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Review Article

Genomic expression in non syndromic cleft lip and palate patients: A review

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ABSTRACT

Cleft lip and palate are common congenital anomalies with significant medical, psychological, social, and economic ramifications, affecting one in seven hundred live births. Genetic causes of non syndromic cleft lip and/or palate (NSCLP) include chromosomal rearrangements, genetic susceptibility to teratogenic exposures, and complex genetic contributions of multiple genes.

Development of the orofacial clefts in an individual will depend on the interaction of several moderately effecting genes with environmental factors. Several candidate genes have been genotyped in different population types, using case parent trio or case control design; also genes have been sequenced and SNPs have been reported. Quantitative and molecular analysis have shown linkage and association studies to be more relevant. Recent literature search shows genome wide association studies using microarray. The aim of this paper was to review the approaches to identify genes associated with NSCLP and to analyze their differential expressions.

Although no major gene has been confirmed, a lot of research is ongoing to provide an understanding of the pathophysiology of the orofacial clefts.

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Cleft lip and palate are common congenital anomalies with significant medical, psychological, social, and economic ramifications, affecting one in seven hundred live births.¹ Its incidence varies according to race, gender and cleft type, being more common among Indian and Oriental populations (2.3 per thousand total clefts) and least common among Afro-Caribbean groups (0.6 per thousand total clefts).² NSCLP is a common congenital anomaly with incidence ranging from 1 in 300 to 1 in 2500 live births.^{3–5} A child is therefore born with a cleft somewhere in the world approximately two and a half minutes. In India, cleft lip/palate occurs in nearly 1 in 500 live

births; majority of which are not surgically corrected.⁶ The number may be much more than expected as most of the births in rural areas are not reported.

As oral clefts are believed to be of multifactorial etiology, both genetic predisposition and environmental influences, such as age, sex, race; smoking, alcohol, caffeine, benzodiazepines, corticosteroids; as well as other occupational exposures, may play a role. The maxillary, medial nasal, and lateral nasal prominences through a complicated process of epithelial bridging, programmed cell death, and subepithelial-mesenchymal penetration lead to a defect of epithelial

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fusion involving many possible genetic loci or intracellular signaling pathways.

Segregation analyses demonstrate that genetic factors are important in the pathogenesis of NSCLP. Genetic causes of cleft include chromosomal rearrangements, genetic susceptibility to teratogenic exposures, and complex genetic contributions of multiple genes. Literature provides an overview of genetic approaches to identify disease genes for genetically complex birth defects.⁷ Several genes that have a combined role in up to 20% of all clefts have been identified. Ongoing human genome-wide linkage studies have identified a major gene on chromosome 9, positive in multiple racial groups.⁸ Population-based candidate-gene studies identify genes involved in the etiology where family-based linkage studies are compromised by lack of access to affected members, low penetrance, and/or genetic heterogeneity.

The aim of this paper is to review the approaches to identify genes associated with NSCLP and to analyze their differential expressions.

1. Review of literature

Literature was searched for key words “genes, genetic, cleft, cleft lip, cleft palate” to include articles published on various genetic studies conducted on cleft lip, palate genomics. The search yielded 850 relevant articles that had information on gene location, size and function and thus we finally, collected 134 references on the genes in relation with NSCLP. Online Mendelian Inheritance in Man catalog was found to list more than 400 single-gene causes of NSCLP.

2. Candidate genes

2.1. MSX1 & TGFβ

Muscle segment homeobox 1, (MSX1) is also known as homeobox 7, homeobox protein MSX-1, HOX7, HYD1, MSH homeobox homolog 1, MSH Homeo Box Homolog 1 (*Drosophila*) Gene, MSX1_Human, OFC5. The MSX1 gene provides instructions for making a protein that regulates the activity of other genes and acts during early development to control the program of craniofacial morphogenesis during development of teeth and craniofacial skeleton.

Mutations in MSX1 have been observed to contribute in NSCLP.^{9–13} Human MSX1 gene maps to 4p16.1 locus and spans 4.05 kb. It contains two exons and an intron. MSX1 gene expression is associated with cyclin D1 up-regulation, thus inhibits cellular differentiation by regulating cell cycle.^{9,14}

Transforming growth factor-beta 3 (TGFβ3) gene encodes a member of the TGF-beta family of proteins, secreted during embryogenesis and cell differentiation. A number of studies suggest show the role of TGFβ3 gene to be related to NSCLP.^{5,15,16}

The TGFβ family is particularly involved in palate development and all isoforms 1, 2 and 3 are expressed during this process. Studies on TGF family genes suggest that their function in the embryonic palate is mediated through the smad signaling system.¹⁷ TGFβ3 is expressed initially in the

epithelial component of the vertical shelves. Later, it is also expressed in the horizontal shelves, but expression becomes undetected once the epithelial seam disrupts. TGFβ1 expression is limited to the horizontal shelves but like TGFβ3, switches off soon after epithelial seam disruption while TGFβ1, 2 accelerate palatal shelf fusion.^{18–20}

MSX1 gene and TGF family gene show mutations and may contribute to NSCLP.²¹ Candidate genes TGFA, MSX1, and TGFβ3 in Vietnamese population have demonstrated transmission distortion for alleles of MSX1 for the whole population and identified two missense mutations including one (P147Q) in approximately 2% of the population.²¹ Case-parent trio and case-control studies on the MSX1 gene in NSCLP from Korea showed results consistent with other studies in the US and Chile in determining the risk of CLP.²² Transmission disequilibrium test analysis for MSX1 and TGFβ3 in South American children showed evidence of association with MSX1. With likelihood ratio test analysis, “cleft lip only” showed association with MSX1 and “cleft palate only” with TGFβ3.²³ In an association study between MSX1 polymorphism and NSCLP from Operation Smile Colombia, four alleles from MSX1 microsatellite sequence were analyzed. Significant statistical difference was found between patients who carried allele 3 and CLP. Allele 4 (heterozygous and homozygous form) was the most frequent in CLP (74%) patients and controls (82%).²⁴ This relationship, when studied by polymerase chain reaction (PCR) and denaturing polyacrylamide gel electrophoresis (PAGE), the allele CA4 frequency in CLP and cleft palate only group was significantly higher than that of controls.²⁵

Candidate gene approaches have identified important roles for MSX1 and genes in the Fibroblast growth factor (FGF) family.^{26,27} Evaluation of association data for four candidate genes using a population from Philippines, however, showed no evidence for association with previously reported allelic variants of transforming growth factor-beta 2 (TGFβ2), homeobox 7 (MSX1), or transforming growth factor-alpha (TGFA), or with the new TGFβ3 variant.²⁸ Linkage analysis performed in five affected pups to study evidence for linkage between the trait and TGFβ3 or MSX1 excluded TGFβ3 and MSX1 as candidate genes.²⁹

Fiquet and colleagues studied the implication and role of angiogenesis-related genes in the etiology of CL/P and found that these genes participate in several biological activities and their implication might not be always related to angiogenesis defects.³⁰

2.2. IRF6

Interferon regulatory factor-6 (IRF6) gene encodes a member of the interferon regulatory transcription factor (IRF) family. Family members share a highly-conserved N-terminal helix-turn-helix DNA-binding domain and a less conserved C-terminal protein-binding domain. Mutations in this gene can cause X-linked CLP, ectodermal dysplasia, Van der Woude's and popliteal pterygium syndromes.³¹ This gene is found to play important role in palate formation. Candidate gene approaches have identified important roles for IRF6 and genes in the FGF family.²⁷ Single nucleotide polymorphism (SNPs) in and around IRF6, when tested for association with NSCLP in Honduran

individuals from families with at least two members affected by cleft and one member with NSCLP, support of linkage was observed for three SNPs-rs1856161, rs2235371, and rs2235377. When analysis was restricted to NSCLP cases, excluding cleft palate only cases, support for association was strengthened.³² Direct sequence analysis of IRF6 on samples from a large European cohort, identified mutations in 27 (80%) families and showed IRF6 as the major causative gene of Van der Woude syndrome in Europe.^{33–35} Mutation in IRF6 was found to be associated with 4–6% increased risk of having a child with CLP. A clear association of IRF6, observed in patients from Belgium, suggests its contribution to the occurrence of isolated CLP, even if no mutation in the gene can be identified.^{33–37} Association between the IRF6 G820A polymorphism and NSCLP in Chinese patients frequently showed 820A allele more in cleft palate only population and conferred an increased risk.³⁸ Candidate genes, when genotyped in two population-based samples from Scandinavia (Norway: 562 case-parent and 592 control-parent triads; Denmark: 235 case-parent triads) identified significant associations with IRF6 in both populations.³⁹ Twelve SNP were screened in a group of 100 patients with NSCLP and 60 controls by next generation sequencing in China. Their results showed that genetic polymorphism of the IRF6 gene is associated with increased risk of NSCLP in a Xinjiang Uyghur population.⁴⁰

2.3. RUNX2

The Runt-related transcription factor 2 (RUNX2) gene has been suggested as a candidate gene for NSCLP. Genetic and molecular studies in humans and mice indicate that RUNX2 is critical transcriptional regulator of bone and tooth formation. Heterozygous mutations in RUNX 2 cause cleidocranial dysplasia, characterized by skeletal defects, supernumerary teeth, and delayed eruption.⁴¹

Using a case-parent trio design, RUNX2 was studied from four populations (Maryland, Taiwan, Singapore, and Korea) and genotyped for 24 SNPs in the gene. When all trios were combined, the transmission asymmetry test revealed 11 SNPs showing excess maternal transmission, plus one SNP (rs1934328) showing excess paternal transmission. For the 11 SNPs showing excess maternal transmission, odds ratios of being transmitted to the case from the mother ranged between 3.00 and 4.00. The parent-of-origin likelihood ratio tested for equality of maternal and paternal transmission were significant for three individual SNPs (rs910586, rs2819861, and rs1934328).⁴²

2.4. TGFA

Transforming growth factor alpha (TGFA) has also shown association between DNA sequence variants at the TGFA genetic locus and NSCLP.⁴³ Gene–gene interaction using markers in TGFA and IRF6 in an NSCLP case-parent trios from four populations for 17 SNPs in TGFA suggested evidence of interaction.⁴⁴

2.5. BCL3

The B-Cell Leukemia/lymphoma 3 (BCL3) gene has been suggested as a candidate gene for NSCLP based on association and linkage studies in some populations.³⁰ Although the role of

BCL3 in the etiology of NSCLP is unknown, BCL3 is related to genes involved in cell lineage determination and cell cycle regulation. It has also been reported to act as a transcriptional activator. Linkage studies for BCL3, located in 19q13.2, in 40 multiplex pedigrees, have supported a role for BCL3 in causing NSCLP, but no evidence of linkage or genetic heterogeneity was found.^{15,45,46}

3. Additional mutations

Direct sequencing suggested that point mutations in these candidate genes were likely to contribute to 6% of isolated clefts. Additional cases, possibly due to microdeletions or isodisomy, may contribute to clefts as well. Sequence analysis alone suggested that point mutations in Forkhead box protein E1 (FOXE1), GLI2, JAG2, LIM/homeobox protein (LHX8), MSX2, SATB2, SKI, SPRY2, and TBX10 may be rare causes of isolated cleft lip with or without cleft palate, and the linkage disequilibrium data supported a larger role for variants in or near MSX2, JAG2, and SKI.⁴⁷ 397 SNPs spanning the 9q22–q33 2-LOD-unit interval showed. Association signals for Caucasians and Asians clustered at 5' and 3' of FOXE1, respectively.⁴⁸ Linkage disequilibrium investigation performed to study candidate genes TP63, JAG2 and MID1, in a study of cleft patients/parents trios, suggested that JAG2 and MID1 may play a role in NSCLP.⁴⁹ Estrogen receptor 1 (ESR1) and fibroblast growth factor receptor 2 (FGFR2) genes from the 6q25.1–25.2 and 10q26.11–26.13, respectively, were identified as likely causative genes using gene prioritization software.⁵⁰ In addition, MTHFR, TGF-beta3, and RAR alpha play a role in cleft onset. In Cleft palate only, one gene (TBX22) has been identified at present, but others are probably involved too.⁵¹ For isolated cleft palate, TRIMM found associations with Aristaless-like 3 (ALX3), Homeobox protein Mohawk (MKX), and platelet-derived growth factor (PDGFC).^{23,37,52} FLNB was identified in cleft palate. Except for FLNB, HIC1 and ZNF189, maternal genes did not appear to influence the risk of cleft.³⁸ Candidate genes sequencing within 9q22–q33 NSCLP in samples from Colombia, USA and Philippines, revealed 32 new variants.⁴⁸

A genome-wide linkage analysis in two large Syrian families, each one with a large number of cases (18 in family 1 and 4 in family 2) identified a strong candidate locus for this common birth defect on chromosome 17p13.⁵³ Multiplex families with NSCLP from Shanghai showed the most significant multipoint linkage results for chromosomes 3q and 4q. However, associations were found for loci on chromosomes 3, 5–7, 9, 11, 12, 16, 20, and 21. Also the most significant association result was found with D16S769 (51 cM).⁵⁴ In a study in Indian population, two Indian pedigrees with isolated NSCLP, were analyzed using genome wide linkage scan that used ~10,000 SNPs. Among those, the most significant evidence was for chromosome 13q33.1–34 at marker rs1830756.³

4. Genome wide association studies

Genome-wide association (GWA) studies and inexpensive sequencing identify disease genes and characterize both gene–environment and gene–gene interactions for risk

counseling, development of preventive therapies and to identify the gene locus responsible for the defect.^{55,56} Genome wide studies are important advances in discovering the genetic variations influencing disease. GWA, through genotyping technologies, assay hundreds of thousands of SNPs.⁵⁷

Results from a genome-wide screen, ascertained through probands with NSCLP in Mexico, Argentina and United States, suggested evidence of linkage to chromosomes 2, 6, 17 and 18. Fine mapping excluded all regions except chromosome 2. With additional evidence for a susceptibility gene for NSCLP on 2q.⁵⁸ A 10-cM genome scan of extended multiplex families with CLP from seven diverse populations revealed genes in six chromosomal regions, including a novel region at 9q21 (heterogeneity LOD score [HLOD] = 6.6). In addition, meta-analyses with the addition of results from 186 more families showed genome wide significance for 10 more regions, including another novel region at 2q32–35.⁵⁹

Genome wide study, to search for the suggestive loci linked to clefts with and without dental anomalies outside the cleft area identified a novel locus on 8p11–23.⁶⁰ Analyses of a 10 cM genome scan in multiplex CLP families, followed by association analyses of 1476 SNPs in candidate genes and regions, demonstrated linkage results for regions 1q32, 2p13, 3q27–28, 9q21, 12p11, 14q21–24 and 16q24. Results were phenotype dependent, IRF6 region results were most

significant for individuals with CL alone, and the FOXE1 region results were most significant in families with CLP.⁶¹ Genome wide scan of 92 affected sib-pairs of CLP using 400 microsatellite markers, showed excessive allele sharing at 1p apart from previously implicated 2p13 and 6p23–24.⁵² Genome-wide association study reported two new loci associated with NSCL/P at 17q22 (rs227731, combined $P = 1.07 \times 10^{-8}$), relative risk in homozygotes = 1.84, 95% CI 1.34–2.53) and 10q25.3 (rs7078160, combined $P = 1.92 \times 10^{-8}$), relative risk in homozygotes = 2.17, 95% CI 1.32–3.56).⁶²

Review of linkage and associations to the various genetic loci and candidate genes shows more than 16 chromosomal regions with evidence of linkage to orofacial clefts or its related phenotypes. These include, genes that regulate transcription factors, growth factors, cell signaling and detoxification metabolisms. Table 1 demonstrates involvement of various genes reported to be associated with the clinical entity of NSCLP.

5. Discussion

Clinically NSCLP causes problems of ingestion, swallowing, dysarthria, occlusal disharmony, hypoplasia of the maxilla during growth and external appearance to differing degrees. Corrective surgery, orthodontic treatment and speech

Table 1 – Reported genetic associations with NSCLP.

Gene	Code	References proposing association	References denying association
Muscle segment homeobox 1	MSX1	9, 14, 23, 25, 26, 29, 30, 36	11, 34
Muscle segment homeobox 2	MSX2	26	
Transforming growth factor-alpha	TGFA	23, 27, 28	11
Transforming growth factor-beta 2	TGFB2		11
Transforming growth factor-beta 3	TGFB3	9, 23, 25	11, 34
T-box transcription factor-10	TBX10	26	
T-box transcription factor-22	TBX22	10	
Poliovirus receptor like-1	PVRL1	10	
Interferon regulatory factor-6	IRF6	10, 15–23, 36	
Aristaless-like 3	ALX3		12–14
Homeobox protein Mohawk	MXK		12–14
platelet-derived growth factor	PDGFC		12–14
Runt-related transcription factor 2	RUNX2	24	
Forkhead box protein E1	FOXE1	26, 33, 39	
	GLI2	26	
	JAG2	26, 31	
LIM/homeobox protein	LHX8	26	
	SATB2	26	
	SKI	26	
	SPRY2	26	
	TP63		31
	MID1	31	
B-Cell Leukemia/lymphoma 3	BCL3	32	
	FST		34
Estrogen receptor 1	ESR1	35	
Fibroblast growth factor receptor 2	FGFR2	35	
	RAR alpha	37	
	MTHFR	37	
	FLNB	13	
	HIC1		13
	ZNF189		13
	C9ORF156	39	
	HEMGN	39	

exercises are required to effect improvement. The burden on patients and their families is therefore heavy. Craniofacial development is highly complex with a large array of genes implicated. Identifying the key genes in human NSCLP represents a major challenge.

Various molecular biology techniques have been employed for a better understanding of developmental biology of the defect. Like the direct analysis of functional candidates, linkage and/or candidate gene directed association studies. The analysis of patients with heterogeneous etiology, dilutes the chances of finding positive gene–phenotype correlations. The candidate gene based studies by the genome wide scan find the up regulated and down regulated genes.

Genome-wide association studies entail the matching of a given human genome sequence with an annotated, high resolution map of common genetic variation. It benefits from a large collection of DNA samples, obtained from a population whose clinical characteristics are well defined, cost effective genotyping and sophisticated statistical analysis.

The primary use of GWA studies is in investigation of biologic pathways of disease causation and normal health. To date four genome wide scans for NSCLP have been published, one using sib pair analysis in an English population,⁵⁴ second using multiplex families of Chinese origin,⁵⁶ third using multiplex families from North and South America⁶¹ and the fourth using two large Syrian families.⁵⁵ These four studies do not generally concur on significant or highly suggestive regions, probably reflecting the diverse populations investigated. In these studies blood DNA was purified, and the whole-genome genotyping scan was performed using the Gene Chip Mapping. The assay was performed using 250 ng of genomic DNA and scan images were processed with Affymetrix Micro Array Suite. Software Data were analyzed with GDAS v2 software. Ped-Check was used for the detection of Mendelian errors. This was followed by parametric linkage analysis, the trait model (mode of inheritance, disease-allele frequency, and penetrance of genotypes).³⁴ Since the parameters of the disease model were uncertain, in the initial genome scan, the evidence of linkage with a nonparametric, penetrance-independent, affected-only, and allele-sharing model was assessed. On finding significant evidence of linkage by exceeding the pre-determined threshold ($P.005$) with an allele-sharing method, a range of parametric models to the data were fitted.³

This review thus shows the various genes involved in the causation of NSCLP, with possible gene-gene and gene-environment interactions.

6. Conclusion

Evidence suggests genetic predisposition of NSCLP, caused by the interactions of multiple interacting genes. Although no major gene has been confirmed, a lot of research is ongoing to provide an understanding of the patho-physiology of the orofacial clefts.

Conflicts of interest

The author has none to declare.

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