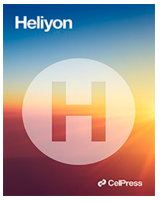
Heliyon 10 (2024) e34929

Contents lists available at ScienceDirect

Heliyon

journal homepage: www.cell.com/heliyon



Case report

A rare homozygous ALX4 mutation in a Bangladeshi girl with frontonasal dysplasia type-2 (FND2)

Barna Goswami [a,1], Asifur Rahman [c,**,1], Iffat Jahan [a], Shahina Akter [a], Tanjina Akhtar Banu [a], Eshrar Osman [b], Mohammad Samir Uzzaman [b], Ahashan Habib [a], Md Shamsul Alam [c], Abu Saleh Mohammad Abu Obaida [c], Md Murshed Hasan Sarkar [a], Salim Khan [a,*]

[a] Bangladesh Council of Scientific and Industrial Research, Dhaka, Bangladesh
[b] SciTech Consulting and Solutions, Dhaka, Bangladesh
[c] Bangabandhu Sheikh Mujib Medical University, Bangladesh

ARTICLE INFO

Keywords:
Frontonasal dysplasia type 2 (FND2)
Whole genome sequencing (WGS)
ALX4
Computed tomography (CT)
Magnetic resonance imaging (MRI)
Online Mendelian Inheritance in man (OMIM)

ABSTRACT

Background: Frontonasal dysplasia type-2(FND2), a rare phenotypically variable and heterogeneous developmental anomaly resulting from mutation of the ALX4 gene, is primarily characterized by malformation of the skull and facial skeleton. This study was designed to showcase a clinical, imaging, and genetic analysis of FND2 in a consanguineous family of Bangladeshi origin.
Methodology: Clinical imaging and whole genome sequencing of mother, father and patient was done by using Nextera DNA flex library preparation kit (Illumina, USA) using Novaseq 6000 next generation sequencer to find out ALX4 mutation which causes FND2 in patient.
Result: We report the clinical as well as molecular findings in an 8-year-old girl with FND2. The child presented with various characteristic features of skull and facial anomalies associated with FND 2 along with numerous other features many of which have not been described in previous literature. The parents also showed some key clinical, radiological, and genetic features of FND 2. The whole genome sequencing (WGS) revealed homozygosity for a 793C-T transition in the ALX4 gene, which resulted in premature termination at codon 265 (p.Arg265Ter). Both of her parents were heterozygous carriers of ALX4 mutation.
Conclusions: This is the first report that associates clinical, imaging, and genomics analyses in a Bangladeshi patient for better understanding the disease FND2. These results will facilitate diagnosis and genetic counseling of the future FND2 patients.

* Corresponding author.
** Corresponding author.
E-mail addresses: barnagdu@gmail.com (B. Goswami), bijoun007@bsmmu.edu.bd (A. Rahman), iffatjahan57@gmail.com (I. Jahan), shupty2010@gmail.com (S. Akter), tanzinabcsir@yahoo.com (T.A. Banu), eshrarosman@gmail.com (E. Osman), samiruzzaman@gmail.com (M.S. Uzzaman), ahashan73@yahoo.com (A. Habib), dr_shamsul@hotmail.com (M.S. Alam), dr_abu_obaida@yahoo.com (A.S. Mohammad Abu Obaida), murshed_mbdu@yahoo.com (M.M. Hasan Sarkar), k2salim@yahoo.com (S. Khan).
[1] These authors contributed equally: Barna Goswami and Asifur Rahman.

https://doi.org/10.1016/j.heliyon.2024.e34929
Received 15 January 2024; Received in revised form 16 July 2024; Accepted 18 July 2024
Available online 20 July 2024

1. Introduction

Frontonasal dysplasia (FND) is a rare yet wide spectrum of developmental anomalies having distinctive features of the skull, face, and brain. Based on the genes responsible, displaying more or less distinctive phenotypes, FND has been classified into the FND1, FND2, and FND3 caused by ALX3, ALX4, and ALX1 genes respectively. Among many other features, alopecia, hypertelorism, anomalous nasal bone causing flat nose with occasional notched alai nasi, genital abnormalities, craniosynostosis, parietal foramina and various brain malformations characterize FND2 [1,2].

ALX4 belongs to the family of aristaless-like homeobox genes, having 2 other members, the ALX1 and ALX3 genes. This particular homeobox family is characterized by a paired type homeodomain and a conserved C-terminal paired tail domain. All the genes of this family encode transcription factors alike in the neural crest-derived mesenchymal cells during embryogenesis. ALX4 particularly plays a vital role in the development of craniofacial development, skin, and hair follicles in humans [1,3].

The emergence of Next Generation Sequencing (NGS) technology radically changed the diagnostic workflow by providing a quick, powerful, and low-cost alternative for genetic analysis in the early stages of the process. On the other hand, Whole-Genome Sequencing (WGS) for rare diseases has enabled doctors to diagnose genetic diseases quickly, and families to avoid long diagnostic odysseys and dilemmas. Of all genomic testing methods, WGS is the most precise one and offers the highest odds of finding a diagnosis. It provides the highest coverage of the human genome, not only in regions not covered by other methods [4–6]. This increased coverage at first-line usage has been shown to reduce the need for unnecessary iterative tests and reduce the length of stay in the NICU [7,8].

In this report, we provide a detailed clinical, imaging and gene analysis of a girl with FND2. Whole genome sequencing of the girl and her parents revealed a novel homozygous stopgained variant (p.Arg265Ter) of the ALX4 gene in affected child from both the parents who are heterozygous carriers.

1.1. Case presentation

The proband, born by lower uterine caesarian section (LUCS) to consanguineous parents at full term, came to us at the age of 8 years, with two soft swellings, one each on both parietal regions with skull bone gap. The swellings were present as small reddish areas on the scalp at birth, which increased in size noticeably with intermittent CSF leak for 8 months prior to presentation. She had facial deformity, skull gap, lack of scalp hair, eyebrows or eyelashes, and an extra digit in her right foot since birth. Owing to financial constraints, the parents could not afford any treatment until they came to us and the baby grew up with some delayed milestones of development. The baby had head injury twice, early in her life.

The mother conceived once more at 5 years of age of the proband and had to have a medical abortion at about 5½ months of gestation for intrauterine death (IUD). Prenatal ultrasonography (USG) at that time revealed an anomalous foetus with a large complex cystic swelling in the occipital region with a large skull gap.

Initially, she had computed tomography (CT) and USG of the head early in her life for the head injuries that she had. Later, when she came to us, we investigated her thoroughly, the findings of which are given in the result section.

We repaired both the parietal meningoceles in the same sitting. After about a month of repair, one of the surgical sites started to leak and a Lumbo-peritoneal (LP) shunt was given, as ventriculoperitoneal (VP) shunt was deemed risky considering her anomalous skull, dura, and brain condition. She did well for the next 1 and ½ years when she started to have headaches and enlargement of the repaired encephaloceles. Unfortunately, because of the COVID-19 pandemic, she could not come and started to have leaking from one of the encephaloceles. When the lockdown was over, she came to us again and we revised the LP shunt, which unfortunately over drained and the baby was having sunken flap syndrome. So, we removed the LP shunt out. However, within 2 days of removal of the LP shunt, the swellings came back and started to leak again. We put an external ventricular drainage (EVD) through one of the meningoencephalocele swellings with an IV cannula which worked well and the leaking stopped and the headache subsided. She developed meningitis following EVD, had several episodes of seizure and became unconscious which improved a little with medical management. As we were planning and waiting to put a VP shunt even with risk, she deteriorated gradually and ultimately succumbed to her illness.

2. Material and methods

This study was approved by the Ethical Review Committee of Bangabandhu Sheikh Mujib Medical University (BSMMU), Bangladesh. Informed written consent, to conduct the study, was obtained from the family members. The girl was clinically examined at BSMMU hospital. Whole genome sequencing (WGS) was performed for research purposes at the Genomic Research Laboratory, Bangladesh Council of Scientific and Industrial Research (BCSIR), Bangladesh.

2.1. Clinical examination and investigations

A detailed family history and pre and postnatal history were taken. A thorough examination and relevant investigations like CT scan and MRI of the head, Magnetic resonance imaging (MRI) screening of the whole spine, USG of the whole abdomen, Electrocardiogram (ECG) and Echocardiogram, X-ray of the skull, chest and hands and feet, routine blood pictures and biochemical analysis were carried out when she came to us. Karyotyping of the proband and WGS of the proband and her both parents were carried out.

3. Genomic analysis

3.1. Isolation of genomic DNA

Peripheral blood of the family members was collected in blood collection tube contain K_3EDTA. Genomic DNA were extracted by using Maxwell RSC whole blood DNA extraction kit (Promega) according to the manufacturer's instructions in Maxwell automatic extractor (Promega) was used as DNA extractor. The gDNA were quantified with Quantus Fluorometer (Promega).

3.2. Library preparation and next-generation sequencing

A trio whole genome sequencing (WGS) analysis of genomic DNA of mother, father and patient was done by using Nextera DNA flex library preparation kit (Illumina, USA) following the instruction recommended by the provider. Sequencing was carried out using NovaSeq 6000 (Illumina, USA) Sequencing System. The sequencing data was analyzed using a BaseSpace Sequence Hub (Illumina, Inc.). Raw sequence reads were processed and aligned against the human NCBI GRCh38 reference genome assembly using the BWA software. To analyze data generated from targeted sequencing, the BaseSpace Variant Interpreter software (Illumina), the BaseSpace Interpreter (Illumina), Sorting Intolerant From Tolerant (SIFT) http://sift.bii.a-star.edu.sg/) [9], Polymorphism Phenotyping v2 (PolyPhen-2) (http://genetics.bwh.harvard.edu/pph2/) [10],ClinVar (https://www.ncbi.nlm.nih.gov/clinvar/) [11], The Human Gene Mutation Database (http://www.hgmd.cf.ac.uk/ac/index.php) [12], Leiden Open Variation Databases (LOVD) (http://www.lovd.nl/3.0/home), and the Database for Annotation, Visualization and Integrated Discovery (DAVID) (https://david.ncifcrf.gov/) were implemented in the fully detailed bioinformatics pipeline.

4. Results

Clinical and imaging features: There was an array of clinical features unique to FND2 in this patient. Moreover, she had a lot more features that had not been described in the literature before that were revealed clinically and through investigations. The baby was delivered through LUCS at full term. She showed many of her features since birth. Although she had delayed milestones of development, she was otherwise an intelligent girl when she came to us. Clinically hypertelorism, telecanthus, up slanting palpebral fissures, short palpebral fissures, blepharophimosis, strabismus, nystagmus, and epicanthal fold were evident. CT scan of the head and X-ray skull demonstrated increased distance between orbits (Fig. 1 A & 2 B). Manifestations of nasal anomalies included bifid nasal tip, depressed nasal tip, broad nasal bridge, broad Nasal ridge, depressed nasal bridge, depressed nasal ridge, wide nasal root, cleft alae nasi, short and broad columella, and broad philtrum (Fig. 1 A). She had thick and everted lips, narrow palate, posterior medial palatal fissure, high arched palate, and prominent median palatal raphe (Fig. 1 B). She had low-set ears (Fig. 1D and E). The only anomaly of her limbs was preaxial polydactyly in her right foot which was evident in her X-ray of the right foot (Figs. 1C & 2 K). Her hair-related anomalies were sparse scalp hair, sparse eyelashes, absent eyebrows, and sparse body hair (Fig. 1A–D, E & F). Ultrasonogram revealed hypoplastic and polycystic ovary. She had a brachycephalic head. (Fig. 2 A & D). CT scan of the head and lateral X-ray skull showed cranium bifidum (Fig. 2A–C, D & I), craniosynostosis (Fig. 2 A & D), prominent forehead with frontal bossing (Fig. 2 A, B, D), frontal

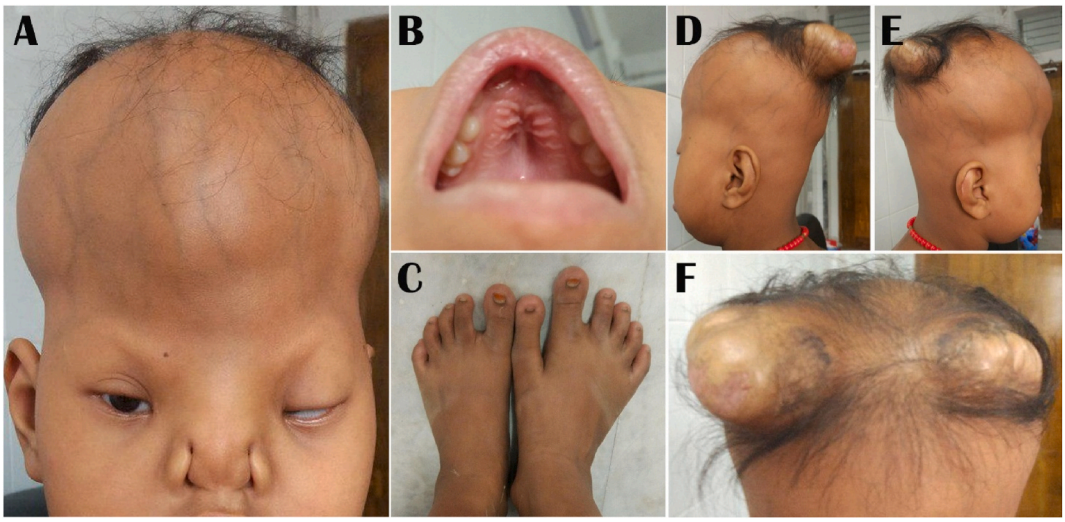


Fig. 1. Image of the patient's face showing the prominent forehead with frontal bossing, sparse scalp hair, sparse eyelashes, absent eyebrows, hypertelorism, telecanthus, upslanting and short palpebral fissures, blepharophimosis, strabismus, epicanthal fold, bifid and depressed nasal tip, broad and depressed nasal bridge, broad and depressed nasal ridge, wide nasal root, cleft alae nasi, short and broad columella, and broad philtrum **(A)**; Thick and everted lips and high arched palate **(B)**; Preaxial polydactyly of right foot **(C)**; Brachycephaly **(A, D)**; parietal swellings **(D, E, F)**.

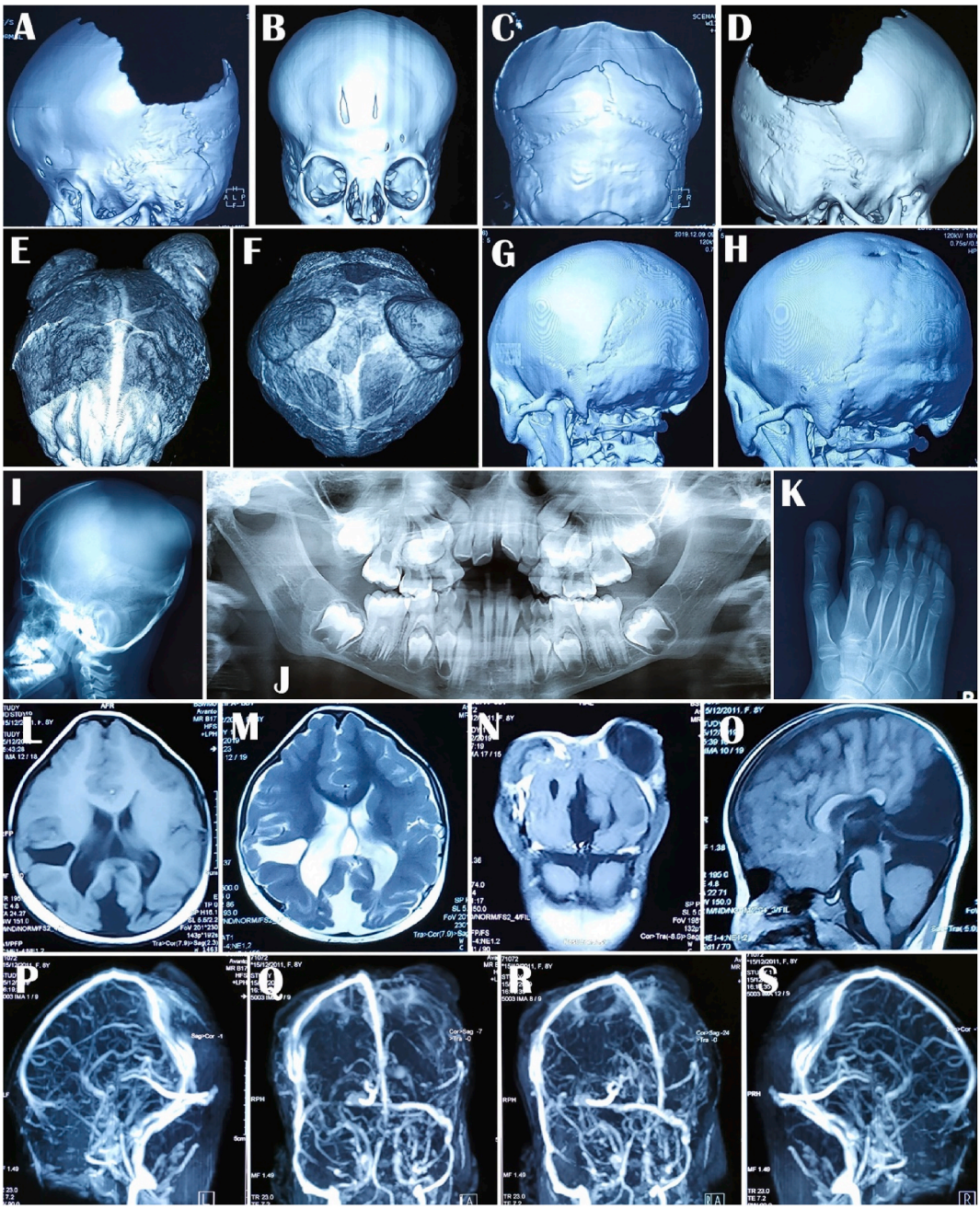


Fig. 2. 3D CT scan of the patient shows Brachycephaly **(A & D)**; cranium bifidum **(A, C, D)**; craniosynostosis **(A, D)**; prominent forehead with frontal bossing **(A, B, D)**; frontal bony gap **(A, B, D)**; multiple woormian bones **(A, C, D)**; increased distance between orbits **(B)**; trifoliate nasal bone **(B)**; wide nasal root **(B)**. Bone subtraction 3D CT scan of the patient showing multiple anomalous dural venous channels **(E, F)**. 3D CT scan of head of the mother shows normal parietal foramina **(G)**. 3D CT scan of head of the father shows enlarged bilateral parietal foramina **(H)**. X-ray skull lateral view of the patient shows the gap in the parietal region **(I)**. The Orthopantomogram (OPG) of the patient shows malocclusion of teeth and diastema of upper incisors **(J)**. X-ray of the right foot of the patient shows preaxial polydactyly **(K)**. Axial T1WI and T2WI images of MRI of the brain of the patient show interdigitated sinuous gyri, cavum interpositum, supratentorial interhemispheric cyst, right parietal porencephalic cyst **(L, M)**. Coronal contrast enhanced MRI of the brain shows supratentorial interhemispheric cyst, supra cerebellar cyst, meningoencephalocele **(N)**. Sagittal T1WI of the brain shows dysgyria, dysplastic occipital lobes, kinked corpus callosum, supratentorial interhemispheric cyst, supra cerebellar cyst **(O)**. MRV of the patient shows absent posterior part of the superior sagittal sinus, absent straight sinus, absent right transverse sinus, and persistent primitive marginal sinus **(P, Q, R, S)**.

bony gap (Fig. 2 A, B, D), trifoliate nasal bone (Fig. 2 B), underdeveloped maxillary bone (Fig. 2 I), and multiple woormian bones (Fig. 2 A, C & D). In 3D CT scan of the head of the parents, the mother had normal bilateral parietal foramina (Fig. 2 G), but the father had bilateral enlarged parietal foramina (Fig. 2H). She had prognathism (Fig. 2 I) and OPG showed malocclusion of teeth, and diastema of upper incisors (Fig. 2 J). From history, examination, and MRI findings many features related to the central nervous system (CNS) were evident. She had delayed developmental milestones although her intellect was at par for her age. MRI of the brain demonstrated biparietal meningoencephalocele (Fig. 2 N), dysgyria (Fig. 2 O), interdigitated sinuous gyri with deficient falx cerebri anteriorly (Fig. 2L and M), dysplastic occipital lobes with wide and deviated posterior interhemispheric fissure (Fig. 2 L, M, N, O), kinked corpus callosum (Fig. 2 O), cavum interpositum (Fig. 2L and M), right parietal porencephalic cyst (Fig. 2L and M), and cerebellar atrophy with large supra cerebellar space (Fig. 2N and O). Bone subtraction 3D CT scan of the patient revealed multiple anomalous dural venous channels also (Fig. 2E and F). Her MRV revealed vascular anomalies like absent posterior part of the superior sagittal sinus, absent straight sinus, absent right transverse sinus, persistent primitive marginal sinus (Fig. 2P and Q, R, S).

4.1. Molecular findings

Whole genome sequencing was done for the patient and parents. The sequencing and mapping produced 81980648, 854,793,941 and 1132649444 reads for each of the samples of the patient, mother and father respectively. Unique reads were found after excluding duplicate marked reads using Basespace Dragen germline software version 3.5.1(https://basespace.illumina.com) Quality control examination of the sequencing reads revealed that >99 % of the sequencing data yielded a PHRED score of 30 or above. Any over-represented adapter sequences in the sequencing libraries were not identified.

The WGS analysis of the patient and both of her parents revealed the mutations of ALX4 gene in all 3 of them. Sequence analysis of the coding exons, regions of ALX4 gene (NM_021926.3) in the patient revealed a homozygous alteration (c.793C > T) resulting in a stop gained change at codon 265 (p.Arg265Ter). The variation from Arginine to Termination was rare. According to clinvar database of Basespace this variation was found pathogenic. Two other mutations in the ALX4 were also detected in the patient's sample. One was (c.304C > T) which is a missense mutation resulting in a change from (p. Pro102Ser) Proline to Serin; and the other was the c.104G > C alteration which is also a missense change from Argenin to Threonine (p.Arg35Thr). All of these mutations in the ALX4 gene are homozygous according to the Clinvar database of Basespace, the latter 2 mutations are benign. Analysis also showed that the father and mother were also heterozygous for the change of pathogenic mutation in the ALX4 gene (c.793C > T). However, there were also pathogenic mutations of 2 other genes, the PRKACB and the NOTCH3. The mutations of both these genes were heterozygous.

5. Discussion

Frontonasal dysplasia (FND), first designated by Sedano et al. [13], is a distinctive albeit heterogenous group of manifestations, limited to the face and head. FND encompasses anomalies in the craniofacial midline in multiple combinations with occasional association with malformations of other parts of the body. Variants of FND are much more specified today with the specifications of genetic characterizations and FND-like syndromes have been specifically categorized accordingly in the Online Mendelian Inheritance in Man (OMIM). FND, has been classified into 3 types based on clinical, radiological, and genetic analysis. After Kariminejada et al. [14]. described a baby of FND and became suspicious of a new syndrome, few more cases were reported and on the basis of genetic analysis it was categorized as FND2 in the OMIM and it is proven now that FND2 is caused by mutation of the ALX4 gene [1,2,15–21].

With newer technologies and discoveries, particularly, with genetic analysis coming into play, the understandings are taking better shape gradually. With the advent and ease of genetic analysis, the spectrum of FND has diversified. Our patient shares a lot of clinical features described in the literature so far as well as had homozygous pathogenic mutation in the ALX4 (c.793C > T) causing a truncated ALX4 expression, making her a definitive case of FND2. The patient was the only child of young healthy consanguineous parents who are carriers of heterozygous ALX4 mutation with recessive trait. The characteristic phenotype and other additional features prompted us to sequence the patient's whole genome to confirm whether this was only an over expression of FND2 caused by ALX4 mutation or if there were any other overlapping expressions of other mutant genes. Along with the rare variation of ALX4 (c.793C > T), 2 other variations of the ALX4, (c.304C > T) and (c.104G > C) reinforce an autosomal recessive transmission of FND2. The clinical and molecular findings of the patient both strongly led us to the diagnosis of FND2. Pathogenic mutations in the patient's sample of the PRKACB gene and the NOTCH3 gene were not manifested clinically per se. Although the patient showed a few common overlapping manifestations caused by these 2 genes, her features did not match significantly to categorize her condition specifically caused by these 2 genes.

As she showed many new features that have not been described in the earlier literature along with many regular recognized ones which we described in the result section, she was a case of FND2 with over expressions of ALX4 mutation as she shares the homozygous mutations from both the parents.

The findings in this patient altogether lay in the extreme end of the spectrum of FND2 resulting from homozygous pathogenic ALX4 which raises the possibility to characterize it as a distinct entity. This particular case has many features sharing similarities in terms of FND2, but the features are not restricted to the craniofacial region only as defined classically. Despite the strong proof of genetic analysis, the many other new features that include anomalies of the CNS, vasculature, limbs, and ectodermal appendages distinguish this from the classical FND2 and warrants the need for a new terminology for this entity other than FND2. Further research would open the opportunity to identify the exact role of the ALX4 gene in the development of different organs during embryogenesis other than the craniofacial region only.

According to the birth history, she is the first child of consanguineous parents, about 5½ years after marriage. The mother was not

exposed to any known teratogenic drug or radiation during the pregnancy with an uneventful gestation period. The mother had only one antenatal check-up and USG screening at 6 & ½ months of gestation, both of which were said to be normal. The child was delivered by LUCS because of prolonged labour. Past Obstetric history of the mother revealed that she had conceived about 3 years before conceiving this child. USG at that time revealed a huge occipital encephalo-meningocele of the foetus with a large gap in the occipital region and IUD. The pregnancy was terminated medically at about 5½ months of gestation. As the WGS identified that the proband harbored the homozygous mutation, ALX4 (c.793C > T) while her parents were heterozygous for this mutation with a autosomal recessive trait, this strongly suggests that the proband had inherited the homozygous mutation from both heterozygous parents. Additionally, it is very likely that the foetus that had to be aborted medically had the same type of mutation as well.

Premarital genetic counseling is the first step to minimize genetic disease transmission from parents to offspring and counseling is imperative to reduce the transmission of autosomal recessive disorders, especially in the context of consanguineous marriages. Awareness campaigns are likely to contribute a major part to the success of premarital counseling in such circumstances. Prenatal screening could be followed by premarital screening as the second step for early diagnosis of any congenital abnormalities. The chromosomal aneuploidies account for around 95 % of live-born chromosomal abnormalities, which often may deem tricky to identify owing to timing, expense, sensitivity, and ethical issues. To date, no prenatal screening system is available in Bangladesh because of social taboos, illiteracy, and financial constraints. Our data highlight the importance of prenatal screening for early diagnosis of any congenital abnormalities by genome-wide testing methods, especially for those children born to consanguineous parents.

6. Conclusion

The proband developed FND2 with manifestations far beyond the usual spectrum of FND2 due to homozygous mutation of ALX4 gene inherited from both the heterozygous ALX4 mutant consanguineous parents with recessive trait. The extreme and rare manifestations related to ALX4 mutation calls for a new terminology as it exceeds the confines of FND2 only. This case also demonstrates the growing importance of comprehensive prenatal screening by whole genome sequencing, especially for those born to consanguineous parents.

Data availability

The raw sequence reads of Patient and Father of patient from Bangladesh were submitted in the NCBI Sequence Read Archive (SRA) under the accession no. SUB14407480, SUB14411182 and SUB14418689 the Bioproject accession number PRJNA1106190 (https://www.ncbi.nlm.nih.gov/sra/PRJNA1106190)

CRediT authorship contribution statement

Barna Goswami: Writing – review & editing, Writing – original draft, Formal analysis, Data curation, Conceptualization. **Asifur Rahman:** Formal analysis, Data curation, Conceptualization. **Iffat Jahan:** Methodology. **Shahina Akter:** Resources. **Tanjina Akhtar Banu:** Resources. **Eshrar Osman:** Supervision. **Mohammad Samir Uzzaman:** Supervision. **Ahashan Habib:** Investigation. **Md Shamsul Alam:** Investigation. **Abu Saleh Mohammad Abu Obaida:** Investigation. **Md Murshed Hasan Sarkar:** Writing – review & editing, Resources. **Salim Khan:** Writing – review & editing, Visualization, Supervision.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:Salim Khan reports financial support was provided by Bangladesh Council of Scientific and Industrial Research. Salim Khan reports a relationship with Bangladesh Council of Scientific and Industrial Research that includes: employment and funding grants. Salim Khan has patent pending to Not applicable. no conflict of interest If there are other authors, they declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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