Concomitant partial exon skipping by a unique missense mutation of *RPS6KA3* causes Coffin–Lowry syndrome

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ABSTRACT

Coffin Lowry syndrome (CLS) is an X linked semi dominant disorder characterized by diverse phenotypes in cluding intellectual disability, facial and digital anomalies. Loss of function mutations in the Ribosomal Protein S6 Kinase Polypeptide 3 (*RPS6KA3*) gene have been shown to be responsible for CLS. Among the large number of mutations, however, no exonic mutation causing exon skipping has been described. Here, we report a male pa tient with CLS having a novel mutation at the 3′ end of an exon at a splice donor junction. Interestingly, this nu cleotide change causes both a novel missense mutation and partial exon skipping leading to a truncated transcript. These two transcripts were identified by cDNA sequencing of RT PCR products. In the carrier mother, we found only wildtype transcripts suggesting skewed X inactivation. Methylation studies confirmed X inactivation was skewed moderately, but not completely, which is consistent with her mild phenotype. Western blot showed that the mutant RSK2 protein in the patient is expressed at similar levels relative to his mother. Pro tein modeling demonstrated that the missense mutation is damaging and may alter binding to ATP molecules. This is the first report of exon skipping from an exonic mutation of *RPS6KA3*, demonstrating that a missense mu tation and concomitant disruption of normal splicing contribute to the manifestation of CLS.

1. Introduction

Coffin Lowry syndrome (CLS) (MIM 303600) is a syndromic form of intellectual disability (Pereira et al., 2010) arising as a result of loss of function mutations in the Ribosomal Protein S6 Kinase Polypeptide 3

(*RPS6KA3*) gene (Trivier et al., 1996). Affected individuals with CLS display a wide range of phenotypes including intellectual disability, ta pering fingers, skeletal abnormalities, distinctive facial dysmorphisms and hearing deficit (Hanauer and Young, 2002). Being an X linked semi dominant disorder, CLS symptoms are more pronounced in male pa tients compared to females (Young, 1988). Typical CLS symptoms are usually manifested gradually during childhood making it difficult to di agnose at birth, particularly in families with no previous CLS history (Pereira et al., 2010).

Over 140 mutations distributed throughout the gene have been reported making *RPS6KA3* a gene with strong allelic heterogeneity (Jacquot et al., 1998a, 1998b; Zeniou et al., 2002; Nishimoto et al., 2014). The protein encoded by the *RPS6KA3* gene (ribosomal S6 kinase 2, RSK2) consists of two non identical kinase domains each containing its own ATP binding site, an extracellular regulated kinase (ERK) docking site and a linker region connecting the kinase domains (Trivier et al., 1996; Jacquot et al., 1998a; Delaunoy et al., 2001; Nguyen et al., 2006). It has been demonstrated that the transcription factor ATF4 acts as a critical substrate of RSK2 and together, they regu late synthesis of type I collagen, the main constituent of the bone

matrix. This suggests that lack of ATF4 phosphorylation by RSK2 may contribute to the skeletal anomalies of CLS (Yang et al., 2004).

Pre mRNA splicing is one of the fundamental processes orchestrated by the spliceosome made up of five small nuclear ribonucleoproteins (snRNPs) and >100 proteins (Faustino and Cooper, 2003). Removal of intronic sequences during RNA splicing is governed by signals embedded in splice sites located at exon intron junctions. Some mutations occurring in intron exon boundaries can interfere with recognition of splice sites causing partial or complete exon skipping. Partial exon skipping in autosomal (Weissschuh et al., 2012) as well as in X linked genes (Disset et al., 2006) has been reported to be the cause of a number of disorders including aniridia (Weissschuh et al., 2012), Menkes disease (Das et al., 1994), congenital contractural arachnodactyly (CCA) (Maslen et al., 1997), and Duchenne muscular dystrophy (Disset et al., 2006). Mutations causing exon skipping of the *RPS6KA3* gene are rare. Only ~7% of mutations in *RPS6KA3* result in exon skipping (Jacquot et al., 1998b; Zeniou et al., 2002; Delaunoy et al., 2006). All documented cases are due to intronic mutations in *RPS6KA3* and there have been no reported case to date of exonic missense mutations within this gene resulting in partial exon skipping (Jacquot et al., 1998b; Zeniou et al., 2002; Delaunoy et al., 2006).

Here we report a male CLS patient with a novel *RPS6KA3* missense mutation (c.631G > C, last base of exon 8) predicted to result in an amino acid substitution (p.D211H). We performed RT PCR followed by cDNA sequencing to determine whether this mutation at the splice donor junction altered the effectiveness of the splicing process. We also performed Western blot analysis to see the expression level of the mutant RSK2 protein in patient DGDP201. Moreover, protein structure analysis was performed for additional supportive evidence of the pathological role of the missense mutant protein.

2. Materials and methods

2.1. Sequencing of RPS6KA3 coding regions

Genomic DNA was isolated from blood samples by standard protocols using phenol chloroform extraction. All 22 exons of the *RPS6KA3* (NM_004586.2) gene were amplified from genomic DNA isolated from patient DGDP201, and exon 8 was amplified from genomic DNA isolated from the patient's mother and grandmother. Amplicons were then analyzed by a standard fluorescent sequencing protocol.

2.2. Cell culture

Lymphocytes were isolated from blood by density gradient centrifugation. Briefly, ~7 ml of blood was loaded on top of 5 ml of lymphocyte separation medium (Fisher) and centrifuged for 30 min at 1700 rpm. The middle phase containing lymphocytes was carefully removed and ~12 ml of PBS (Fisher) was added to the recovered lymphocytes. The mixture was then centrifuged for 10 min at 1300 rpm. This washing step was repeated twice prior to immortalization of the cells. Transformation was carried out by adding cyclosporin, Epstein Barr virus (EBV) and 20% FBS media (100 ml Fetal bovine serum, 5 ml antibiotic, 5 ml ʟ glutamine, 390 ml RPMI) sequentially to the isolated lymphocytes.

2.3. Western blot

Protein was isolated from lymphoblastoid cell lines derived from patient DGDP201 and his mother. RSK2 antibody (Sigma) targeting the N terminal end of the protein was used to detect the level of expression of wild type and mutant proteins. Detection was carried out using the Amersham™ ECL™ Western Blotting Analysis Systerm (GE Healthcare).

Table 1
Primer sequences.

Target	Forward primer (5′–3′)	Reverse primer 5′–3′
RSK2ex6A-ex10A[*]	GAGGAGATTTGTTTACACGCTTATCCAAA	CCTTGGAAAGGGAGTGTACCAGTAAGC
RSK2ex6B-ex10B[*]	TCAGGGGAGGAGATTTGTTTACACGC	TTGGAAAGGGAGTGTACCAGTAAGCATT
RSK2ex8[**]	CTTTGCCATCTTATTCACAGTCTAAAGCA	CTACGCCTGGCCCTAATACAAGTTT

[*] cDNA.
[**] genomic DNA primers.

2.4. X inactivation

The X chromosome inactivation (XCI) pattern was determined by PCR analysis of a polymorphic CAG repeat in the first exon of the androgen receptor (*AR*) gene. Methylation of sites close to this short tandem repeat has been demonstrated to correlate with X chromosome inactivation (Allen et al., 1992). In this assay, amplification of the *AR* gene both before and after digestion with the methylation sensitive HpaII restriction enzyme was used to determine the methylation status of the maternal and paternal X chromosome. XCI degree threshold patterns are classified as random (XCI < 80%), moderately skewed (80% < XCI ≤ 90%), and highly skewed (>90%).

2.5. RT PCR and sequencing

Cell culture from selected cell lines was first centrifuged for 10 min at 1000 rpm. Total RNA was isolated from the pellets using the RNeasy Plus Mini kit (Qiagen) following the manufacturer's protocol. cDNA synthesis was then carried out from 1 μg of total RNA using the RevertAid First cDNA Synthesis Kit (Thermo Scientific) following the manufacturer's instructions. The target loci within the *RPS6KA3* gene were amplified using primers designed from *RPS6KA3* exon 6 and 10, respectively (Table 1). All reactions were carried out in a total volume of 25 μl using the Taq DNA polymerase (New England Biolabs). The PCR conditions included an initial denaturation for 4 min, 40 cycles consisting of a denaturation at 95 °C for 30 s, annealing at 60 °C for 1 min and extension at 68 °C for 1 min. A final extension for 5 min at 95 °C was included at the end. Only 8 μl of the PCR product was run on a 1% agarose gel stained with ethidium bromide (100 mg/ml). After confirmation of the size of the amplicons, the PCR products were purified by standard protocol and sequenced using the Big Dye® Version 3.1 Cycle Sequencing kit (Applied Biosystems).

2.6. Clinical report

The affected individual (DGDP201) is a Caucasian male born full term via Cesarean section secondary to decreased fetal heart tones and failure to progress. Birth parameters were within normal limits with weight of 2.61 kg (3rd to 10th percentile), length of 49 cm (25th to 50th percentile) and a head circumference of 35.1 cm (25th to 50th percentile). A prenatal ultrasound in the second trimester detected right renal agenesis.

Shortly after birth, the patient was found to possess additional anomalies. He failed his newborn hearing screen and displayed dysmorphic features including retrognathia, hypertelorism, low set ears, short palpebral fissures, high arched palate, hypoplastic nipples and hypotonia. An echocardiogram revealed a small perimembranous ventricular septal defect (VSD) and a cranial ultrasound showed bilateral subepidermal matrix cysts. He was diagnosed with kyphoscoliosis and at 5 weeks of age required a double hernia repair. Genetic testing in the newborn period revealed a normal male karyotype, 46 XY.

At 9 years of age, the patient was first examined at a Genetics Clinic to determine whether he was showing symptoms of CLS. He had severe

developmental delay and was non verbal. He was unable to utilize sign language to communicate. He was not able to walk normally and pre ferred to crawl or scoot. He was using a walker for mobility and could only take a few independent steps. At that time, he was 93.5 cm (<3rd percentile), weighed 22 kg (<3rd percentile), and his head circumfer ence was 53.5 cm (50th percentile). He had been followed for kyphoscoliosis for a number of years for which he wore a brace; he had 50° scoliosis and 70° kyphosis. He wore prescription glasses for hy peropia. It was also reported that when his teeth fall out, they usually come out at the root and take a long time to grow back again. The pa tient displayed significant hearing loss and had been wearing bilateral hearing aids since childhood. He displayed repetitive hand movements but no additional unusual behaviors or problems. He had issues with constipation and was not yet toilet trained. He had brachydactyly with tapering fingers and a double occipital hair whorl. He displayed down slanting palpebral fissures (Fig. S1A and B) and hypertelorism with an innercanthal distance of 4.5 cm (>98th percentile). He possessed bilat eral Darwinian tubercles, a flat nasal bridge and mild nasal anteversion (Fig. S1A and B). He had thick upper and lower lips as well as broad hands (Fig. S1C). Based on these clinical findings, he was clinically diag nosed with CLS.

The patient was re examined at age 12 at which time he weighed 31.8 kg (3rd 5th percentile) and had just entered puberty. He displayed inguinal and axillary hair and had started to develop acne. He had a number of skin rashes and infections, although no additional tests were performed. His mother reported that his attempts at walking had dimin ished considerably, possibly due to progressive kyphoscoliosis. He contin ued receiving physical and occupational therapy because of his severe developmental delay. An array comparative genomic hybridization (aCGH) using a custom oligonucleotide platform (Agilent) revealed no copy number variants.

At age 13, the patient underwent surgery for progressive kyphoscoliosis. At that time, he was also diagnosed with unilateral hip dysplasia. At 14, he continued to show psychomotor delays and athetotic movements. He would have occasional staring spells but no formal seizures or drop attacks as observed in other patients with CLS.
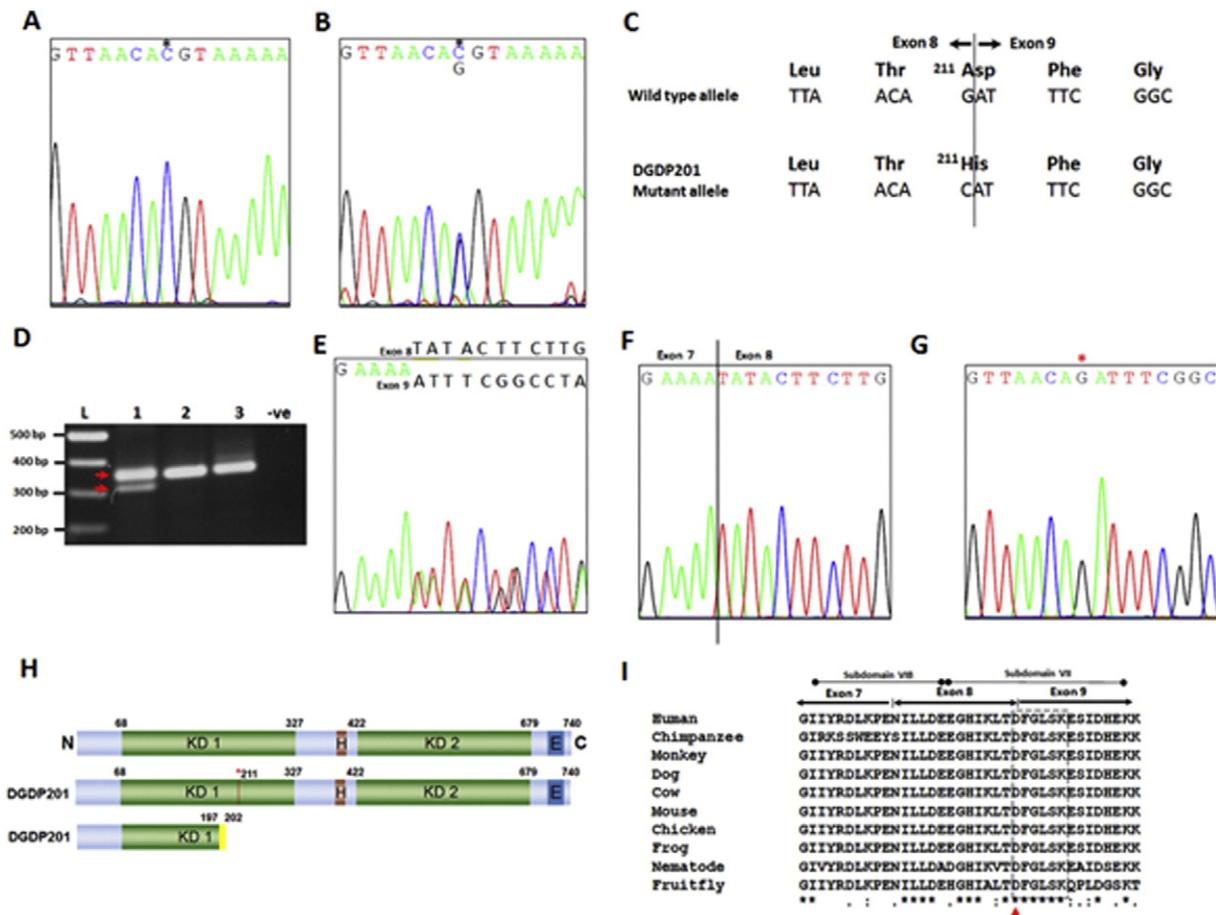

Fig. 1. Mutation in the last nucleotide of exon 8 causes partial exon skipping and an amino acid substitution. Chromatograms showing the mutation in (A) DGDP201 and (B) carrier mother. The position of nucleotide change is marked by an asterisk. The mother is hererozygous for this variant (c. 631G > C) displaying two peaks corresponding to a 'C' and 'G' nucleotide. (C) The resulting amino acid substitution (Asp to His) due to the missense mutation at the last nucleotide of exon 8 is shown. (D) RT-PCR showing transcripts amplified in DGDP201 (lane 1) carrier mother (lane 2) and white male control (lane 3). A bright band is present in all samples assayed, except negative control (-ve). Two types of transcripts are produced in DGDP201 on lane 1. The letter 'L' designates the lane containing the ladder, while the arrows in red indicate positions of the longer and shorter transcripts. (E) A chromatogram showing two types of tran scripts produced in DGDP201 as a result of partial exon 8 skipping due to the missense mutation (c.631G > C, NM_004586.2). The lower sequence corresponds to the transcript produced as a result of exon skipping, while the upper sequence represents the normal transcript (F) Chromatogram showing no exon 8 skipping in carrier mother. (G) Chromatogram showing wild-type transcript only in carrier mother. Asterisk denotes the position of the last base of exon 8 followed by exon 9. (H) Schematic representation showing the predicted RSK2 protein structure (NP_004577). Two types of proteins are predicted to be produced in DGDP201. The full length mutant protein contains the mutation (p.D211H) depicted by the red asterisk, while exon skipping produces a truncated protein. The five aberrant amino acids produced in the truncated RSK2 protein are shown in yellow. The truncated protein is predicted to con tain 202 amino acids. Kinase 1 (KD 1) indicates the position of the N-terminal kinase domain, Kinase 2 (KD 2) shows the position of the C-terminal kinase domain. KD; kinase domain, H; hydrophobic motif, E; ERK docking site. (I) Comparison of amino acid sequences of RSK2 in diverse species including human, chimpanzee, monkey, dog, cow, mouse, chicken, frog, nematode, and fruitfly. All amino acids encoded by exon 8 are displayed in the middle. Amino acids derived from exon 8 constitute parts of the subdomain VIB and subdomain VII of KD 1. The red arrow marks the invariant position of the amino acid substitution (D^{211}), while the box highlights a highly conserved region within subdomain VII.

His head circumference was 56.3 cm (50th to 98th percentile). He was able to feed himself independently with a spoon and was able to chew well, although his adult teeth were not emerging.

The patient's mother had mild thickening of the lower lip and bulbous nasal tip (Fig S1D and E) with fleshy hands and tapering fingers (Fig. S1F) indicative of a CLS carrier. She also had mild upslanting palpebral fissures (Fig S1D) and was placed in special education from fourth grade through high school. She has three sisters who did not have any learning disabilities. Other than the patient's first cousin, who required special education classes, all other relatives were reported as healthy. Written informed consent was obtained from the patient's mother for the publication of this report and any accompanying images of this family.

3. Results

3.1. Mutation in RPS6KA3 exon 8

Sequencing of all 22 RPS6KA3 coding exons revealed that the patient has a mutation in the last base of exon 8 (c.631G > C, NM_004586.2). This mutation is also present in his mother, but not in the maternal grandmother. We designed primers targeting exon 8 (Table 1) and amplified the genomic region encompassing the mutation to generate chromatograms. Sequencing confirmed the mutation (g. 78136 G > C) in the patient and carrier mother who is heterozygous (Fig. 1A and B). This mutation is expected to result in an aspartic acid being replaced by a histidine at amino acid 211 (p. D211H) (Fig. 1C).

3.2. RT PCR and cDNA sequencing

To determine any effects of the mutation on RPS6KA3 transcription, we performed RT PCR, amplifying a region of RPS6KA3 encompassing exons 6 10. Interestingly, a shorter transcript was produced in the affected individual in addition to the normal transcript corresponding to the targeted region (Fig. 1D). Sequencing of the amplified fragments revealed that the mutation (c.631G > C, NM_004586.2) causes partial exon skipping by producing aberrant transcripts missing exon 8 in

addition to the mutant full length transcript (Fig. 1E). Despite being heterozygous for the mutation at the genomic level, DGDP201's mother did not demonstrate exon 8 skipping for the mutant allele in mRNA (Fig. 1F and 1G) and only expressed the normal transcript (Fig. 1D). X inactivation analysis revealed that the patient's mother had moderately skewed X inactivation with a ratio of 90:10 while the maternal grandmother had random X inactivation. Based on the RNA studies, DGDP201's mother is skewed towards the normal X chromosome being preferentially active (Fig. S2).

3.3. RSK2 protein structure and amino acid alignment

Analysis of the position of the mutation in the peptide sequence revealed that the mutation (p.D211H) is located in the N terminal kinase domain of the RSK2 protein (Fig. 1H). Sequence analysis of the transcript missing exon 8 showed that it has a predicted premature termination codon TAA shortly after producing five aberrant amino acids KFRPK (p.Asn198Lysfs*6) (Fig. 1H). We postulate that a truncated protein consisting of only 202 amino acids is produced as a result of exon 8 skipping (Fig. 1H). Comparison of RSK2 orthologs from nine other species of mammal, vertebrate, insect, and nematode revealed that substitution of aspartic acid (D^{211}) by histidine in DGDP201 occurs in a highly conserved region in the amino acid sequence (Fig. 1I).

3.4. Western blot

Western blot analysis showed expression of the mutant RSK2 protein (~84 KDa) in patient DGDP201 (Fig. 2). Densitometry analysis revealed similar RSK2 protein levels in DGDP201, his mother, white male (WM6) and white female controls (WF115) (Fig. 2). The truncated RSK2 protein (~23 KDa) expected to be produced in the DGDP201 was not detected, possibly because it is rapidly degraded due to its instability.

3.5. Protein modeling

We performed a domain search on the protein with the GenBank accession number NP_004577.1 using NCBI Blast and SMART (Letunic
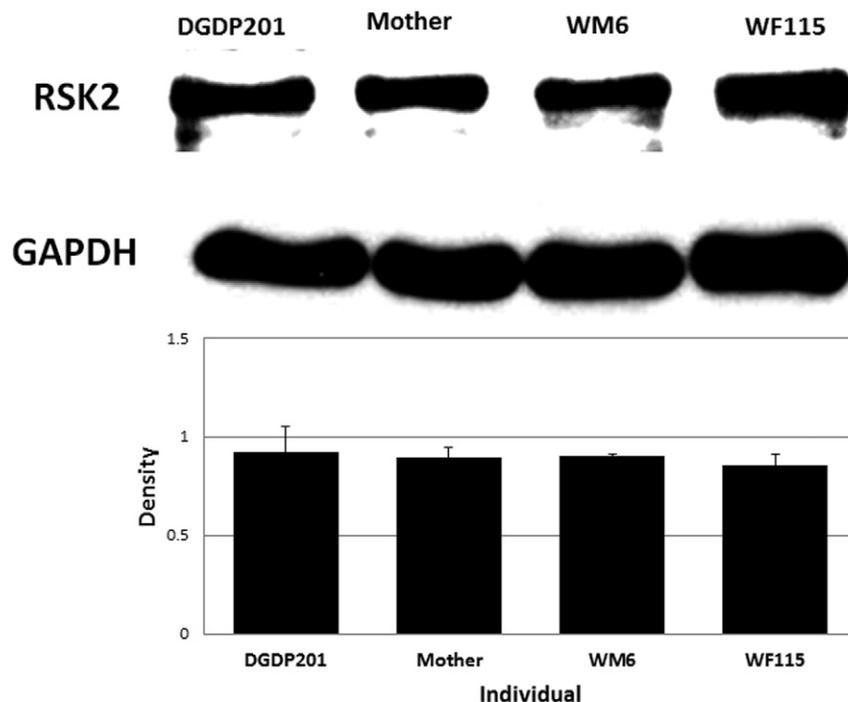

Fig. 2. Western blot showing expression of the mutant RSK2 protein in DGDP201. The RSK2 expression levels in the carrier mother, white male (WM6) and white female controls (WF115) are also displayed. Densitometry analysis revealed similar RSK2 protein levels across all individuals assayed.
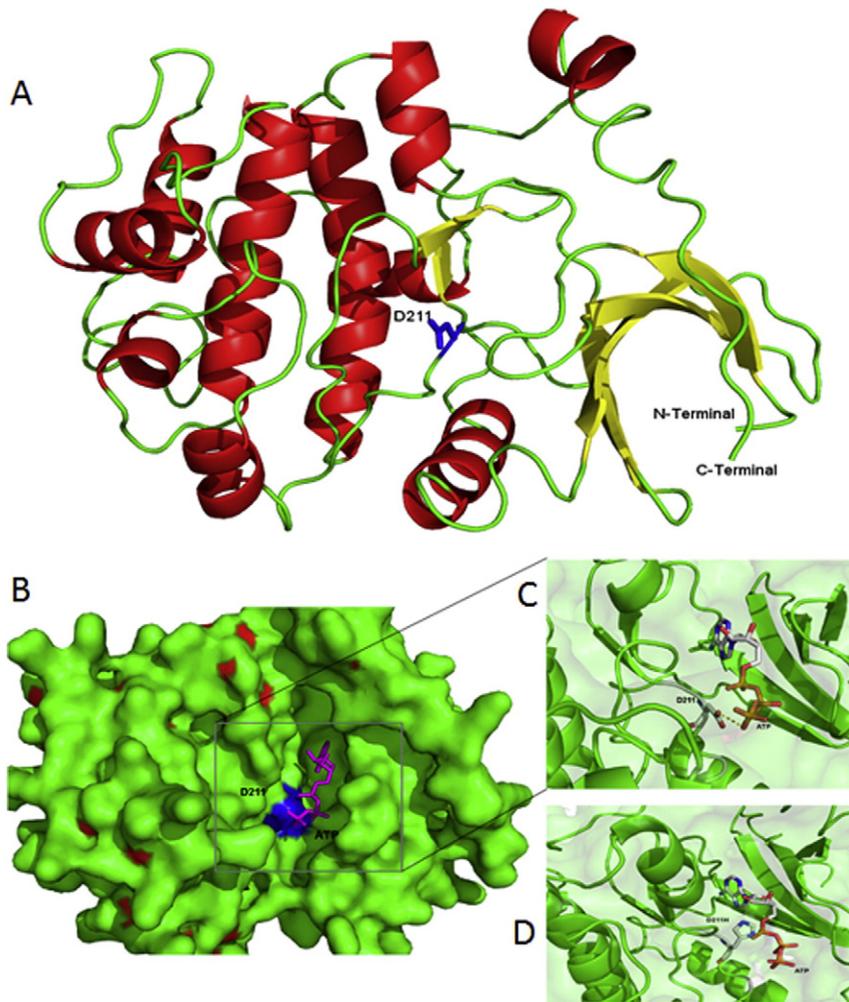
Fig. 3. (A) Homology model for the segment spanning amino acids 65–370 of the protein encoded by the *RPS6KA3* gene using sequence alignment based on homology to Protein Kinase C beta II (pdb id: 3PFQ). Color code is red for helix, yellow for strands and green for loops, blue represents the position of the mutation site D211. (B) Homology model for the segment spanning amino acids 65–370 of the protein encoded by the *RPS6KA3* gene with ATP (shown by sticks in magenta) close to the D211 residue in the binding pocket (in blue). (C) Enlarged picture of the selected box showing the interaction of the Asp (D211) amino acid with the ATP molecule. (D) p.D211H (Asp to His) point mutation model suggesting change in the polar contacts with the ATP molecule.

et al., 2012), which predicted two serine/threonine protein kinase cata lytic domains in the amino acid regions 68 327 (Fig. 3) and 422 679 and an additional Ser/Thr type protein kinases around 328 389. We then performed a SIFT analysis for the gene sequence NP_004577.1 to investigate potentially deleterious effects of the p.D211H missense mu tation. The SIFT (Kumar et al., 2009) analysis suggests that the mutation is non tolerable with a tolerance index score of 0.0 (on a scale of 0 1, where amino acid substitution is predicted to be damaging if the score is ≤0.05, and tolerable if the score is >0.05) and will affect protein func tion. We further analyzed this mutation using PolyPhen2 (Adzhubei et al., 2010), which designated the mutation p.D211H as being "proba bly damaging" with score 1.00. PolyPhen2 designates the mutation as being "benign", "possibly damaging", and "probably damaging" with the respective scores of <1.5, >1.5 and >2.0 respectively. We also used the support vector machine based tool I Mutant 2.0, where the p.D211H mutation has a negative score with DDG value −1.31 (pH: 7 and temperature 25 °C) which indicates that this mutation may de crease protein stability. SPPIDER (Porollo and Meller, 2007) predicts the p.D211 residue as an interfacial residue in the soluble domain. We also used the HMM based evolutionary approach PANTHER to investi gate the impact of point mutation on protein function. PANTHER (Mi2013) designated the point mutation p.D211H as deleterious with a subPSEC score of −11.36 (protein sequences having subPSEC scores ≤−3 are said to be deleterious).

To support this analysis with structure based data we also generated a homology model for the amino acid region 63 370 of the sequence NP_004577.1, using the crystal structure of Protein Kinase C beta II (pdb id: 3PFQ) and G protein coupled receptor kinase 1 bound to ATP (pdb : 3C4W), as homologous template proteins (Fig. 3A). Aligning the homology model with the homologous protein (crystal structure of G protein coupled receptor kinase 1 bound to ATP (pdb: 3C4W)), Fig. 3B and C shows that the p.D211 residue is involved in the interac tion with an ATP molecule in the binding pocket, and mutating this res idue might affect the function of protein (Fig. 3D).

4. Discussion

The patient (DGDP201) investigated in the current study has a nu cleotide substitution in the last base of *RPS6KA3* exon 8 (c.631G > C, NM_004586.2) which causes partial exon skipping. We postulate that c.631G, located at a splice junction immediately adjacent to the splice donor site of intron 8, is important for splicing as an invariant sequence. Due to this mutation, it is likely that cleavage at the 5′ splice junction does not take place properly and consequently intron 8 along with the attached exon 8 are discarded in the splicing process. RT PCR revealed two types of transcripts generated in the affected male as a result of this missense mutation p.D211H. While exon 8 skipping creates shorter transcripts missing that entire exon, full transcripts containing the

nucleotide substitution are also concomitantly produced. Amino acid sequence analysis of the shorter transcript revealed that it has a premature stop (p. Asn198Lysfs*6) due to a frameshift, indicating that a truncated RSK2 protein is likely produced. The full length RSK2 protein carries an amino acid substitution (p.D211H) in the N terminal kinase domain (Fig. 1H). The deleterious effects of the mutation are anticipated because of its position within this functionally important domain. This changes the amino acid character dramatically by replacing the conserved aliphatic and negatively charged residue aspartate D^{211} (Fig. 1C) with the aromatic and positively charged histidine H^{211}.

Accordingly, protein modeling revealed that the amino acid substitution (p.D211H) likely affects RSK2 protein function by altering protein stability as well as by affecting the binding of ATP molecules to the RSK2 protein. This mutation is therefore likely to abolish the kinase activity of RSK2 since the truncation eliminates part of the subdomain VII in the N terminal kinase 1 domain (Fig. H and I) and the ensuing functional domains including the C terminal kinase 2 domain (Fig. H).

It is possible that both the truncated and mutant proteins are non functional, which would explain the broad spectrum of the CLS phenotype manifested by our patient. The mutation observed in subject DGDP201 is inherited from the carrier mother who is heterozygous for the mutation (c.631G > C, NM_004586.2) and displays only a mild CLS phenotype. Only the wild type transcripts were detected in DGDP201's mother, however, suggesting that the mutant allele was most likely silenced by skewed X inactivation, and, indeed, X chromosome inactivation studies showed moderate skewing. Based on her mild CLS phenotype, we believe the small proportion of transcripts synthesized from the active chromosome X carrying the mutant allele were undetected by RT PCR due to its limitations.

More than 140 mutations have been reported across the RPS6KA3 gene with the majority of the alterations being missense mutations (Pereira et al., 2010; Nishimoto et al., 2014). Exon skipping are rare events occurring in only ~7% of mutations in RPS6KA3 (Jacquot et al., 1998b; Zeniou et al., 2002; Delaunoy et al., 2006). Skipping of exons 4 6, 9, 10, and 18 (Jacquot et al., 1998a, 1998b; Zeniou et al., 2002) and exon 14 (Delaunoy et al., 2006) due to intronic mutations or large deletions have been reported. To our knowledge, the present study is the first report of exon skipping due to an exonic mutation in RPS6KA3. More interestingly, this missense mutation is at the last base of an exon and causes concomitant partial exon skipping. The skipped exon 8 consisting of 38 bp is the smallest exon within the RPS6KA3 gene. Only two mutations located within exon 8 (c.598 600delCTT and c.605 607delATG) have been previously reported (Delaunoy et al., 2001; Delaunoy et al., 2006). Exon 8 of RPS6KA3 constitutes part of subdomains VIB and VII which are both integral components of the eukaryotic kinase domain (Fig. 1I) (Hanks and Hunter, 1995). The amino acid residue aspartic acid D211 is part of the highly conserved DFG triplet in kinases which helps to orient the γ phosphate of ATP for impending transfer (Fig. 1I) (Hanks and Hunter, 1995). The importance of this amino acid is highlighted in the present study through the detrimental phenotypic consequences in subject DGDP201.

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Conflict of interest

The authors declare no conflict of interest.

Acknowledgments

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