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ORIGINAL ARTICLE

Novel heterozygous sequence variant in the *HOXD13* gene underlie non-syndromic syndactyly

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ABSTRACT

Background: The term "Syndactyly" refers to an inherited deformity of the hand or foot marked by the persistence of the webbing between adjacent digits that are more or less completely attached and primarily inherited in an autosomal dominant manner. Pathogenic variants in the Homeobox D13 (*HOXD13*) gene, located on chromosome 2q31.1, have been associated with syndactyly type 5, brachydactyly type D, E, and synpolydactyly type 1 phenotype. The study's objective was to clinically and genetically define syndactyly as a notable aberrant trait identified in an inbreed of Pakistanis recruited from a remote region of the nation.

Methods: Whole exome sequencing (WES) coupled with Sanger sequencing was carried out to uncover the disease-associated variant/s, followed by 3D protein modeling to check the variant-related effect on protein level.

Results: WES data analysis revealed a novel-heterozygous *HOXD13* gene missense variant (c.969G>T; p.Trp323Cys) that might explain the disease parthenogenesis. According to American College of Medical Genetics and Genomics, the identified variant is likely pathogenic (Class 2). In addition, in 3D protein modeling of the normal and mutant proteins, the protein structure became unstable due to the mutation, potentially impairing the protein's final function.

Conclusion: Our findings extend the mutation spectrum of *the HOXD13* gene and provide additional evidence that *HOXD13* plays an important role in limb development.

Keywords: HOXD13, syndactyly, missense mutation, webbing.

Introduction

Syndactyly is a digital malformation in which two or more digits are fused (webbed) as they fail to separate during limb development. It is one of the most frequent congenital limb abnormalities and occurs as an isolated anomaly or a part of a complex syndrome (1). Among the most common hereditary limb deformities, syndactyly has a prevalence of 3-10/10,000 births, with higher estimates of 10-40/10,000 reported (2). Syndactyly exhibits high inter- and intra-familial clinical variability among different families and within the same individual. The degree of phenotypic variability can be unilateral or bilateral and symmetrical or asymmetrical (3). At least nine non-syndromic syndactylies with additional subtypes have been characterized. They are inherited in an autosomal dominant fashion, while two autosomal recessive and an X-linked recessive entity have also been described (4). Of the non-syndromic syndactylies, the most common type is Syndactyly type 1 (SD1), following an autosomal dominant inheritance pattern with variable phenotypes. Webbing affecting fingers and/or toes may be bilateral or unilateral, bony or cutaneous, and might reach the nail or just affect the proximal segments of the phalanges. According to the current classification and clinical observations, SD1 can be categorized into

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four subtypes (SD1-a, SD1-b, SD1-c, and SD1-d) (4). Syndromic syndactylies have been associated with a wide range of phenotypic spectra such as polydactyly, ectodermal dysplasia, Split-hand foot malformation, ophthalmology, Ellis-van Creveld syndrome, Bardet-Biedl syndrome and other severe manifestations (5-8). Using the term "syndactyly" in the OMIM gives us 531 different entries, thus emphasizing its association with different severe disorders.

The present study investigated the Pakistani consanguineous family with syndactyly (SD-1) phenotypes. We identified a novel missense variant within the Homeobox D13 (*HOXD13*) gene (c.969G>T; p.Trp323Cys) in the affected member, thus adding to the **variant** spectrum and further enlightening our knowledge about the pathogenesis of *the HOXD13* **variant** in human limbs deformities.

Subjects and Methods

Written informed consent was obtained from the patient and other members to publish this article in compliance with the Helsinki Declaration. The study was approved by the Institutional Review Board (IRB) of the University of Management and Technology (UMT), Lahore, Pakistan. Following consent and approval to the study, venous blood samples were collected from all the available members of the family.

Genomic DNA extraction from all the available members and quantification was performed using standard methods (9). The target sequences of the gDNA samples were fragmented by the sonication method. Illumina adapters were ligated to generate fragments for subsequent sequencing on the HiSeq 2500 platform using standard methods (Blueprint Genetics) (10,11). Sequencing involves amplification, partial digestion, adapter ligation, and purification. The amplicon size of 150 bp length for reads generation was adjusted. These reads were then subjected to bioinformatics analysis. Filtration steps for whole exome sequencing (WES) were followed as described previously (12). Filtration was followed using ACGM guidelines. First, OMIM-linked known genes were considered during the variant filtration process and later ClinVar, HGMD, and other databases were searched.

Variant-specific primers were designed using Primer 3 online software. The identified variants were appropriately segregated in all available members using standard bi-directional Sanger sequencing (13,14).

HOXD13 protein modeling was performed-using methods described previously (15, 16). The amino acid sequence of Homeobox protein Hox-D13 (HOXD13), encoding protein, was retrieved from the UniProt Knowledgebase database with accession number P35453 in FASTA format. Protein's 3D structure from its amino acid sequence predicted by AlphaFold (AF-P35453-F1) AlphaFold Protein Structure Database (ebi. ac. UK). The 3D structure of the mutated protein was generated by MODELLER (9.19).

Different evaluation tools were used for the assessment of protein structure. RAMPAGE and ERRAT further processed the model. RAMPAGE generates a Ramachandran plot to assess models and the distribution of residues in favored, allowed, and outlier regions. ERRAT generated a plot indicating the confidence and overall quality of the model. UCSC Chimera is used for visualization purposes.

Results

Clinical synopsis

We investigated an affected individual recruited from the remote Punjab province of Pakistan with distinctive syndactyly phenotypes (Figure 1A). The present family has five affected individuals in three generations, while three affected individual (III-1, III-2, and III-5) deceased in an earthquake calamity (Figure 1A). Digital images and radiographs were obtained from the single affected individual (II-2). Affected individual (II-2), 87 years old, showed complete bilateral webbing in the third and fourth finger of the right hand accompanied by fused nails, while complete webbing of the third, fourth, and fifth finger of the left hand (fused nails; Figure 1B and C) was observed. Toes were normal in affected individuals II-2. The three deceased individuals also had bilateral syndactyly phenotypes restricted to hands, as described by their father. The cutaneous webbing phenotype is variable and ranks from partial/incomplete unilateral to complete bilateral cutaneous finger webbing. Phenotype

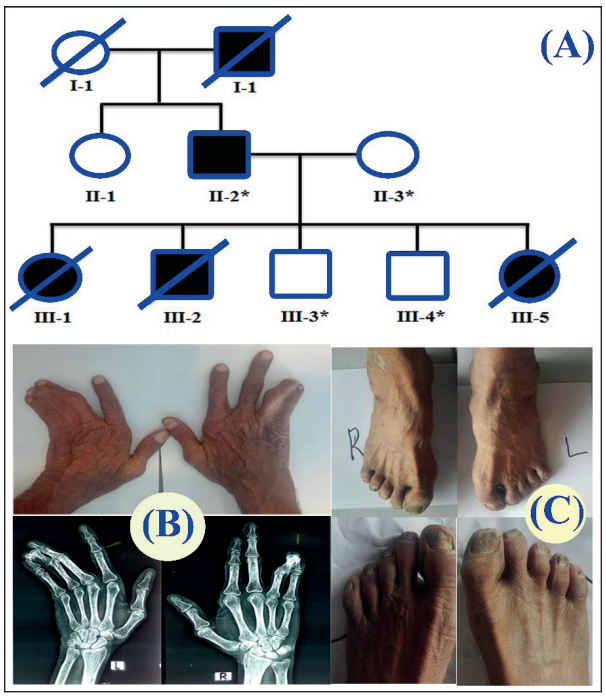

Figure 1. *(A) Pedigree of family A depicting autosomal dominant inheritance. Circles and squares represent females and males, respectively. Unfilled symbols represent unaffected members while filled black symbols signifying affected members. The individual numbers labelled with asterisks indicate the samples available for this study. (B-D) Affected individual (II-2), age 87 years, showed complete bilateral webbing in the third and fourth digits of right hand along with fused nails, while complete webbing of third, fourth and fifth finger of the left hand (fused nails) with normal toes.*

such as clinodactyly, polydactyly, brachydactyly, and contractures were not observed. Apart from syndactyly, no other abnormality was observed.

Genetic and in silico analysis

WES, followed by the downstream Bioinformatics analysis, found a novel heterozygous missense variant [c.969G>T; p.Trp323Cys] in the exon 2 of the *HOXD13* gene. Sanger sequencing of *the HOXD13* gene (NM_000523.4; NP_000514.2) revealed a novel heterozygous missense variant (c.969G>T; p.Trp323Cys) causing G to T transition in the exon two at position 969 in the affected individual, substituting Tryptophan into Cysteine at amino acid position 323 (Figures 1C and 2A). The identified missense variant was not found in any unaffected family members (Figure 2B). According to American College of Medical Genetics and Genomics, the identified variant was classified as "Likely-Pathogenic-class 2" [PP3, PM1, PM2]. The variant identified in the present study (c.969G>T; p.Trp323Cys) was considered disease-causing using several online available bioinformatics tools (Table 1). The variant was also not observed in ExAC, genomAD, and 165 in-house exomes datasets. The 323Trp amino acid is also conserved across different species.

In this study, *in silico* methodologies such as homology modeling for wild-type and mutant were carried out. The predicted structure of HOXD13 has a good degree of accuracy. Different evaluation programs assessed the final refined model. Using homology modeling, online structure analysis tools predicted and evaluated three-dimensional models of wildtype and mutated HOXD13 (p. Trp323Cys). Ramachandran plot indicated that approximately 94% and 99% of residues in the wildtype and mutant structure lie in allowed regions of torsion angles, respectively. 3D structure of both wildtype and mutant proteins was subjected to the Errat protein structure verification server. ERRAT provided an overall quality factor of wildtype and mutant structure model range between 94% and 89%, respectively, which is very satisfactory.

Changes (ΔΔG) are for predicting the impact of single amino acid replacements on protein stability due to thermal denaturation. The predicted structure of the 3D model of mutant HOX13 showed that substituting Tryptophan for Cysteine would not change the protein structure. However, using ENCoM, DUET, SDM, and mCSM, we predicted that Trp323Cys mutation would cause a -0.955, -0.994, -1.270, and -1.171 kcal/mole change in the ΔΔG, respectively. The protein structure will become unstable due to the mutation in HOXD13 (Trp323Cys), according to the negative value of ΔΔG (Figure 3B and C).

Discussion

SD1 is clinically a heterogeneous genetic malformation of the limb. The typical clinical presentation includes webbing the 3/4 or 4/5 fingers and webbing of 2/3 toes. The affected individual in the family showed syndactyly of the third, fourth, and fifth digits in the left hand and syndactyly of the fourth and fifth digits in the right hand,

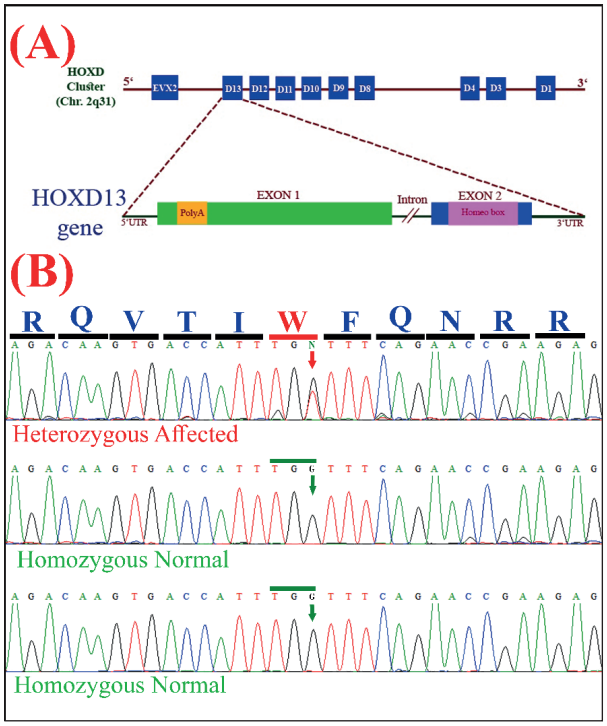

Figure 2. *(A) Genomic structure of HOXD13, schematic representation of the HOXD13 gene, showing the polyalanine tract in exon 1 (blue box), the homeobox in exon 2 (green box) and red arrows showing the variants identified in the present study. (B) Sanger sequence analysis showing segregation of novel heterozygous missense variant (c.969G>T) in the HOXD13 gene.*

respectively (4). Phenotypes include brachydactyly, digital duplication within the syndactylous web, camptodactyly, pre/post-axial digital duplication, duplicated metatarsals, extra phalangeal creases, and broad hallux have not been observed in the present report. Features such as the joining of the distal phalange within the syndactylous web were similar to those reported earlier (17). The feet were normal, and no other abnormalities were noted. The Clinical presentation of SD varies among different families and individuals within the same family (17,18).

SD1 and synpolydactyly (SPD) have some common associations. SD1-c is characterized by isolated syndactyly of 3/4 fingers, and typical features of SPD, including webbing of 3/4 fingers and 4/5 toes, with digital duplication (partial or complete) within the syndactylous web. The webbing phenotype was considered a milder phenotype in several SPD families reported. In addition, both phenotypes, such as 3/4 finger and 2/3 toes webbing, are characteristic of SD-1b, whereas the characteristic phenotype of SD1a includes isolated webbing of 2/3 toes (19). The close association between SD1-a, SD1-b, SD1-c, and SPD inspired us to perform direct Sanger sequencing of *the HOXD13* gene.

To explore the genetic basis of the disorder WES plus Sanger sequencing was performed. In the present family, a novel missense variant (c.969G>T) was identified in the *HOXD13* gene, substituting tryptophan for cysteine amino acid at position 323. The identified variant was predicted to be highly pathogenic/disease-causing,

Table 1. *Pathogenicity of the mutation (p.Trp323Cys) using different online tools.*

S. No	Online tool	Prediction
1.	SIFT	Damaging
2.	Provean	Damaging
3.	MutationAssessor	High
4.	MetaIR	Damaging
5.	MetaSVM	Damaging
6.	FATHMM-MKL	Damaging
7.	FATHMM	Damaging
8.	MutationTaster	Disease-causing
9.	DANN	Pathogenic (0.9893)
10.	Varsome	Uncertain Significance (Pathogenic, PM1, PM2, PP3)

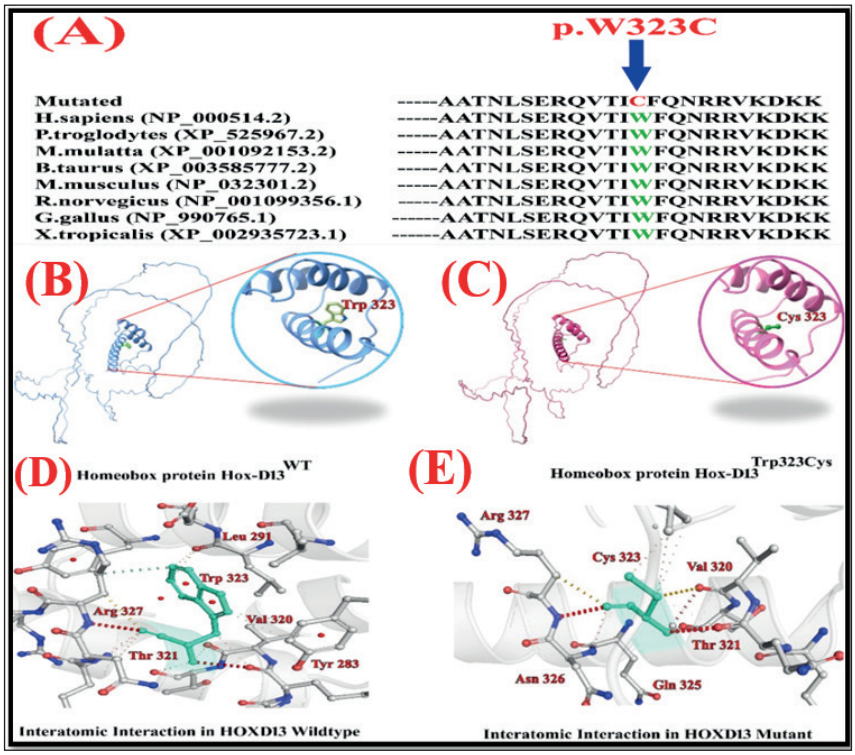

Figure 3. *(A) Amino acid sequence comparison of human HOXD13 protein with other orthologs, showing tryptophan (W) amino acid conservation across different species. (B-E) 3D protein structure showing the effect of (W323C) mutation on the interaction and protein structure of HOXD13 protein.*

revealed by different online available tools, and conserved across different species (Figure 3D). To date, 62 variants have been reported in the *HOXD13* gene causing several limbs malformation, including SPD, brachydactyly, oligodactyly, cryptorchidism, and other syndromic limb malformations (OMIM).

HOXD13 plays a very important role in the embryogenesis of limb development. The pathophysiology of the mutant/abnormal HOXD13 is postulated such that the mutant HOXD13 protein having additional polyalanine repeats leads to aggregation due to abnormal protein conformation, thus obstructing the translocation of proteins from the cytoplasm to the nucleus (20). Although the identifying mechanism is still unclear, the missense variant in the *HOXD13* gene causes a 50% reduction in protein levels (21).

As transcription factors, HOXD13 plays a major role in limb development. They are required at a certain dosage and time to initiate the correct morphogenic process. Thus disturbance or any type of mutation in the *HOXD13* gene could lead to abnormal limb/digits development. Pathogenic mutations in the *HOXD13* may also cause polydactyly by indirectly or directly inducing extraneous intradigital condrogenesis through retinoic acid reduction (22). The expression levels may differ in individuals within the same family, leading to phenotypic variability among heterozygous individuals from mild to clinically normal appearance (23).

Disorders like syndactyly associated with other severe disorders can be easily detected earlier using ultrasound procedures that might give the clinicians and doctors to perform proper genetic and molecular diagnosis that might help in the future management of the disorders. In addition, proper genetic counseling of the family having severe skeletal disorders might help eradicate the disorder in future poignancies. Furthermore, introducing a newborn screening program in a developing country like Pakistan will be the first step in screening some severe genetic disorders. Parental diagnosis can significantly reduce the burden of such severe disorders (24,25). This can be accomplished by prenatal genetic testing for monogenetic disorders (PGT-M). PGT and *in vitro*, fertilization are options for parents wishing to have future pregnancies (26,27). Furthermore, in a developing country like Pakistan, the identification of variants in genes using WES should be implemented in the hospitals that might help control severe genetic disorders in Pakistan (28-31).

In conclusion, we have reported a novel heterozygous disease-causing sequence variant in the *HOXD13* gene, resulting in an autosomal-dominant form of syndactyly. Our results expand the mutational spectrum of *HOXD13* and enhance our knowledge of the molecular basis of SD1. Furthermore, the present knowledge can be applied to embryotic or fatal genetic screening in other families suffering from syndactyly.

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List of Abbreviations

HOXD13	Homeobox D13
SD1	Syndactyly type 1
SPD	Synpolydactyly

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None.

Declaration of conflicting interests
The authors of this article have no affiliations with or involvement in any organization or entity with any financial interest or non-financial interest in the subject matter or materials discussed in this manuscript.

Consent to participate
Informed consent was obtained from the patients.

Ethical approval
The study was approved by the IRB of the UMT, Lahore, Pakistan. Ethical approval number: RC2022-R3, dated: January 14, 2022

Author contributions
MU and FA performed experiments and wrote the manuscript. SA did clinical tests and blood sampling. All authors reviewed and approved the final draft of the manuscript.

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References

1. Sato D, Liang D, Wu L, Pan Q, Xia K, Dai H, et al. A syndactyly type IV locus maps to 7q36. J Hum Genet. 2007;52:561–64. https://doi.org/10.1007/s10038-007-0150-5
2. Castilla EE, Paz JE, Orioli-Parreiras IM. Syndactyly: frequency of specific types. Am J Med Genet. 1980;5: 357–64. https://doi.org/10.1002/ajmg.1320050406
3. Malik S. Syndactyly: phenotypes, genetics and current classification. Eur J Hum Genet. 2012;20:817–24. https://doi.org/10.1038/ejhg.2012.14
4. Umair M, Ahmad F, Bilal M, Abbas S. Syndactyly genes and classification: a mini-review. J Biochem Clin Gene. 2018;1(1):34–47. https://doi.org/10.24911/JBCGenetics/183-1532177257
5. Alfadhel M, Umair M, Almuzzaini B, Asiri A, Al Tuwaijri A, Alhamoudi K, et al. Identification of the TTC26 splice variant in a novel complex ciliopathy syndrome with biliary, renal, neurological, and skeletal manifestations. Mol Syndromol. 2021;12(3):133–40. https://doi.org/10.1159/000513829
6. Ullah A, Ali RH, Majeed AI, Liaqat K, Shah PW, Khan B, et al. A novel insertion and deletion mutation in the BHLHA9 underlies polydactyly and mesoaxial synostotic syndactyly with phalangeal reduction. Eur J Med Genet. 2019;62(4):278–81. https://doi.org/10.1016/j.ejmg.2018.08.005
7. Ahmad F, Nasir A, Thiele H, Umair M, Borck G, Ahmad W. A novel homozygous missense variant in NECTIN4 (PVRL4) causing ectodermal dysplasia cutaneous syndactyly syndrome. Ann Hum Genet. 2018;82(4):232–8.
8. Ullah A, Umair M, Ahmad F, Muhammad D, Basit S, Ahmad W. A novel homozygous variant in the SMOC1 gene underlying Waardenburg anophthalmia syndrome. Ophthalmic Genet. 2017;38(4):335–9. https://doi.org/10.1080/13816810.2016.1227456
9. Umair M, Rafique A, Ullah A, Ahmad F, Ali RH, Nasir A, et al. Novel homozygous sequence variants in the GDF5 gene underlie acromesomelic dysplasia type-grebe in consanguineous families. Congenit Anom (Kyoto). 2017;57(2):45–51. https://doi.org/10.1111/cga.12187
10. Waqas A, Nayab A, Shaheen S, Abbas S, Latif M, Rafeeq MM, et al. Case Report: biallelic variant in the tRNA methyltransferase domain of the AlkB homolog 8 causes syndromic intellectual disability. Front Genet. 2022;13:878274. https://doi.org/10.3389/fgene.2022.878274
11. Ullah A, Umair M, Majeed AI, Abdullah, Jan A, Ahmad W. A novel homozygous sequence variant in GLI1 underlies first case of autosomal recessive pre-axial polydactyly.

Clin Genet. 2019;95(4):540–1. https://doi.org/10.1111/cge.13495

12. Ahmad F, Ahmed I, Alam Q, Ahmad T, Khan A, Ahmad I, et al. Variants in the PNPLA1 gene in families with autosomal recessive congenital ichthyosis reveal clinical significance. Mol Syndromol. 2021;12(6):351–61. https://doi.org/10.1159/000516943
13. Ullah A, Gull A, Umair M, Irfanullah, Ahmad W. Homozygous sequence variants in the WNT10B gene underlie split hand/foot malformation. Genet Mol Biol. 2018;41(1):1–8. https://doi.org/10.1590/1678-4685-gmb-2016-0162
14. Khan S, Rawlins LE, Harlalka GV, Umair M, Ullah A, Shahzad S, et al. Homozygous variants in the HEXB and MBOAT7 genes underlie neurological diseases in consanguineous families. BMC Med Genet. 2019;20(1):199. https://doi.org/10.1186/s12881-019-0907-7
15. Nayab A, Alam Q, Alzahrani OR, Khan R, Sarfaraz S, et al. Targeted exome sequencing identified a novel frameshift variant in the PGAM2 gene causing glycogen storage disease type X. Eur J Med Genet. 2021;64(9):104283. https://doi.org/10.1016/j.ejmg.2021.104283
16. Umair M, Ballow M, Asiri A, Alyafee Y, Tuwaijriet AA, Alhamoudi KM, et al. EMC10 homozygous variant identified in a family with global developmental delay, mild intellectual disability, and speech delay. Clin Genet. 2020;98(6):555–61. https://doi.org/10.1111/cge.13842
17. Deng H, Tan T. Advances in the molecular genetics of non-syndromic syndactyly. Curr Genomics. 2015;16(3):183–93. https://doi.org/10.2174/1389202916666150317233103
18. Dai L, Liu D, Song M, Xu X, Xiong G, Yang K, et al. Mutations in the homeodomain of HOXD13 cause syndactyly type 1-c in two Chinese families. PLoS One. 2014;9(5):e96192. https://doi.org/10.1371/journal.pone.0096192
19. Albrecht AN, Schwabe GC, Stricker S, Boddrich A, Wanker EE, Mundlos S. The synpolydactyly homolog (spdh) mutation in the mouse - a defect in patterning and growth of limb cartilage elements. Mech Dev. 2002;112(1–2):53–67. https://doi.org/10.1016/S0925-4773(01)00639-6
20. Albrecht AN, Kornak U, Böddrich A, Süring K, Robinson PN., Stiege AC, et al. A molecular pathogenesis for transcription factor associated poly-alanine tract expansions. Hum Mol Genet. 2004;13(20):2351–9. https://doi.org/10.1093/hmg/ddh277
21. Fantini S, Vaccari G, Brison N, Debeer P, Tylzanowski P, Zappavigna V. G220V substitution within the N-terminal transcription regulating domain of HOXD13 causes a variant synpolydactyly phenotype. Hum Mol Genet. 2009;18(5):847–60. https://doi.org/10.1093/hmg/ddn410
22. Kuss P, Villavicencio-Lorini P, Witte F, Klose J, Albrecht AN, Seemann P, et al. Mutant Hoxd13 induces extra digits in a mouse model of synpolydactyly directly and by decreasing retinoic acid synthesis. J Clin Invest. 2009;119(1):146–56. https://doi.org/10.1172/JCI36851
23. Umair M, Hayat A. Non-syndromic split hand-foot malformation (SHFM): recent classification. Mol Syndromol. 2019;10:243–54. https://doi.org/10.1159/000502784
24. Alfadhel M, Umair M, Almuzzaini B, Alsaif S, AlMohaimeed SA, Almashary MA, et al. Targeted SLC19A3 gene sequencing of 3000 Saudi newborn: a pilot study toward newborn screening. Ann Clin Transl Neurol. 2019;6(10):2097–103. https://doi.org/10.1002/acn3.50898
25. Alyafee Y, Alam Q, Altuwaijri A, Umair M, Haddad S, Alharbi M, et al. Next-generation sequencing-based pre-implantation genetic testing for aneuploidy (PGT-A): first report from Saudi Arabia. Genes (Basel). 2021;12(4):461. https://doi.org/10.3390/genes12040461
26. Alyafee Y, Al Tuwaijri A, Alam Q, Umair M, Haddad S. Next generation sequencing based non-invasive prenatal testing (NIPT): first report From Saudi Arabia. Front Genet. 2021;12:630787. https://doi.org/10.3389/fgene.2021.630787
27. Alyafee Y, Al Tuwaijri A, Umair M, Alharbi M, Haddad S, Ballow M. Non-invasive prenatal testing for autosomal recessive disorders: a new promising approach. Front Genet. 2022;13:1047474. https://doi.org/10.3389/fgene.2022.1047474
28. Umair M, Ahmad F, Ullah A. Whole exome sequencing as a diagnostic tool for genetic disorders in Pakistan. PJMR. 2018;57(2);90–1.
29. Umair M, Ahamd F, Bilal M, Asiri A, Younus Y, Khan A. A comprehensive review of genetic skeletal disorders reported from Pakistan: a brief commentary. Meta Gene. 2019;20(1–7):100559. https://doi.org/10.1016/j.mgene.2019.100559
30. Hayat A, Hussain S, Bilal M, Kausar M, Almuzzaini B, Abbas S, et al. Biallelic variants in four genes underlying recessive osteogenesis imperfect. Eur J Med Gent. 2020;63(8):103954. https://doi.org/10.1016/j.ejmg.2020.103954
31. Umair M. Rare genetic disorders: beyond whole-exome sequencing. J Gene Med. 2023:e3503. https://doi.org/10.1002/jgm.3503