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Research Article

Variant Analysis of *FOXE1* Gene in Non-Syndromic Cleft Lip and Palate Patients of Peshawar, Pakistan

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Abstract

One of the most common structural birth defects is the cleft lip and palate, having an incidence rate of 1:700 live births worldwide. It can be isolated cleft lip, isolated cleft palate or a combination of both cleft lip and palate. The orofacial cleft can be caused by a variety of genetic and environmental factors or through an interaction between these factors. The gene Fork-head Box protein E1 (*FOXE1*) has been documented to be involved with cleft lip and palate. The present study aims to screen mutations in *FOXE1* and to correlate phenotype and genotype of affected individuals. After obtaining ethical approval, peripheral blood samples were collected from 100 affected individuals, irrespective of age and gender. The genomic DNA was extracted, followed by quality control through spectrophotometer and gel electrophoresis. Polymerase chain reaction (PCR) was carried out to perform the targeted Sanger sequencing of *FOXE1* gene. A point mutation (c.274C>T, rs3021526) in *FOXE1* gene was detected in 15 samples. Based on phenotype, males and isolated cleft palate type was commonly involved,with bilateral occurrence, and moderate cases were frequently seen among the enrolled patients. In this study, a missense substitution was observed in gene *FOXE1*. This study provides a foundation for genetic counseling for families at risk and offer valuable insights for prevention and management plans.

Keywords: Cleft lip and palate, *FOXE1* gene, Pakistani population

1. Introduction

Globally, isolated cleft lip (ICL), isolated cleft palate (ICP), and a combination of cleft lip and palate (CLP) are among the most common structural birth defects . They affect 1 in 700 births worldwide, posing a major public health problem resulting from genetic variations, exposure to environment or due to interactions between these factors (Liu et al., 2015; Nikopensius et al., 2011; Vieira et al., 2005). Maternal smoking, alcohol consumption, lack of multivitamin, folic acid, and poor nutrition during pregnancy are significant risk factors for oral clefts (Lina M Moreno et al., 2009). Drugs like phenytoin, sodium valproate, benzodiazepines, and corticosteroids during gestation may also be involved in the formation of CLP (Faiza Sharif et al., 2019). Individuals affected with CLP need multidisciplinary comprehensive treatments ranging from neonatal nursing, psychosocial counseling, multiple maxillofacial, plastic surgeries, and speech therapy from birth and through adult life (Dodhia et al., 2017; Habel et al., 1996).

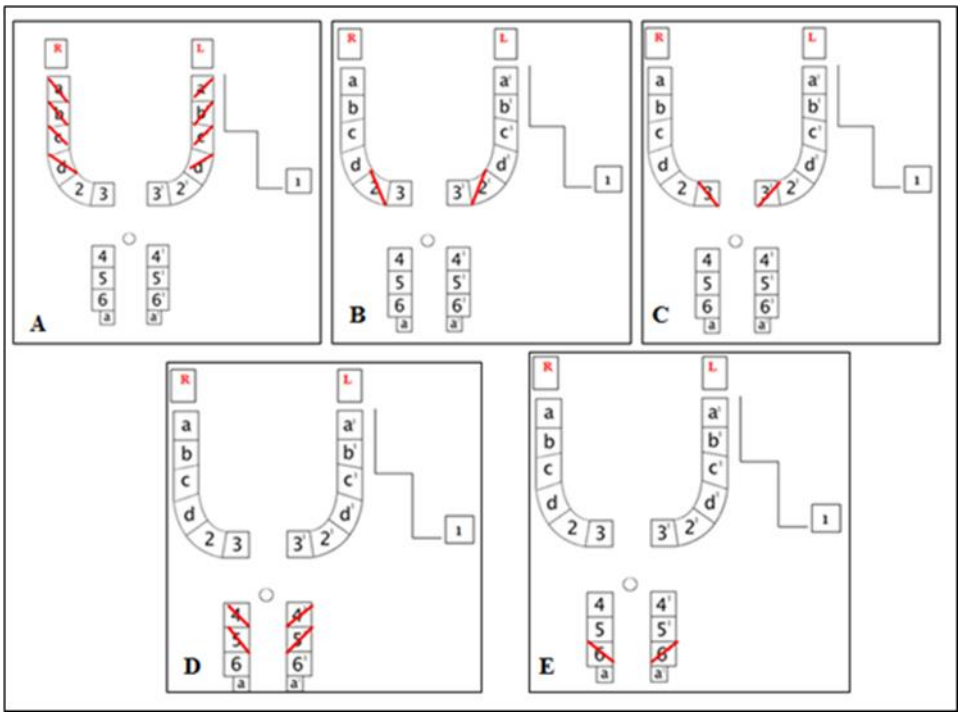

Figure 1: Classification of cleft lip and palate. A: Cleft of lip, B: Cleft of alveolar bone, C: Cleft of primary palate, D: Cleft of secondary palate, E: Cleft of soft palate

The orofacial cleft is the result of non-fusion between the facial prominences or embryonic processes (Millard Jr, 1994). The fusion that forms the total palate initiates in front during the 8th week, the premaxillary and palatine process in the 9th week, the hard palate and the soft palate by the 11th week (Millard Jr, 1994). The expression of *FOXE1* is observed in oral pharynx epithelium, medial nasal process, and maxillary process showing consistent results of its involvement in lip development (L. M. Moreno et al., 2009).

Orofacial clefts are divided into two types on the basis of inheritance pattern and epidemiologic record into syndromic orofacial clefts (SOC) and non-syndromic orofacial clefts (NSOC) (F. Sharif et al., 2019). CLP consists of 70% non-syndromic CLP and 30% syndromic CLP associated with Van der woude syndromes and Pierre robin syndrome (Demeer et al., 2019; Dodhia et al., 2017). Based on epidemiologic, embryonic and genetic bases, Orofacial clefts are categorized into ICL, ICP, and CLP (Figure 2) (Khan et al., 2012; Leslie et al., 2012). In 1922, the first CLP classification was proposed by Davis and Ritchie (Smith et al., 1998) which was later modified by Kernahan in 1958. Due to limitations, Smith further modified the striped Y Kernahan classification (Smith et al., 1998) . The classification of CLP is still going on in order to achieve the most comprehensive classification that is not only easily understood by patients, hospital administrators, care givers, clinicians and epidemiologists but also serve as an effective tool of communication (Millard, 1994). Approximately, 400 syndromes are characterized by features of CLP including cytogenetic abnormalities (trisomy 13, 18), single gene mendelian syndrome like Van der woude syndrome, Wolf Hirschhorn syndrome, Treacher Collins syndrome, stickler syndrome (Marazita et al., 2009).

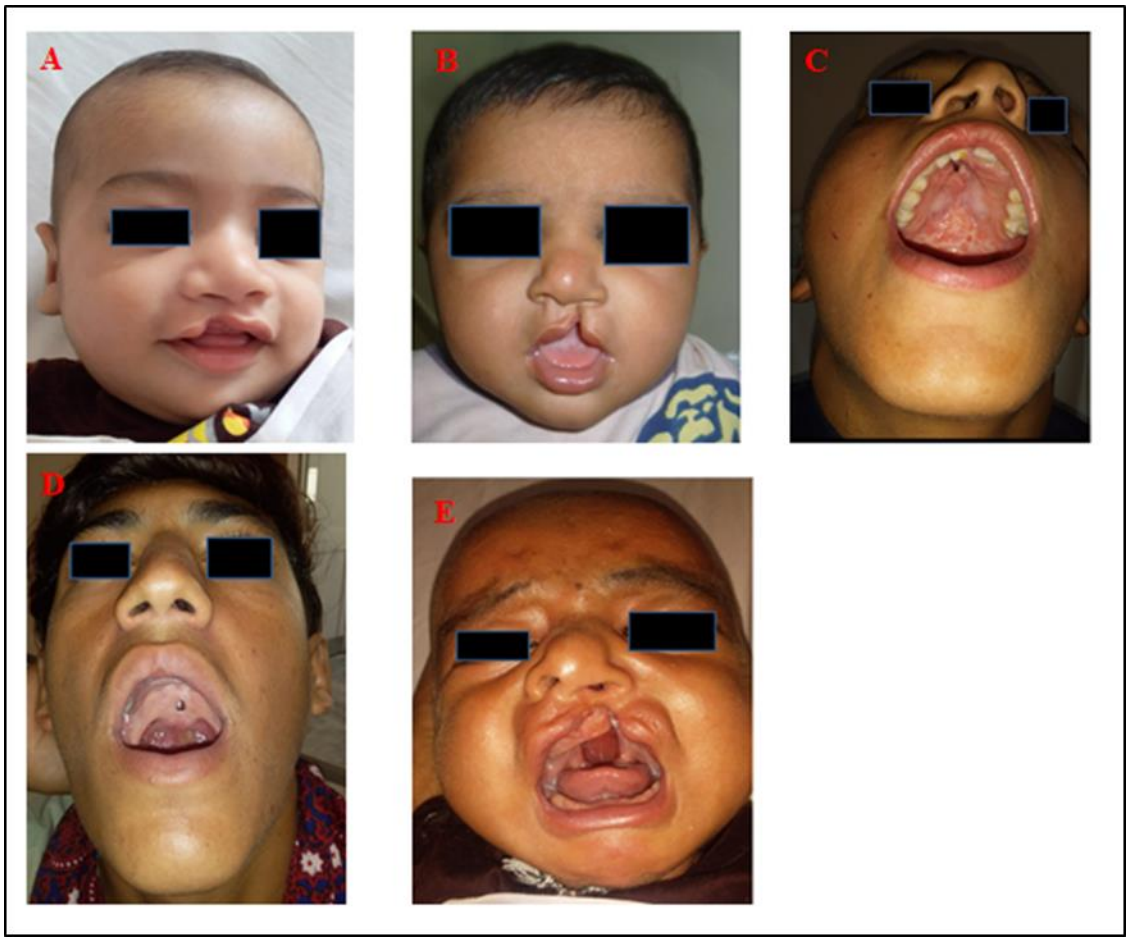

Figure 2: Types of CLP. A: Cleft lip on right side, B: Cleft lip and alveolus on left side, C: Cleft of primary palate, D: Cleft of secondary palate, E: Cleft of lip-alveolar ridge-primary palate-secondary palate-soft palate respectively.

In the last two centuries, despite the advancement in research techniques, the genetics of orofacial clefts remains debatable due to unclear inheritance pattern and heterogeneity among the population (Xie et al., 2018). Genetic studies have proved that a single gene, either dominant or recessive, is not directly involved in CLP development. Rather, it proposes that 2-14 interacting genes/loci are likely to be present in CLP (Kini, 2023) . In non-syndromic CLP, a positive association was identified on several human chromosomes (chromosome 1,2,4,6, 14,17,19) (Marazita et al., 2004; M. Venza et al., 2009).

In humans, *FOXE1* is a member of transcriptional family and consists of single exon gene present on chromosome 9q22 encoding for 367 amino acid and proteins, having a molecular weight of 42kD, contains DNA binding forkhead domain. It binds and opens chromatin structures also facilitates the binding of transcription factors to DNA (M. Venza et al., 2009). *FOXE1* is a transcription factor playing an important role in development of thyroid, lips and palate. It is also found in testis, epidermis, brain, heart, lung, hair follicles, thymus, kidney, placenta, liver, skeletal muscle, pancreas, colon and small intestine. It is involved in palate formation, most likely through the expression of *MSX1* and *TGFB3* genes, which are direct targets of transcriptional family (I. Venza et al., 2011). *FOXE1* gene affects the thyroid gland at this locus and is related with

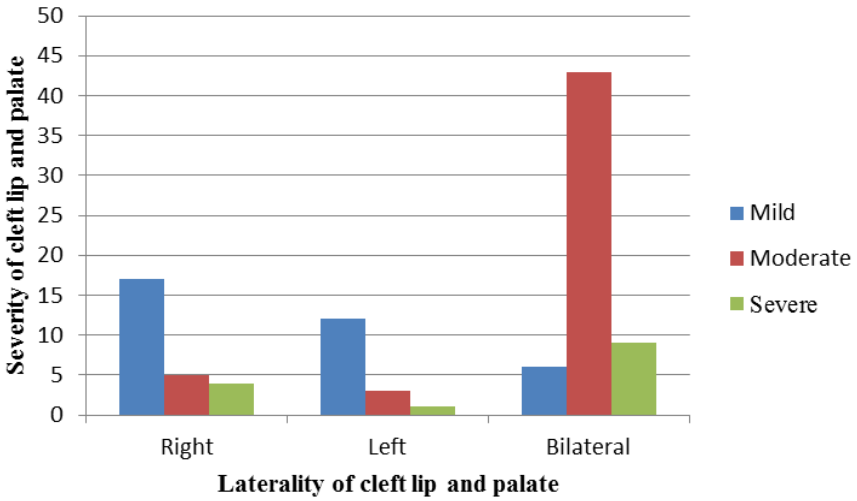

Figure 3: Comparison of severity with laterality of cleft lip and palate

numerous thyroid biomarkers. Additionally, it causes a number of disorders like hypothyroidism, goiter and a variety of thyroid cancers (Bonora et al., 2014). In 2004, the first positive association between CLP and *FOXE1* gene was identified by conducting genome wide association study (GWAS) on 388 families from nine different populations, suggesting 9q22-q33 gene region was linked with CLP (Lina M Moreno et al., 2009).

In Pakistan, the crude birth rate of CLP is 25.6 per 1000 individuals (F. Sharif et al., 2019). According to Smile Train (world's largest cleft-focused organization), approximately 50,000 corrective surgeries were executed in more than 25 centers of Pakistan, in 2008-2015 (F. Sharif et al., 2019) . Pakistan is the 4th most affected country in the world by CLP, following China, India, Indonesia (Fayyaz et al., 2015). According to a study of 123 children in Peshawar, one in every 523 births was affected by CLP, ICP being more common among females, and ICL/CLP was frequently found in males (F. Sharif et al., 2019). There is a paucity of knowledge on malformations associated with CLP individuals in Pakistan (F. Sharif et al., 2019). Few genetic studies have been published on CLP in Pakistan.

The available literature has revealed that CLP might occur as a result of genetic mutations in developmental period. Despite various developments and years of research, the genetics of clefts remains elusive due to unclear inheritance pattern and heterogeneity among the population. Due to high rate of consanguinity in Pakistani population, common inheritance pattern of the disease is autosomal recessive inheritance. There is high probability, to find genetic variants in *FOXE1* gene among CLP individuals. Understanding the mutation in *FOXE1,* underlying CLP will help us with comprehending the physiological and etiological process of the gene in humans. Moreover, after discovering the mutation and correlation with CLP, genetic counseling can be arranged for families at risk. This approach will aid in prevention and management of CLP. This study will help to understand the genetics of CLP in Pakistan, as no other targeted studies were conducted for *FOXE1* gene.

2. Material and Methods

Current study was conducted at Institute of Basic Medical Sciences (IBMS) Khyber Medical

Table 1: Gender distributions of types of clefts

S #	Gender	ICL	ICP	CLP	Total
1.	Male	13	21	25	59
2.	Female	05	22	14	41
Total		18	43	39	100

Table 2: Classifications of cleft lip and palate patients based on severity

Group number	Severity	Smith Classification	Description	Number (n)
Group 1	Mild	1,2	Lip, alveolus	37
Group 2	Moderate	3,4,5	Primary and secondary palate	49
Group 3	Severe	1-6	Lip, alveolus, primary and secondary palate, soft palate	14

University, Peshawar. Individuals with non-syndromic CLP were identified in Burn and plastic surgery center, Rehman Medical Institute (RMI), Khyber Teaching Hospital (KTH) Peshawar. This study was approved by the ethical committee at the Institute of Basic Medical Sciences, KMU. Written informed consent (English and Urdu) was obtained from both the guardians and the patients. Clinical data was collected by completing a questionnaire. The classification pattern used in this study is Smith modified Kernahan classification.

Venous blood sample (~3ml) was collected from patients in an EDTA tube. Thermofischer scientific DNA extraction kit was used for extraction of DNA from the blood samples. This kit is designed for rapid and efficient purification of high-quality DNA and takes about 45-60 minutes to yield purified DNA. NanoDrop spectrophotometer (Titertek Berthold) was used for DNA quantification to determine the average concentration of DNA in each sample. The sequence for *FOXE1* gene involved in non-syndromic CLP was obtained from online database NCBI (National center for biotechnology information). Primer blast online database was used for primer designing. PCR was used to amplify the target sequence. For visualization of amplified products, agarose gel electrophoresis "Major science MP-300 V" was used that works on the principle of molecular weight of DNA fragments. The samples were sequenced from Beijing TsingkeXinye Biotech, China. All of the clinical and genetic data (demographics and phenotypes) obtained from current work was recorded in Microsoft Excel (2010) and SPSS statistical software version 20. For genotype and inheritance pattern, the analysis of Sanger sequencing were performed using software like "Finch TV and blast NCBI", "4Peaks" respectively

. **Table 3: Family history and cleft types**

S.No	Family history	Isolated cleft lip (ICL)	Isolated cleft palate (ICP)	Cleft lip and palate (CLP)	Total
1.	Positive	03	05	02	10
2.	Negative	14	39	37	90
Total		17	44	39	100

Table 4: Comparison of demographics/phenotype with genetic variation

S#	Variation	Sample #	Age (M/Y)	Gender (M/F)	Laterality (R/L/Bi)	Classification (Mild, Moderate, Severe)
1.	c.274C>T	A3	7M	M	Bi	Mild
2.	c.274C>T	A11	8M	M	Bi	Moderate
3.	c.274C>T	A12	9M	F	Bi	Moderate
4.	c.274C>T	A18	6M	F	Bi	Moderate
5.	c.274C>T	A22	4Y	M	Bi	Moderate
6.	c.274C>T	A26	4Y	F	Bi	Moderate
7.	c.274C>T	A29	2Y	F	Bi	Moderate
8.	c.274C>T	A31	3Y	M	R	Severe
9.	c.274C>T	A40	2Y	M	Bi	Moderate
10.	c.274C>T	A42	10Y	M	Bi	Moderate
11.	c.274C>T	A60	4Y	M	Bi	Moderate
12.	c.274C>T	A64	5Y	F	Bi	Moderate
13.	c.274C>T	A67	5M	M	R	Mild
14.	c.274C>T	A72	6M	F	Bi	Mild
15.	c.274C>T	A79	1Y	M	R	Mild

3. Results

This study was performed on 100 individuals with non-syndromic CLP. The age of the patients ranged from 3 months to 10 years with the mean age of 1-2 years. In this study, 59% of the patients were males, while 41% were females **(table 1)** . This study focused on "Smith modified Kernahan's Y classification", based on this classification, there are five types of clefts (*figure 1)*. There are 5 types of clefts on the basis of "Smith modified Kernahan's Y classification" of the patients involved in this study *(figure 2).* The patients were divided into three groups (mild, moderate, severe) on the basis of severity of deformity. Our results showed that a majority of the affected individuals belonged to the moderate category (**table 2**).

In terms of severity, when compared with laterality, most common form is moderate, affecting the primary and secondary palate, frequently seen on bilateral side (*figure 3).* Moreover, the severity, when compared between genders, the males are 23% mild, 27% moderate, and 11% were severe. While, in case of females, 14% mild, 22% moderate, and 3% were severe. In both the genders, most frequently occuring form was moderate, followed by mild, and the least frequent form was severe. Ten patients with deformity had history in the family, while their parents were cousins, which supports the claim of influence of consanguinity on CLP. The cleft types among parents are shown in **table 3**.

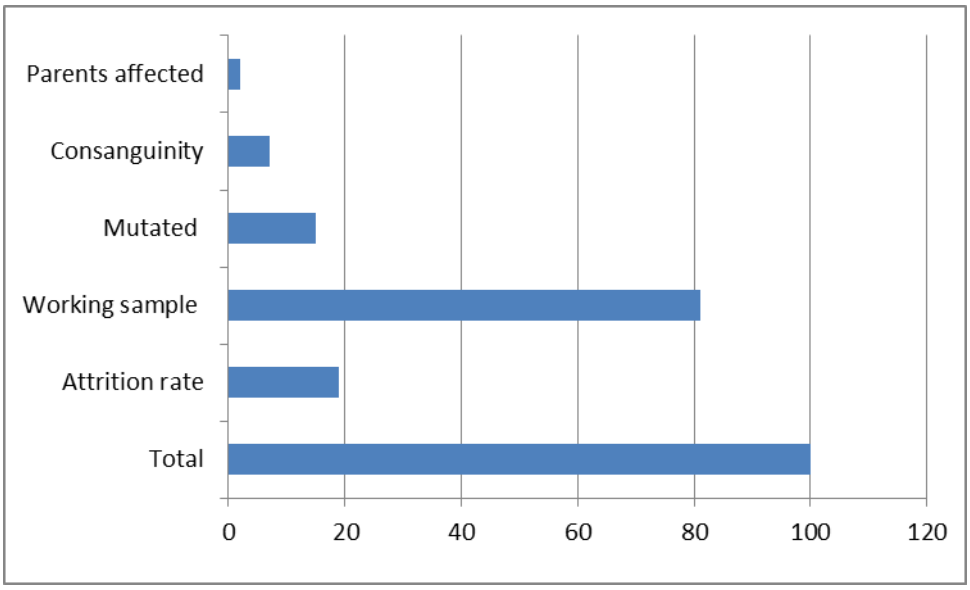

Figure 4: Detailed genetic results of 100 patients affected with CLP

Two different set of primers were used for PCR amplification, followed by Sanger sequencing. A complete genetic result of the whole sample size consisting of 100 patients affected with CLP is shown in *figure 4.* Due to machine error or bad sequencing results, 19 DNA sequences were excluded from genetic analysis, while 15 showed variations/SNPs by analyzing on "Finch TV", "4Peaks" and "Blastn" software. We found the association of SNP (rs3021526) in *FOXE1* gene in CLP in 15 samples, while seven of them showed positive consanguinity among parents. Moreover, two of the parents were also affected. The genotype results derived by using Finch TV, 4. Peaks and blastn NCBI software are shown in *figure 5*.

In the current study, no obvious genotype-phenotype correlation was observed. On the basis of laterality, bilateral deformity is more common followed by right side; furthermore, moderate form is more common than mild and severe form. The summarized results of the 15 samples are shown in **table 4.** Among the affected individuals, males are more common in comparison to females.

4. Discussion

The aim of this study was to identify genetic variants in *FOXE1* gene in CLP patients and to determine an association between genotype and phenotype of the affected patients. Although, no clear association was seen between phenotype and genotype of the affected individuals, having point mutations in *FOXE1* gene, yet bilateral deformity and moderate form of CLP were more common. Orofacial clefts are one of the most common congenital birth defects worldwide. It is not only a major reason of long term incapacity in children but also affects their emotional and physical health, in addition to the financial stress for families (Khan et al., 2012; Nes et al., 2014; Yu et al., 2009). Cousin marriages, lack of prenatal counseling, lagging behind in monthly doctor checkups, maternal malnutrition especially folic acid defeciency are known to be some of the causative factors associated with CLP in Pakistan (Lina M Moreno et al., 2009). In this study of 100 cases, 2-3 medical checkups during the pregnancy were performed by only five mothers, but no nutritional supplements or folic acid had been used. In the early stages during the

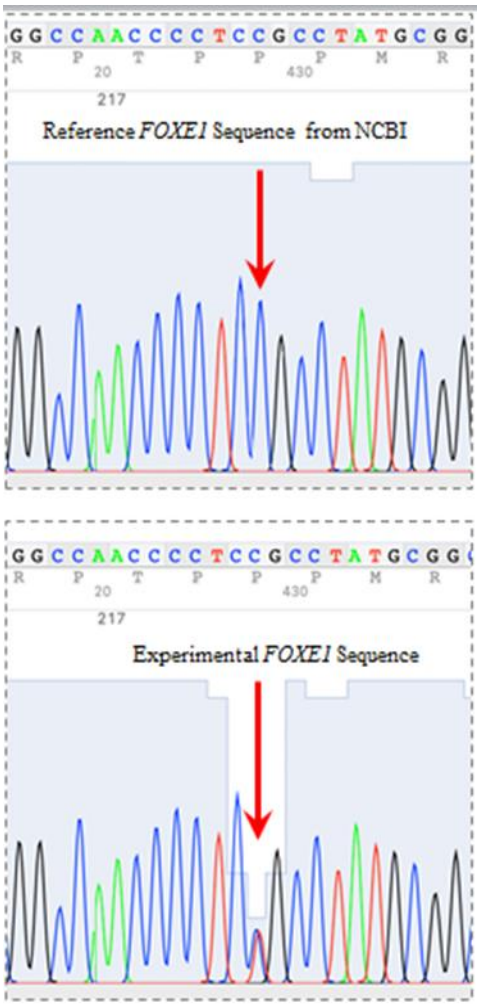

Figure 5: Sequence variant (c.274 C>T) of *FOXE1* gene of sample A3

development of head and neck region, several processes appear and eventually disappear (Yoon et al., 2000). During embryonic development, if tissue prominences successfully merge by mesenchyme proliferation, normal structures form. However, if fusion fails, it results in clefts, such as cleft lip or palate. (Yoon et al., 2000). Few studies on phenotype and incidence rates of CLP in Pakistan have been performed but no genetic study performed the genetic correlation so far.

In the present study, a total of 100 individuals of different types of non-syndromic CLP were studied. In the study, the age presentation ranged from 3 months to 10 years with a mean age of 1-2 years. In another study conducted in Pakistan, most common age was 3.5 years (Khan et al., 2012). A study in China, revealed the most common age was less than 1-year in the patients. In the current study, CLP prevalence in the males (59%) was more common than females (41%) which is consistent with the earlier studies performed in Saudi Arabia (Aljohar et al., 2008). On the basis of phenotypes, bilateral deformity was more common followed by right side and then left side. When gender and laterality were compared, males were more prone to bilateral deformity when compared to females. In a study in Pakistan, the males were commonly affected on left side and according to types of clefts, females were commonly affected with ICP (Khan et al., 2012). A study in Saudi Arabia revealed right side to be more commonly involved, while in Chinese population left side was more common followed by right side and lastly bilateral deformity (Aljohar et al., 2008). Our study resulted in more isolated cleft palate patients followed by combination of CLP and lastly isolated cleft lip. Similar to our results, another study in Pakistan also revealed isolated cleft palate to be more common than CLP (F. Sharif et al., 2019). On the other hand, CLP was more commonly seen in Chinese population (F. Sharif et al., 2019). In India, isolated cleft lip was more commonly involved followed by CLP and lastly ICP (F. Sharif et al., 2019). As stated by Smith modified Kernahan classification used in our study, most common type seen is moderate form (cleft of primary and secondary palate) respectively.

In different populations of the world, the group of genes involved in CLP may be different. Earlier studies have identified the association of genetic variants of *FOXE1* with non-syndromic CLP. *FOXE1* is a transcription factor playing an important role in development of lips and palate and works by binding and opening chromatin structures, also it facilitates the binding of transcription factors with DNA (Liu et al., 2015).

FOXE1 affects the expression of other genes, for instance mutated *FOXE1* could decrease the *MSX1* and *TGF-b3* expression, resulting in failed palate-shelf formation. Moreover, *FOXE1* also affects *GLI2* in 'Shh/Gli pathway' that is involved in development of palate (Jezewski et al., 2003). Consanguinity is a common practice in Pakistan, such similar marriage patterns are also seen in other Asian and mid-eastern countries. A study in Pakistan revealed 17% of affected CLP parents were in a cousin marriage, reinforcing the strong genetic association. (Elahi et al., 2004). In another local study 61.6% of CLP parents were having marriages within family, these results were also in conformity with those from Saudi Arabia where the consanguinity was 56% in CLP families. This high frequency of consanguinity calls for genetic counseling and investing in education in Pakistan (Khan et al., 2012). In our study, 10 patients were born to parents with consanguineous marriage, so consanguinity plays a critical role in the development of orofacial clefts and should be discouraged. Another study from Tanzania showed 15% family history in CLP and from Ethiopia 4.8% showed family history (Buyu et al., 2012; Khan et al., 2012).

In 2004, a highly significant association was suggested between CLP and 9q22-q33 region, where *FOXE1* gene is present. The two most associated SNPs were rs3758249 and rs4460498 (Lina M Moreno et al., 2009). Subsequently, to study the role of *FOXE1* in CLP, cases were genotyped for SNPs/mutations around and near *FOXE1*.The exon coding region of *FOXE1* was sequenced and association with CLP was found in 15/100 cases (c.274C>T, rs3021526) signifying that *FOXE1* is involved in etiology of CLP in patients of Peshawar. Sequencing of the coding regions in *FOXE1* showed missense substitution. In another study from China showed that *FOXE1* (rs3021526) was highly associated with papillary thyroid carcinoma (Lidral et al., 2015; Liu et al., 2015).

5. Conclusions

The aim of this study was to identify the genetic variants in *FOXE1* gene in CLP patients and also to correlate the phenotype with genotype in Peshawar population. We concluded that 15 samples showed missense substitution in *FOXE1* gene. The family history was observed in 10 patients in the enrolled cases, which establishes a strong correlation between family history and CLP probability in their offsprings. Moreover, the study found CLP more common in males as compared to females. Whereas, the most commonly observed type was ICP followed by CLP and then ICL. On the basis of laterality, bilateral deformity was frequently seen, followed by right side. The study recommends counselling for those carrying the *FOXE1* genetic mutation, nutritional balance, and regular medical checkup during gestation period to prevent cleft development.

Conflict of Interest

The authors declare that they have no competing interests.

Funding

No external funding was received for this research project.

Study Approval

The study was ethically conducted in accordance with the World Medical Association Declaration of Helsinki. It was approved by Ethical committee at Institute of Basic Medical Sciences, Khyber Medical University Peshawar, Pakistan. All the participants (or their guardians) gave written informed consents to perform current study and publish research data.

Consent Forms

Consent forms are available with the authors.

Data Availability

All data generated or analysed during this study are included in this article. Further inquiries can be directed to the corresponding author.

Authors Contributions

SM was responsible for mmethodology and writing original draft; FKB performed clinical investigation and diagnosis of patients; JA, INK did data analyses; ZH was responsible for conceptualization, supervision, review and editing of the manuscript. All authors read and approved the final manuscript.

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