



Clinical Presentation and WES Analysis of a Large Iranian Pedigree in Five Successive Generation Affected to Sever Multiple Synostosis 2 (SYNS2, Farhud Type)

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Abstract

Background: Bone Morphogenetic Proteins and the related Growth and Differentiation Factors (GDFs) are much conserved signaling proteins. GDF5 is pivotal for skeletal development. Several skeletal dysplasia and malformation syndromes are known as a result of mutations in *GDF5*. Multiple Synostosis Syndrome2 (SYNS2) is characterized by tarsal-carpal coalition, humeroradial synostosis, brachydactyly, and proximal symphalangism. In this study, we analyzed a large Iranian pedigree affected with a new type of SYNS2 (Farhud Type) in five successive generations.

Methods: In this family-based study (1982-2022), Genetic linkage analysis of the pedigree (58affected, 62healthy) excluded the locus on chromosome 17q21-q22 in our previous study. Thus, we focused on 20q11.22 locus and *GDF5* gene. Genetic investigations were performed on 16 patients with SYNS2 and 40 healthy individuals.

Results: Whole-exome-sequencing results identified a heterozygote missense mutation in exon2 of *GDF5* (NG_008076.1:g.9239G>A, NM_000557.2:c.1424G>A, S475N, rs121909347). This mutation was found in all patients but not in the unaffected individuals. This missense mutation is notable because S⁴⁷⁵ is strictly conserved among different species, and it is located in a highly conserved and active mature domain of GDF5 (phyloP100way=7.64). The corresponding defect in GDF5 may have unknown interaction with normal active 3rd and 4th structure of the product. Further bioinformatics study (amino acid multiple alignments) showed that the S⁴⁷⁵ is a much-conserved residue in many different species.

Conclusion: These results introduce a new role of *GDF5* in pathogenesis of a SYNS2 (Farhud Type), considered in genetic counseling, prenatal diagnosis, and as a potential target for molecular therapy, if possible.

Keywords: Multiple synostoses syndrome2; Iranian pedigree; Genetics



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Introduction

Bone Morphogenetic Proteins (BMPs) and the related Growth & Differentiation Factors (GDFs) are much-conserved signaling proteins belong to the Transforming Growth Factor beta (TGF β) superfamily (1, 2). Mutations in BMPs and their receptors have been well recognized in a wide variety of congenital and postnatal diseases (3, 4). BMPs functions are different; however, they have a common signaling mechanism (5, 6).

Growth Differentiation Factor 5 (*GDF5*), also known as cartilage-derived morphogenetic protein 1 (CDMP1) or BMP14, a member of BMP and TGF β families is a secreted growth factor, which is pivotal for skeletal development, chondrocyte differentiation, chondrogenesis, cartilage, bone, and joint formation (5, 7). The mutations in *GDF5* in both mouse and humans are known to cause abnormal joint development (8-10). This protein is predominantly expressed in cartilaginous tissues of the developing long bones and the more distal elements of the appendicular skeleton that develop from the budding limb (7, 11). Lin et al. (12) mapped the *GDF5* to 20q11.2 chromosome containing two exons. The mature GDF5 contains 120 amino acids and has a molecular mass of 13.6KD (13).

Several skeletal dysplasia and malformation syndromes in humans are known as a result of mutations in *GDF5*, including Acromesomelic Dysplasia (Hunter-Thompson) (8, 9), Chondrodysplasia (Grebe-Type) (10), Brachydactyly Type-C (11, 14), Fibular Hypoplasia and Complex Brachydactyly (Du-Pan) (15), Brachydactyly Type-A2 (16-18), Proximal Symphalangism (18-20), Osteoarthritis Susceptibility-5 (21), and Multiple Synostosis Syndrome-2 (21).

Noggin (*NOG*) is one of the best-characterized extracellular BMP signaling antagonists (22). Mutations in *NOG* are responsible for Multiple

Synostosis Syndrome-1 (SYNS1) (23). Furthermore, the fusion of the proximal interphalangeal joints (proximal symphalangism, SYM1), is the most recognized hallmark feature caused by *NOG* mutations. It displays minimal genetic heterogeneity, as mutations in only two other genes, *GDF5* and *FGF9*, have been reported in a small number of families (24). Multiple Synostosis Syndrome2 (SYNS2), has an autosomal dominant inheritance pattern which for the first time was identified in a large Iranian family (25, 26). SYNS2 is characterized by the tarsal-carpal coalition, humeroradial synostosis, brachydactyly, and proximal symphalangism (25, 27). Although Akarsu et al. studied a proband and some patients of this family, their results were presented only as a lecture in a genetic congress, and just published as an abstract (27).

Here, we report a complete clinical and molecular genetic study of this large Iranian pedigree with SYNS2, Farhud Type in five successive generations and their 40-year-long follow up.

Materials and Methods

Subjects

The family was ascertained from seven different villages collectively known as Chamestan, located between Amol and Noor in Mazandaran province, north of Iran. This family came to attention for the first time in 1982, when proband was referred to the Dr. Farhud Genetic Clinic in Tehran for consultation. The oldest people in this family remembered their ground mother as being the founder of this pedigree. Both of the founder's parents and sisters were normal. This large Iranian pedigree consists of 120 individuals (58 patients and 62 relatives) and extends over five generations (Fig.1).

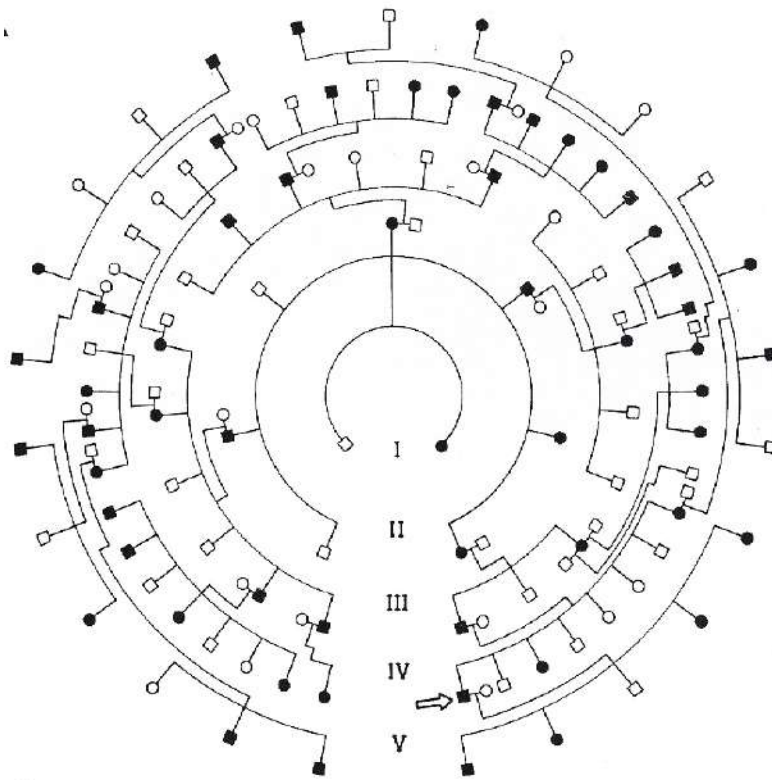


Fig.1: The pedigree of a large Iranian family with SYNS2 Farhud type (26)

Overall, 120 individuals were studied. Approval for this study was obtained from the Committee of Ethics. The informed consent was signed, all patients and family members were evaluated and examined by a team of experienced orthopedists and radiologists.

The diagnosis was confirmed on the basis of clinical and radiographic evaluations and history taking. In addition, X-rays were obtained from 12 patients.

Molecular Genetics Study: Peripheral whole-blood samples were collected from all participants and genomic DNA was extracted and stored at -20°C . Genetic linkage analysis of this pedigree had been performed by DNA markers of chromosomal regions 17q21-q22 (where the other types of multiple synostosis had previously been mapped) but found no linkage to known loci on chromosomes 17q22 (*NOG*). Further investigation by Akarsu et al.(1999) mapped the multiple synostosis

syndrome2 (Farhud Type) in this family to markers on chromosome 20q11.2, with the highest load score observed at D20S200 ($Z=13.58$, $\theta=0.0$) (27). Both exonic and exon/intron junctions of *GDF5* and *NOG* were amplified by PCR, using specific primers:

F:5'CGCTGCTGCCGCTGTCTCT3' and R:5'GCCCTCCATTCATGCAG3' (*GDF5*, exon1), F:5'GAATGGGG-CAGAGGTGAAAG3' and R:5'CCTGACCCCTCTGTGATICCA3' (*GDF5*, exon2),

F:5'CTCGGCGTGCTCTCCTC3' and R:5'GAACTGGTT-GGAGGCCGG3' (*NOG*, exon1). After PCR of the genetic regions, all amplicons were directly sequenced.

For more investigation and to find other likely variant(s) related to our patients' phenotype, we suggested WES analysis in the proband. After quality control of the extracted genomic DNA from proband sample preparation by Agilent SureSelect Human All Exon V6 protocol, WES was carried

out. FastQC and Qualimap are the two-software used for statistical analysis, removing unnecessary data, and checking the quality of reads of the raw fastq files. BWA, Picard Tools, and Genome Analysis ToolKit (GATK) software packages have been used for mapping, manipulating the bam, sam, and VCF files, and Variant Calling in the process of analyzing, respectively. The final detected variant of WES was confirmed by Sanger sequencing method in the proband and also in other 57 patients of this family.

Results

After several years of extensive investigations, the disorder was classified and reported as a new autosomal dominant condition with severe multiple synostosis type2 (Farhud Type).

Clinical findings: The observed phenotype was very similar in most of the patients. Both hands and feet were bilaterally affected in all cases. Based on the results of radiography in different patients, the following were observed: fusion of the head of the elbow joint bones (humerus and radius at an angle of 110-140 degrees) (Figs.2-3), clubfoot, severe abnormalities of the leg bones and ankle joint, fusion of the talus and calcaneus bones, which has led to supination of the legs and walking on the outer side of the foot (Figs.4-5), hyperplasia of the humerus and ribs, defects of the spine (Fig.6).



Fig.2: (A-B): Brachydactyly of fingers (C-D): The synostosis of the humerus to the ulna, fixed at an angle of 110°-140° in flexion supination position and elbow range of motion limitation.

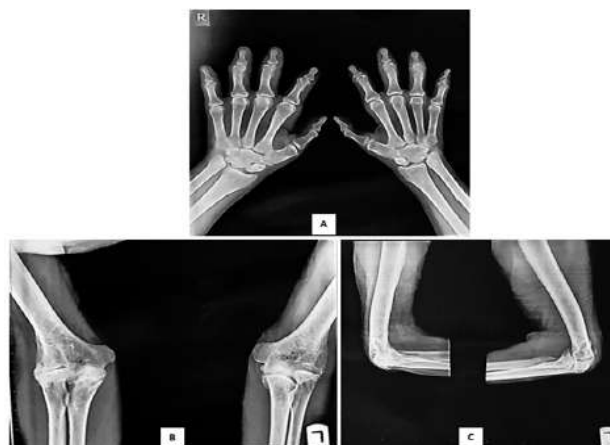


Fig.3: (A): The second to fifth fingers have two joints (brachydactyly), and the first joint is twice as long as the second joint. Fusion of the wrist bones of both hands is observed (except the scaphoid bone). (B-C): The fusion of the humerus and ulna bones is seen in both elbows (with greater intensity on the right side) along with degenerative changes.



Fig.4: (A-B): Brachydactyly of fingers and flat feet are appear. (C-D): fixation of the feet in supination position, because of partial synostosis (physis) of calcaneus and talus, causing a very difficult walking style on the outer edges of the feet. (E-F): Limited rotation of ankle in both internal and external direction is observed



Fig.5: (A-B): The second to fifth toes have two joints (brachydactyly), and the first joint is twice as long as the second one. The fusion of the first and second thumb of both feet is seen. There is a fusion of the ankle bones. Flat foot is seen in both feet. (C): No bone lesions were observed in the knees.

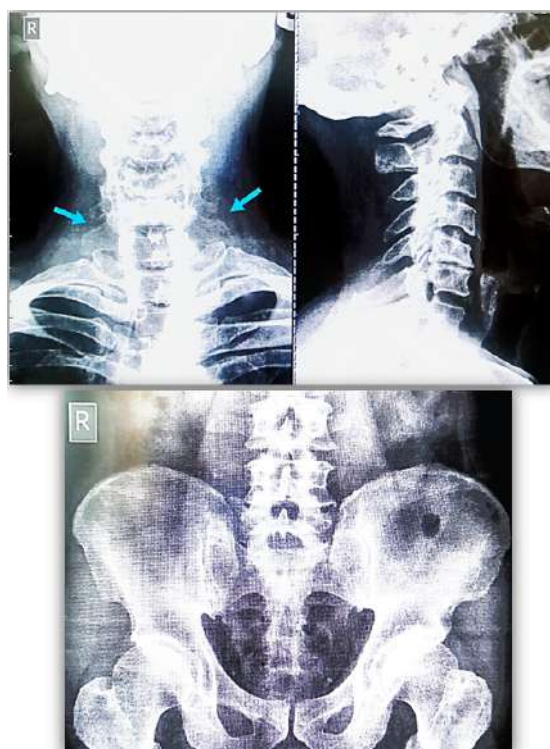


Fig. 6:Partial accessory ribs, and Partial hip bones synostosis

All patients (30females, 28males) had Brachydactyly (Figs.3, 5-7) together with limited extension and flexion of both elbows (the main clinical presentation of the upper limbs) and some degree of flexion and pronation abnormalities in different joints. In both hands, loss of proximal flexion creases was observed. Variations in the function of elbow had a spectrum as near normal range of motion, bilateral fixation of elbow joints, and unilateral fixed and contra-lateral limited range of motion, and bilateral limited movement. There was no impairment of wrist function in general, however, ulnar deviation of 30degree or more was observed in most of patients.

Fingers had only two segments which appeared as brachydactyly. The synostosis of the humerus to the ulna (Figs.2-3), fixed at an angle of 110° - 140° in flexion supination position resulted in elbow range of motion limitation in any direction which made a particularly difficult lifestyle, especially in

the hand to face/mouth movements. This event ended in anatomical adaptive changes in shoulder joints and cervical vertebrae. Moreover, atrophy of most muscles involved, particularly in hands produced compensatory hypertrophy of some others. A limitation of supination and pronation in the wrist of both sides are notable in most individuals and aplasia on distal and segment of 4th phalanges in some patients. Moreover, several minor anomalies such as hyperplasia of the humerus, shortness of toes and thumbs were seen with variation in form and intensity in each individual (Table 1). As the radiographs showed, the patients of this family had some major abnormalities such as brachyphalangia of both sides, synostosis of elbow joint, especially humerus and ulna-radius, talus-calcaneus fusion on both sides (Table 2). Partial accessory ribs and partial hip bone synostosis exist in some individuals' radiography images as well (Fig.6).

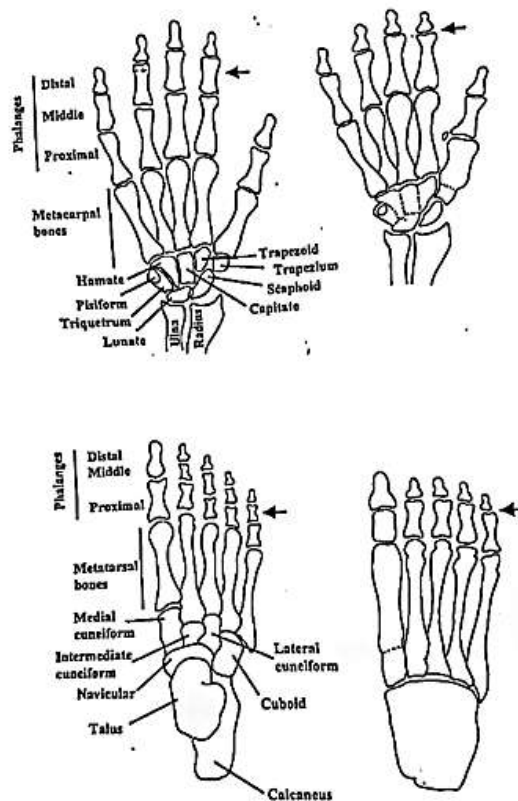


Fig.7: Illustration of bone structures in SYSN2 (Farhud Type) resulted in brachydactyly in hand and foot of the patients. Sketches of normal hand and foot are shown on the left (above and below). Arrows represent missing phalanges of both hand and feet. The bones involving in the synostotic complex indicated by dotted line in both carpal tarsal regions.

Table 1: Clinical findings in patients affected with SYNS2, Farhud Type.

<i>Clinical Findings</i>	<i>Total % (n=44)</i>
Facial abnormalities	None
Deafness	None
Brachydactyly	100%
Loss of proximal flexion creases in hands	100%
Loss of distal flexion creases of 2 nd finger in one or both hands	29.54%
Agensis of distal parts and toe nails	22.7%
Limited movement of the wrist	None
Limited pronation/supination of forearms	95.4%
Limited extension and flexion of forearms	95.4%
Limited internal and external rotation of ankle	100%
pes planus varus	100%
Syndactyly of 3 rd and 4 rd fingers	2.27%
Restricted movement of knee	None

Examination of the X-rays from both hands and feet revealed carpal and tarsal coalition in all patients. In hands, the scaphoid bone was usually not involved in the synostotic complex of the carpal bones. The synostosis between the trapezium and 1st metacarpal bones was remarkable. The 1st metacarpal bone was short and bone modeling was defective. Loss of middle phalanges or complete fusion between proximal and middle phalanges was consistent. In addition to brachydactyly, proximal symphalangism was also observed in patients. Radial head sublocation and radio-humoral synostosis was observed in the elbow joints.

More severe abnormality was observed in the feet of these patients. Bilateral varus deformity and flat feet were apparent. The patients represented a fixation of the feet in supination position, because of partial synostosis (physis) of calcaneus and talus, causing a very difficult walking style on the outer edges of the feet. Limited rotation of ankle in both internal and external direction was observed. Lack of distal phalanges (in all patients) and toenails (in some patients) was also observed in the postaxial side of the feet 3rd and/or 4th toes).

Table 2: Radiological findings in patients affected with SYNS2 Farhud Type

Radiological findings	Total% (n=12)
Hand:	
-Carpal synostosis	100%
-Synostosis of trapezium and first metacarpal bones	100%
-Short and broad 1 st metacarpals	100%
-Middle phalanges aplasia or complete fusion between middle and proximal phalanges	100%
Elbow:	
-Radial head subluxation	16.6%
-Humero-Radial synostosis	83.3%
-Humero-Radio-Ulnar synostosis	8.3%
Feet:	
-Synostosis of Tarsal bones	100%
-Synostosis of medial cuneiform and 1 st metatarsals	58.3%
-Middle phalanges aplasia or complete fusion between middle and proximal phalanges	100%
Knee:	
-Aplasia of intercondyloid eminence of tibia	100%
-Flattening of medial and lateral condyle of tibia	100%

In the feet, synostosis between medial cuneiform and first metatarsal bone was observed in most of the patients. Middle phalanges aplasia was also remarkable in the feet. Aplasia of intercondyloid, prominence of tibia and flattening of the medial and lateral condyle of tibia were noticed. Comparison of two X-ray pictures taken within the period of 14 years from the proband showed that the motion of the elbow joint progressively becoming more limited.

The mode of inheritance of this family was undoubtedly autosomal dominant with complete penetrance and no apparent skip generation. All

patients are physically active and of normal intelligence. They have adapted well to their limb malformations, but could not perform certain functions. There was no history of deafness in patients and facial appearance was normal.

Mutation Findings: Unexpectedly, genetic linkage analysis of this pedigree excluded the locus on chromosome 17q21-q22 in the previous studies (21-24, 27-29) and DNA sequencing results showed no mutation in *NOG* in this locus.

Akarsu et al (27) mapped the multiple synostosis syndrome 2 (Farhud Type) in this Iranian family to markers on chromosome 20q11.2, with the highest load score observed at D20S200

($Z=13.58$, $\theta=0.0$). By mutation screening of a proband with SYNS2, they identified a heterozygous missense mutation in exon2 of *GDF5* (NG_008076.1:g.9239G>A, NM_000557.2:c.1424G>A, p.S475N, rs121909347). This mutation was found in patients but not in the unaffected individuals. Ser475 lies in a highly conserved region of the protein. The mutant allele has an autosomal dominant mode of inheritance (Fig.1).

Interestingly, bioinformatics investigation on amino acid sequence alignments in 22 distant different species showed that the S475 residue is a much-conserved amino acid in *GDF5*, which demonstrates the importance of this residue during evolution (phyloP100way=7.64).

In addition, WES data showed this mutation in the *GDF5* in proband (*GDF5*:NM_000557:exon2:c.G1424A:p.S475N, rs121909347). Based on several lines of computational evidences such as BayesDel_addAF, DANN, DEOGEN2, EIGEN, FATHMM-MKL, LIST-S2, M-CAP, MutationTaster, PrimateAI, and SIFT, this variation is predicted to be deleterious. The frequency of this variant is unknown in population genetic databases such as Iranome, 1000 Genomes, gnomAD, and ExAC. There was no other mutation related to this disorder or the clinical features detected in the WES result. Moreover, this variant was confirmed by Sanger sequencing in the proband and 46 individuals of his family members.

Discussion

In this study, a large Iranian pedigree with a new genetic syndrome multiple synostosis type2 (SYNS2, Farhud type) with confirmed mutation in *GDF5* was presented.

The phenotypic expression in this family is similar to the family reported by Pearlman et al. referred to as Nivergelt-Pearlman Syndrome (28). In their pedigree, a mother and daughter were affected with brachydactyly, radial head subluxation, synostosis in carpal and tarsal bones, and varus deformity of the feet. The malformation in this original pedigree is distinct from the other types of multiple synostosis phenotype mainly because of the lack of symphalangism and progressive ankylosis in various joints. However, the clinical evaluation of the entire Iranian kindred suggested that this family is in a borderline between the typical multiple synostosis phenotype and the family reported by Pearlman et al (28), because: 1) in addition to the symptoms compatible with Pearlman syndrome, progressive synostosis in humeroradial joint and proximal symphalangism were observed; 2) malformation was more severe in the postaxial side of the lower limbs and the distal phalanges were missing between the 3rd and/or 4th toes, these manifestations have frequently been reported in multiple synostosis phenotypes (29-31), but not in the family reported by Pearlman et al (28); 3) progressive ankylosis in the metacarpophalangeal joints which is common in synostosis was not observed; and 4) this family had no hearing impairment and facial development frequently reported in other types of multiple synostosis syndrome. In Table 3, various clinical features of three different types of multiple synostosis syndromes are compared.

Table 3: Various clinical features of three different types of multiple synostosis syndromes 1, 2, 3 (OMIM)

<i>SYNS1, NOG</i>	<i>SYNS2 (Farhud-Type), GDF5</i>	<i>SYNS3, FGF9</i>
HEAD & NECK	HEAD & NECK	HEAD & NECK
Face		Head
-Narrow face	Nose	- Dolichocephaly (in some patients)
-Short philtrum	- Broad hemicylindrical nose	Eyes
Ears		- Proptosis (in some patients)
-Progressive conductive deafness		Mouth
-Stapes ankylosis		- Cleft palate (in 1 patient)
Eyes		
-Strabismus		
Nose		
-Hemicylindrical nose		
-Hypoplastic alae nasi		
-Hypoplastic nasal septum		
Mouth		
-Thin vermilion border of upper lip		
SKELETAL	SKELETAL	SKELETAL
Spine	Spine	Skull
-Vertebral anomalies	-Vertebral fusions	-Sagittal suture fusion (in some patients)
-Spinal canal stenosis		Spine
-Hypoplastic spinal processes (cervical vertebrae)	Limbs	-Fusion of lumbar joints (L2-3 and L4-5, in some patients)
Limbs	-Humeroradial synostosis	Limbs
-Short upper arms		-Humeroradial synostoses
-Cubitus valgus	Hands	-Semidislocation of elbow joint
-Dislocated radial head	-Carpal fusions	-Cubitus valgus
-Limited forearm	-Carpal coalition	Hands
pronation/supination	-Tarsal-carpal coalition	-Fusion of the interphalangeal joints
-Short legs	-Progressive symphalangism	-Fusion of the first metacarpal and trapezium
Hands	-Proximal symphalangism	-Limitation of finger joint flexion
-Proximal symphalangism (2,3,4)	-Brachydactyly	-Limitation of carpal and interphalangeal joint movement, progressive
-Fusion of midphalangeal joints	-Loss of proximal flexion creases in hands	-Broad thumbs (in some patients)
-Clinodactyly	Feet	-Radially deviated thumbs (in some patients)
-Brachydactyly	-Tarsal fusions	Feet
-Cutaneous syndactyly (2,3,4)	-Tarsal coalition	-Fusion of the first metatarsal, cuneiform, and navicular
-Carpal fusions	-Talipes equinovarus (in some patients)	-Fusion of interphalangeal joints
-Hypoplastic/aplastic middle phalanx		-Limitation of tarsal and interphalangeal joint movement, progressive
-Hypoplastic/aplastic distal phalanx	CHEST	-Broad halluces (in some patients)
-Single palmar creases	Ribs	-Medially deviated halluces (in some patients)
Feet	-Partial accessory ribs	-Cutaneous syndactyly of toes (in some patients)
-Short feet		
-Short halluces		
-Tarsal fusions		
-Cutaneous 2,3 toe syndactyly		
-Absent distal phalanges		
-Proximal symphalangism 2,3,4		
SKIN, NAILS, & HAIR		
Skin		
-Absence of skin creases over proximal interphalangeal (PIP) joints		
-Absence of skin creases over distal interphalangeal (DIP) joints		
-Single palmar creases		
Nails		
-Aplastic/hypoplastic fingernails		
-Aplastic/hypoplastic toenails		
CHEST		
Ribs Sternum Clavicles & Scapulae		
- Anteriorly positioned shoulders		
- Short sternum		
- Pectus excavatum		
- Prominent costochondral junction		

Another interesting observation in this Iranian family is the fact that all the observed malformations are always bilateral and there is minimal intrafamilial variation between the affected family members. The most variable parameter is the severity of mobility of elbow joints within a range of mildest to complete fixation of elbows bilaterally with only a small variation in the angle of elbow. However, this variation is largely due to the age of the patients, as it was observed that evaluation of two sets of X-ray films taken in a period of 14 years from a patient revealed that malformation in the elbows is getting more severe with older age. Asymmetry and variation of expression is a well-known entity in various limb malformation syndromes. However, similar to the reported observations in other types of multiple synostosis phenotype, less variation between left and right was also noticed in this pedigree. The knees were less affected than elbows, and the malformation does not restrict the movement of knees. Syndactyly between 2nd and 3rd fingers is known as type I syndactyly, and is frequently associated with multiple synostosis phenotype. In this pedigree, one case with mesoaxial syndactyly in hands was observed. The 3rd -4th syndactyly in hands is also known as a type I syndactyly which is the most common form of syndactyly in different populations. Therefore, it could be a coincidental presentation in these kindred. Thus, this presentation is taken into account as one of the main clinical features of the multiple synostosis in this Iranian family.

All patients unexceptionally showed signs and symptoms of the disease neonatally. Regardless of the age and sex, all of them have been involved with severe hands anomalies such as carpal synostosis, synostosis of the trapezium, and short and broad 1st metacarpals with aplasia of middle phalanges. Moreover, one many notes of feet and knees deformities clinically and radiologically evident in all of them like tarsal bone synostosis and aplasia of the intercondyloid eminence of tibia. Flattening of medial and lateral condyles of tibia has been also found in all of them. There were other deformities in elbow like radial head of sub-location and radial synostosis ended in less limitation

of elbow range of motion with less frequency. Partial accessory ribs and partial hip bone synostosis exist in some individuals' radiography images as well.

Up to now, some pathogenic mutations in the *GDF5* have been found in the patients with Multiple Synostosis (18, 20). Our previous genetic linkage study excluded the locus on chromosome 17q21-q22 as a likely site of multiple synostosis (25-27). Further investigations revealed no mutations in the *NOG* in any of the patients, hence, prompting us to consider the secondary responsible gene namely *GDF5* according to Akarsu and Dawson (27, 32). DNA sequencing of *GDF5* showed only a change as S475N as the only finding in patients with preservation of the other normal members of the family. Assuring to rule out the possibility of polymorphism in Iranian ethnicities a cohort of 40 healthy Iranian individuals of the age, sex, and origin matched have been studied which showed no such mutation.

This substitution has been located in a highly-conserved and active mature domain of the protein, and may have unknown interaction with normal active 3rd and 4th structure of the product.

For this reason, it could have the potency of causative for the disease. Dawson reported five sporadic cases of Multiple Synostosis patients with only one case of such a mutation and without any further discussion on its effect (32).

Conclusion

Altogether, after several years of extensive investigation, the disorder was identified as a new type of Multiple Synostosis Syndrome 2 (SYNS2, Farhud Type) with autosomal dominant inheritance with full penetrance and 100% expressivity. The gene responsible for this disease was also discovered (*GDF5*). Currently, prenatal diagnosis is performed for patients of this large family.

Journalism Ethics considerations

Ethical issues (Including plagiarism, informed consent, misconduct, data fabrication and/or falsification, double publication and/or submission, redundancy, etc.) have been completely observed by the authors.

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Conflict of interest

The authors declare that there is no conflict of interests.

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