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# Identification and expression analysis of a novel intragenic *EFNB1* mutation causing craniofrontonasal syndrome☆

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ABSTRACT

Craniofrontonasal syndrome (CFNS) is an X-linked disorder caused by mutations in the *EFNB1* gene and characterized by distinctive craniofacial and digital malformations. In contrast with most X-linked traits, female patients with CFNS display a more severe phenotype than males. In this report, the clinical, molecular and RNA expression analyses of a female subject with CFNS are described. A novel c.445_449delGAGGG deletion in exon 3 of *EFNB1* was demonstrated in this patient. To assess the effect of this novel mutation at the transcript level, the expression of *EFNB1* mRNA was studied by quantitative RT-PCR. To our knowledge, this is the first time that an *EFNB1* transcript carrying a truncating mutation in exon 3 is demonstrated to undergo degradation by nonsense-mediated mRNA decay. Our results expand the mutational spectrum of CFNS and add to the functional consequences of truncating *EFNB1* mutations.



## Introduction

Craniofrontonasal syndrome (CFNS; *OMIM* # 304110) is an X-linked malformative disorder identified by Cohen in 1979 (Cohen, 1979) and characterized by distinctive craniofacial and digital anomalies. Clinical findings in CFNS include coronal synostosis, hypertelorism, a broad bifid nose, a low posterior



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hairline, an anterior widow's peak, longitudinally grooved fingernails, and other digital anomalies (Cohen, 2001; Orr et al., 1997; Saavedra et al., 1996). CNFS has a paradoxical clinical expression as it is more severe in heterozygous females than in males, a phenotypic pattern not usually seen in X-linked inheritance (Grutzner and Gorlin, 1988; Twigg et al., 2004). The disorder is caused by mutations in the *EFNB1* gene, located at Xq13.1 and encoding Ephrin B1, a protein that interacts with Eph tyrosine kinase receptors and plays a critical role in cell migration and pattern formation during embryonic development (Twigg et al., 2004, 2006; Wieland et al., 2004). To date, approximately 100 different *EFNB1* mutations have been identified in CFNS patients (www.hgmd.cf.ac.uk/) and about 55% of them are frameshift, nonsense, and splice site mutations that lead to premature termination codons (PTCs). Missense mutations constitute about 42% of all *EFNB1* mutations and lead to the exchange of amino acid residues that are important for receptor-ligand interaction and signaling (Twigg et al., 2004; Wieland et al., 2005). Truncating mutations are associated with extremely reduced transcript levels via a mechanism known as nonsense-mediated RNA decay that prevents the translation of transcripts carrying PTCs (Lejeune and Maquat, 2005). Only a minority of *EFNB1* truncating or splice-site mutations have been investigated at the expression level (Makarov et al., 2010; Wieland et al., 2008). Here, we describe a case of CFNS due to a novel *EFNB1* truncating mutation and report the results of *EFNB1* expression by RT-qPCR.

## Clinical report

### *Subject*

A 33-year-old woman was referred to the Genetics Department due to cranio-facial dysmorphism. She is the first child of healthy, non consanguineous parents and has 3 younger healthy siblings. Her medical family history was unremarkable for congenital malformations or inherited diseases. She was delivered by caesarean section at 38 weeks of pregnancy and birth examination revealed hypertelorism, left frontal hollow (depression), left coronal synostosis, facial asymmetry, broad and flattened nasal bridge, bifid nasal tip, and cleft palate. Growth and development were normal and her menarche occurred at l5 years of age. She underwent cranio-facial surgeries to correct her unilateral coronal synostosis and cleft palate when she was 3-years-old. Upon examination, she was 150 cm tall (<3rd centile for age), weighed 44 kg (<3rd centile), and had an OFC circumference of 53 cm (between 10th and 25th centile). A mild widow's peak, brachycephaly, frontal bossing, prominent metopic ridge, orbital asymmetry, and bilateral palpebral ptosis were also seen (Fig. 1A). Ophthalmologic examination disclosed bilateral lagophthalmos, exotropia and lacrimal tract blockage, as well as ectropion and epicanthic folds in her left eye. The patient also exhibited thick and wiry hair, short neck, *pterygium colli*, low posterior hair line (Fig. 1B and C), Sprengel's deformity, severe thoracic scoliosis, and cubitus valgus (Fig. 1E). In her hands, bilateral short 4th and 5th metacarpals, bilateral camptodactyly on F4-5, bilateral clinodactyly on F5 (Fig. 1F and G), grooved fingernails (Fig. 1H), and aberrant dermatoglyphics were seen. A gynecological examination revealed hypoplastic breasts, adequate gynecological hair distribution, and normal female external genitalia. No anomalies were found in the abdominal and pelvic ultrasonographies. Spinal X-rays showed odontoid hypoplasia, dorsal-lumbar scoliosis (Fig. 2A), lumbar hyperlordosis (Fig. 2B), and vertical position of the ribs due to severe scoliosis. Several anomalies in the right forearm including distal bowing (Fig. 2C) and dislocation in the radius as well as radial-cubital dissociation (Fig. 2D) were seen. Right carpal dislocation and short fourth and fifth metacarpals (Fig. 2E) in both hands were also found. Pain, decreased range of motion, and deformity were associated with these forearm anomalies (Fig. 1D).

### *EFNB1 mutational analysis*

The study was approved by the Institutional Review Board and the patient gave her written consent to participate. Genomic DNA was extracted from peripheral blood leukocytes using a semiautomated Quickgene system (Fujifilm, Tokyo, Japan). The 5 exons of *EFNB1* and adjacent intronic sequences were amplified by PCR using pairs of primers derived from gene normal sequences (*Ensembl ID 00000204961*). Primer sequences and annealing temperatures are available on request. Each 25 μl PCR amplification reaction contained 1× buffer, 200 ng of genomic DNA, 0.2 mM of each dNTP, 2U Taq polymerase, 1 mM of forward and reverse primers, and 1.5 mM MgCl2. PCR products were analyzed in 1.5% agarose gels from

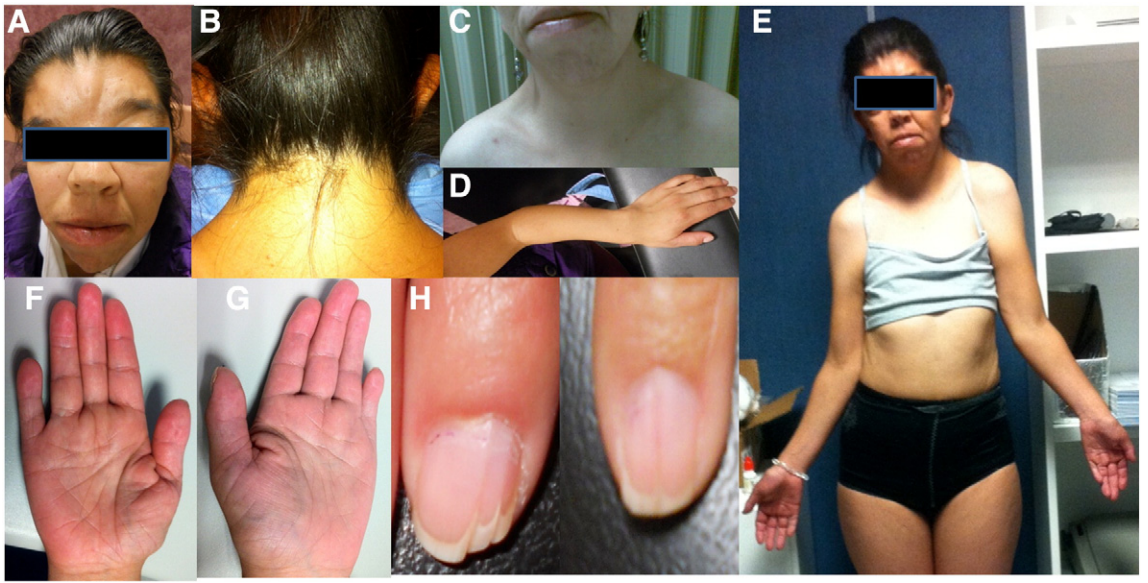


**Fig. 1.** Clinical features in the CFNS patient. (A) Frontal facial view showing orbital and facial asymmetry, hypertelorism, bilateral palpebral ptosis, frontal bossing, and prominent metopic ridges. (B, C) Short and webbed neck and, low posterior hair line were also seen. (D) Decreased range of motion and deformity of the right forearm were detected. (E) Cubitus valgus (F, G) Bilateral shortening and clinodactily of the 5th finger were evident. Note aberrant dermatoglyphics. (H) Longitudinally split nails.

which the bands with the amplified templates were excised and the DNA was subsequently purified with the help of Qiaex II Gel Extraction kit (Qiagen, Hilden, Germany). Direct automated sequencing of *EFNB1* was performed with the BigDye Terminator Cycle Sequencing kit (Applied Biosystems, Foster City, CA). All samples were analyzed on an ABI Prism 3130 Genetic Analyzer (Applied Biosystems). Wild-type and mutated *EFNB1* sequences were compared manually.

### *Fluorescence in situ hybridization (FISH)*

Cells were dropped onto slides and then immersed in 85% acetic acid for 6 min. Subsequently, slides were placed through a series of ethanol washes of 70%, 85%, and 100% for 2 min each and allowed to air dry at room temperature. Working probe solutions were prepared using a centromeric X probe (Vysis/Abbott Molecular Inc., CEP X SpectrumGreen DNA Probe) according to manufacturer's specifications. Slides were counterstained with DAPI/Antifade (Vysis/Abbott Molecular Inc., Abbott Park, IL, USA) and analyzed in an epifluorescence microscope (LEICA DMRXA2, Leica Microsystems, Wetzlar, Germany).

### *RNA isolation and EFNB1 RT-PCR and RT-qPCR*

Whole blood samples from the CFNS patient and from a healthy female donor (HFD) were obtained. Briefly, 2.5 mL of whole blood was collected in PAXGene Blood RNA tubes (PreAnalytiX, Hombrechtikon, Switzerland) and stored at room temperature for a minimum of 2 h. After, total RNA isolation was performed using the PAXgene Blood RNA Kit, in accordance with the manufacturer's instructions. RNA purity and concentration were determined using a NanoDrop 2000 Spectrophotometer (Thermo Fischer Scientific, Waltham, MA). Total RNA was reverse transcribed using the Omniscript RT Kit (Qiagen Valencia, CA, USA) as recommended by the supplier. In brief, a mix containing 50 ng/μL RNA, 1× Buffer Mix, 0.5 mM dNTPs, and 0.2 U/μL Omniscript RT was incubated for 60 min at 37 °C. The expression of *EFNB1* was investigated using primer pair 5′- AGG CCA GAG CAG GAA ATA CG -3′ and 5′- GAT CTT CAT GGT GCG TGT GC -3′ to amplify a 174 bp product encompassing exons 2 and 3 of *ENFB1*; constitutive *RPL4* expression was analyzed as assay control, using primers 5′- AGG CTC CTA TTC GAC CAG ATA -3′ and 5′-TTT GTTGGT GCA AAC ATT CG -3′ to amplify a 234 bp product. PCR was performed using KAPA2G Fast HotStart (Kapa, Biosystems, Boston, USA) as instructed by the manufacturer. PCR conditions were as follows: pre-denaturalization for 3 min at 95 °C; 38 amplification cycles for 15 s at 95 °C, 15 s at 57 °C, and 7 s at 72 °C; a final cycle of 7 min at 72 °C was used. *EFNB1* and *RPL4* products were resolved in 2% agarose gels stained with ethidium bromide. Total RNA of HeK293T cell line (kindly donated by Dr. Sergio Juarez-Mendez, National Pediatric Institute, Mexico City, Mexico) was used as positive control of *EFNB1* expression. For RT-qPCR, total RNA was subjected to expression by quantitative PCR using KAPA SYBR Fast Universal qPCR kit according to the manufacturer's recommendations. Primers were the same as described

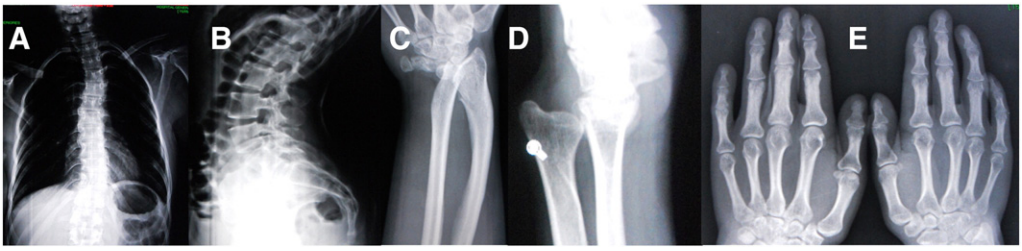


**Fig. 2.** (A) AP plain X-ray of the spine demonstrating dorsal and lumbar scoliosis. (B) Lateral plain X-ray of spine showing severe lumbar lordosis. (C) X-ray film of right forearm evidencing distal bowing of the radius. (D) AP plain X-ray film of right forearm showing radius dislocation and radial-cubital dissociation. (C) AP plain X-ray film of hands demonstrating short 4th and 5th metacarpals.

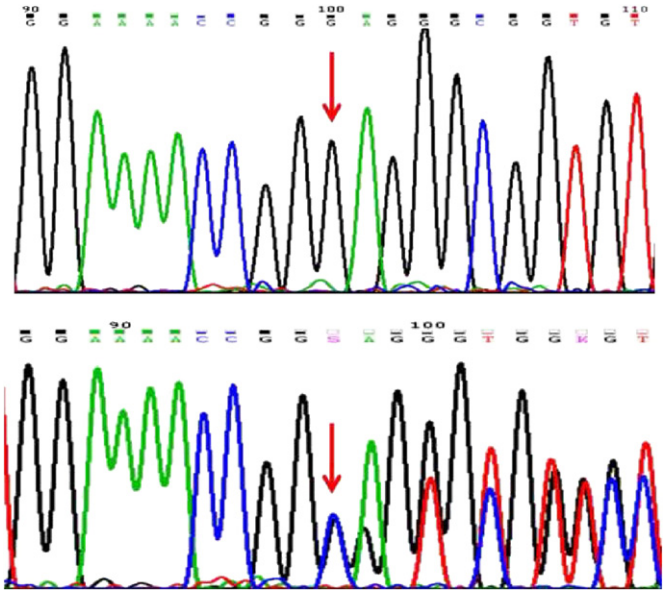

**Fig. 3.** Partial DNA sequence of *EFNB1* exon 3 from a control DNA (top) and from CFNS patient DNA (bottom). A novel frameshift c. 445_449 del GAGGG heterozygous mutation predicting a premature stop signal (E149G*fs**24), was observed in patient's DNA.

above for the *EFNB1* RT-PCR. The *OAZ1* gene (primer pairs 5′- ACG TCC AAC GAC AAG ACG AGG ATT -3 and 5′- TCA GCA CTG TTC GCC AGT TAA TGC -3 amplifying a 82 bp product) expression was used as control for normalization. Amplification was carried out in a Rotor-Gene 6000 thermocycler (Corbett Research, Sydney, Australia). Melting curves were generated for each gene to ensure the purity of the amplification product. A 10 μL aliquot of the RT-qPCR reaction was incubated for 10 min at 95 °C followed by 40 amplification cycles of 15 s at 95 °C and for 45 s at 57 °C for *EFNB1* and at 64 °C for *OAZ1*. *EFNB1* expression was determined by relative quantification, which was calculated using 2 e($-\Delta\Delta$Ct) method, where $\Delta Ct = Ct_{(EFNB1)} - Ct_{(OAZ1)}$ and $\Delta\Delta Ct = \Delta Ct_{(CFNS)} - \Delta Ct_{(control)}$ (Livak and Schmittgen, 2001).

## Results

Nucleotide analysis disclosed a novel heterozygous mutation (c.445_449del GAGGG) in exon 3 of *EFNB1*. Direct sequencing of this region showed a pattern of peak overlapping characteristic of superimposed sequences due to heterozygosity for a deletion event. This mutation originated a frame-shifting and caused a premature stop signal (TGA) 24 codons downstream (E149G*fs**24) (Fig. 3). No additional changes were detected in the remaining exons. FISH's analysis in 1000 interphase cells identified 3.2% cells with a single X, 1.3% cells with triple X, and 95.5% cells with a normal 46,XX karyotype. Standard RT-PCR expression analysis showed that *EFNB1* was expressed in blood cells of the CFNS patient (Fig. 4A). As this expression could arise from the normal allele, the assessment of the relative expression of *EFNB1* was performed by real time-quantitative RT-PCR. Compared to control female blood cells, *EFNB1* was expressed 70% less in blood cells from the CFNS patient carrying a novel *EFNB1* deletion (Fig. 4B).

## Discussion

We described a female who had severe dysmorphic features compatible with a diagnosis of CFNS and in whom a novel *EFNB1* mutation was demonstrated. She had clinical characteristics consistent with craniofrontonasal syndrome. CFNS shows a very unusual pattern of inheritance since most affected

patients are females and obligated male carriers demonstrate either a mild manifestation such as hypertelorism or no typical features at all [Wieland et al., 2005). It has been hypothesized that *EFNB1* mutations result in more severe abnormal craniofacial developments in females due to a process called cellular interference (Feldman et al., 1997; Wieacker and Wieland, 2005).

Mutations in the *EFNB1* gene have been identified in the majority of familial and sporadic CFNS patients. Currently, the mutation detection rate for the *EFNB1* gene is 92% (Wieland et al., 2005). *EFNB1* has 5 exons and almost 100 distinct mutations have been reported. The major types of molecular alteration, accounting for up to 55% of all mutations, consist of nonsense, frameshifting, and splice site mutations that lead to PTCs (Makarov et al., 2010; Wieland et al., 2008). Frameshift and nonsense mutations of exons 1–4 (as our patient's mutation) as well as splice site mutations and intragenic deletion are predicted to result in PTCs and a complete loss of *EFNB1* gene function due to nonsense-mediated RNA decay. However, this is not a strict correlation as Wieland et al. reported a deletion in exon 4 of *EFNB1* which generated a PTC, but the transcript escaped NMD (Wieland et al., 2008). Quantitative RT-PCR in blood cells from our patient carrying a novel *c.445_449delGAGGG* deletion in exon 3 showed that *EFNB1* expression level was less than half (0.3) compared with a normal control. This result strongly suggests that transcripts from the novel *c.445_449delGAGGG* truncating allele are degraded by NMD. To our knowledge, this is the first time that an *EFNB1* transcript carrying a truncating mutation in exon 3 is demonstrated to undergo degradation by NMD.

Recently, Baker et al. reported a patient with similar abnormalities to CNFS, in whom mosaicism for an additional ring X chromosome was found (Baker et al., 2010). In our patient, we failed to detect relevant sex chromosome aneuploidy as the 3% of 45,X cells detected by FISH falls within the normal range for age (Guttenbach et al., 1995).

In conclusion, we present a patient with craniofrontonasal syndrome due to a novel *EFNB1* frameshift mutation (*c.445_449delGAGGG*) and add to the expression analysis of truncating *EFNB1* mutations.

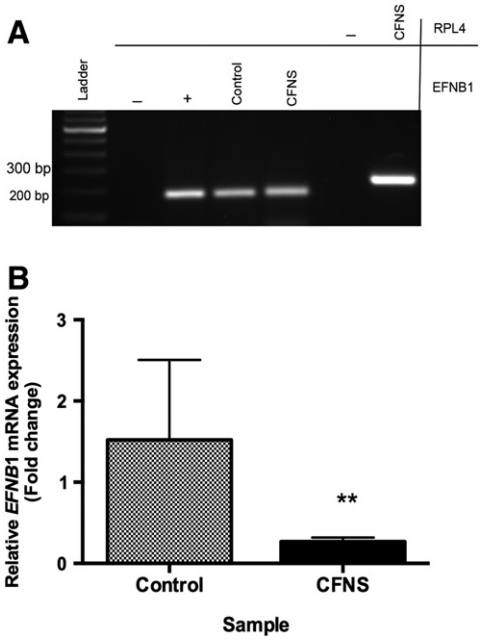


**Fig. 4.** *EFNB1* expression analysis in a CFNS patient carrying a novel gene deletion. A) Expression of *EFNB1* mRNA in the CFNS patient and in a healthy female donor (control) showed gene expression by standard RT-PCR. RPL4 is the RT-PCR "housekeeping" control; (−) = Negative control (+) = Positive control. B) Quantitative RT-PCR revealed a significant decrease in EFNB1 expression level in CFNS compared to the control (confidence interval: $p < 0.0049$, asterisked *). The reduction is about 70% compared to control blood cells.

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