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BRIEF COMMUNICATION

Exome sequencing revealed a novel nonsense variant in *ALX3* gene underlying frontorhiny

Asmat Ullah[1] · Muhammad Umair[1] · Umm e-Kalsoom[1] · Shaheen Shahzad[2] · Sulman Basit[3] · Wasim Ahmad[1]

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Abstract

Frontorhiny is one of the two forms of mid-facial malformations characterized by ocular hypertelorism, wide and short nasal ridge, bifid nasal tip, broad columella, widely separated nares, long and wide philtrum and V-shaped hairline. Sometimes these phenotypes are associated with ptosis and midline dermoid cysts. Frontorhiny inherits in an autosomal recessive pattern. Sequence variants in the Aristaless-like homeobox 3 (*ALX3*) gene underlying frontorhiny have been reported previously. Here, in the present study, we have investigated four patients in a consanguineous family of Pakistani origin segregating frontorhiny in autosomal recessive manner. Genome scan using 250k Nsp1 array followed by exome and Sanger sequence analysis revealed a novel homozygous nonsense variant (c.604C>T, p.Gln202*) in the *ALX3* gene resulting in frontorhiny in the family. This is the first mutation in the *ALX3* gene, underlying frontorhiny, in Pakistani population.

Introduction

Frontonasal dysplasia (FND1; MIM 136760) is a heterogeneous group of facial malformations. Clinical features associated with FND are limited to face and head. It is clinically diagnosed on the basis of the presence of at least two of the features including ocular hypertelorism, median facial cleft, broad nasal bridge, missing or underdeveloped nasal tip, widened philtrum, median cleft upper lip, and widow's peak frontal hairline [1, 2]. Most of the reported cases of FND are sporadic or resulted from the effect of teratogens, but few familial cases have been reported as well. In familial cases of FND, the disease segregates in autosomal recessive fashion. Three forms of FND have been clinically characterized. *ALX1*-related FND has been characterized by anomalies of face and head associated with mild mental retardation [3, 4]. *ALX3*-related FND, also known as frontorhiny, is less severe form and is characterized by the presence of prominent philtral ridge [5]. Patients with *ALX4*-related FND shows skull defects associated with alopecia and genital anomalies [6, 7]. Up till now, two sequence variants in *ALX1*, seven in *ALX3*, and 22 in *ALX4* gene have been reported in families of different ethnic origins segregating FND [3–8]. Loss of function variants in the *ALX1* cause more severe phenotypes than *ALX3*- and *ALX4*-related FNDs [3, 4]. Mouse genes *Alx1, Alx3*, and *Alx4* show expression in the facial mesenchyme [9, 10]. Dee et al. [11] reported that *Alx1* in zebra fish regulates migration of cranial neural crest cells into the frontonasal primordia. Disruption of genes controlling migration of cranial neural crest cells into the frontonasal primordia result in the frontofacionasal dysplasia, characterized by ocular and midface defects with the midline facial cleft.

In the present study, we have presented first case of frontorhiny diagnosed in Pakistani population. On the basis of the pedigree information, inheritance pattern of the disease was predicted to be autosomal recessive. SNP microarray and exome sequencing were performed to search for disease causing gene in the family.

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✉ Wasim Ahmad
wahmad@qau.edu.pk

[1] Department of Biochemistry, Faculty of Biological Sciences, Quaid-i-Azam University Islamabad, Islamabad, Pakistan

[2] Department of Biotechnology, International Islamic University, Islamabad, Pakistan

[3] Center for Genetics and Inherited Diseases, Taibah University Al Madinah, Al Munawarah, Saudi Arabia

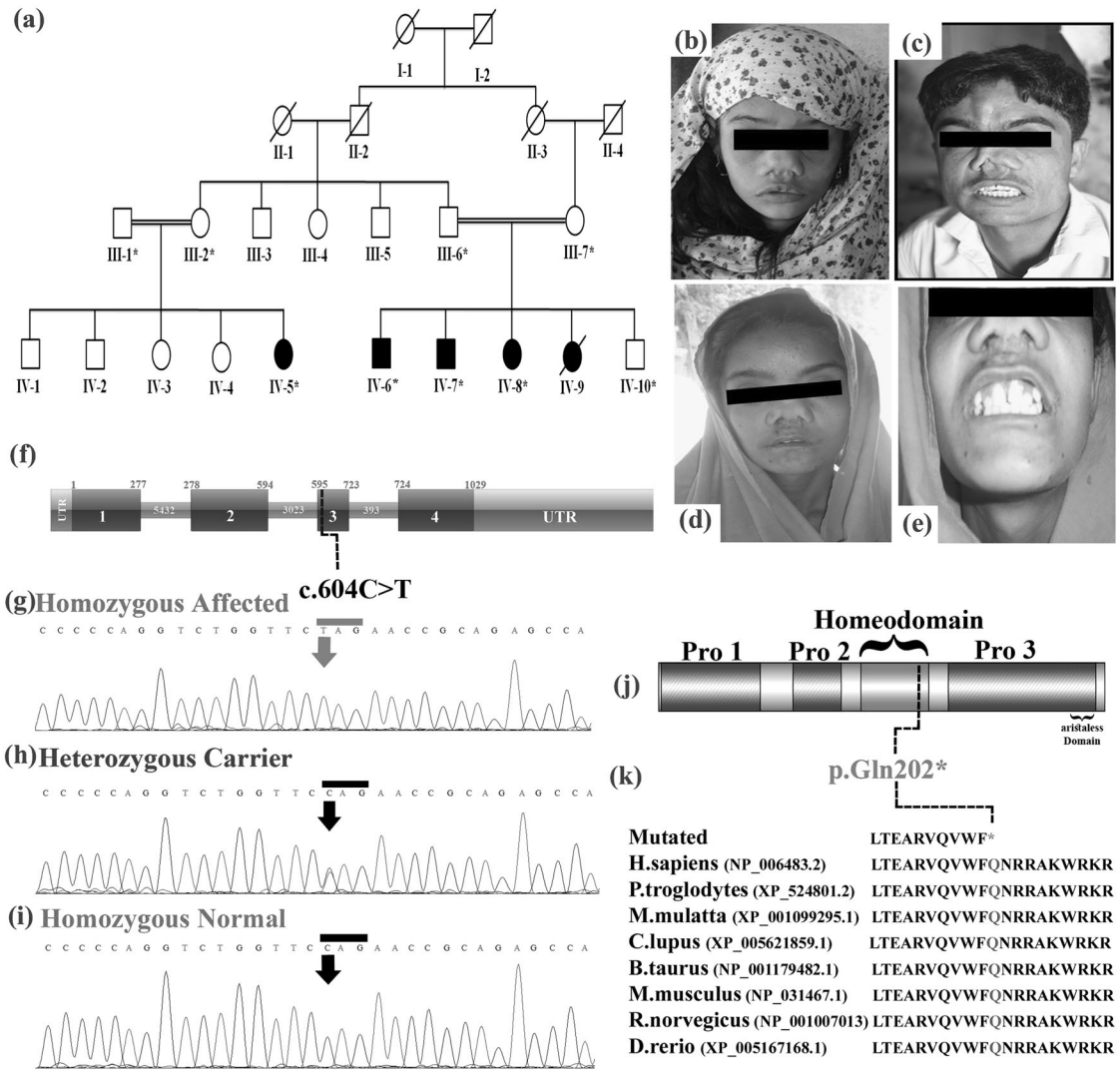

Fig. 1 **a** Pedigree of a consanguineous Pakistani family segregating frontorhiny in autosomal recessive manner. Asterisk above squares and circles represent individuals who were available to participate in the study. **b–e** Clinical features of affected individuals (IV-5, IV-6, IV-8) showing ocular hypertelorism, broad nasal bridge, broad bifid nasal tip, widened prominent philtral ridge, and crowded teeth. **f** Diagrammatic presentation of the *ALX3* gene with UTRs, exons, and introns. A nonsense variant in exon 3, found in our study, is pointed out. **g–i** Sequence results of exon 3 of *ALX3*. Point of variation is represented by an arrow. **j** Domains of human *ALX3*. **k** Conservation of residue glutamine202 among different species

Materials and Methods

Ethical approval and blood collection

Approval to conduct the study was obtained from Institutional Review Board of Quaid-i-Azam University Islamabad, Pakistan. A consanguineous family of Pakistani origin with five affected individuals, segregating frontorhiny, was visited at its residence, in a small town in Sindh province. Family elders and all other participating individuals were briefed about objectives and benefits of the study. Written informed consents for blood collection and publication of photographs were obtained from participating individuals. An informative pedigree was drawn with the information provided by elders of the family (Fig. 1a). For DNA extraction, 5 ml blood from both unaffected and affected individuals was collected in EDTA containing tubes.

DNA extraction and Genome-wide homozygosity mapping

DNA was extracted from blood collected from five normal (III-1, III-2, III-6, III-7, IV-10) and four affected individuals (IV-5, IV-6, IV-7, IV-8) using the standard phenol-chloroform method. DNA of two normal (III-1, III-2) and three affected individuals (IV-5, IV-6, IV-8) was processed for genome-wide homozygosity mapping using the Affymetrix GeneChip Genome-Wide Human SNP 250 K array

(Affymetrix, Santa Clara, CA). Genomic DNA of affected and unaffected individuals was digested with restriction enzyme (NspI). PCR amplification using single primer set was performed after DNA-ligation to the adaptor. Amplified products were purified, quantified, fragmented, and labeled stepwise. Amplicons labeled with magnetic beads were hybridized to the 250 K SNP mapping array. SNP genotypes were determined using BRLMM algorithm incorporated in Affymetrix Genotyping Console. Homozygous regions shared by all the three affected individuals were determined using Homozygosity Mapper (http://www.homozygositymapper.org/) [12].

Exome sequencing

For exome sequencing, DNA of an affected individual (IV-6) was prepared using instructions provided by Agilent SureSelect Target Enrichment Kit. Illumina HiSeq 2000/2500 sequencer was used for sequencing. Reads were analyzed using the Burrows–Wheeler Aligner (BWA) Enrichment application of Base Space (Illumina Inc. 5200 Illumina Way San Diego, CA, 92122, USA). Alignment was performed with BWA [13] and the variants were called with Genome Analysis Toolkit (GATK) [14]. All the called Variants were annotated with Illumina Variant Studio v2.2. On the basis of the prediction of autosomal recessive form of disease, exome data was filtered for homozygous variants. All the suspected homozygous variants were searched in available databases of human genetic variations.

Sanger sequencing

To verify segregation of the variant (c.604 C>T), exon 3 of the *ALX3* was sequenced in DNA of available normal and affected individuals in the family. Primers for amplification of exon 3 of the *ALX3* (forward: 5′-CATCCTGGAG CCTGCTCC-3′; reverse: 5′-GTCATACAGAACGCTGAC GCT-3′) were designed using Primer-3 software. PCR conditions to amplify exon 3 of the *ALX3*, purification of PCR-amplified products and Sanger sequencing were performed, as described previously Ullah et al. [15]. Pathogenicity of identified variant was checked using MutationTaster (http://www.mutationtaster.org/).

Results

Clinical features

Four affected individuals (IV-5, IV-6, IV-7, IV-8) were diagnosed with frontorhiny. They showed ocular hypertelorism, broad nasal bridge, absent nasal tip, widened philtrum, and crowded teeth (Fig. 1b–e). Affected individuals were mentally normal but were able to participate in daily life activities. Parents of the affected individuals were phenotypically normal.

Genome-wide homozygosity mapping and exome sequencing

Analysis of SNP genotyping results using Homozygosity Mapper (http://www.homozygositymapper.org) [12] identified single-homozygous region on chromosome 1p13.3–p13.2 (110,091,271–112,269,872 bp) flanked by SNPs rs530565 and rs3738298 in affected individuals (IV-5, IV-6, IV-8).

Exome sequencing results were analyzed carefully. Steps for variants prioritization and number of variants obtained at each step are listed in Table S1. Total 124 biallelic variants were found in the homozygous region on chromosome 1p13.3–p13.2. Variants with frequency >0.01 in human genetic variation databases (1000 Genome, dbSNP, ExAc, gnomAD) were filtered out. Analysis of the variants led to identification of a homozygous nonsense sequence variant (c.604C>T, p.Gln202*) in the *ALX3* gene located on chromosome 1p13.3. Other exonic sequence variants with frequency <0.01 were not found in the homozygous region (1p13.3–p13.2). As sequence variants in the *ALX3* gene was earlier reported in causing FND phenotypes, therefore the variant (c.604C>T), identified in our family, was further analyzed for segregation in the family. Analysis validated correct segregation of the variant with disease phenotype in the family. The variant was not found in 1000 Genomes, Exome Aggregation Consortium (ExAc) and 150 ethnically matched control individuals. Mutation prediction tool "MutationTaster" predicted the identified variant as disease causing. The mutant residue glutamine, at amino acid position 202 (p.Gln202*), was found conserved among various species (Fig. 1k).

Discussion

Frontonasal dysplasia is a human developmental anomaly affecting head and face. In the present study, we have investigated a large consanguineous family with five individuals segregating frontonasal dysplasia in autosomal recessive pattern. Clinical characteristics of affected individuals including ocular hypertelorism, broad nasal bridge, absent nasal tip, and widened philtrum are the same as reported previously by Twigg et al. 5] However, cleft palate, ptosis and strabismus reported by these authors were not observed in our family. Affected individual (IV-8) in our family had shown crowded teeth (Fig. 1e), a feature not reported by Twigg et al. 5] Recently, Ullah et al. 4] reported another Pakistani family segregating frontonasal dysplasia, which resulted due to a recessive variant in the *ALX1* gene.

Affected individuals in this family showed severe abnormalities of widened philtrum, bifid nasal tip, opened nostrils, broad nasal bridge, ocular hypertelorism, misshaped eyebrows, and protrusion of teeth.

Following SNP-based homozygosity mapping to map the disease gene on chromosome 1p13.3–p13.2, exome sequencing led to the identification of a novel nonsense sequence variant (p.Gln202*) in the *ALX3* gene. This is predicted to result in loss of function of the ALX3 protein either through nonsense-mediated mRNA decay or resulting in production of truncated ALX3 protein, subsequently affecting signaling pathways in ALX3 target cells.

The *ALX3* gene encodes 343-amino acids long homeodomain protein and acts as a transcriptional regulator. ALX3 contains three proline-rich domains (Pro1, Pro 2, Pro 3) and a homeodomain (Fig. 1j). Pro 1 and Pro 2 are located at amino-terminus and Pro 3 at carboxyl-terminus of the protein. The variant (p.Gln202*) was found located in homeodomain (Fig. 1j) predicting complete loss of function of ALX3. Interruption of homeodomain disturb binding of ALX3 to target DNA 16]. The ALX3 is expressed in neural crest-derived mesenchyme of developing craniofacial regions and in the mesenchyme of developing limbs 17]. Single knock out of *Alx3* in mice did not show any skeletal abnormality, but *Alx3/Alx4* double mutant mice have shown severe craniofacial malformations 18]. In humans, recessive variants in the *ALX3* caused craniofacial deformities termed as frontorhiny [5, 19].

Up till now, seven sequence variants in the *ALX3* gene causing frontorhiny in families of different ethnic origins (Morocco, Ireland, Algeria, Netherland, Turkey, India) have been reported 5]. However, clinical features resulting from sequence variants in the *ALX3* gene are less severe as compared with those resulting from loss of function variants in the *ALX1* gene, which cause slightly different condition known as frontonasal dysplasia [3, 4]. It is important to note that the sequence variant (p.Gln202*) in the *ALX3* gene resulting in frontorhiny, identified in the family presented here, is the first mutation reported in the Pakistani population. This study will be helpful in genetic counseling and prenatal testing for families with history of similar anomalies in the Pakistani population.

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Compliance with Ethical Standards

Conflict of interest The authors declare no conflict of interest.

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