MOLECULAR CHARACTERIZATION OF CLEFT LIP WITH OR WITHOUT CLEFT PALATE IN PAKISTAN

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Dedicated to the Prophet Muhammad (Peace be upon Him)

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LIST OF ABBREVIATIONS:

$(NH_4)_2SO_4$	Ammonium Sulphate
ABI	Applied Biosystems
AP-2α	Activator protein-2
ARMS	Amplification refractory mutation system
BMPs	Bone Morphogenetic proteins
BSL-II	Biosafety level 2
$CaCl_2$	Calcium chloride
cc	Cubic centimeter
CH_3COONa	Sodium acetate
CL	Cleft lip
CL/P	Cleft lip with or without cleft palate
CLAPP	Cleft lip and palate association of Pakistan
CLP	Cleft lip and cleft palate
cM	Centi-Morgan
CP	Cleft palate
DLX	Distalless
DNA BD	DNA binding domain
DNA	Deoxyribonucleic acid
dNTPs	Deoxyribonucleotide
EDTA	Ethylene-diamine tetra acetic acid
EGF	Epidermal growth factor
FGF	Fibroblast growth factor
FOXE1	Forkhead box protein E1
GABRB3	Gamma-aminobutyric acid receptor subunit β3
GLI2	Zinc finger protein
GSC	Goosecoid
IPTG	Isopropyl β-D-1-thiogalactopyranoside
IRB	Institution Review Board
IRF6	Interferon regulatory factor 6

KCl	Potassium chloride
LB	Luria-Bertani
LD	Linkage disequilibrium
LOD	Log of odds ratio
M	Molarity
µM	Micro molar
$MgCl_2$	Magnesium chloride
mM	Milli molar
MSX	Muscle segment
MTHFR	Methylenetetrahydrofolate reductase
Na_2EDTA	Disodium ethylene-diamine tetra acetic acid
NaCl	Sodium chloride
NaOH	Sodium hydroxide
nm	Nano meter
OFC	Orofacial clefts
OTX	Orthodontical
PBD	Protein binding domain
PCR	Polymerase chain reaction
PIC	Polymorphism information content
pmole	Pico mole
PPS	Popliteal pterygium syndrome
PVRL1	Poliovirus receptor-related 1
RARA	Retinoic acid receptor alpha
SATB2	Special AT-rich sequence binding protein 2
SDS	Sodium dodecyl sulfate
SHH	Sonic hedgehog
SKI	Sloan-Kettering Institute
SMIR	Smad-interferon regulatory factor-binding domain
SNP	Single nucleotide polymorphism
SPRY2	Sprouty homolog 2
SUMO1	Small ubiquitin-like modifier

T.E	Tris EDTA
T/A	Thymine/Adenine
Taq	*Thermus aquaticus*
TBX10	T-box developmental gene 10
TDT	Transmission disequilibrium test
TGFα	Transforming growth factor alpha
TGFβ	Transforming growth factor beta
TP53	Tumor protein 53
TP73L	Tumor protein p73-like
UV	Ultraviolet
VWS	Van der Woude syndrome
X-gal	5-bromo-4-chloro-3-indolyl-beta-D-galacto-pyranoside

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ABSTRACT

Orofacial clefts including cleft lip and cleft palate are birth defects which may be present either alone as isolated or non-syndromic cleft, or they may be present in association with other anomalies as a syndrome. Van der Woude syndrome (VWS) is one of the most common syndromes which involves orofacial clefts and is usually one of the less severe syndromes of clefting. The most characteristic feature of VWS is the presence of lower lip pits which are present in about 85% of the cases. Mutations and polymorphisms in the gene Interferon regulatory factor 6 (*IRF6*) are known to cause both syndromic as well as non-syndromic forms of cleft lip and/or cleft palate in humans.

To date, no research has been performed on genetics of cleft lip with or without cleft palate in Pakistan. In the current study, 23 VWS patients were recruited from different areas of Punjab, Azad Kashmir and Balochistan. Mutational screening of *IRF6* gene was performed on all collected samples. An association study between single nucleotide polymorphisms of *IRF6* and non-syndromic cleft lip with or without cleft palate (CL/P) in single affected individuals was also performed. Additionally, 3 large consanguineous families with an apparent recessive mode of inheritance were recruited in search of novel loci for non-syndromic CL/P. The frequency of VWS identified in the current study was calculated to be 1.17% from the Punjab province and 1% from Azad Kashmir which was lower as compared to other world populations. Additionally 15 mutations including 7 novel and 8 known mutations within the coding region of *IRF6* were identified which cause VWS. These mutations were found in 18 of 23 unrelated families segregating VWS from Pakistan. The newly identified mutations include 3 missense mutations (p.M1T, p.G99S, and p.S407P) while the 4 novel frameshift mutations are p.R7fsX52, p.A190fsX34, p.S212fsX12, and p.209fsX15. The known pathogenic variants included 6 missense and 2 nonsense mutations including p.R84C, p.V321M, p.R250Q, p.C374W, p.R400W, and p.R400Q, p.Q204X, and p.R412X. The present findings add to the spectrum of *IRF6* mutations which are responsible for VWS. Additionally, the identification of the same mutations responsible for VWS in Pakistan, as reported in other populations worldwide, marks these residues as mutational hotspots.

In the association studies between single nucleotide polymorphisms in and around *IRF6* (*rs2235543*, *rs2235375*, *rs2013162*, and *rs1319435*) and non-syndromic CL/P in case-parent trios from Punjab, the transmission disequilibrium test (TDT) showed highly significant association of the 'C' allele of *rs2235375* with non-syndromic CL/P (P=0.008). These results identify *IRF6* as a significant contributor to non-syndromic CL/P in Pakistan.

Three large consanguineous families with non-syndromic CL/P and recessive mode of inheritance were also analyzed in search of novel loci. Linkage analysis suggests that regions on chromosome 9 are likely implicated in orofacial clefting in family CLP-SM1. Other regions, such as those on chromosome 5, may also be significant in the inheritance of CL/P in family CLP-SM1. Beyond the dubious region on chromosome 12, family CLP-SM2 did not provide any obvious candidates of linkage. Based on these findings, it is expected that there will be more complexity in the inheritance of CL/P than single allele acting through a classical recessive or dominant model.

The results of this study have demonstrated for the first time the important role of *IRF6* in clefting in the Pakistani population. It has also further revealed the genetic complexity of non-syndromic cleft lip with or without cleft palate even in families which seem to segregate the disorder in an apparent Mendilian inheritance pattern. Identification of responsible genes will reveal pathways necessary for normal orofacial development.

CHAPTER 1

INTRODUCTION