ORIGINAL ARTICLE DOI:https://doi.org/10.52206/jsmc.2023.13.3.756 OPEN ACCESS

Mutation Screening for Molecular Pathogenicity of MSX1 gene in Human Cleft lip Subjects from Khyber Pakhtunkhwa

Amin Jan,[1] Seher Obaid,[1] Susan Kakakhel,[1] Saifullah Khalil,[2] Tehmeed Ullah,[3] Musharraf Jelani[4]

ABSTRACT

Background: Cleft lip palate is a genetically heterogeneous group of diseases. Cleft lip-palate can cause deleterious effects during growth on the appearance, language and hearing abilities. Multidisciplinary approach to the care for children born with these defects is required.

Objective: To investigate a selected MSX1gene for the molecular pathogenicity in the two families of cleft lip and palate segregating disease phenotype in autosomal recessive fashion.

Material and Methods: After getting informed written consent and approved from the Institutional Review Board, KMU Peshawar, clinical Investigations of the selected two families were performed in the HMC, Peshawar. Blood Sampling was done in EDTA tubes and DNA was extruded through standard procedure. PCR was performed. Primers were designed using Primers 3 Software and DNA Sequencing done. Sequencing was performed. Bioedit Software and ClustalW were used for Sequence Alignment. Mutation analysis of the pathogenic variant in families A and B demonstrating autosomal recessive non-syndromic cleft lip palate phenotype was performed.

Results: while analyzing bidirectional sequencing of MSX1 gene including all coding exons and intron-exons boundaries in two affected individuals of family A and an affected individual of family B, the affected individuals in each family did not reveal any pathogenic variant. Thus pathogenicity of MSX1 gene in the patients of families A and B exhibiting autosomal recessive non-syndromic cleft lip palate phenotype was excluded.

Conclusion: Mutations screening for molecular pathogenicity of MSX1 gene in affected individuals of families with cleft lip palate phenotypes were investigated and revealed that MSX1gene is not the culprit pathogenic variant in the two families understudy.

Keywords: Autosomal recessive, Heterogeneous group, Non-syndromic, Pathogenic variants, Phenotype,

This article may be cited as: Jan A, Obaid S,Kakakhel S, Khalil S, Ullah T, Jelani M. Mutation Screening for Molecular Pathogenicity of MSX1 gene in Human Cleft lip Subjects from Khyber Pakhtunkhwa. J Saidu Med Coll Swat 2023;13(3):93-102. DOI:https://doi.org/10.52206/jsmc.2023.13.3.756

INTRODUCTION

Cleft lip palate or cleft lip without cleft palate (CL/P, MIM 119530) is a genetically heterogeneous group of diseases involving upper lip, and oral cavities. These are non-fatal birth defects which are multi-factorial in origin, as both genes and environmental factors contribute to their etiology. On the basis of diverse ethnic and geographic distribution it is one of the commonest congenital craniofacial defects.[1]

Isolated cleft lip and cleft palate are usually grouped together as cleft lip-palate due to epidemiological as well as embryological similarities.[2] Cleft lip anomaly is established as a unilateral (either right or left side) or as a bilateral (both left and right). The line of the cleft usually emerge on the lateral portion of the upper lip and descends through philtrum to the alveolus between canine tooth and lateral incisor, following the line of incisive suture up to incisive foramen.[3]

1. Northwest School of Medicine, Peshawar Pakistan.
2. Swat Medical College, Swat Pakistan.
3. Hayatabad Medical Complex, Peshawar.
4. Islamia College University, Peshawar Pakistan.

Correspondence: Dr. Amin Jan
Northwest School of Medicine, Peshawar Pakistan.
Email: dr.aminjan@hotmail.com

Received: October 27th, 2022 - 1st Revision: July 11th, 2023
2nd Revision: July 14th, 2023 - Accepted: August 20th, 2023

Cleft lip-palate manifests when cleft lip extends from incisive foramen to the palatine suture in central part of the palate, again having different ranges of severities. The line of cleft may be disturbed by soft tissue bridges like skin or mucosa, or hard tissues like bone, or both. It results to establish an incomplete cleft, which may lead to unilateral and bilateral cleft lip-palate.[4] There is wide variation of severity of cleft lip, ranging from a minimal notch located on lateral part of the lip to the most serious form: a bilateral cleft-lip and alveolus, separating upper lip philtrum and pre-maxilla from the maxillary arch.[5]

In spite of its treatable nature, cleft lip-palate can cause deleterious effects during the growth on the appearance, language and hearing abilities.[6] These problems can cause psychological difficulties for the affected individuals as well as their families which ultimately results in complications in social integration. These patients need a multidisciplinary care right from birth or infancy till their puberty, because they contribute to increased mortality and morbidity ratio as compared to the general population.[7]

Multidisciplinary approach to the care for children born with these defects, include nursing, maxillofacial surgery, plastic surgery, otolaryngology, audiology, speech therapy,

genetics, psychology, counseling, orthodontics and dentistry i.e. the clinical care has been fragmented among different disciplines, which definitely leads to substantial variations in management and hence cause controversy.[8] Anyhow care standard for patients with cleft lip, cleft lip palate and cleft palate only, in both the developed as well as the developing countries, still remains a cause for concern.

Classification of Cleft Lip Palate

On the basis of association with specific malformative patterns or occurrence as isolated defects, cleft lip palate can broadly be categorized into two subtypes, syndromic and non-syndromic.[9] Both of these forms exhibit genetics as a strong component.

1. Genetics of Non-Syndromic Cleft Lip Palate

Non-syndromic cleft lip palate, a multifactorial disorder, is caused by interaction between genes and environment. The environmental factors play role demonstrating variable prevalence of the disease in newborns in various countries.[10] Diverse potential agents have been identified, while focusing their attention to the causative environmental risk factors for cleft lip palate, in several studies such as addiction of mother during pregnancy to alcohol, smoke, exposure to viral infection, malnutrition or diet deficiency, use of drugs or other teratogenic agents.[11] Physical or emotional stress implication in clefting has also been suggested very recently.[12] Followings factors are the probable causes of non-syndromic cleft lip palate.

a. Transforming Growth Factor α
b. Transforming Growth Factor β-2
c. Transforming Growth Factor β-3
d. Muscle Segment Homeobox Drosophila Homolog 1
e. Methylene-Tetrahydro-Folate Reductase

2. Genetics of Syndromic Cleft Lip-Palate

Syndromic clefts have strong association with chromosomal syndromes (more than 350 Mendelian disorders), known teratogenic exposure (alcohol, caffeine and smoking) and uncategorized syndromes.[13] Several syndromes involving cleft lip with or without cleft palate have been reported previously.

a. Van der Woude syndrome
b. Popliteal Pterygium Syndrome
c. X-linked Clefting and Ankyloglossia
d. Gorlin Syndrome
e. Stickler Syndrome

The purpose of this study is to investigate the molecular pathogenicity of MSX1gene in the two families of cleft lip and palate segregating disease phenotype in autosomal recessive fashion, through Sanger sequencing.

MATERIAL AND METHODS

Study comprising of clinical and molecular analysis of two families with cleft lip palate, is presented in this study. Informed written consent from each family was taken for their participation and publishing their photographs for clinical presentations in the present study. The Institutional Review Board (IRB), Khyber Medical University Peshawar, Pakistan approved this study according to the ethical committee's guidelines.

Families' Description

The research subjects were visited at their residential area at village Khero Khel Pacca, district Lakki Marwat. Families' elders were interviewed for getting information about the disease history in each family. Details of cousins' marriage between the parents of the affected individuals were sorted out. Pedigrees were drawn. Squares were used to indicate male individuals in a pedigree drawing. Shaded squares were for male patients and un-shaded for normal male individuals, while shaded circles were used for female patients and un-shaded for normal females. Consanguineous unions were shown by double marriage lines between the partners. Crossed symbols were meant for deceased members of the families. Roman (I, II, III...) numerals were used for representing generation breakup, whereas Arabic (1, 2, 3...) numerals to show persons in each generation.

Family A

Family A belonged to a remote district, Lakki Marwat, of Khyber Pakhtunkhwa province. It was a five generations family demonstrating non-syndromic cleft lip palate phenotype segregating in autosomal recessive fashion. Affected individuals, born to a consanguineous couple, were present in the fifth generation only and there was no previous history of the disease in the forefathers of parents.

Venous blood samples of six family members, including parents (III-1 and IV-1), two affected, a male (V-1) and a female (V-4) and two unaffected (V-2 and V-3) siblings, were collected for the study.

Family B

Family B belonged to a remote village of Miran Shah, tribal areas of North Waziristan Agency near Bannu district, Khyber Pakhtunkhwa province. It was a three generations family demonstrating non-syndromic bilateral cleft lip palate phenotype segregating in autosomal recessive fashion. Affected individuals, born to a consanguineous couple, were present in the third generation only and there was no previous history of the disease in the forefathers of parents.

Venous blood samples of three family members, including parents (III-1 and III-2) and an affected daughter (VI-1), were collected for the study. Sample of venous blood (5-7ml) was obtained from selected persons, willing to participate in the study, in respective families. Ethylene-diamine tetra-acetic acid (EDTA) tubes (BD Vacutainers®, USA) were used to store the blood samples at 3-5°C in refrigerator.

Clinical and Radiological Examinations

Clinical and radiological examinations of all the affected individuals were performed in the plastic surgery department, Hayatabad Medical Complex, Peshawar. Surgical interventions of the affected individuals of cleft lip palate of families A and B were performed at plastic surgery department, Hayatabad Medical Complex.

Extraction of Genomic DNA

A method used by Lõoke M et al; (2011) was used with some modifications.[14] Genomic DNA was extracted from venous blood, stored in ethylene-diamine tetra-acetic acid tubes, using Purelink TM Genomic DNA Extraction Kit (Invitrogen, USA). After the lysis of cells and nuclei, absolute ethanol (Sigma-Aldrich, Germany) of 200 µl amount was added to the lysis product and mixed thoroughly by vortexing up to 8-10 seconds. The solutions were poured over the GD columns. Dried GD columns were transferred to fresh 2 ml micro-centrifuge tubes and preheated elution buffer of 72°C were added in amount of 100 µl to each GD column and kept at room temperature for five minutes. The matrix of GD column absorbed the elution buffer which was then centrifuged at 13000 rmp for one minute. The microcentrifuge tubes then contained genomic DNA dissolved in elution (Tris-EDTA) buffer. The DNA concentration was measured using automated spectrophotometer, Nanodrop-1000 (Thermo-Scientific, United States) at optical density 260 nm. The DNA was diluted to 100 ng/µl and stored at 2-8°C for further use and analysis.

Polymerase Chain Reaction

A method used by Schochetman G et al; (1988) was used with some modifications.[15] In 200 µl small tubes (Axygen, USA), 50 ng template DNA was used for polymerase chain reaction (PCR) in 50 µl of reaction mixture, using 20-25 pmol of primer each primer's set, 0.25 mM free nucleotides or dNTPs, 1-2 units Taq polymerase, 1-2 mM magnesium chloride and 3 µl of 10X ammonium sulphate buffer (Invitrogen, USA). The reaction mixture was centrifuged for thirty second at 600 rpm for thorough mixing. Standard reaction conditions were used for DNA amplification. The steps included first cycle of DNA denaturation at 95°C for 3 minutes, followed by 35 cycles of denaturation at 95°C for thirty seconds, annealing temperature at 55-59°C for thirty seconds and extension or polymerization at 72°C for thirty seconds, and final extension for 8 minutes at 72°C. The reaction was performed in TPersonal thermo-cycler (Biometra® Germany).

Gel Electrophoresis

A method used by Aaij C et al; (1972) was used with some modifications.[16] The products of PCR were analyzed through visual inspection on 2% agarose gel stained with ethidium bromide. The composition of agarose gel was 100 ml 1X Tris-EDTA buffer (Tris 89.1 mM, EDTA 2.5 mM, Boric acid 88.9 mM) 2 grams of agarose and 10 µl of ethidium bromide (10 mg/ml). Prior to electrophoresis the samples were stained with 3 µl loading dye (0.25% bromophenol blue in 40% sugar solution). The electrophoresis conditions included 100 volts current for 30 minutes in horizontal gel electrophoresis apparatus (Bio-Rad, USA) having 1X Tris-EDTA running buffer. The size of amplified products was determined by using DNA ladder of 50-base pairs (Fermentas, Life Sciences, UK). After completion of electrophoresis, the gel was placed on UV trans-illuminator (Biometra, Germany) to visualize amplified products of polymerase chain reaction.

Primers Designing

Primers sets (Table 01) were designed for PCR amplification from intronic regions of targeted DNA sequences using online Primers3 software (http://frodo.wi.mit.edu/). Breslauer et al, (1986)

devised Primers3 default chemistry parameters for thermodynamic stability of primers were selected.[17] The size of each primer ranged from 18-22bp long with an average GC contents of 45-60%. The product size of each set was less than 1000bp.

Mutation Screening

Mutation screening of potential candidate genes in the related phenotypes was performed through bidirectional sequencing. The targeted sequences of MSX1 genes were amplified through standard PCR conditions. After 1st amplification PCR and exons purification, the products were subjected to 2nd or sequencing PCR using BigDye® Terminator v1.1 Cycle Sequencing Kit according to instructions of the supplier (ABI Life Technologies, USA). The ethanol purified products of 2nd or sequencing PCR were loaded to automated DNA sequencer ABI 3730XL DNA Analyzer (ABI Life Technologies, USA).

Table 01. Primers sequences selected for PCR amplification of MSX1 gene

No	Name	Primers Sequence	Product	Ta
1	MSX1-2F	5'-CCTCGCCTTATTAGCAAGTTC-3'	412 bp	60°C
2	MSX1-2R	5'-ATCAGATGAGGGATCATGGAC-3'		
3	MSX1-2F	5'-CTCCTGGAATCTTAGTTTCTTC-3'	519 bp	61°C
4	MSX1-2R	5'-TACTAAGGGACTCTTCCAGCC-3'		

Ta = annealing temperature, oC = degree centigrade, F = forward primer, R = reverse primer, bp = base pairs

Sequencing Data Analysis

Sequencing raw data analysis was performed by using ABI Sequence Scanner software v 1.0 (ABI Life Technologies, USA). For finding mutations in candidate genes, two softwares Bioedit sequences aligner 6.0.7 editor version and Mutation Surveyor V4.0.5 (SoftGenetics Inc.) were used to align wild type and patients' sequences. The ClustalW tool (http://www.ebi.ac.uk/Tools/msa/clustalw2/) was used for multiple sequence alignment.

RESULTS

Pedigrees Analyses and Clinical Features

Family A

Family A (Figure 01), demonstrated a non-syndromic cleft lip palate phenotype. Pedigree analysis revealed autosomal recessive segregation of the disease. In six generations pedigree the disease phenotype had appeared only in the sixth generation without any previous history of forefathers. The patients were born to a consanguineous couple and at the time of study an affected male (VI-1) and an affected female (VI-4) were of 10 and 4 years of ages respectively.

Both patients exhibited unilateral cleft lip palate in association with hypodontia and irregular dentition (Figures 2 and 3). Radiological examinations of the affected male (VI-1) revealed that canines were missing in primary and secondary dentition. Both affected individuals were operated for correction of cleft lip and cleft palate at Plastic Surgery Department Hayatabad Medical Complex, Peshawar at two years of ages. Both parents (IV-1 and V-1) of the affected individuals and two of the siblings (a female VI-2 and a male VI-3) in the sixth generation were phenotypically normal.

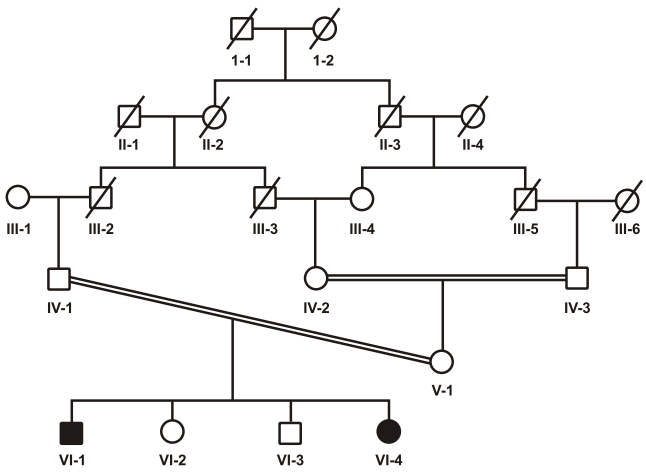


Figure 01:
Pedigree analysis of family A demonstrating a non-syndromic cleft lip palate phenotype with autosomal recessive mode of inheritance. Affected males and females are indicated by filled squares and circles, respectively. Double lines between individuals represent consanguineous unions. The deceased individuals are indicated by crossed symbols.

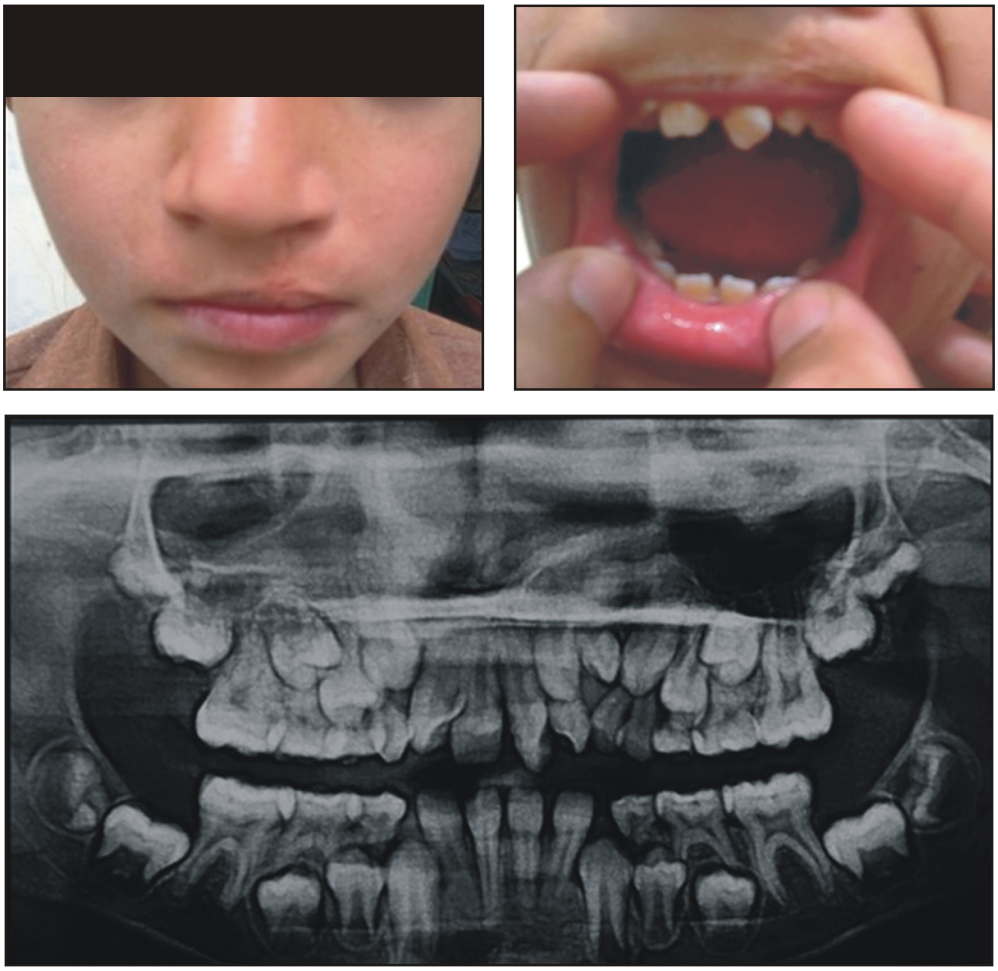


Figure 02: Clinical presentation of an affected male (VI-1) of family A. Note post-operative unilateral cleft-lip and the associated sign of hypodontia (missing canines). OPG radiograph represent abnormal dentition and hypodontia. Note the irregular incisors and missing canines in primary and secondary dentition *(Picture are reproduced with patient/attendant consent).*



Figure 03: Clinical presentation of an affected female (VI-4) of family A. Note post-operative unilateral cleft-lip and both pre and post-operative cleft-palate *(Picture are reproduced with patient/attendant consent).*

Family B

Family B (Figure 4) demonstrated a non-syndromic unilateral cleft lip phenotype. Pedigree analysis revealed the disease segregation in autosomal recessive fashion. In three generations pedigree the disease phenotype had appeared only in the third generation without any previous history of forefathers. The affected female patient (III-1) was born to a consanguineous couple and was eight months old at the time of study. The patient represented unilateral cleft lip without involving palate or other associated anomalies. She was operated for correction of cleft lip at Plastic Surgery Department Hayatabad Medical Complex, Peshawar. Both parents (II-1 and II-2) of the affected individual were phenotypically normal.

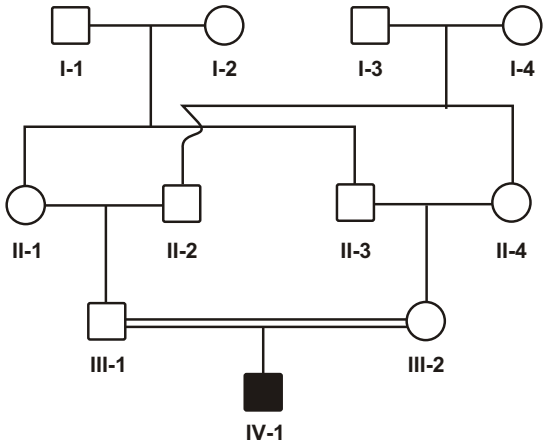


Figure 04: Pedigree analysis of family B demonstrating an autosomal recessive cleft lip phenotype. Affected males and females are indicated by filled squares and circles, respectively. Double lines between individuals represent a consanguineous union.

Mutation Analysis of MSX1 Gene in families A and B

Mutation analysis of the pathogenic variant in families A and B demonstrating autosomal recessive non-syndromic cleft lip palate phenotype was performed by direct sequencing of MSX1 gene as a potential candidate. Bidirectional sequencing of MSX1 gene including all coding exons and intron-exons boundaries in all members of family A (Figure 5 and 6) and B (Figure 7 and 8) was performed by automated sequencer ABI 3730XL DNA Analyzer (Life Technologies, USA). However sequencing analyses of two affected individuals (V-1 and V-3) of family A and an affected individual of family B did not reveal any pathogenic variant. Thus pathogenicity of MSX1 gene in the patients of families A and B exhibiting autosomal recessive non-syndromic cleft lip palate phenotype was excluded.

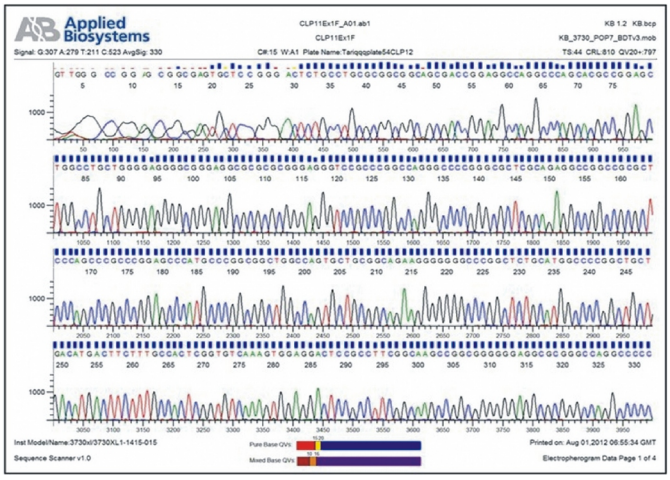


Figure 05: Electropherogram of MSX1 gene sequence of exon 1 (5' end) with primer 1F in unaffected parent (IV-1) of family A.

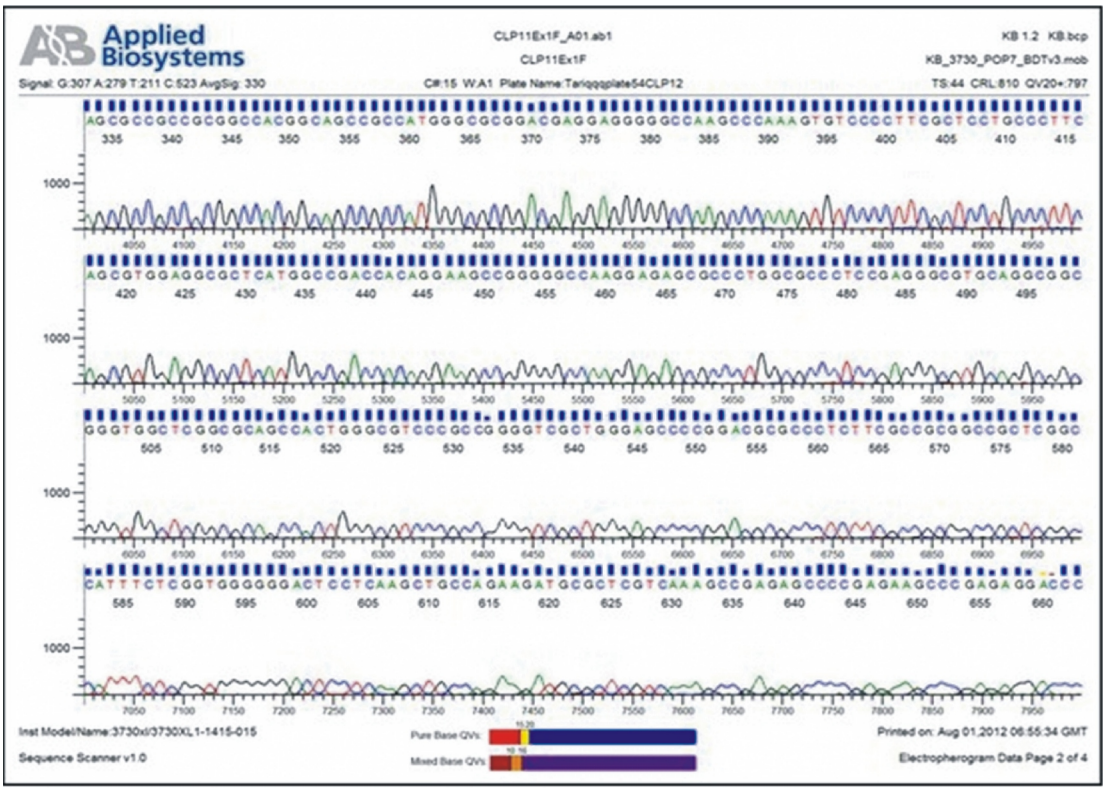


Figure 06: Electropherogram of MSX1 gene sequence of exon 1 (3' end) with primer 1F in unaffected parent (IV-1) of family A.

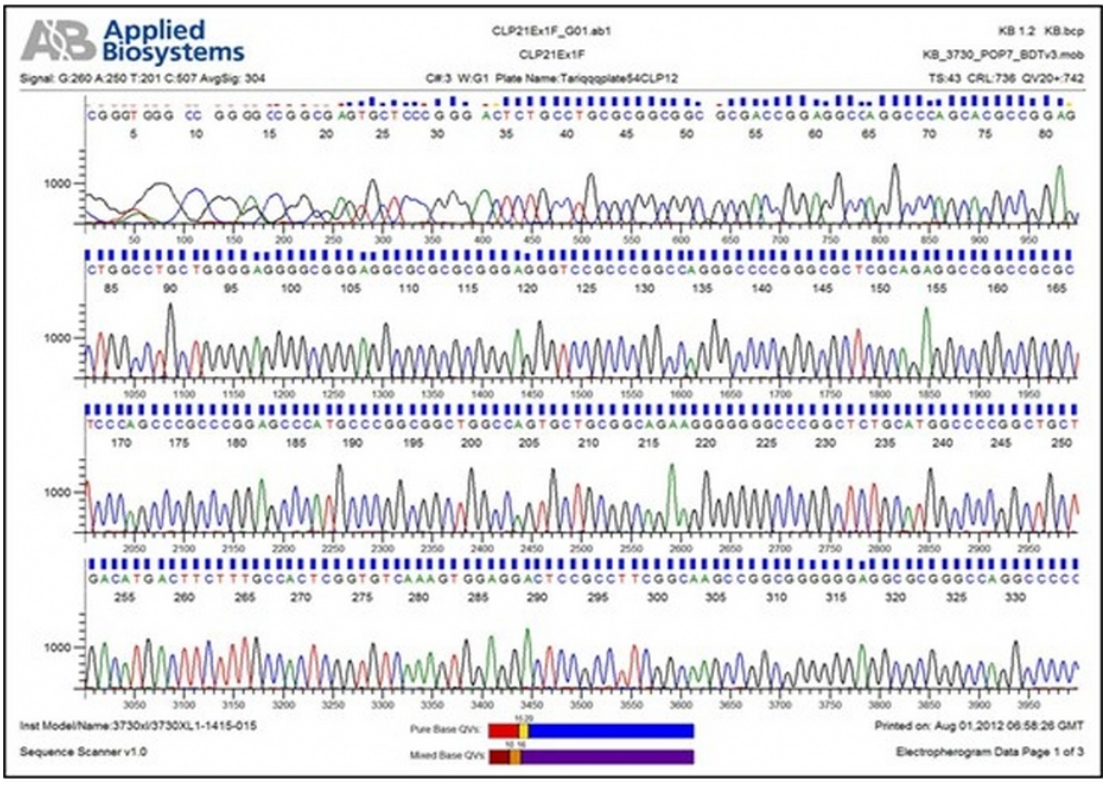


Figure 07: Electropherogram of MSX1 gene sequence of exon 1 (5' end) with primer 1F in an affected individual (IV-1) of family B.

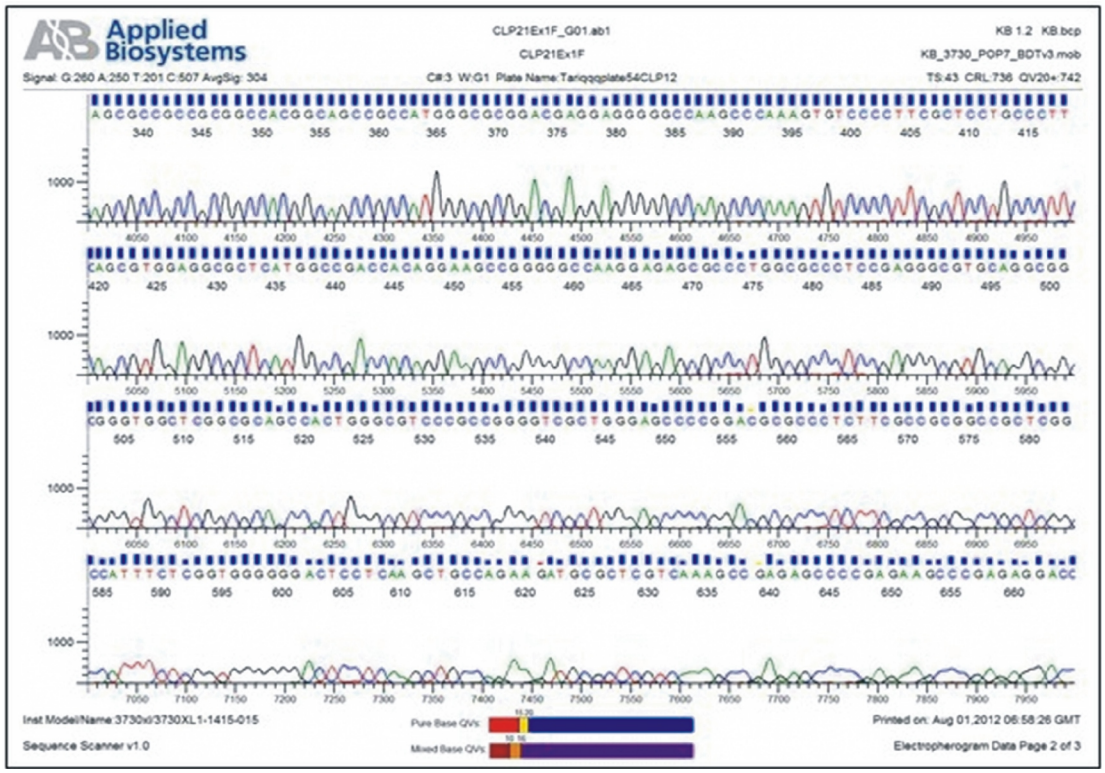


Figure 08: Electropherogram of MSX1 gene sequence of exon 1 (3' end) with primer 1F in an affected individual (IV-1) of family B.

DISCUSSION

Identification of the genes involved in the development of the human craniofacial region can serve as a first step towards developing a better understanding of diagnosis, treatment and preventions of developmental anomalies of this region. The genetic background of NSCL/P is complex and most causes remain yet to be identified.

Mutations screening of familial cases has been widely used as a diagnostic tool. In present study two families with non-syndromic cleft lip palate were investigated for molecular pathogenicity. The mammalian MSX gene family consists of 3 physically unlinked members, named MSX1, MSX2 and MSX3. However in developing vertebrate embryos, MSX 1 and MSX 2 are widely expressed at the sites where epithelial mesenchymal interactions take place.

The process of palatal fusion is also controlled by interactive signaling from the mesenchyme to the epithelium, which is mediated by growth factors and extracellular matrix proteins. One of the key components in this process is MSX 1 expression, which coordinates palatogenesis. Mutations screening of MSX1 in affected individuals of cleft lip palate were performed through Sanger sequencing. Mutation analysis of the pathogenic variant in families A and B demonstrating autosomal recessive non-syndromic cleft lip palate phenotype was performed by direct sequencing of MSX1 gene as a potential candidate.

Patients of families A and B exhibited unilateral cleft lip palate in association with hypodontia and irregular dentition. Radiological examinations revealed missing canines in primary and secondary dentitions. Affected individuals were operated for cleft lip and cleft palate corrections. Mutation analysis of MSX1 gene in affected individuals of these families did not reveal pathogenic variant.

Bidirectional sequencing of MSX1 gene including all coding exons and intron-exons boundaries in all members of family A (Figure 5 and 6) and B (Figure 7 and 8) was performed by automated sequencer ABI 3730XL DNA Analyzer (Life Technologies, USA). However sequencing analyses of two affected individuals (V-1 and V-3) of family A and an affected individual of family B did not reveal any pathogenic variant. Thus

pathogenicity of MSX1 gene in the patients of families A and B exhibiting autosomal recessive non-syndromic cleft lip palate phenotype was excluded.

Gene-gene interactions of PAX9 and MSX1 have been thoroughly studied in cleft lip palate development in mouse models. Double heterozygous mutants of PAX9 and MSX1 exhibit consistently missing lower incisors, however transgenic BMP4 expression compensates the disease phenotype to some extent [18]. Similarly according to a previous study Shh and Bmp2 expression in these mutants indicate that dental epithelial growth is significantly reduced after transition of bud to cap stage, thus leading to a smaller "incisor field" and this defect is followed by diminished mesenchymal expression of FGF3 and FGF10 [19]. The products of FGF3 and FGF10 genes function as stimulators of epithelial growth during tooth development.[20]

Reduced cell proliferation in dental epithelium, mesenchyme and lack of mesenchymal Notch1 expression was also observed in double heterozygous mutants at the bud stage and exhibited abnormal differentiation of ameloblast on labial and lingual surfaces.[21] Concerning oligodontia phenotype MSX1 and PAX9 are frequently associated with absence of first premolars and molars respectively.[22] Thus a shared reduction of PAX9 and MSX1 in humans may enhance the risk for orofacial clefting and oligodontia. A candidate gene-based association study reported significant linkage disequilibrium between both CLP and CP with polymorphisms in MSX1.[23] Contrary to these results in our study it was observed that there was no significant association of NSCL/P with the MSX1 gene variant.

CONCLUSION

Candidate gene sequencing of cleft lip palate was performed in familial cases from remote areas of Khyber Pakhtunkhwa, Province Pakistan. A single candidate gene sequencing following Sanger method did not reveal pathogenic variant.

Recommendations

Determining the relative risk of CLP on the basis of genetic background and environmental influence (including smoking, alcohol use, and dietary factors) will be useful for genetic counseling and the development of future preventive measures. We suggest further sequencing of candidate genes and preferably whole exome analysis through next generation or solid phase sequencing.

Conflict of Interest

Authors declare no conflict of interest

Grant Support and Financial Disclosure

NIL

REFERENCES

1. Worley ML, Patel KG, Kilpatrick LA. Cleft lip and palate. Clin Perinatol. 2018;45(4):661-78.
2. Wilson-Nagrani C, Richmond S, Paternoster L. Non-syndromic cleft lip and palate polymorphisms affect normal lip morphology. Frontiers in Genetics. 2018;9(2):413-8.
3. Almas F, Cote V, Ramly EP, Kantar RS, Hamdan U. Premaxillary setback with posterior vomerine ostectomy: outcomes of single-stage repair of complete bilateral cleft lip with a severely protruding premaxilla. Cleft Palate Craniofac J. 2019;56(4): 471-8.
4. Vyas T, Gupta P, Kumar S, Gupta R, Gupta T, Singh HP. Cleft of lip and palate: Areview. J Fam Med Prim Care. 2020;9(6):2621-5.
5. Deepak C, Ramanathan A. Role of MSX1 Gene in Orofacial Clefting: A Systematic Review. Biomed Pharmacol J. 2017;10(3):1071-3.
6. Neela PK, Gosla SR, Husain A, Mohan V, Thumoju S, Rajeshwari R. Analysis of single nucleotide polymorphisms on locus 13q33. 1-34 in multigenerational families of cleft lip palate using MassArray. Indones Biomed J. 2021;13(1):27-33.
7. Manojkanna K, Chandana CS, Priya VV, Gayathri R. Role of magnesium in the expression of MSX-1 and PAX9 molecules during early tooth development. Drug Invent Today. 2018;10(2):38-43.
8. Janaki CS, Lokanadham S. Role of MSX1 Gene Mutation as Predictive Factor in Cleft Palate/Lip-A Mini Review on Facial Skeletal Malformations. J Mol Genet Med. 2017;11(309):174708-62.
9. Gulnaz H, Majeed N, Ijaz S. Patterns of Orofacial Clefts in Patients with Consanguineous Parents. Pak J Med Health Sci. 2017;11(2):791-4.
10. Gargya I, Chawla JPS. The enigma of genetics on development of human dentition. Clin Surg. 2017;2:14-86.
11. Suazo J, Santos JL, Colombo A, Pardo R. Gene-gene interaction for nonsyndromic cleft lip with or without cleft palate in Chilean case-parent trios. Arch Oral Biol. 2018;91:915-23.
12. Gu M, Zhang Y, Liu H, Liu J, Zhu D, Yang X. MSH homeobox 1 polymorphisms and the risk of non-syndromic orofacial clefts: A meta-analysis. Eur J Oral Sci. 2018;126(3):180-5.
13. Haynie AS, English JD, Paravina RD, Moon A, Hanigan J, Abu Al Tamn MA, et al. Colour stability of 3D-printed resin orthodontic brackets. Journal of Orthodontics. 2021 Sep;48(3):241-9.
14. Lõoke M, Kristjuhan K, Kristjuhan A. Extraction of genomic DNA from yeasts for PCR-based applications. Biotechniques. 2011;50(5):325-8.
15. Schochetman G, Ou CY, Jones WK. Polymerase chain reaction. J Infect Dis. 1988;158(6):115-47.

16. Aaij C, Borst P. The gel electrophoresis of DNA. Biochim Biophys Acta BBA-Nucleic Acids Protein Synth. 1972;269(2):192-200.
17. Breslauer KJ, Frank R, Blöcker H, Marky LA. Predicting DNA duplex stability from the base sequence. Proc Natl Acad Sci. 1986;83(11): 3746-50.
18. Hong Y, Xu X, Lian F, Chen R. Environmental risk factors for nonsyndromic cleft lip and/or cleft palate in Xinjiang Province, China: A multiethnic study. Cleft Palate Craniofac J. 2021;58(4):489-96.
19. Askarian S, Gholami M, Khalili-Tanha G, Tehrani NC, Joudi M, Khazaei M, et al. The genetic factors contributing to the risk of cleft lip-cleft palate and their clinical utility. Oral Maxillofac Surg. 2022; 110-18.
20. Saleem K, Zaib T, Sun W, Fu S. Assessment of candidate genes and genetic heterogeneity in human non syndromic orofacial clefts specifically non syndromic cleft lip with or without palate. Heliyon. 2019;5(12):572-77.
21. Weng M, Chen Z, Xiao Q, Li R, Chen Z. A review of FGF signaling in palate development. Biomed Pharmacother. 2018;103:240-47.
22. Consolaro A, Medeiros MCM, Miranda DAO, Oliveira IA de. Supernumerary teeth in patients with cleft lip and palate: the tooth germs do not separate. Dent Press J Orthod. 2021;26(8):324-29.
23. Lupo PJ, Mitchell LE, Jenkins MM. Genome-wide association studies of structural birth defects: A review and commentary. Birth Defects Res. 2019; 111(18):1329-42.

DATA SHARING STATEMENT: The data that support the findings of this study are available on request from the corresponding author. The data is not publicly available due to privacy or ethical restrictions.

AUTHOR'S CONTRIBUTION

The following authors full fill authorship criteria as per ICMJE guidelines;

Jan A, Obaid S:	Substantial contribution to conception and design, drafting the manuscript, final approval, agreed to be accountable for all the work.
Kakakhel S, Khalil S:	Substantial contribution to conception and design, data acquisition, drafting the manuscript, final approval, agreed to be accountable for all the work.
Ullah T:	Analysis and interpretation of data, drafting the manuscript, final approval, agreed to be accountable for all the work.
Jelani M:	Analysis and interpretation of data, critical revision,final approval, agreed to be accountable for all the work.

This is an Open Access article distributed under the terms of the Creative Commons Attribution-NonCommercial 4.0 International License, which permits unrestricted use, distribution & reproduction in any medium provided that original work is cited properly.