

An unusual cause for Coffin-Lowry syndrome: three brothers with a novel microduplication in *RPS6KA3*

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Running title

Novel duplication in *RPS6KA3* causing Coffin-Lowry syndrome

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ABSTRACT

Coffin-Lowry syndrome (CLS) is a rare X-linked disorder characterized by moderate to severe intellectual disability, hypotonia, craniofacial features, tapering digits, short stature, and skeletal deformities. Using whole exome sequencing and high-resolution targeted comparative genomic hybridization array analysis, we identified a novel microduplication encompassing exons five through nine of *RPS6KA3* in three full brothers. Each brother presented with intellectual disability and clinical and radiographic features consistent with CLS. q-RT-PCR analyses performed on mRNA from the peripheral blood of the three siblings revealed a marked reduction of *RPS6KA3* levels suggesting a loss-of-function mechanism. PCR analysis of the patients' cDNA detected a band greater than expected for an exon 4 - 10 amplicon, suggesting this was likely a direct duplication that lies between exons 4 through 10. This microduplication is only the third intragenic duplication of *RPS6KA3* and the second and smallest reported to date thought to cause CLS. Our study further supports the clinical utility of methods such as next-generation sequencing and high-resolution genomic arrays to detect small intragenic duplications. These methods, coupled with expression studies and cDNA structural analysis have the capacity to confirm the diagnosis of CLS in these rare cases.

KEYWORDS

Coffin-Lowry syndrome, intragenic duplication, microduplication, X-linked intellectual disability, *RPS6KA3*

INTRODUCTION

In 1966, the first identified patient to have Coffin-Lowry syndrome (CLS) was described by Coffin, Siris & Wegienka, and then independently described again by Lowry, Miller, & Fraser in 1971 (Coffin et al., 1966; Lowry et al., 1971). The condition is a rare, X-linked inherited disorder that is characterized by dysmorphic features and intellectual disability in males, with mild expression in some females (Pereira et al., 2010). Characteristic facial features include prominent forehead and ears, downward slanting palpebral fissures, ocular hypertelorism, broad based nose with anteverted nares, thick nasal septum, wide mouth, full lips, and dental anomalies (Delaunoy et al., 2001; Pereira et al., 2010; Rogers & Abidi, 1993; Rojnueangnit et al., 2014). These facial features are typically mild during infancy and become more pronounced in late childhood and adolescence (Delaunoy et al., 2001). Other features include behavioral problems, growth restrictions, and various skeletal anomalies including progressive childhood-onset kyphoscoliosis, pectus anomalies, narrow pelvis, tapered fingers, and metacarpal pseudoepiphyses (Pereira et al., 2010; Rogers & Abidi, 1993). In addition, there can be neuropsychiatric concerns including progressive spasticity, sleep apnea, loss of strength, and stimulus-induced drop attacks, which can be triggered by multiple events including unexpected tactile or auditory stimuli, or excitement (Rogers & Abidi, 1993).

Pathogenic mutations in the gene *RPS6KA3* (MIM:300075), located at Xp22.12, cause Coffin-Lowry syndrome (MIM:303600) (Pereira et al., 2010). *RPS6KA3* is composed of 22 exons and encodes a protein of 740 amino acids (Delaunoy et al., 2001; Jacquot et al., 1998; Pereira et al., 2010). The protein, in turn, functions as a growth-factor regulated protein kinase, ribosomal S6 kinase 2 (RSK2). Related to RSK2 are three other proteins, RSK1, RSK3, and RSK4; all of which

are from four different genes (Delaunoy et al., 2001). This family of genes is part of the Ras-MAPK signaling pathway (Delaunoy et al., 2001; Pereira et al., 2010; Wang et al., 2006). There is a broad range of cellular signals that activate these proteins including insulin, growth factors, neurotransmitters, and UV-irradiation. Once activated, the proteins are involved in cellular proliferation, differentiation, and apoptosis (Delaunoy et al., 2001; Wang et al., 2006). *RPS6KA3* expression is seen at its highest level in regions of the brain that have high synaptic activity (Rojnueangnit et al., 2014).

The types of mutations in *RPS6KA3* vary and either lead to loss of phosphotransferase activity of the protein or a premature termination of translation (Delaunoy et al., 2006). The most commonly reported pathogenic variants include missense mutations, nonsense mutations, splicing errors, or small deletion or insertion events leading to frameshift. There have been a few reported intragenic deletions and a few multiple gene duplications that include *RPS6KA3* (Pereira et al., 2010). No genotype/phenotype correlations have been established for CLS (Wang et al., 2006).

We describe three brothers with the clinical and radiographic findings of CLS, each of whom possess a duplication of approximately 7 kb encompassing exons 5 through 9 in *RPS6KA3*. This is the third intragenic duplication to be described in *RPS6KA3* and the second as causative for CLS. The duplication reaffirms that intragenic rearrangement, not detectable by common exon screening, can cause CLS. Therefore, in cases where suspected CLS is not confirmed by traditional methods, additional testing for intragenic duplications in *RPS6KA3* should be considered.

FAMILY A

Individuals III-1, III-2, and III-3 (Figure 1A) are brothers with similar clinical and radiographic features that are typical for CLS, and each possess the same duplication of exons 5 through 9 in *RPS6KA3*. They are the only children of non-consanguineous Caucasian parents. The patients' mother, II-4, has a history of developmental delay and mild intellectual disability that required special education. She also has unilateral deafness and mental health issues, including suspected bipolar disorder and depression. The patients' father, II-3, has an early-stage macular degeneration but no intellectual disability. A maternal half-uncle, II-8, has autism spectrum disorder, attention-deficit/hyperactivity disorder (ADHD), learning disabilities, and complete deafness. However, he is not thought to have CLS, and the etiology of his problems is unknown. There also are three other maternal uncles, II-5, II-6, II-7, with no intellectual disability or learning difficulties.

Individual III-1

Individual III-1 (Figure 1B) is a 12-year-old male, born full-term, after an uncomplicated pregnancy. The biological mother smoked tobacco during the pregnancy but reportedly did not use other substances. At birth he had a submucous cleft palate, laryngomalacia, and vesicoureteral reflux and subsequently experienced aspiration. Shortly after birth, in 2006, a chromosome analysis at band level G636 determined his karyotype to be 46,XY. At age 4 years, he started having febrile seizure during sleep, which did not reoccur after being treated with topiramate (Topamax). At 5, he was first evaluated by clinical genetics because of global developmental delay, marked speech and language delay, short stature, microcephaly, and dysmorphic features. At that time, in 2011, a comparative genomic hybridization (CGH) array using the SignatureChipOS v2.0 3-plex array which included 134811 oligonucleotide probes,

was normal with no significant copy number changes detected. Prader-Willi syndrome methylation studies along with 22q11.2 deletion syndrome FISH, Prader-Willi syndrome FISH, and subtelomere FISH studies were normal. On physical examination, he had short palpebral fissures, ptosis, hypodontia, bifid uvula, supernumerary nipples, pectus carinatum, and broad tapered fingers (Figure 1B and Table 1). A hearing evaluation identified a possible conductive hearing loss. A spinal radiograph indicated moderate neuromuscular scoliosis, which has required bracing. A spinal MRI revealed thoracolumbar spinal cord syrinx extending from T6-T12, two small annular fissures at C5-C6 and C6-C7, and a small central disc protrusion at C6-C7. There was no significant spinal canal stenoses or other cord abnormalities. A sleep study revealed mild obstructive sleep apnea. At age 11 years, his height was 132.8 cm (-2.2 SD). At age 12 years, his height was 149.0 cm (19 percentile), weight was 38.40 kg (18 percentile), and head circumference was 51.0 cm (3 percentile). He still does not have any speech. Cognitively, he is more severely affected than his brothers.

Individual III-2

Individual III-2 (Figure 1C) is an 11-year-old male, born full term, after an uncomplicated pregnancy. He was first evaluated by clinical genetics at the age of 9 because of his brother's history and his history of developmental delay, short stature, and dysmorphic features. A physical examination revealed downward slanting palpebral fissures, telecanthus, widely-spaced teeth, hypodontia, eversion of the lower lip, pectus carinatum, supernumerary nipples, inverted nipples, and broad tapered fingers (Figure 1C and Table 1). He also has experienced some exercise intolerance. A hearing evaluation showed normal hearing in both ears, and an ophthalmology exam found pseudo-esotropia. A spinal radiograph revealed minimal

levoscoliosis. At age 8 years, his height was 117.20 cm (-2.4 SD). At age 11 years, his height was 137.5 cm (10 percentile), weight was 41.70 kg (65 percentile), and head circumference was 56.0 cm (97 percentile). His intellectual disability is less severe than his older brother.

Individual III-3

Individual III-3 (Figure 1D) is a 9-year-old male, born at 37 weeks gestation after a pregnancy complicated by controlled gestational diabetes and intrauterine growth restriction. He was small for gestational age at birth and subsequently developed stridor. He was evaluated by clinical genetics at the age of 5 years because of his brothers' and his history of developmental delay, short stature, failure to thrive, and dysmorphic features. On physical examination, he had short palpebral fissures, ptosis, prominent lips, hypodontia, prominent pectus carinatum, supernumerary nipple, and broad tapered fingers (Figure 1D and Table S1). A hearing evaluation was normal. A spinal radiograph revealed moderate neuromuscular scoliosis, which also has required bracing. He also has a history of stimulus-induced drop attacks. At age 9 years, his height is 121.0 cm (-2.4 SD), weight is 23.6 kg (-1.6 SD), and head circumference is 52.5 cm (50 percentile). Developmentally, he is able to speak in short sentences and can hold simple conversations. Cognitively, he is more advanced than his two older brothers.

METHODS AND MOLECULAR FINDINGS

In 2011, Individual III-1 underwent a CGH array, using the SignatureChipOS v2.0 3-plex array including 134811 oligonucleotide probes, which was normal. In 2015, Individual III-1 had *RPS6KA3* sanger sequencing of exons 1 through 22 which was negative. In 2016, an X-linked intellectual disability next-generation sequencing panel with 114 genes also was negative for a causative variant. In 2017, whole exome sequencing of the same patient was performed at the

Greenwood Genetic Center, using the Agilent SureSelect^{XT} Clinical Research Exome Kit, on a research basis and an in-house algorithm used to detect potential deletions and duplications identified the hemizygous, likely pathogenic, duplication of exons 5 through 9 of *RPS6KA3* gene (GenBank: NM_004586.2) region ChrX:20205946-20213263 (based on GRCh37/hg19 build) (Figure 2A). Although the exact breakpoints and intragenic location could not be determined, the duplication of exons 5-9 was confirmed by genomic quantitative PCR. The duplication was subsequently confirmed in all three brothers by targeted high resolution aCGH 60K oligonucleotide array at GeneDx (Figure 2B).

In order to assess the effect of the duplication on gene transcription, we analyzed the expression profile of *RPS6KA3* by quantitative real time-PCR (qRT-PCR). Total RNA was extracted from peripheral blood cells of the patients and healthy controls using the *quick-RNA Miniprep kit* (ZYMO research) or Sigma Gen Elite Mammalian Total RNA Miniprep Kit. An RNA sample from the biological father and mother were not available. One μg of total RNA was reverse-transcribed with the High Capacity RNA-to-cDNA Kit (Applied Biosystems, Thermo Scientific). qRT-PCR was performed using commercial predesigned Taqman probes mapping in the non-duplicated region: *RPS6KA3* Hs01078601_g1 (located in exon 21–22 boundary) (Applied biosystem, Thermofisher); *ABL1* probe (Hs01104725) was used for normalization (Applied biosystem, Thermofisher). To calculate the relative changes in gene expression, we applied the $2^{-\Delta\Delta\text{CT}}$ method as previously described (Livak et al., 2001). The *RPS6KA3* transcript in the brothers compared with the healthy controls, showed a significant reduction in all three (Figure 2C).

Subsequently, we analyzed the cDNA of the patients and controls by RT-PCR to determine if the duplication was present in a transcript of *RPS6KA3*. We designed multiple primers yielding eight overlapping PCR products covering the entire coding sequence of *RPS6KA3* (data not shown). A PCR product obtained from Individual III-1 with a forward primer in exon 4 (RSK2_Ex4f 5'-CTCAGGCTCTGATGCTAGGC) and a reverse primer in exon 10 (RSK2_E10R 3'-TTTTCCCTGGAAAGGGAGTG) generated an abnormal ~ 1000bp size product (Figure 2D – lane 2) larger than the expected 550 bp product observed in the control (Figure 2D – lane 1), which is consistent with the introduction of 450bp expected size of the duplication of exons 5-9 within the exons 4-10 genomic region. The direct duplication is predicted to cause a frameshift with consequent introduction of a novel amino acid sequence (FETFGQKWNVISW) followed by a premature stop codon (Figure S1).

DISCUSSION

We describe three brothers with classic clinical and radiographic findings of CLS including moderate intellectual disability, broad tapered fingers, skeletal anomalies, and characteristic facial features. Evaluating these brothers, we identified a novel intragenic duplication in the *RPS6KA3* gene as the cause for their CLS.

Previously, Newman et al. (2015) has shown that most interstitial duplications are tandem and often lie in the proper orientation causing gene reading frame disruption and lead to loss-of-function (LOF) mutations. Two previous groups have identified direct duplications in *RPS6KA3* that caused an alteration in the coding region leading to LOF either by mRNA level reduction or impacting the protein function (Bertini et al., 2015; Pereira et al., 2007). Therefore, we hypothesize that the duplication observed in each of the three brothers presented here has

impacted the *RPS6KA3* gene function through one of the aforementioned mechanisms. Overall, the decreased level of *RPS6KA3* transcript together with the deduced location of the duplication (Figure 2D), which is predicted to disrupt the reading frame and create a premature stop codon (Figure S1), strongly suggest nonsense-mediated decay (NMD) as the most likely mechanism explaining the LOF phenotype.

In 2007, Pereira et al. reported a patient with the typical clinical findings of CLS but who did not have a sequence-detected mutation in *RPS6KA3*. Subsequently, western blot analysis revealed that the RSK2 protein was much larger than expected (Pereira et al., 2007). Further molecular studies found an in-frame tandem duplication of exons 17-20, resulting in a non-functioning protein, which the authors postulated caused the CLS. Subsequently, Bertini et al. (2015) described a male patient with a 625 kb duplication of Xp22.12, which includes only one gene, *RPS6KA3*. Although the exact intragenic location of the duplication was not identified, the mutation involved an in tandem partial duplication of exons 1 through 7. Bertini et al. (2015) then suggested that the partial duplication inhibited the expression of the gene resulting in LOF of the protein kinase activity. The patient had mild intellectual disability, short stature, and orthodontic problems. However, his phenotype was not typical of CLS having an absence of the characteristic facial appearance (Bertini et al., 2015).

Other duplications have been reported to cause syndromic and non-syndromic X-linked intellectual disability. In 2013, Matsumoto et al. described a family with individuals who possessed 584 kb microduplications of Xp22.12. The microduplications contained seven genes, six of which were uncharacterized and seventh, the *RPS6KA3* gene. Molecular analyses demonstrated that the *RPS6KA3* protein levels in lymphoblasts of these individuals were

increased between 1.53-to-2.14 fold compared to controls. The phenotype was variable among the affected individuals but Matsumoto et al. (2013) stated that the duplication was likely causing their mild non-syndromic intellectual disability. Other larger duplications with multiple genes, causative for non-syndromic X-linked intellectual disability, have been described by Madrigal et al. (2007), Thorson et al. (2010), and Tejada et al. (2011).

As presented above and summarized in Table 1, all three brothers have classical characteristics of CLS including moderate intellectual disability, global developmental delay, speech delay, dental abnormalities, prominent lips, pectus carinatum, short stature, spinal abnormalities, and broad tapered fingers. However, these siblings have other features not described as characteristic findings of CLS and include short palpebral fissures, ptosis, pseudo-esotropia, bifid uvula, and supernumerary and inverted nipples. On the other hand, other characteristics that are seen in 80-99% of individuals with CLS were not found in these brothers including prominent forehead, depressed nasal bridge, anteverted nares, open-mouth appearance, delayed skeletal maturation, craniofacial hyperostosis, large palms, brachydactyly, joint hyperflexibility, and abnormal diaphysis morphology (Table 1).

To the best of our knowledge, the novel mutation in our patients is the third intragenic microduplication reported affecting *RPS6KA3* mRNA levels and resulting in LOF. The LOF in our patients appears causative for their CLS and supports the notion that microduplications in *RPS6KA3* can lead to CLS by this mechanism. In the future, analyses of the genomic rearrangement in similar situations, the definition of the exact breakpoints, and orientation of the duplication within exons 4 through 10 would be warranted to better understand the dynamic underlying the recombination and its consequences.

Analyses of the intronic sequences of *RPS6KA3* by UCSC genome browser (<http://genome.ucsc.edu/>) reveals the presence of a multitude of *Alu* repeats, which may be responsible for deletion/duplication events as a plausible mechanism underlying the duplication observed in our patients. The intragenic duplication described by Pereira et al (2007), was likely mediated by *Alu* recombination, and although it did not shift the reading frame, the protein analysis, nonetheless, showed an aberrant partially duplicated RSK2 protein with reduced kinase activity. Therefore, our findings, along with previous published studies, Pereira et al. (2007) and Bertini et al. (2015), provide further evidence that molecular analyses capable of detecting intragenic duplications coupled with methodologies that assess mRNA or protein function might be needed for patients clinically diagnosed with CLS but in whom classic exon targeted panels failed to detect a mutation in the *RPS6KA3* gene.

When sequencing analysis of *RPS6KA3* does not find a pathogenic mutation in a patient suspect to have CLS, then exon-targeting deletion and duplication analysis represents a valuable approach, considering the high number of *Alu* repeats present within the gene. More data about the frequency of these pathogenic duplications, often missed by conventional molecular testing, are needed in order to improve the diagnostic rate and genotype-phenotype correlations in CLS. Finally, our study provides data supporting that microduplications in *RPS6KA3* can cause CLS due to LOF mechanism.

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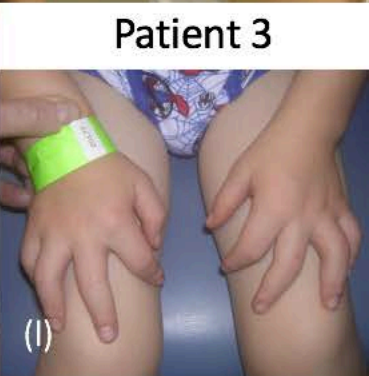
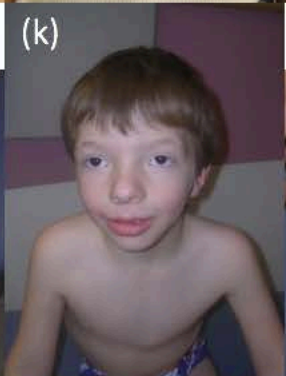
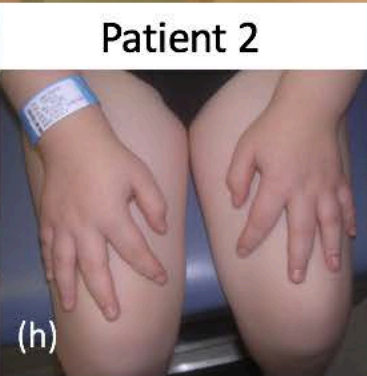
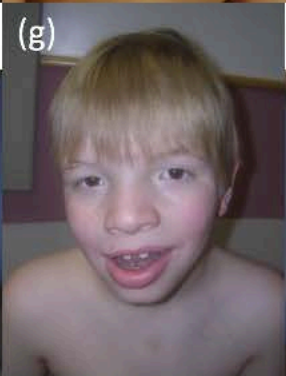
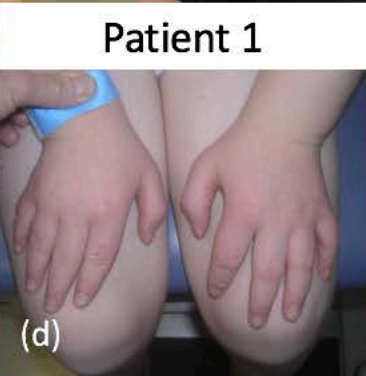
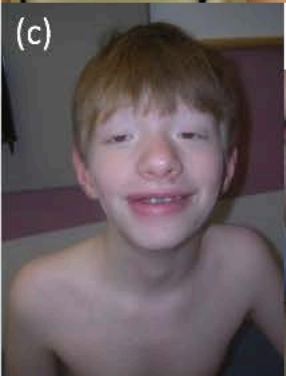
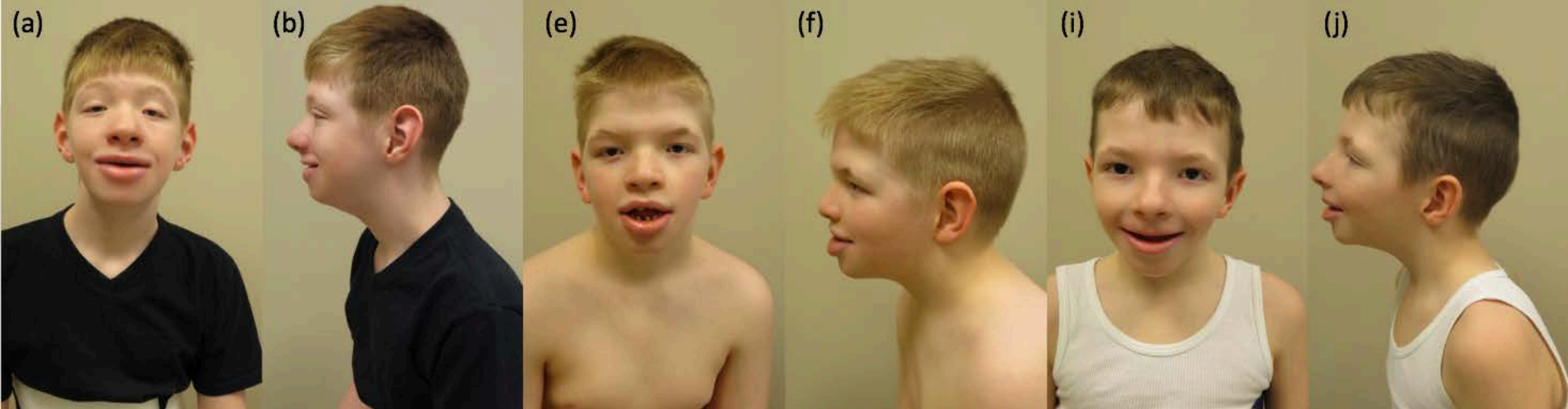
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FIGURE LEGENDS

Figure 1 – (A) A three-generation pedigree displaying our patients with Coffin-Lowry syndrome; Individuals III-1, III-2, and III-3. Our proband is identified by the arrow. (B) Individual III-1, frontal view (upper left), and lateral view (upper right) at age 12, frontal view (lower left), and hands (lower right) at age 10. (C) Individual III-2, frontal view (upper left) and lateral view (upper right) at age 11, frontal view (lower left), and hands (lower right) at age 9. (D) Individual III-3, frontal view (upper left), and lateral view (upper right) at age 9, frontal view (lower left), and hands (lower right) at age 7.

Figure 2 - (A) Ideogram of chromosome X and the Xp22.12 region with a schematic representation of the genomic structure of *RPS6KA3* gene. Exons are shown to scale with the coding sequence in grey and untranslated regions in dark blue. The exons contained within the detected microduplication are colored red and are outlined by the box. Previously reported intragenic duplications are underlined in black (see text). (B) Array CGH profile of the chromosome X where the abnormal profile showing the duplication (chrX:20205622_20213526; GRCh37/hg19 build) in Individual III-1 is boxed in red. (C) Real-time qPCR performed on RNA extracted from peripheral blood of the affected brothers and a healthy control showed a significantly reduced expression of *RPS6KA3* in the three brothers. (D) RT-PCR using a forward primer in exon 4 and a reverse primer in exon 10 led to the production of a 1000 bp fragment (lane 2) suggesting the presence of the duplication within the exon 4-10 region in the patient. This additional fragment was absent in the normal control (lane 1). Lane 3 is the water control which contains no DNA.

Figure S1 - (A) Genomic structure of *RPS6KA3* gene. Exons are shown to scale with the coding sequence in grey and untranslated regions in dark blue. The exons contained within the detected microduplication are colored red and are outlined by the box. (B) Predicted splicing mechanism leading to duplication of exon 5-to-9 in direct orientation according to the RT-PCR gel analysis in Figure 2 that showed an increased band size of 450bp. (C) *In silico* prediction of the *RPS6KA3* open reading frame (ORF) resulting from the duplication reveals the introduction of a stretch of 14 amino acids FETEFGQKWNVISW (underlined in red) preceding a premature stop codon in position p.273 (c.819) (boxed in red).



Patient 1

Patient 2

Patient 3

