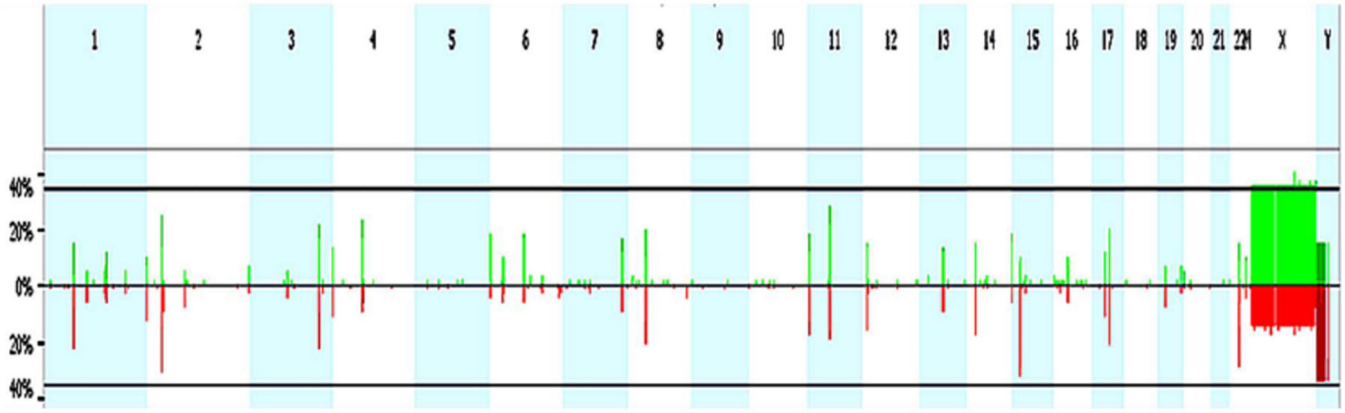



**Figure 1. Genomic distribution and frequencies of copy number variations (CNVs) called in 30 pediatric multiple sclerosis (MS) trios**

Each patient has two assays, one vs each parent. Green bars above the line indicate duplications, and red bars below the lines indicate deletions. The size of the bars indicates the frequencies of the CNVs in the population (y-axis). Chromosome is indicated across the top of the figure.

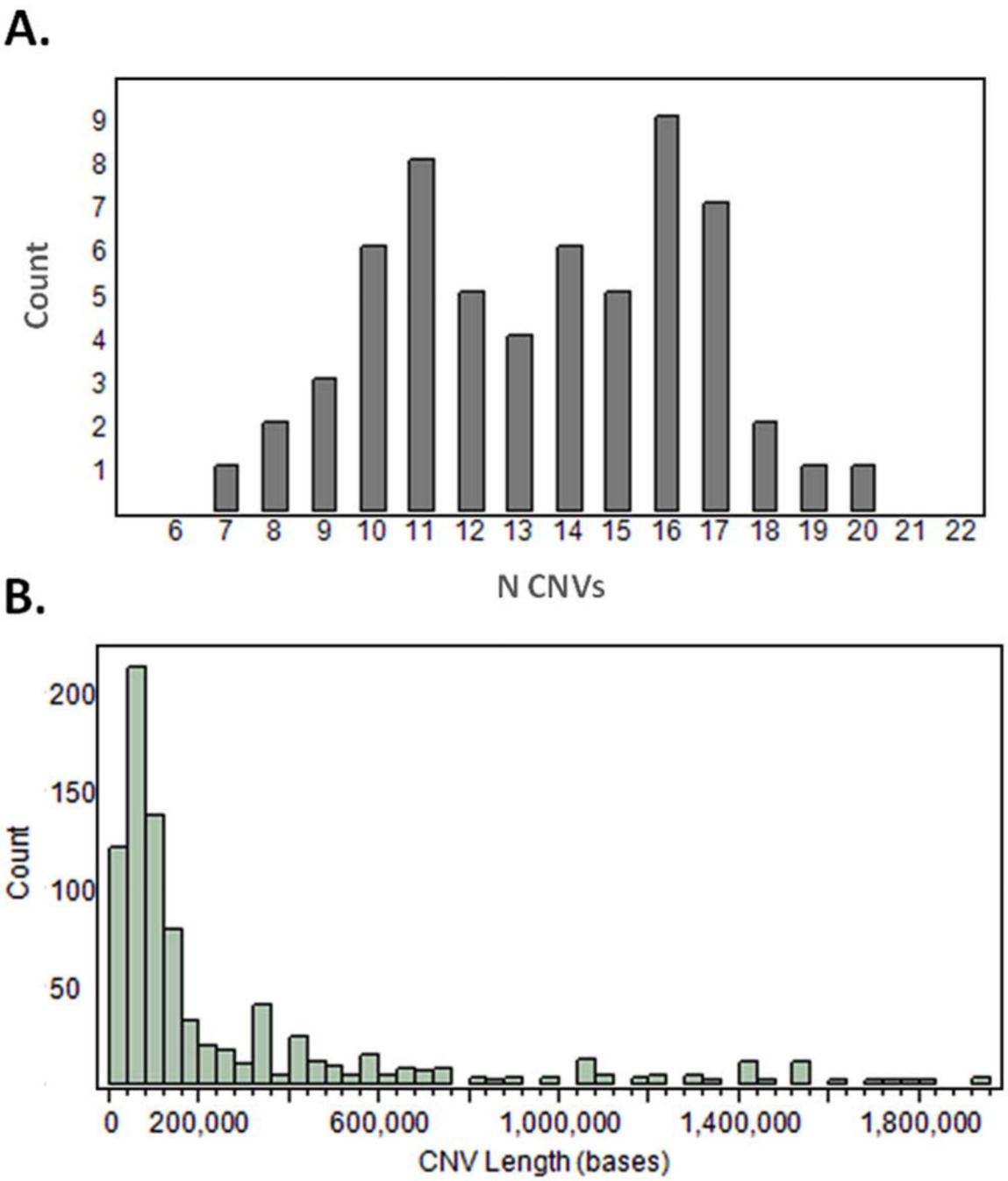



**Figure 2. Distribution of number of copy number variations (CNVs) per array and CNV lengths**

(A) X-axis is number of CNVs per array, y-axis is the number of assays having a given number of CNVs; (B) x-axis is CNV length, y-axis is the number of assays having a given CNV length.

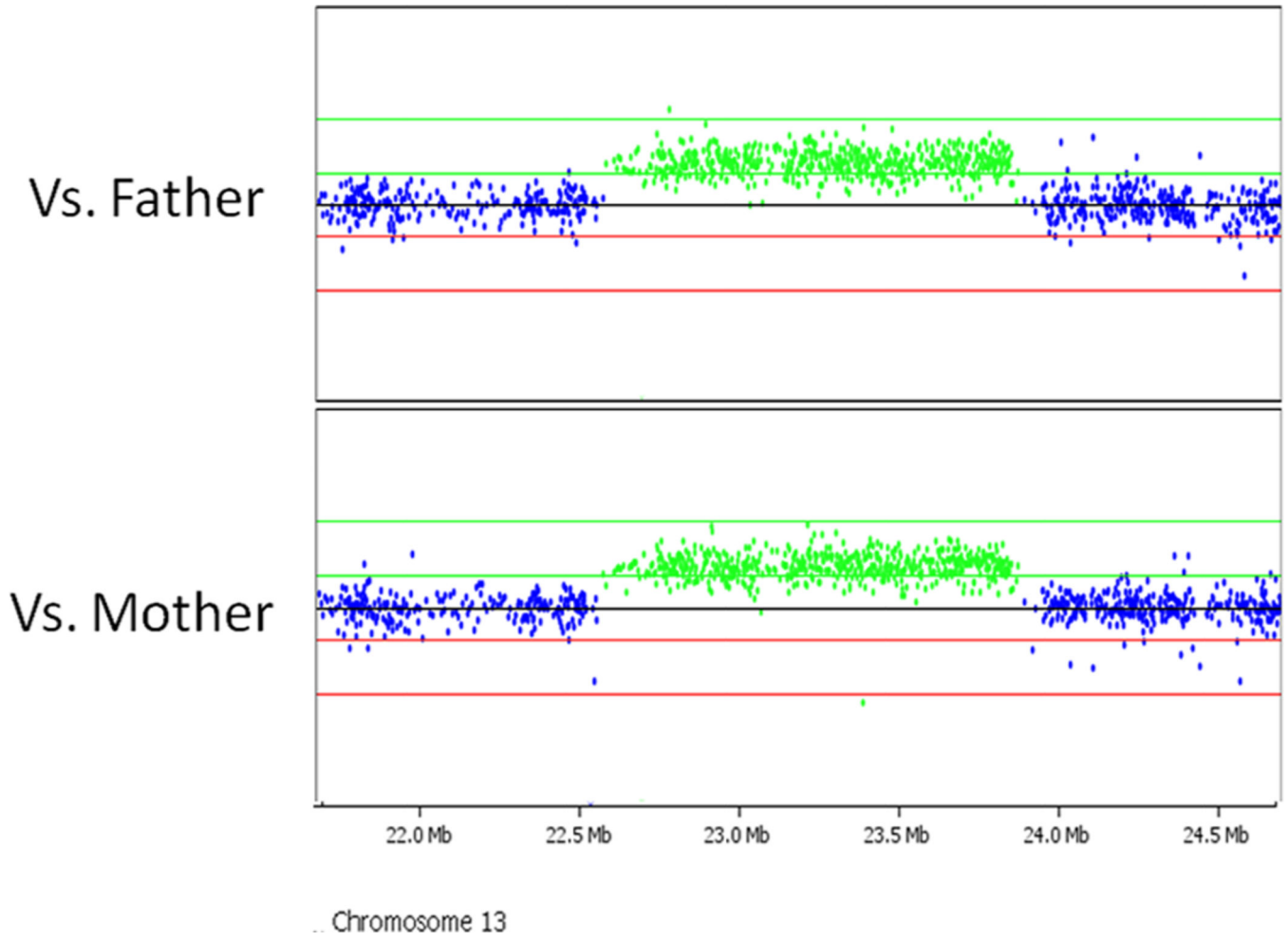



**Figure 3. De novo chromosome 13 duplication contains nine genes including SACS**

Duplication in a patient that includes SACS gene. The duplication is apparent vs both parents. The y-axis gives the log *R* ratio. Dots are log *R* ratios for individual probes. Green lines are cutoffs for three copies and >three copies, red lines are cutoffs for one copy and zero copies. (Top panel) Father reference; (Bottom panel) Mother reference.

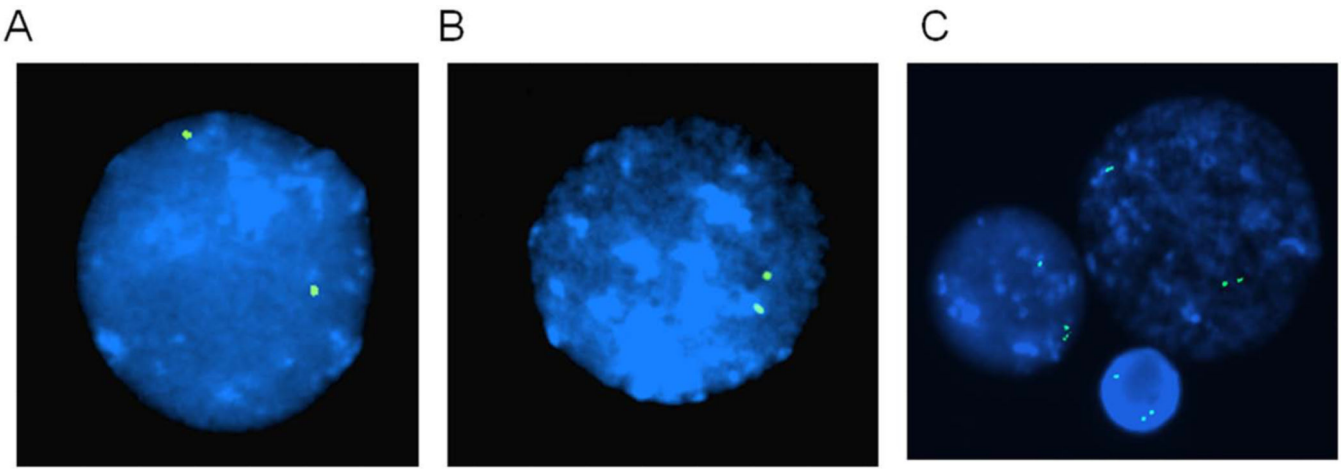
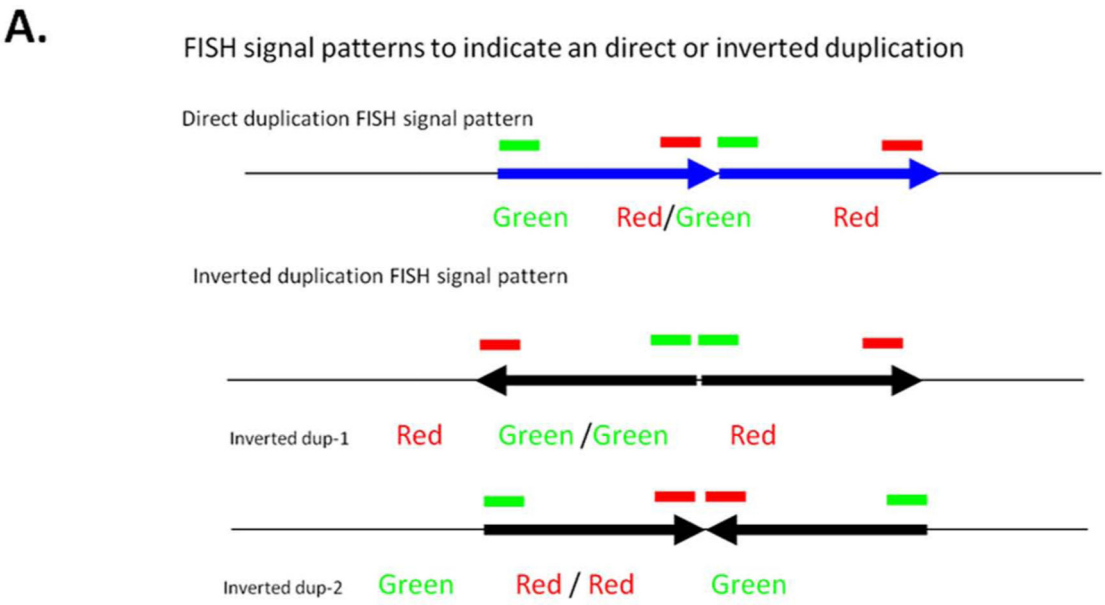



**Figure 4. Fluorescence in situ hybridization (FISH) confirms that duplication of the region containing SACS gene is de novo**

(A) Mother with two copies of region; (B) Father with two copies of region; (C) Patient with three copies of the region resulting from a duplication of the probe region on one chromosome 13 homologue. Probe, BAC RP11-88F2 (green).



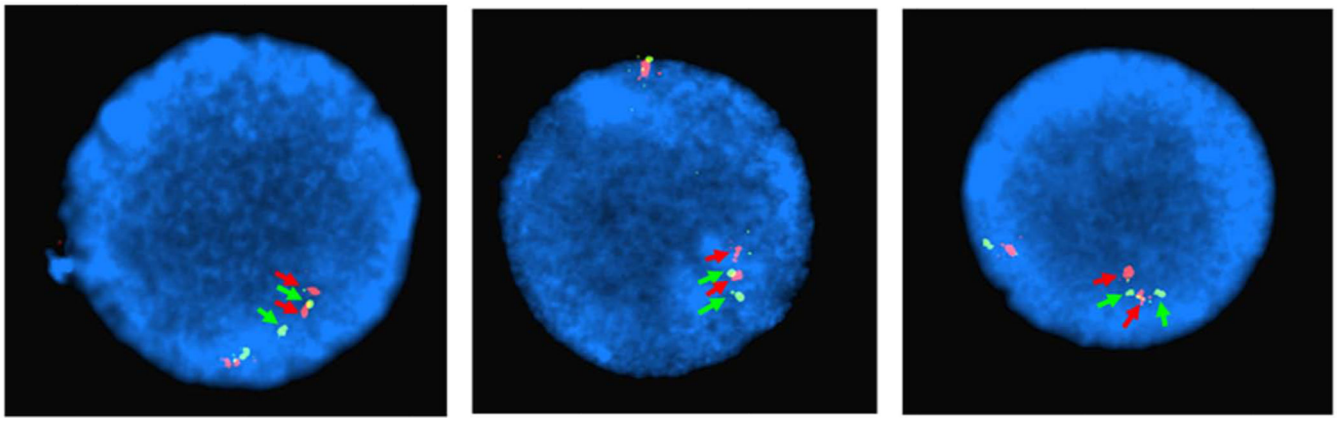




**Figure 5. Fluorescence in situ hybridization (FISH) of three interphase cells of the patient reveals no inversion**

FISH using two probes at each end of the duplication. (A) FISH signal pattern to indicate a direct or inverted duplication; (B) FISH of three interphase cells in the patient. The order of the probes (red-green-red-green) indicates a non inverted duplication.

**Table 1**

Identified CNVs having no overlap with the Database of Genomic Variants.

| Chromosome Region | Event | Length (bps) | Cytoband | N Probes | Genes in Region |
|---|---|---|---|---|---|
| chr7:139,762,267–139,810,927 | Duplication | 48661 | q34 | 25 | RAB19, MKRN1 |
| chrX:36,476,805–36,517,179 | Deletion | 40375 | p21.1 | 18 | No Genes |
| chr9:80,101,965–80,124,770 | Deletion | 22806 | q21.2 | 14 | PSAT1 |
| chr2:134,904,980–134,945,289 | Duplication | 40310 | q21.3 | 24 | MGAT5, LOC151162, TMEM163 |
| chr16:22,978,311–23,025,826 | Duplication | 47516 | p12.1 | 23 | USP31 |
| chr6:2,173,516–2,208,248 | Duplication | 34733 | p25.3 | 16 | GMDS |
| chr8:53,550,004–53,681,430 | Duplication | 131427 | q11.23 | 42 | FAM150A |
| chr5:112,584,877–112,626,468 | Duplication | 41592 | q22.2 | 21 | MCC |
| chr14:66,120,972–66,189,040 | Duplication | 68069 | q23.3 | 25 | GPHN |
| chr13:106,978,755–107,001,243 | Duplication | 22489 | q33.3 | 25 | FAM155A |