CLINICAL REPORT

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Vertical Transmission of a Frontonasal Phenotype Caused by a Novel *ALX4* Mutation

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Frontonasal dysplasias (FND) comprise a spectrum of disorders caused by abnormal median facial development. Its etiology is still poorly understood but recently frontonasal dysplasia phenotypes were linked to loss-of-function mutations in the *ALX* homeobox gene family, which comprises the *ALX1*, *ALX3*, and *ALX4* genes. All ALX-related frontonasal phenotypes till date had been compatible with an autosomal recessive mode of inheritance. In contrast, heterozygous loss-of-function mutations in *ALX4* had been only associated with isolated symmetrical parietal ossification defects at the intersection of the sagittal and lambdoid sutures, known as enlarged parietal foramina. We report a family with vertical transmission from mother to son of mild frontonasal dysplasia phenotype caused by a novel *ALX4* gene mutation (c.1080-1089_delGACCCGGTGCinsCTAAGATC TCAACAGAGATGGCAACT, p.Asp326fsX21).This is the first report of a frontonasal phenotype related to a heterozygous mutation in *ALX4*. This mutation is predicted to cause the loss of the aristaless domain in the C-terminal region of the protein and preserves the homeodomain. We speculate that a different mechanism, a dominant-negative effect, is responsible for the distinct phenotype in this family. © 2013 Wiley Periodicals, Inc.

Key words: ALX4; parietal foramina; frontonasal dysplasia; dysmorphology

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INTRODUCTION

Frontonasal dysplasias (FND) comprise a spectrum of heterogeneous disorders caused by abnormal median facial development. The presence of two or more of the following clinical findings could define FND: True ocular hypertelorism, broadening of the nasal root, median facial cleft affecting the nose and/or upper lip and palate, unilateral or bilateral clefting of the alae nasi, lack of formation of the nasal tip, anterior cranium bifidum occultum, and a V-shaped or widow's peak frontal hairline [Sedano and Gorlin, 1988]. Additional characteristics are responsible for a myriad of other disorders within the FND spectrum, such as craniofrontonasal dysplasia (OMIM 304110), oculoauriculofrontonasal syndrome (OMIM 601452), frontofacionasal dysplasia (OMIM 229400), cerebrofrontofacial syndrome (OMIM 608578), acromelic frontonasal dysostosis (OMIM 603671), acrofrontofacionasal dysostosis 1 (OMIM 201180) and 2 (OMIM 239710), among others.

The majority of these cases are sporadic and their etiology is generally poorly understood. A genetic cause (*EFNB1* gene mutations) has been identified in X-linked craniofrontonasal dysplasia [Wieland et al., 2004]. Recently, patients with autosomal recessive FND were linked to homozygous loss-of-function mutations in the *ALX* homeobox gene family, which comprises *ALX1*, *ALX3*, and *ALX4* genes [Twigg et al., 2009; Kayserili et al., 2009; Uz et al., 2010]. This gene family belongs to the Paired-class homeoproteins and plays an important role in the complex processes of cranial development as well as neural tube closure and limb development [McGonnell et al., 2011]. Biallelic mutations in *ALX4* were identified in a patient with severe FND with additional findings of alopecia, coronal craniosynostosis, hypogonadism, and intellectual disability, named ALX4-related FrontoNasal Dysplasia with Alopecia and Genital abnormality phenotype (ALX4-related FNDAG) [Kayserili et al., 2009]. More recently, an attenuated FND phenotype, with enlarged parietal foramina and abnormalities of the corpus callosum and cerebellum was described in a boy with a homozygous mutation in *ALX4* [Kayserili et al., 2012].

Conflict of interest: None.

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In contrast, heterozygous loss-of-function mutations in *ALX4* were originally associated with isolated symmetrical parietal ossification defects at the intersection of the sagittal and lambdoid sutures, known as enlarged parietal foramina, without facial features characteristic of FND [Wuyts et al., 2000]. We report on the first vertical transmission of a mild FND phenotype caused by a novel heterozygous *ALX4* gene mutation.

CLINICAL REPORT

The proband, the second child of non-consanguineous parents, whose mother presented with arterial hypertension during pregnancy, was born preterm by cesarean with a birth weight of 2,650 g and length of 45 cm. He developed jaundice and required phototherapy. He evolved with normal developmental milestones. Because of bilateral cryptorchidism, the patient underwent orchidopexy at the age of 4 years, without success. He underwent two additional surgical procedures, but his left testis was considered dysgenetic. He has used corrective lenses for hyperopia since the age of 8 years.

He was referred to the Genetics Unit because of craniofacial dysmorphisms and cryptorchidism. He was evaluated at the age of 10 years and 4 months showing normal anthropometric measurements (W: 38.5 kg ~75th centile; H: 136 cm ~25th centile, and OFC: 53.5 cm ~50th centile); craniofacial features comprised frontal alopecia, sparse eyebrows, widely spaced eyes (ICD = 4 cm, OCD = 8.8 cm, IPD = 6.4 cm), telecanthus, lack of formation of the nasal tip, broad and elongated columella (Fig. 1A,E,F); undescended left testis, and broad thumbs (Fig. 1G).

His mother shared a similar pattern of facial features, including sparse eyebrows, widely spaced eyes, telecanthus (ICD = 3.7 cm, OCD = 8.5 cm, IPD = 6.1 cm), median cleft at the tip of the nose, and a broad and elongated columella (Fig. 1C).

Complementary exams in the proband, including abdominal ultrasound, echocardiogram, audiologic, and ophthalmologic evaluations (fundoscopy and slip-lamp exam), were normal. His cranial X-ray, as well as his mother's, displayed forme fruste parietal foramina (Fig. 1B,D).

The probands sister and a female maternal cousin (Fig. 2A) were normal upon physical exam and cranial X-rays.

We concluded that the craniofacial features presented by this family were suggestive of FND and once mutations in *ALX4* gene have been recently described in patients with a mild phenotype, this specific gene was selected to be sequenced.

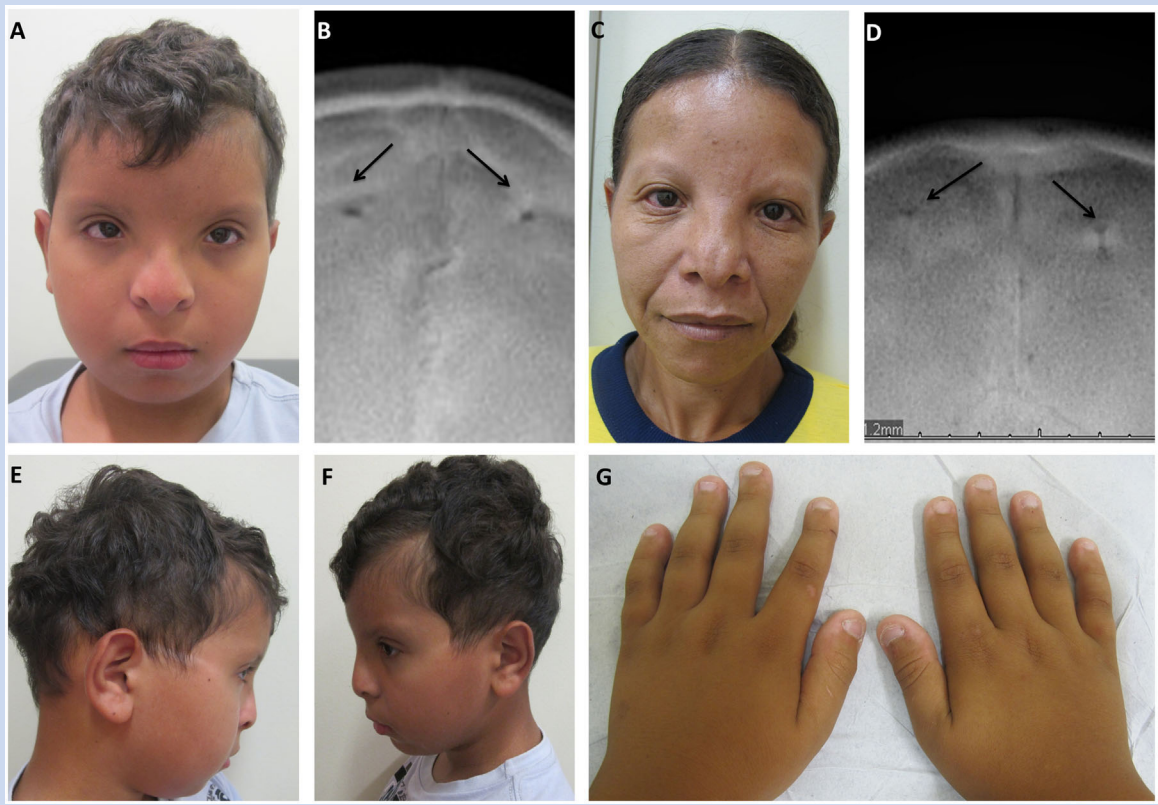

FIG. 1. Frontal view of the proband (A) and his mother (C): Note the sparse eyebrows, widely spaced eyes, telecanthus, broad nasal bridge, lack of the tip of their nose, with broad, elongated columella, and frontal alopecia in the proband (E,F). B,D: Cranial X-ray of the proband (B) and his mother (D) showing parietal foramina (dark arrows). G: Note broad thumbs in the probands hands. [Color figure can be seen in the online version of this article, available at http://wileyonlinelibrary.com/journal/ajmga]

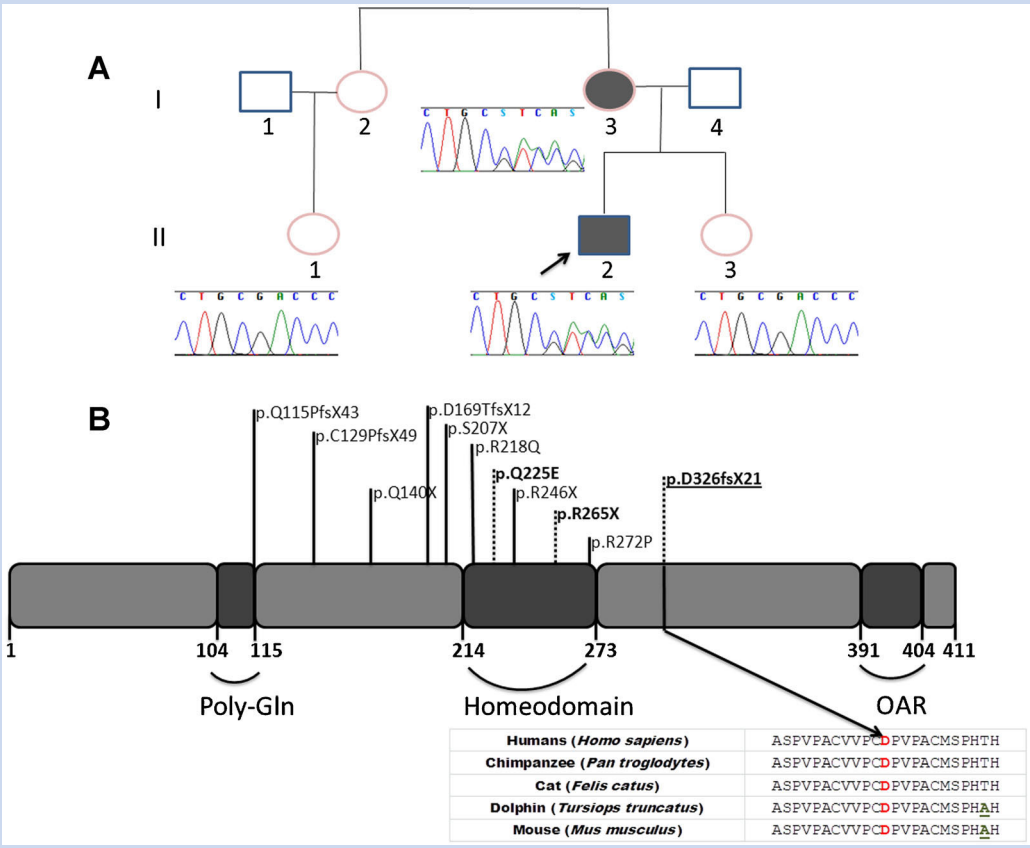

FIG. 2. A: Family pedigree: Observe that only the proband (II-2) and his mother (I-3) harbor the frameshift mutation in *ALX4*. B: The *ALX4* gene product showing the distribution of the mutations according to the localizations of the protein domains: p.Asn326fsX21, in bold and underlined, is the mutation described here; p.Gln225Glu and p.265X, in bold, are the mutations described in the literature related to a frontonasal involvement in homozygosity; the other mutations shown are related to parietal foramina [Wuyts et al., 2000; Mavrogiannis et al., 2001; Gentile et al., 2004; Mavrogiannis et al., 2006]. The arrow depicts the conservation of the region harboring the mutation here described among different species. [Color figure can be seen in the online version of this article, available at http://wileyonlinelibrary.com/journal/ajmga]

MATERIALS AND METHODS

For DNA sequencing analysis, we amplified the four *ALX4* exons of the proband and his mother as well as those of his sister and a female maternal cousin. The pair of primers used were described elsewhere [Mavrogiannis et al., 2001]. Bi-directional sequencing was performed using the BigDye Terminator v3.1 Cycle Sequencing kit, (Applied Biosystems, Carlsbad, CA) on the ABI PRISM® 3037 DNA Analyzer (Applied Biosystems) sequencer.

RESULTS

Exon sequencing of *ALX4* disclosed a novel heterozygous mutation carried by the proband and his mother: p.Asp326fsX21 (c.1080-1089_delGACCCGGTGCinsCTAAGATCTCAACAGAG ATGGCAACT; cDNA reference: NM_021926.3; protein reference: NP_068745.2). The analysis in the proband's sister and cousin yielded normal results (Fig. 2A).

DISCUSSION

We have presented the first FND phenotype associated with a heterozygous mutation in the *ALX4*. This illustrates the phenotypic heterogeneity of ALX4-related phenotypes, ranging from severe frontonasal involvement associated with homozygous loss-of-function mutations to enlarged parietal foramina caused by haploinsufficiency. The phenotype expressed by our family is placed in the middle of this spectrum.

The facial features observed in this group of patients are the main clinical finding. Kayserili et al. [2012] pointed out that the nasal configuration with notched alae nasi was shared by all the three described patients and this dysmorphic feature could be used as a good clinical discriminator between *ALX4* group and the other *ALX* gene phenotypes. Nevertheless, the mother and son reported here lacked the notched alae nasi, but shared a broad and elongated columella, providing a different gestalt (Fig. 1A,C). Thus, the facial features, within the spectrum of frontonasal dysplasia, are more diverse than previously thought and further clinical reports will be

important to fully discover the full range of phenotypes associated with *ALX4* mutations.

Genital anomalies are a frequent finding and restricted to males, although the mother of the proband has shown normal reproductive capacity. Three out of four male patients with a FND phenotype, including the proband described here, presented bilateral cryptorchidism [Kayserili et al., 2009]. The follow-up of these affected individuals would be important to determine if the gonadal abnormality will interfere in their ability to bear children.

Ectodermal involvement is a variable characteristic in patients with FND. It ranges from universal alopecia, described in the two original patients [Kayserili et al., 2009], to normal hair pattern [Kayserili et al., 2012]. The proband reported here had frontal alopecia and sparse eyebrows (Fig. 1A,E,F). The latter was also observed in the mother (Fig. 1C). These findings indicate an essential role of *ALX4* in the development of skin structures, such as the hair follicle [Kayserili et al., 2009].

Another point of interest is the presence of calvarial bone defects. It is known that enlarged parietal foramina are caused by heterozygous loss-of-function mutations in either *ALX4* or *MSX2*. Typically, they present as bilateral oval or round openings in the parietal bones on either sides of the posterior sagittal suture. When the bone defect extends between the anterior and posterior fontanelles, it is termed cranium bifidum. They are anatomically distinct from the normal minute foramina that transmit anastomotic vessels [Mavrogiannis et al., 2006]. Patients with the autosomal recessive FND phenotype have also shown the spectrum of cranium bifidum/enlarged parietal foramina. Interestingly, three parents of probands, heterozygous for the mutations, were also evaluated for this specific abnormality and only one of them showed smaller enlarged parietal foramina. Two others showed a normal parietal foramina, also observed in the mother–son described here (Fig. 1B,D). This could be explained by an incomplete penetrance of this trait, already observed on several patients carrying heterozygous mutations that do not show the bone abnormalities on their X-rays [Mavrogiannis et al., 2006].

The genetic background underlying the phenotype in the family reported here is distinct from the previous case reports. *ALX4* has two different isoforms, which may be transcribed from two different translation initiation sites, and encodes a homeodomain transcription factor required for many developmental processes. Identifiable motifs include a poly Pro/Gln sequence encoded by exon 1, a homeodomain DNA-binding region encoded by exons 2 and 3 and an aristaless/OAR domain encoded by exon 4 [Mavrogiannis et al., 2001].

All the three patients with ALX4-related FNDAG phenotype reported in the literature showed mutations in a homozygous status, severely impairing the protein function. Two of them exhibited the same mutation (p.Arg265X), and were from nearby cities in the Black Sea region of Turkey, suggesting a founder effect. The p.Arg265X mutation, located on helix III of the homeodomain (Fig. 2B) that is known to interact with DNA major groove, probably disrupts part of this highly conserved domain and causes the complete loss of the C-terminal paired tail domain. The authors also demonstrated that the protein was mislocalized to the cytoplasm, giving further support to a loss-of-function mechanism [Kayserili et al., 2009].

A milder ALX4-related FNDAG phenotype was described in a patient presenting another homozygous missense mutation (p.Gln225Glu) on the helix I of the homeodomain (Fig. 2B). The fact that in silico studies showed that the mutation could interfere in the protein folding and that there are other mutations in the same highly conserved Gln12 residue of other Paired-class homeodomain coding genes, reported as causing different disorders, support the pathogenicity of the mutation described here. Furthermore, the authors speculated that a residual ALX4 activity could be responsible for the milder phenotype of this patient compared to the two previously ones [Kayserili et al., 2012].

On the other hand, heterozygous mutations either in the homeodomain or in the N-terminal region adjacent to the homeodomain (Fig. 2B), causing protein haploinsufficiency, were only associated with the presence of enlarged parietal foramina [Wuyts et al., 2000; Mavrogiannis et al., 2001; Gentile et al., 2004; Mavrogiannis et al., 2006].

The family reported here has a heterozygous frameshift mutation in the C-terminal region adjacent to the homeodomain (p.Asp326fsX21), a site not overlapping with the ones in enlarged parietal foramina. This mutation was considered pathogenic: It predicts a premature stop-codon, the region is highly conserved among different taxa, it segregates only in the affected individuals within this family, and it predicts the loss of the OAR domain (Fig. 2B).

The OAR or aristaless domain is conserved in a subset of the large class of Paired-class homeoproteins [Brouwer et al., 2003]. Its function in ALX4 has never been addressed. Studies on the function of the OAR domain in other Paired-class homeoproteins, such as ALX1 and ARX have shown different activities: The deletion of the aristaless domain in ALX1 unleashes its teratogenic potential, functioning to restrain activity of this transcription factor in vivo, partially or completely through its effect on DNA binding. The same study showed that this pattern was not shared by ALX3 [Brouwer et al., 2003]. On the other hand, McKenzie et al., [2007] demonstrated that the transcription repression activity of ARX is modulated by two strong repression domains and one activator domain, the aristaless domain. The authors of these studies suggested that each transcriptional factor could have distinct forms that may be activated or repressed. Until the precise role of the OAR in ALX4 is understood, we speculate that the loss of this OAR domain with the maintenance of the homeodomain in the allele carrying the mutation in the family reported here will generate a product that is not subject to nonsense mediated decay (NMD), as the mutation is located in the last exon of the gene. In mammalian cells, this mechanism of RNA degradation requires at least one intron downstream of a premature termination codon in order for the transcript to be targeted for NMD [Inoue et al., 2004]. Therefore, the generated product in the present family could interfere with the normal allele, in a dominant-negative manner. This distinct mechanism could explain the differences observed in the family when compared to the other reported cases, once triggering or escaping NMD is known to cause different phenotypes, besides contributing to a variable phenotypic expressivity [Inoue et al., 2004]. On the other hand, residual function of the normal *ALX4* allele

could be an explanation for preventing the present patients from developing the ALX4-related FNDAG, the severe end of the ALX4-spectrum disorders, originally described only in patients with homozygous loss-of-function mutations.

In summary, we have described two related individuals with a heterozygous mutation in *ALX4* presenting a distinct phenotype of FND that may be placed in the middle of the severity scale of the ALX4-spectrum disorders. We suggest that the loss of the ALX4 OAR domain with the maintenance of the homeodomain impairs the function of the normal allele in a dominant-negative effect, explaining the increased severity when compared to patients with heterozygous mutations. This report strengths the argument that mild FND phenotypes could be caused by *ALX4* gene mutations, contributing to uncover the etiology of this heterogeneous group of disorders.

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