Journal of Human Genetics (2019) 64:867–873
https://doi.org/10.1038/s10038-019-0638-9

ARTICLE

Four novel mutations in *EFNB1* in Indian patients with craniofrontonasal syndrome

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Received: 18 February 2019 / Accepted: 21 June 2019 / Published online: 8 July 2019

Abstract

Craniofrontonasal syndrome (CFNS) (OMIM #304110) is a very rare, X-linked developmental disorder characterized by facial stigmata, including hypertelorism, frontonasal dysplasia, craniosynostosis, bifid nasal tip, and digital abnormalities. CFNS is caused by mutations in the Ephrin 1 gene (*EFNB1*) located at Xq13.1, which encodes the transmembrane protein Ephrin B1. Interestingly, heterozygous females are more severely affected than hemizygous males. We report on four individuals from four unrelated Indian families with mild-to-severe CFNS. All patients had variable degrees of hypertelorism and nasal bridge depression, which did not correlate with changes in other tissues. Although patients 3 and 4 showed the most severe facial dysmorphism and syndactyly, there were no structural CNS changes or developmental delay. In contrast, patient 1 displayed agenesis of corpus callosum and developmental delay, although facial and finger abnormalities were milder. Patients 1, 2, and 4 showed different degrees of clefting. DNA sequencing revealed four previously undescribed heterozygous mutations in exons 1 and 2 of *EFNB1*. Patient 1 carried the second single amino acid deletion reported up to date. The other three affected individuals harbored frameshift mutations, leading to premature termination codons. Our findings broaden the spectrum of *EFNB1* mutations and illustrate the absence of an obvious correlation between mutation type, severity, and expression of symptoms.

Introduction

Craniofrontonasal syndrome (CFNS) (OMIM #304110) has first been described in 1979 by Cohen [1]. It is an X-linked disorder caused by mutations in the Ephrin B1 gene *(EFNB1)*, which is located on the short arm of the X chromosome (Xp13.1) [2]. The paucity of male CFNS patients has been described in the literature [3]. It is known that heterozygous females are more severely affected than hemizygous males [4–6]. In females, characteristic abnormalities include severe hypertelorism, depressed nasal bridge, and coronal synostosis. Occasionally, cleft lip and palate, diaphragmatic hernias, and corpus callosum agenesis or dysgenesis are present. Hemizygous males show no or mild signs, such as hypertelorism. Longitudinal ridging of the nails, syn- or poly-dactyly, and wiry hair commonly occur in CFNS [2, 5–7].

Supplementary information The online version of this article (https://doi.org/10.1038/s10038-019-0638-9) contains supplementary material, which is available to authorized users.

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EFNB1 codes for Ephrin B1, which is a transmembrane protein of 346 amino acids (ENST00000204961.4). Ephrin B1 binds to Eph receptor kinases [8]. Eph/ephrin complexes play crucial roles in neural development and plasticity, as well as morphogenesis through formation of boundaries [9, 10]. Twigg et al. [11] proved that the *Efnb1* expression is particularly high in murine neural crest cells. While the complete loss of *Efnb1* in mice is perinatally lethal, female *Efnb1*$^{+/-}$ mutants exhibit dysmorphic features characteristic of CNFS, which are absent in hemizygous males [12]. This phenomenon might be explained by difficulties establishing boundaries in the mosaic state arising from X inactivation intermingling *EFNB1*-negative and *EFNB1*-positive cells [13]. In hemizygous males, the function of EFNB1 might be taken over by a related ephrin.

To date, a total of 116 mutations in *EFNB1* have been described, the majority of which are missense mutations, followed by the occurrence of small deletions and splice-site mutations. Here, we report on four additional patients from four different families who carry four novel *EFNB1* mutations and demonstrate clinical variability in CFNS.

Materials and methods

Sanger sequencing

Written informed consent of the patients was acquired prior to genetic testing. DNA was obtained from the whole-blood samples. The five exons and exon–intron boundaries of *EFNB1* (NM_004429) were amplified using oligonucleotide primers. Primer pairs were 5′-AGAAGAGCGACAC CGAAGC-3′ and 5′- AGACCTCCCCACATGCACT-3′ yielding a 379-bp product for exon 1, 5′- CCTGAGGCTG ACCATCTTCT-3′ and 5′- GTTAAGCCCAGGGAGAG AGC-3′ resulting in a 357-bp product for exon 2, 5′-TGGG AGTTTCTGGGTAATGC-3′ and 5′-CTGTTCCAAAGGT CAAACAGG-3′ yielding a 223-bp product for exon 3, 5′-A TGACTGAGGGCACCTATGC-3′ and 5′-GGGCCTAAC AAGGTGACAGA-3′ yielding a 250-bp product for exon 4, and 5′-GCCTGAAATCTGCTGTGTGT-3′ and 5′-AA ATACAAAGGTGGGCACAG-3′ yielding a 585-bp product for exon 5. The PCR products were sequenced on an ABI3730xl DNA Analyzer (Applied Biosystems).

Mutation analysis

The sequencing data were compared with the *EFNB1* cDNA reference sequence GenBank accession number NM_004429 and analyzed using the software Geneious [14]. Further in silico analysis of the mutations was performed by the prediction tools MutationTaster [15] and Human Splicing Finder [16].

Ephrin ectodomain 3D model

The ectodomain structure of the closely related Ephrin B2 (1iko) was uploaded to the CCP4 software [17, 18]. The residues Ile63, Cys64, and Cys101 were projected as ball-and-stick structures onto the ribbon-type protein model.

Results

Clinical presentation

The clinical findings of the four unrelated female patients are summarized in Table 1 and the corresponding images are shown in Figs. 1 and 2. All patients described in this paper are the first children of Indian origin born to non-consanguineous parents, who did not show any signs of CFNS.

Patient 1 was referred for dysmorphic evaluation and developmental delay. Hypothyroidism was diagnosed in the newborn period and treated with 25 µg thyroxine. On examination at the age of 6 months, she presented with a weight of 5.5 kg (<3rd centile), length of 64 cm (3rd centile), and head circumference of 39.5 cm (<3rd centile). Head control was attained. She had a coarse face, a hoarse voice, a small anterior fontanel, significant hypertelorism, bilateral epicanthic folds, bilateral low-set ears, macrostomia, a tongue tie, a thick upper midline frenulum, a microform cleft upper lip, and a short neck. She had an extremely flat nasal bridge, anteverted nares, and a midline crease of the tip of the nose (Fig. 1a). Brachydactyly and hyperelasticity of finger joints were evident (Fig. 2a). The inspection of the feet revealed a bilateral splintering of the big toes (Fig. 2b, c) and a medially deviated third toe with bilateral undertoeing (Fig. 2c). Magnetic resonance imaging (MRI) showed agenesis of corpus callosum.

Patient 2 was born at term by normal delivery (birth weight 3.85 kg (75−90th centile)) and started to crawl at 5 months of age. Clinical examination demonstrated a head circumference of 39 cm (3rd centile), a small anterior fontanel, a coarse face, hypertelorism, downslanting palpebral fissures, brachycephaly, a small cleft of the upper lip, low-set overfolded pinna, a high-arched palate, and a short neck (Fig. 1b). No interdigital webbing or abnormalities of fingers and toes were present. A brain MRI at the age of 5 months revealed a corpus callosum (callosal) dysgenesis. She had a unicoronal synostosis (anterior plagiocephaly) and underwent fronto-orbital advancement and hypertelorism correction. At 2 ½ years of age, she had a weight of 10 kg (<3rd centile), height 85 cm (3rd centile), and head circumference of 44 cm (<3rd centile). She could walk without support but had unclear speech.

Table 1 Clinical features and genotype of four individuals with craniofrontonasal syndrome

	Patient 1, female	Patient 2, female	Patient 3, female	Patient 4, female
Age at examination	5 months	2 years 6 months	4 years 6 months	16 years
Consanguinity	No	No	No	No
EFNB1 mutation screening				
DNA level	c.186_188delCAT	c.404_405insTACATTAC	c.196_197insC	c.43_43delG
Protein level	p.Ile63del	p.Ser136Thrfs*26	p.Arg66Profs*9	p.Ala15Argfs*31
Exon (E); zygosity	E 2; heterozygous	E 2; heterozygous	E 2; heterozygous	E 1; heterozygous
Clinical manifestations (HPO terms)				
Hypertelorism (HP:0000316)	++	++	+++	+++
Epicanthus (HP:0000286)	Yes	No	No	No
Downslanting palpebral fissures (HP:0000494)	+	+	++	++
Anteverted nares (HP:0000463)	Yes	Yes	No	No
Depressed nasal bridge (HP:0005280)	Yes	Yes	Yes	Yes
Midline nasal groove (HP:0004112)	Yes	Yes	Yes	Yes
Abnormality of the pinna (HP:0000377)	No	Yes	No	No
Low-set ears (HP:0000369)	Yes	Yes	Yes	Yes
Coarse facial features (HP:0000280)	Yes	Yes	No	No
Midface retrusion (HP:0011800)	No	No	Yes	No
Micrognathia (HP:0000347)	Yes	Yes	Yes	Yes
High palate (HP:0000218)	No	Yes	Yes	Yes
Anterior open bite (HP:0200095)	No	No	Yes	Yes
Cleft upper lip (HP:0000204)	Yes	Yes	No	Yes
Bilateral cleft lip and palate (HP:0002744)	No	No	No	No
Ankyloglossia (HP:0010296)	No	No	No	No
Hoarse voice (HP:0001609)	Yes	No	No	No
Short neck (HP:0000470)	Yes	Yes	Yes	Yes
Small anterior fontanel (HP:0000237)	Yes	Yes	No	No
Dysgenesis of corpus callosum (HP:0006996)	No	Yes	No	No
Agenesis of corpus callosum (HP:0001274)	Yes	No	No	No
Plagiocephaly (HP:0001357)	Yes	Yes	Yes	No
Craniostenosis (HP:0001363)	Yes	Yes	Yes	Yes
Global developmental delay (HP:0001263)	Yes	No	No	No
Brachydactyly (HP:0001156)	Yes	No	Yes	No
Broad thumb (HP:0011304)	No	No	Yes	No
3,4 syndactyly (HP:0006097)	No	No	Yes	Yes
Partial duplication of the distal phalanx of the fourth finger (HP:0009981)	No	No	No	Yes
Longitudinal ridging of toe nails (HP:001807)	Yes	Yes	Yes	Yes
Longitudinal ridging of finger nails (HP:001807)	No	No	Yes	Yes
Shoulder girdle muscle atrophy (HP:0003724)	No	No	Yes	Yes
Limited Shoulder movement (HP:0006467)	No	No	Yes	Yes
Low-set nipples (HP:0002562)	No	No	Yes	Yes

Patient 3 had an unremarkable family history. She showed normal development and intelligence. On examination at the age of 4 ½ years, her height was measured at 98 cm (3rd centile) and the head circumference at 48 cm (<3rd centile). Examination of the face revealed plagiocephaly and severe hypertelorism, a broad nasal root and tip, antimongoloid slant of the eyes, prognathism, and an anterior open bite (Fig. 1c). Cranial imaging showed unilateral left-sided coronal craniostenosis. She underwent fronto-orbital advancement for correction of unilateral coronal synostosis and facial bipartition for correction of hypertelorism. The neck was short and showed webbing,

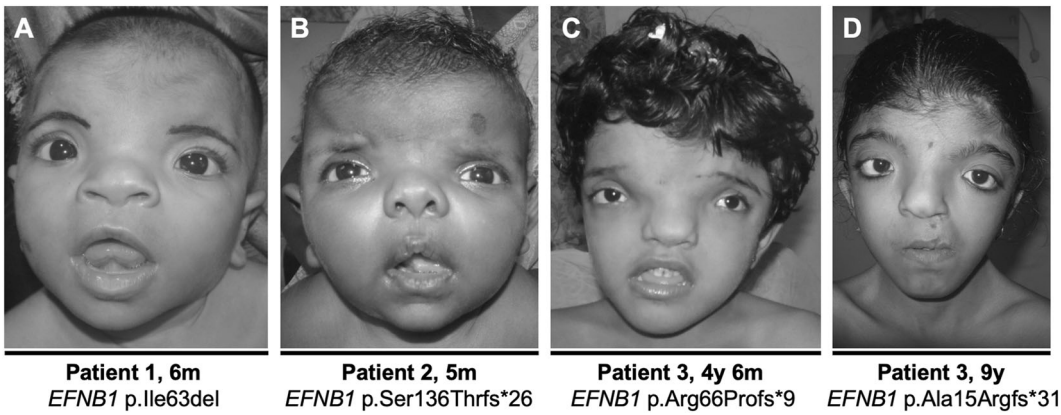

Fig. 1 Variable craniofacial features of four female CFNS patients. All patients present with hypertelorism, depressed nasal bridge, and low-set ears. Patient 1 (**a**) and patient 2 (**b**) present with anteverted nares, patient 2 additionally with a high palate and a cleft upper lip. Patient 3 (**c**) and patient 4 (**d**) show downslanting palpebral fissures. Patient 3 additionally presents with midface retrusion, anterior open bite, and short neck, patient 4 with cleft upper lip and orbital dystopia

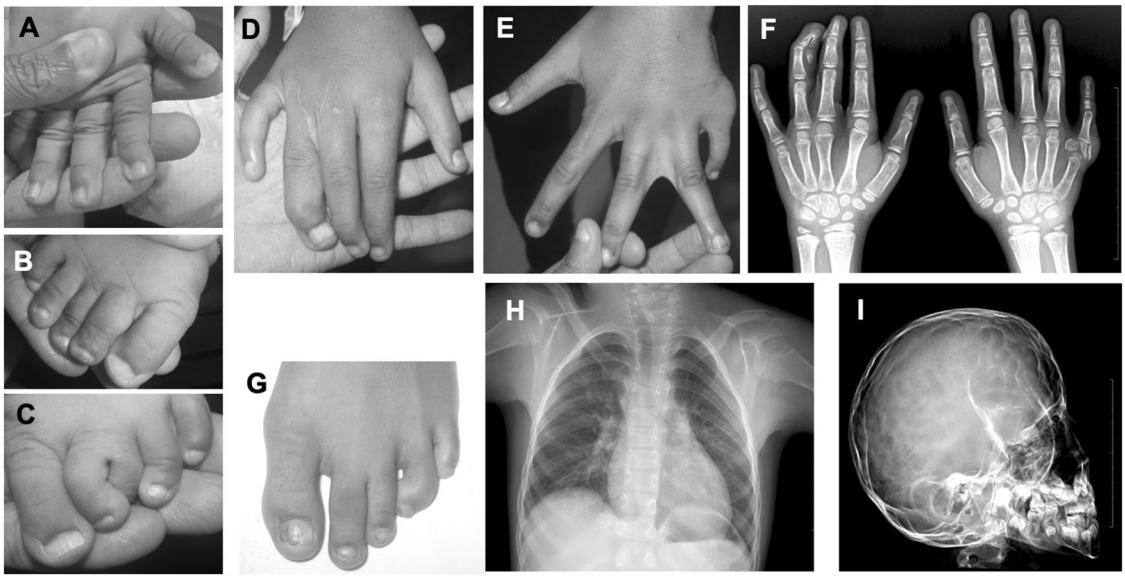

Fig. 2 Additional skeletal findings. Upper and lower extremities of patient 1 (**a–c**) and patient 4 (**d–h**). Patient 1 presents with **a** brachydactyly and a broad thumb, **b** bilateral undertoeing, and **c** a medially deviated third toe of the left foot. Patient 2 presents with **d** complete III–IV syndactyly of the right hand (operated) with fused nails at the fourth finger and **e** partial cutaneous III/IV syndactyly of the left hand. **g** Longitudinal ridging of the left big toe. **f** Radiographs revealed an osseous duplication of the middle and distal phalanges of the fourth finger of the left hand, **h** high ridging scapula with elongated clavicles, and **i** lateral view of skull shows severe craniostenosis with copper beaten appearance

the chest was flattened, and the nipples were low placed. The patient was unable to elevate the shoulders completely, with prominent wasting of infraclavicular muscles. Dysmorphic features of the hands included a complete III/IV syndactyly of the left hand and a partial cutaneous syndactyly of the fingers 2–5 on the right hand. Longitudinal ridging of big toes and other toes was detected and significantly more distinct on the left side.

Patient 4 presented with a broad nasal root and tip, downslanting palpebral fissures, severe hypertelorism, and anterior open bite with normal intelligence (Fig. 1d). She had a webbed neck, dropping of shoulders, pectus excavatum, and low-lying and asymmetrical nipples. The inspection of the extremities revealed complete cutaneous III–IV syndactyly of the right hand, which was surgically treated (Fig. 2d), partial cutaneous III/IV syndactyly of the left hand (Fig. 2e), and longitudinal ridging of third fingers of both hands. Longitudinal ridging of the big toes and other toes was also evident and more distinct on the left side (Fig. 2g). Radiographs revealed an osseous duplication of middle and distal phalanges of the fourth finger of the right hand (Fig. 2f). She had left postal axial polydactyly, which

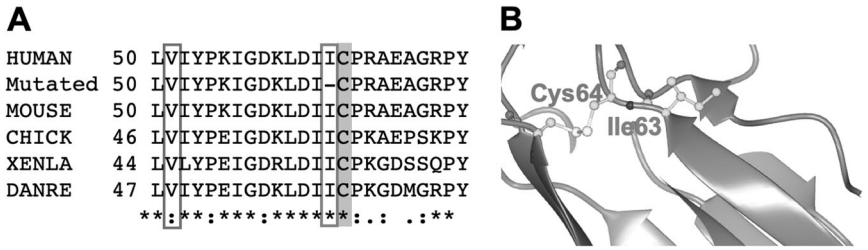

Fig. 3 Consequences of the p.Ile63del mutation. **a** Alignment of the p.Ile63del mutation (red box) identified in patient 1 and the previously found single amino acid deletion p.Val51del (blue box). The adjacent Cys64 cysteine residue is marked in orange. Note the high evolutionary conservation of the sequence harboring both mutations. **b** A 3D model of the EFNB1 ectodomain showing the secondary structure as ribbon and side chains of the amino acids Ile63 and Cys64 as ball and stick (green = carbon, red = oxygen, and blue = nitrogen). The deleted Ile63 lies at the end of a β-sheet (gray arrow) and the adjacent cysteine forms an essential disulfide bridge (yellow), which—by prediciton—will get lost as a consequence of the deletion

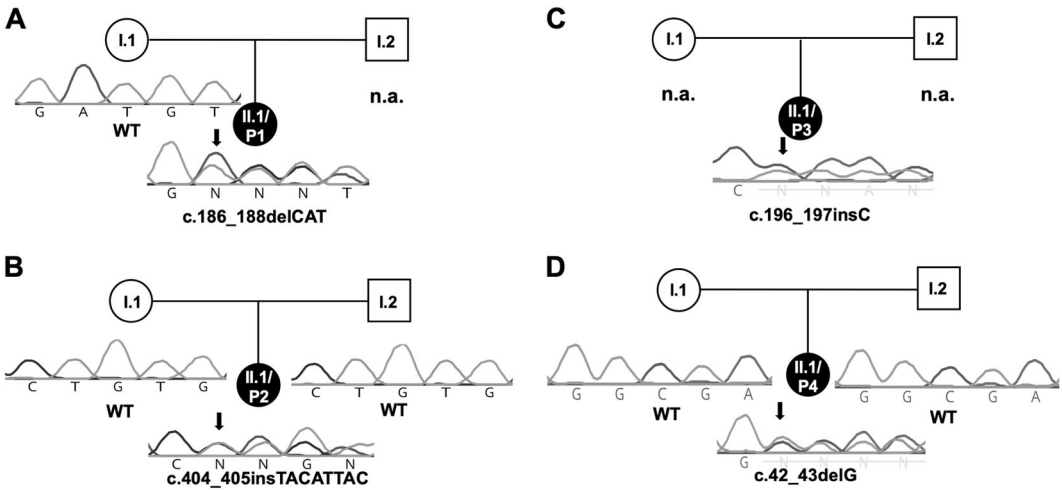

Fig. 4 Pedigrees and segregation of mutations found in CFNS patients. Sanger validation and segregation of the *EFNB1* mutations c.186_188delCAT in patient 1 (**a**), c.404_405insTACATTAC (p.S136Tfs*26) patient 2 (**b**), c.196_197insC (p.Arg66Profs*9) in patient 3 (**c**), and c.42_43delG (p.Ala15Argfs*31) in patient 4 (**d**)

was excised, and left-sided cleft lip and palate, which was surgically repaired during infancy. She had a large fossa ovalis, an atrial septal defect which was corrected by device closure at 9 ½ years of age. The patient also had a high-ridging scapula with elongated clavicles (Fig. 2h) and severe craniostenosis with copper-beaten appearance (Fig. 2i). Bicoronal synostoses with brachycephaly were evident and she underwent hypertelorism correction by facial bipartition technique and maxillary distraction.

In all four cases, CFNS was the suspected diagnosis, but for patient 2, the differential diagnosis of Teebi-type hypertelorism (OMIM #145420) was also considered.

Mutation analysis

Since the clinical findings were suggestive of CFNS, we amplified *EFNB1* by PCR and analyzed the gene by Sanger sequencing. This revealed four heterozygous mutations in exons 1 and 2 of *EFNB1* in the index patients, which were not listed in ExAC, gnomAD, or the 1000 genomes project. Investigation of the available parent DNAs revealed wild-type sequences. An overview of the Sanger sequencing and segregation testing is shown in Fig. 4, and bioinformatic pathogenicity predictions are summarized in Supplementary Table 1.

The heterozygous mutation c.186_188delCAT (p.Ile63del) in exon 2 detected in **patient 1** affects a highly conserved amino acid in the extracellular ephrin domain next to a cysteine residue forming a disulfide bridge (Fig. 3a, b). The mutation was confirmed by Sanger sequencing, but could not be detected in the index' mother (Fig. 4a). DNA of the father was not available. The variant is ranked pathogenic due to high amino acid conservation, but no splice alterations are predicted (ACMG class: likely pathogenic).

Patient 2 carries the mutation *EFNB1* c.404_405insTACATTAC (p.Ser136Thrfs*26) (Fig. 4b). In silico analysis by the Human Splicing Finder indicates a possible alteration of splicing (ACMG class: pathogenic). In **patient 3**, the mutation *EFNB1* c.196_197insC

(p.Arg66Profs*9) was detected (Fig. 4c). No DNA from other family members was available. According to in silico analysis with the Human Splicing Finder, the original exonic splicing enhancer site is altered or broken in the setting of the mutation, most likely causing an alteration of splicing (ACMG class: likely pathogenic). The mutation c.42_43delG (p.Ala15Argfs*31) was found in **patient 4** (Fig. 4d). Sequencing of the parents revealed a wild-type sequence. The activation of an exonic cryptic donor splice site is predicted by the Human Splicing Finder (ACMG class: pathogenic).

Discussion

In this study, we describe four female individuals from four different Indian families with characteristic stigmata of CFNS. By a targeted screening approach through Sanger sequencing, we found four novel disease-causing mutations in the *EFNB1* gene, three of them are frameshift mutations and one is a small deletion.

All mutations described were neither annotated in ExAC nor in the 1000 genomes project. The heterozygous mutation *EFNB1* c.186_188delCAT (p.Ile63del) in patient 1 is located in the highly conserved extracellular ephrin domain, which is crucial for receptor ligand recognition and complex formation [19]. This mutation is the only one of our series, for which no significant effect on splicing is predicted. The only single amino acid deletion reported so far is c.151_153delGTG (p.Val51del) described by Twigg et al. [3], which is in the vicinity of p.Ile63del. The two female carriers of this mutation exclusively revealed typical facial features and coronal craniosynostosis. The phenotype of patient 1 is considerably more severe, especially due to corpus callosum agenesis and developmental delay. In how far the latter might also be attributable to hypothyroidism is currently unknown. The strong effect of the p.Ile63del mutation is surprising, since it deletes one of the two consecutive isoleucine residues. We hypothesize that due to this deletion, the cysteine at position 64 loses its ability to form a disulfide bridge with cysteine 101, which is a strong stabilizer of the tertiary protein structure. The variant most similar to c.404_405insTACATTAC (p.Ser136Thrfs*26) found in patient 2 is c.296_297delTCinsGGTGCTCG (p.Thr100Valfs*62) reported by Inoue et al. [20]. The affected patient had hypertelorism, depressed nasal bridge, bifid nasal tip, bicoronal synostosis, and bilateral cleft lip and palate. No mutations similar to c.196_197insC (p.Arg66-Profs*9) and c.42_43delG (p.Ala15Argfs*31) detected in patients 3 and 4, respectively, have been reported to date.

Mutations with premature termination codons (PTCs) are known to cause nonsense-mediated mRNA decay (NMD) or lead to severely truncated, instable proteins. In some cases, an escape of NMD is possible. We predict that the frameshift mutations described in patients 2, 3, and 4 lead to alternative splicing and result in NMD, with an overall loss-of-function effect. The mutations p.Val51del and p.Ile63del demonstrate how sensitive the Ephrin B1 protein is toward alterations of such kind and support the prediction that even if alternative splicing leads to in-frame products, the resulting larger deletions will also have a loss-of-function effect. Chacon-Camacho et al. [21] analyzed the truncating mutation EFNB1 c.445_449delGAGGG in exon 3 at the expression level and detected a severe decrease in the expression level of EFNB1 mRNA, which confirmed the degradation by NMD. Since no patient RNA was available for our studies, we could not perform investigations on the RNA level.

The facial changes in patients 3 and 4 are more pronounced than in patients 1 and 2. On the other hand, patients 1 and 2 had corpus callosum anomalies. An explanation for the phenotype variability could be the random X inactivation in females, which has been described in CFNS patients [22]. In heterozygous females, the X inactivation causes a somatic mosaicism, in which cells with defective *EFNB1* on their active X chromosome are functionally *EFNB1* null mutants. In this mosaic pattern, a higher share of mutated cells brings about a more severe phenotype and can thus explain the range in CFNS severity. Carrel and Willard [23] showed that the escape of X inactivation could contribute to the interindividual phenotypic differences of heterozygous females.

Some syndromes caused by mutations in different genes have overlapping features with CFNS. Three types of frontonasal dysplasia (FND) are caused by mutations in different genes. Type 1 (FND1, OMIM #136760) is caused by mutations in *ALX3*, Type 2 by mutations in *ALX4* (FND2, OMIM #613451), and Type 3 by mutations in *ALX1* (FND3, OMIM #613456) [24]. Acrofacial dysostosis (AFD1, OMIM #154400), also known as Nager syndrome, is caused by mutations in *SF3B4*. In addition to craniofacial features similar to CFNS, they have characteristic upper-extremity deformities, but no lower-extremity involvement [25]. Acromelic frontonasal dysostosis (OMIM #603671) is another rare syndrome similar to CFNS caused by heterozygous mutations in *ZSWIM6*. It comprises craniofacial malformations similar to CFNS (e.g., FND and corpus callosum agenesis), as well as nonfacial traits, e.g., brain and limb malformations (tibial hemimelia and preaxial polydactyly) [26].

In conclusion, CFNS can be accounted as part of a spectrum disorder of craniofacial and limb anomalies. Our findings broaden the spectrum of *EFNB1* mutations and demonstrate that there is no obvious genotype–phenotype correlation, since the deletion of a single N-terminal amino acid has the same effect as mutations leading to PTCs.

Acknowledgements We would like to acknowledge Prof. Raoul C. Hennekam, Department of Pediatrics, AMC, The Netherlands) for providing the expert opinion on the phenotypic evaluation of the index cases. We are grateful to the patients and family members, whose cooperation made this study possible. This study was supported by the Berlin Institute of Health.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Web resources: The URLs for data presented herein are as follows: Ensembl Genome Browser, http://www.emsembl.org. Mutation Taster, http://www.mutationtaster.org/. Online Mendelian Inheritance in Man (OMIM), http://www.ncbi.nlm.nih.gov/omim. Human Splicing Finder, http://www.umd.be/HSF3/HSF.shtml. Geneious, https://www.geneious.com/. Protein Data Bank in Europe, https://www.ebi.ac.uk/pdbe/entry/pdb/1iko

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