Review article

Assessment of candidate genes and genetic heterogeneity in human non syndromic orofacial clefts specifically non syndromic cleft lip with or without palate

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ARTICLE INFO

ABSTRACT

Non syndromic orofacial clefts specifically non-syndromic cleft lip/palate are one of the most common craniofacial malformation among birth defects in human having multifactorial etiology with an incidence of 1:700/1000. On the basis of association with other congenital malformations or their presence as isolated anomaly, OFC can be classified as syndromic (30%) and nonsyndromic (70%) respectively. The major cause of disease demonstrates complex interplay between genetic and environmental factors. The pathogenic mechanism of underlying factors have been provided by different genetic studies on large-scale with significant recent advances in genotyping technologies usually based on linkage or genome wide association studies (GWAS). On the basis of recent studies, new tools to identify causative genes involved in NSCL/P reported approximately more than 30 genetic risk loci that are responsible for pathogenesis of facial deformation. Despite these findings, it is still uncertain that how much of variance in NSCL/P predisposing factors can be explain by identified risk loci, as they all together accounts for only 20%–25% of NSCL/P heritability. So there is need of further findings about the problem of rare low frequency coding variants and other missing responsive factors or genetic modifiers. This review will described those potential genes and loci reported in different studies whose involvement in pathogenesis of nonsyndromic OFC has wide scientific evidence.

1. Introduction

Nonsyndromic cleft lip and/or palate (or orofacial cleft, OFC) is a congenital malformation characterized by an incomplete separation between nasal and oral cavities without any associated abnormality (Carinci et al., 2007). Non-syndromic cleft lip with or without cleft palate (NSCL $\pm$ P) is most severe sub-phenotype of nonsyndromic orofacial clefts (NSOFC), which are most common craniofacial malformations among birth defects in human (Yoshiura et al., 1998) with multifactorial etiology (Mossey and Modell, 2012). Orofacial clefts particularly cleft lip with or without cleft palate (CL $\pm$ P) being a major public health problem, affecting 1 in every 700 to 1000 births worldwide (Moreno et al., 2004) with an incidence rate of around 0.1% depending on population (Zheng et al., 2015). Overall, highest birth incidence rates of OFCs are reported in Asia (particularly in China and Japan), with a frequency of 1 in 500 affecting more than 2.6 million people in China (Dixon et al., 2011).

2. Clinical diagnosis

The classification of orofacial clefts with or without other genetic defects was described in International Perinatal Database of Typical Oral Clefts (Group, 2011) and successively modified in 2013 by addition of new cleft subgroups (Luijsterburg et al., 2014). On the basis of strong evidence whether the patients have cleft in addition to other congenital anomalies, a recommended grouping of OFCs into syndromic (usually associated with underlying genetic syndrome) and non-syndromic forms (isolated anomaly) was provided (Yu et al., 2017). According to reported classification, CL $\pm$ P being a component of Mendelian syndromes (such as autosomal dominant van der Woude syndrome, VWS) can occur as a result of chromosomal modifications and exposure to known teratogens. However, these etiologies are related to only 10–20% of individuals with CL $\pm$ P (Beiraghi et al., 2003).

In syndromic cases, incidence of cleft with other anomalies differs between studies and also vary between populations but is collectively reported to be approximately 30% (Beriaghi et al., 2009). While the incidence in non-syndromic cleft lip with or without cleft palate (CL ± P) cases approximately constitute 70% of which 20% are familial and 80% are sporadic (Figure 1) (Mossey and Modell, 2012). In contrast, 50% of CP only (CPO) cases reported as syndromic and 50% as non-syndromic (Dixon et al., 2011). It is reported that CP is more frequently associated with other congenital abnormalities than CL ± P (Mossey and Modell, 2012). Over 200 specific genetic disorders, including a number that are either Mendelian or chromosomal in origin, have CL ± P as a component. While CPO is reported as a feature of more than 400 such syndromes (F. K. Wong and Hagg, 2004). Based on anatomical morphology, NSOFC is further categorized into non-syndromic cleft lip with palate (NSCLP), non-syndromic CL only (NSCLO) and non-syndromic CPO (NSCPO). In general, NSCLO and NSCLP share common epidemiology and occur during the same embryological period, so they are assembled together as non-syndromic CL with or without CP (NSCL ± P) different only in severity (Leslie and Marazita, 2013). However, there are some evidences presenting that NSCLO and NSCLP might harbor different congenital etiologies (Leslie and Marazita, 2013; Ludwig et al., 2016).

The basic genetic mechanism of underlying multifactorial etiology have been studied on large-scale with significant progress mainly due to advances in genotyping technologies. More recently, genome-wide association studies have reported approximately 37 genetic risk loci for NSCL ± P that can only describe a small fraction of heritability (Leslie, Carlson, Shaffer, Butali, et al., 2017; Ludwig et al., 2017; Moreno et al., 2004; Nikopensius et al., 2009; Yu et al., 2017). A considerable fraction of reported loci account their effects in different group of populations (Leslie et al., 2016), but strength of association for single variant in different populations differ as a result of difference in locus heterogeneity and risk allele frequencies also revealed variable prevalence rates for NSCL ± P in different populations (Leslie et al., 2016; Mossey and Modell, 2012). Despite these successes, it remains unclear that how much of variance in NSCL ± P can be accounted by common genetic variations or risk loci identified to date. In general, the identification of further novel genetic factors contributing to NSCL ± P susceptibility have been expected. It is therefore of considerable importance to elucidate genetic, non-genetic and their associated causative factors contributing to more common nonsyndromic forms of CL ± P. According to previous studies, it has been reported that CL ± P being heterogeneous shows relatively multifaceted etiology. Different chromosomal loci such as 1p, 1q, 2p, 3p, 3q, 4q, 6p, 8q, 10q, 13q, 14q, 15q, 16p, 17p, 17q, 19q and 20q have been proposed to contain a clefting locus. Practically it is obvious that these regions could contribute to some extent in facial malformation and possibly different results found by many research groups could be due to

variable genetic expressivity in different populations studied (Carinci et al., 2007).

Orofacial clefts are characterized by their position and descriptive terms for instance unilateral, bilateral, or midline and complete, incomplete (Abbott, 2014). Most of cleft lip (CL) cases reported are unilateral (80%–85%) of which 33% being left-sided clefts (Jensen et al., 1988). It is observed that unilateral clefts (48%) are less associated with other syndromes than bilateral clefts (72%) leading to better survival rate (52%) of fetus with unilateral CL ± P than with a bilateral CL ± P (35%). The frequency and type of chromosomal aberrations varied with type of cleft. The highest frequency of different genetic mutation was observed in midline clefts (82%) with high fatal outcomes (Berge et al., 2001). Around the world, CL ± P is more common in males with a sex ratio of 2:1 (male: female) while CP is more common in females (female: male-2:1) (Mossey and Modell, 2012). Males with CL ± P have more serious cleft than females and familial CL ± P is normally less severe than sporadic cases (Ferretti et al., 2011). However, prevalence in males is found to be lower for syndromic forms where the child presents with other anomalies in addition to CL ± P (Mitchell and Risch, 1992). While the incidence of syndromic CL ± P in females is higher when father is greater than 40 years (Rittler et al., 2004). To understand the developmental mechanisms in orofacial clefts it is important to review a large and diverse field of research. Generally, causative factors can be grouped into genetic and environmental or association of both. Environmental factors that can seriously disturb the fetus development, range from maternal age to utilization of medications such as corticosteroids or antiepileptic agents, smoking and alcohol consumption during pregnancy (Honein et al., 2007). In some cases, maternal illness (specially asthma or other respiratory disorders) was suggested to increase the chances of CL ± P (Dietz et al., 2012) while metabolic or nutritional complications such as obesity, lack of dietary folic acid (Wilcox et al., 2007) and malnutrition or diabetes may also be linked (Correa et al., 2008). Genetic components involve mutant gene variants inherited from father or mother that are directly responsible for causing CL ± P or might be a risk factor to increase the chances of developing a cleft. Most CL ± P cases are probably because of a combined effect of genetic and environmental factors during first weeks of pregnancy. It is observed that unaffected carriers who also inherited same underlying variation but escaped phenotypic outcomes by encountering with favorable uterine environment or co-inheriting a protective hereditary background. In other words, pathogenic etiology behind CL ± P appears to follow a complex, multifactorial and unpredictable mode of inheritance more frequently than Mendelian mode of inheritance leading to clinically diverse and genetically heterogeneous group of disorder.

A great deal of research has focused on NSCL ± P, especially with a target to distinguish the underlying genetic factors behind pathogenicity of disease (Mangold et al., 2009). Many reviews have been published depicting the most recent advances including the prenatal and postnatal incidence of chromosomal aberration and related anomalies in CL, CP and CL ± P (de Aguiar et al., 2015). There has been a substantial progress in identification of different molecular pathways with genetic defects responsible for facial deformities which resulted in identification of mutations in different genes but underlying genes are still unknown for large number of families. It now appears to be obvious that NSCL ± P etiology may include many more genes than previously reported, making the study more challenging and confusing for large scale genome-wide association studies (GWAS). Another plausibility is that most of mutations in different candidate genes are generally private and not recognizable by GWAS studies. Moreover, multiple gene interactions are also found to be involved as a causative factor behind different facial disorders. It is evident that underlying mutations or cytogenetic disturbance affecting particular cis-acting regulatory regions may also play a definitive role. In that case, LOF of coding variants resulting in a disorder usually with a cleft (syndromic cases) while down regulation of expression generally leads only to an isolated cleft (Seto-Salvia and Stanier, 2014).
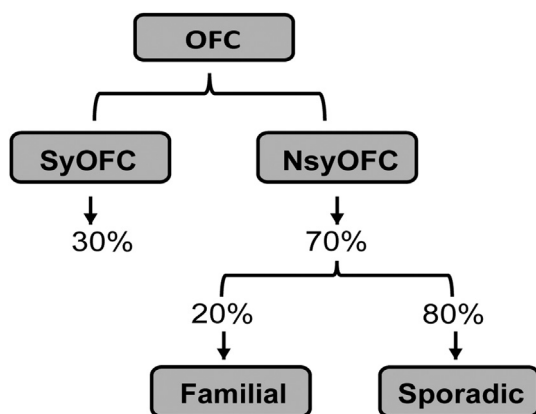

Figure 1. Depicting the percentage record of SyOFC & NsyOFC incidence rate.

Study of NSCL ± P has been significantly proven more refractory to the finding of causal mutations. In spite of fact that it is usually predicted that syndromic cases can be more easily grouped into homogeneous studies than non-syndromic cases that are more heterogeneous because of lack of accomplishment on various levels. Genes play a vital role in the development of non-syndromic clefts but in comparison to syndromic clefts, prevalence of isolated clefts is due to multifactorial or complex inheritance. Various factors interact to support or disturb the complex embryologic growth of craniofacial structures and a complex association of genetic, environmental, and unknown associated factors results in development of a cleft. Furthermore, genetic heterogeneity may also results in population heterogeneity of CL ± P affecting genes (Field et al., 2004). On the account of multiplex nature and genetics heterogeneity of nonsyndromic OFC's affected by number of genetic factors in association with environmental factors and type of inheritance, this review will discuss potential importance of those identified genes and loci whose genetic evidence is strong and found in human genetic studies (Table 1).

2.1. Locus 6p24-p23 (OFC1)

Different studies in different populations support the evidence of localization of a CL ± P gene on chromosome 6 defining it as OFC1 clefting locus. It is observed that orofacial clefting has been mostly related to genetic mutations encompassing short arm of chromosome 6 (Kormann-Bortolotto et al., 1990) but different studies providing contradictory results. A previous study described three individuals with orofacial defect having genetic variation in 6p24.3 near *AP2* and *HGP22* genes (Davies et al., 1995). Linkage of CL ± P to *HLA* locus mapped in 6p21.3 (Watanabe et al., 1984) and *F13A* locus mapped in 6p24 (Eiberg et al., 1987) has also been observed. While three independent case studies found no linkage of CL ± P and 6p locus between *HLA* and *F13A* genes (Blanton et al., 1996; Hecht et al., 1993; Vintiner et al., 1993). A study examined 21 Italian families with nonsyndromic CL ± P of which 14 families were found to be linked to 6p23 region (Carinci et al., 1995). These studies support the localization of CL ± P locus on 6p with confirmation of genetic heterogeneity and linkage analysis. Linkage of different unrelated OFC families to the genes (*ECE1*, *EDNRA*, and *EDNRB*) associated with endothelin-1 (*EDN1*) pathway in 6p23 region was investigated and animal models also confirm linkage of *EDN1* associated genes to OFC (Pezzetti et al., 2000). But study outcomes by mutation analysis excluded the chance of these genes to play any part for

causing nonsyndromic CL ± P in humans. Different studies confirm the linkage of 6p23 as clefting locus (Moreno et al., 2004).

2.2. TGFα-2p13 (OFC2)

Transforming growth factor alpha (*TGFα*) located on chromosomal region 2p13 has been reported to be associated with CL ± P and known for OFC2 (Ardinger et al., 1989). The association of particular C allele of *TGFα* with CL ± P has been confirmed by some studies using restriction fragment length polymorphism (RFLPs) (Sassani et al., 1993) but not confirmed by other studies (Jara et al., 1995). Significant linkage disequilibrium results also supports C allele of *TGFα* in a family based association study (Feng et al., 1994). Linkage analysis studies found the role of *TGFα* in initial stages of CL ± P development and observed that population association can occur even for unrelated loci. But some studies found negative results regarding linkage analysis studies. Failure to identify linkage with *TGFα* could be due to small number of families studied and genetic heterogeneity observed for CL ± P (Stein et al., 1995). Furthermore, association between *TGFα* and CL ± P was confirmed by a meta-analysis study but conclusive data was not found because of genetic heterogeneity (Mitchell, 1997). Allelic interaction between *TGFα* and CL ± P also not found but a strong association between *TGFα* genetic variant and maternal smoking was identified as a risk factor for cleft development (Shaw et al., 1996). Linkage of 2p13 with OFC2 was also observed in a previously reported sample study including 38 families studied for 6p23 locus (OFC1) (Pezzetti et al., 1998). An evidence of genetic heterogeneity in 14 multigenerational families was reported and shown that markers in region of 2p13 are strongly linked to CL ± P. It was observed that mostly the genetic regions with positive results in Caucasian families might not be linked in Chinese with CL ± P (Marazita et al., 2002). From these results it is concluded that *TGFα* could only be a nearby gene to a disease loci.

2.3. BCL3-19q13 (OFC3)

BCL3, a proto oncogene located on 19q13.2 has been found to play a key role in facial formation. Evidence of linkage to *BCL3* as a clefting locus has been identified in 17 multigenerational families out of 39 examined (Stein et al., 1995) and categorized this locus as OFC3. *BCL3* role in orofacial development has also been supported by a study of 30 sporadic CL ± P cases by means of linkage disequilibrium (Amos et al.,

Table 1. List of Potential Genes and Loci Responsible for Non-syndromic CL ± P Described in this Review.

Nonsyndromic CL ± P loci	Chromosomal location	Phenotype/Disease type	Phenotype MIM Number	Genotype MIM number	Inheritance
OFC1/OFCC1	6p24.3	Orofacial cleft I	119530	614287	AD
OFC2	2p13	Orofacial cleft II	602966	602966	-
OFC3	19q13	Orofacial cleft III	600757	600757	AD
OFC4	4q21-q31	Orofacial cleft IV	608371	608371	-
MSX1/OFC5	4p16.1	Orofacial cleft V	608874	142983	-
IRF6/OFC6	1q32.3-q41	Orofacial cleft VI	608864	607199	IC/AD
PVRL1/OFC7	11q23.3	Orofacial cleft VII	225060	600644	AR
TP63/OFC8	3q27	Orofacial cleft VIII	129400	603273	AD
OFC9	13q33.1-q34	Orofacial cleft IX	610361	610273	-
SUMO1/OFC10	2q33	Orofacial cleft X	613705	601912	-
BMP4/OFC11	14q22	Orofacial cleft XI	600625	112262	AD
OFC12	8q24.3	Orofacial cleft XII	612858	612858	AR
OFC13	1p33	Orofacial cleft XIII	613857	613857	AD
OFC14	1p31	Orofacial cleft XIV	615892	615892	AR
DLX4/OFC15	17q21	Orofacial cleft XV	616788	601911	AD
MTHFR	1q36	CL ± P	-	607093	-
CRISPLD2	16q24.1	CL ± P	-	612434	-
CLPTM1	19q13.32	CL ± P	-	604784	AR

1996). A highly polymorphic marker, D19S574 in the range of *BCL3* gene has also shown strong linkage to nonsyndromic orofacial cleft (Martinelli et al., 1998). Different studies in different populations have confirmed the linkage of *BCL3* in development of NSCL ± P. On the basis of results which indicates that *BCL3* has functions related to cell adhesion and its downregulation can cause disruption of facial formation (Figure 2) (Lace et al., 2012). Hence, *BCL3* or an associated gene prompts to be related with congenital facial deformation. Mutation screening in *PVR* and *PVRL2* (closely related to *PVRL1*) located on 19q has also been reported to be linked with CL ± P in five different group of populations. Direct sequence analysis of *PVR* and *PVRL2* found rare variants which were not found in controls. But it was still uncertain that rare or low frequency coding variants in *PVR* and *PVRL2* exhibiting statistically significant association in some populations are sufficient to cause nonsyndromic cleft (Warrington et al., 2006). Different findings in different studies related to association between 19q and orofacial clefting provide supporting evidence that it might contribute to nonsyndromic orofacial clefts.

2.4. Locus 4q21-q31 (OFC4)

Likewise other chromosomal positions reported for orofacial malformation, different studies also support the linkage of chromosome 4q with nonsyndromic CL ± P (Beiraghi et al., 2003). Clefting locus in the region of 4q25-4q31.3 was known for OFC4. Genome scan study of 36 multiplex families with CL ± P show association with 4q locus via linkage disequilibrium test and significant multipoint linkage results with D4S1629 were also obtained (Marazita et al., 2002). Possible localization of 4q locus for cleft lip and palate has also been reported in a father and son with cleft lip by identification of balanced pericentric inversion on chromosome 4p13q21 (Beiraghi et al., 2003). Some studies didn't find positive outcomes for 4q locus (Blanton et al., 1996). But significant results from different studies support the fact that 4q locus has some role in disruption of normal facial formation.

2.5. MSX1 (OFC5)

MSX1 located on 4q16 is a member of muscle segment homeobox gene family and encodes for two coding exons. The encoded protein functions as a transcriptional repressor during embryogenesis through interactions with components of core transcription complex and other homeoproteins. It plays a vital role in craniofacial development, limb-pattering and tumor growth inhibition. Different mutations in *MSX1* have been reported to be associated with nonsyndromic cleft lip with or without cleft palate 5 (OFC5). Because of abnormal morphogenetic and patterning events, failure in fusion of craniofacial primordia occur as a result of misalignment due to decreased mesenchymal growth. Failure in fusion and development can be affected by mutations in genes (like *MSX1*) that control mesenchymal cell proliferation (Green et al., 2015). Such gene variants could therefore increase the risk for OFC development.

MSX1 has been reported as a candidate gene for causing nonsyndromic cleft and different studies also supported the correlation between *MSX1* and environmental factors. Interaction between certain allelic variants of *MSX1* and environmental factors such as alcohol consumption and cigarette smoking during pregnancy increase the chances of CL ± P (Romitti et al., 1999). It is also observed that *MSX1* mutations are linked with both syndromic as well as nonsyndromic cleft CL ± P. Different studies reported in different populations found *MSX1* as a causative risk factor for CL ± P. A recent study based on literature review of genome wide association studies also showed significant association of *MSX1* with variation in normal range craniofacial morphology (Indencleef et al., 2018).

2.6. IRF6 (OFC6)

Interferon regulatory factor-6 (*IRF6*) located on chromosome 1 belongs to interferon regulatory transcription factor (*IRF*) family and regulates the expression of interferon alpha and beta after pathological infection. Family members share a highly-conserved N-terminal helix-turn-helix DNA binding domain and a less conserved C-terminal protein binding domain. The encoded protein may be a transcriptional activator. Most of mutations in *IRF6* were reported in autosomal-dominant van der Woude syndrome, a syndromic form of cleft lip or palate. Furthermore, linkage and genome wide association studies confirmed the association of *IRF6* variants with syndromic as well as nonsyndromic forms of CL ± P (Little et al., 2009). A study proposed that one specific sequence variant found within an enhancer site of *IRF6* results in disruption of a binding site for the transcription factor AP2α (*TFAP2α*) and is significantly over transmitted in NSCLO (Rahimov et al., 2008). It is reported that *IRF6* is responsible for determining the keratinocyte proliferation differentiation switch. But *IRF6* mutant mice fails to exhibit terminal differentiation that results in a hyper proliferative epidermis, leading to multiple epithelial adhesions that can block the oral cavity and in turn causing CL ± P (Richardson et al., 2006). A study reported that *PBX* proteins bind to midfacial regulatory element in order to regulate the *Wnt9b-Wnt3* expression. *Wnt9b-Wnt3* in turn controls *p63*, which directly regulates *IRF6*. Any disruption in the regulation of this pathway leads to localized suppression of apoptosis and yields midfacial clefting i.e. CL ± P in mouse model (Ferretti et al., 2011) (Figure 3). It is evidenced that p63 has role in activation of *IRF6* transcription via *IRF6* enhancer element and mutation within enhancer element increase the chances of cleft lip only. All these outcomes revealed that mutation in *IRF6* may effect a different biological process during lip and palate development while depending on different molecular mechanisms.

As one of major candidate gene, mutations in *IRF6* gene are associated with non-syndromic orofacial cleft type 6 (OFC6). However, some studies observe contradictory outcomes so excluded chromosome 1q32 to be linked with non-syndromic CL ± P (Houdayer et al., 2001) while some studies supported the contribution of 1q32 locus in nonsyndromic CL ± P in a polygenic way instead of monogenic by using transmission disequilibrium test (Blanton et al., 2005). A significant linkage of NSCL ± P was found with genetic alterations in *IRF6*, specially polymorphism rs2235371 (820G > A) that substitutes a valine by an isoleucine at amino acid position 274 (V274I) in SMIR binding domain of *IRF6* (Assis Machado et al., 2018).
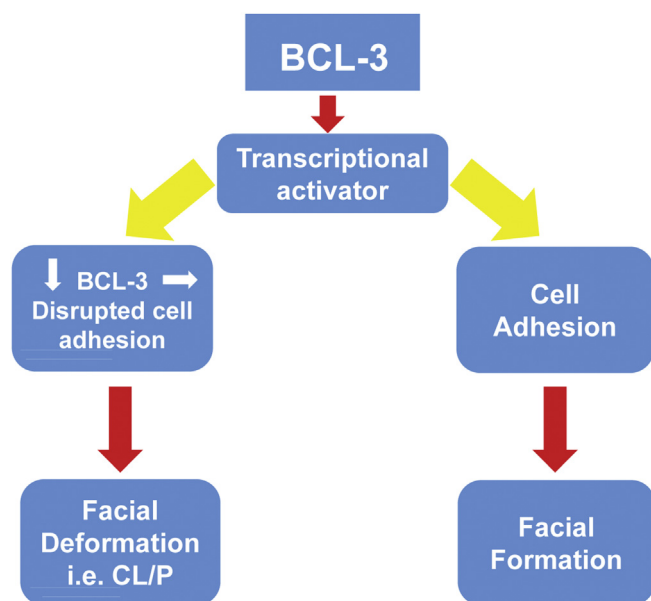

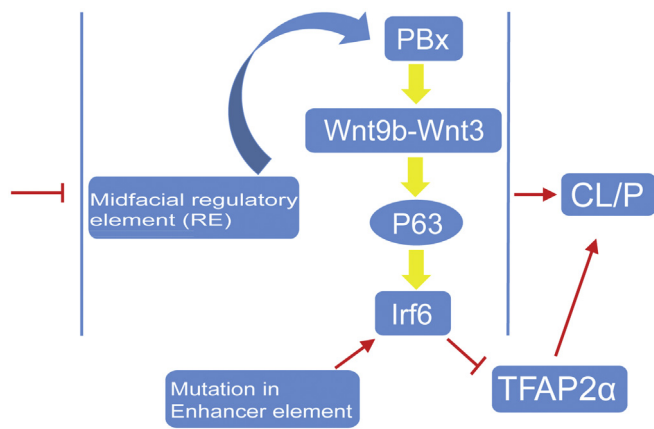**Figure 2.** Illustrating dysregulation of BCL-3 leads to orofacial malformation.

Figure 3. Pbx-directed Wnt-p63-Irf6 regulatory module (Wnt signaling) for midfacial morphogenesis.

2.7. PVRL1/NECTIN1 (OFC7)

PVRL1 located on chromosome 11q23.2 is a member of adhesion family of cell surface proteins that encodes an immunoglobulin related structure important for cell-cell adhesion in epithelia. The important function of *PVRL1* is associated with development of tight junction between epithelial cells (Sozen et al., 2001). During developmental process pair of palatal shelves must be developed along with the development of tongue, after which palatal epithelium comes in close contact and fuse together. *PVRL1* plays a major role in these developments and genetic variations reported to have a significant relationship with CL ± P.

A rare syndromic form of CL ± P clinically categorized as cleft lip/palate with ectodermal dysplasia (*CLPED1*) was found to be linked with 11q23 by linkage mapping (K. Suzuki et al., 2000). *CLPED1* was named as *PVRL1* that encodes nectin-1 and is a part of NAP cell adhesion system playing role as primary cell surface receptor. A highly significant association of W185X mutation in *PVRL1* was identified in sporadic nonsyndromic CL ± P cases showing heterozygosity pattern (Sozen et al., 2001). However, a review study obtained negative results for **W185X** mutation to be associated with nonsyndromic CL ± P but reported new mutations in *PVRL1* as risk factors for CL ± P (Scapoli et al., 2006). Furthermore, a conclusive role of *PVRL1* in orofacial clefting in different populations pay little contribution to nonsyndromic cleft (Avila et al., 2006).

2.8. TP63 (OFC8)

TP63 belongs to *p53* family of transcription factors known as *p63*, a master regulator of epidermal morphogenesis. It is known for its function in proliferation, development and potential role in stratified epithelial tissues characterized both in humans and other animal model. The main target of transcription factor *TP63* is *IRF6* gene. A mutation in binding site of *TP63* located upstream to *IRF6* has been reported in a family as a contributing factor for VWS (Fakhouri et al., 2014). But such mutations are not reported to affect *IRF6* providing evidence that disorder may be caused by disruption in an upstream region which does not directly disrupt target gene sequence.

Different studies linked different syndromic forms of CL ± P caused by mutations in *TP63* encoding tumor protein p63. A distinct genotype-phenotype correlation was also found in different studies to review underlying *TP63* mutations causing human developmental disorder with CL ± P. Although heterozygosity pattern is different for each missense mutation reported but specific for each syndrome, giving insight into molecular data to support the clinical overlap between different syndromic CL ± P (Ianakiev et al., 2000). Mutation analysis of all coding exons of *TP63* performed in 100 Thai patients with nonsyndromic CL ± P also supported the role of *TP63* in CL ± P (Leoyklang et al., 2006).

Incomplete penetrance and variable expressivity was also observed in a family with 3q28del (Ponzi et al., 2015). Recently, Basha et al. support the fact that *TP63* being a transcription factor is a key regulator of epithelial lineage progression during and after development. Different mutations in *TP63* spread in functional domains with a fixed genotype–phenotype correlation. This evidence supports that *TP63* haploinsufficiency can be a causative and dynamic pathomechanism behind clefting (Basha et al., 2018).

2.9. Locus 13q33.1-q34 (OFC9)

A study reported two Indian families with an autosomal dominant nonsyndromic CL ± P and confirmed their linkage to locus 13q33.1-q34 (Radhakrishna et al., 2006). Variable phenotypic features was observed, ranging from unilateral to bilateral CL ± P. It is observed that mostly trisomy patients with CL ± P as associated anomaly also show linkage to chromosome 13 (Berge et al., 2001).

2.10. SUMO1 (OFC10)

Orofacial cleft 10 (OMIM: 613705) is caused by chromosomal aberration of *SUMO1* (OMIM: 601912). *SUMO1* encodes a protein that belongs to SUMO (small ubiquitin-like modifier) protein family. SUMO proteins play a key role in modification and disruption of various cellular proteins and their functional and metabolic process. *SUMO1* functions like ubiquitin in that it bounds to target proteins as a part of post-translational modification system. But unlike ubiquitin which affect proteins for degradation, *SUMO1* contributes in a variety of cellular processes such as nuclear transport, transcriptional regulation, apoptosis, and protein stability (Su and Li, 2002). A girl reported with nonsyndromic unilateral cleft lip and palate with no other associated disorder. Her karyotype 46, XX, t (2; 8) (q33.1; q24.3) was identified with balanced translocation that was thought to disturbed the *SUMO1*. It is reported that a balanced translocation which affects *SUMO1* always observed in patients with NSCL ± P. Significant haploinsufficiency of *SUMO1* was also found to be major causative aspect that is responsible for development of NSCL ± P. Mouse model studies observed *SUMO1* to be expressed in upper lip, primary palate and secondary palate and supported the role of *SUMO1* in palate formation (Alkuraya et al., 2006). Furthermore, it was confirmed that *SUMO1* play a role in regulation of *MSX1* which is one of the causative gene reported for clefting (Gupta and Bei, 2006). It was also found that *TBX22* is a target for *SUMO1* and this modification is required for repression of *TBX22* activity. Loss of *SUMO1* modification was observed in all pathogenic X-linked cleft palate missense mutations. This implied a general mechanism linking the loss of *SUMO1* conjugation to loss of *TBX22* function. The sumoylation process is profoundly affected by environmental factors (Andreou et al., 2007). Hence, it was suggested that *SUMO1* modification may represent a common pathway that regulates normal craniofacial development and is involved in pathogenesis of orofacial clefting.

2.11. BMP4 (OFC11)

BMP4 (bone morphogenetic protein 4) is an important regulatory molecule that plays essential role during development in mesoderm induction, bone induction, limb formation, tooth development and facial development. Loss of function of *BMP4* in mice results in a series of craniofacial malformation including cleft lip and palate (Juriloff and Harris, 2008). *BMP4* is basically a member of BMP family and transforming growth factor beta 1 (OMIM: 190180) superfamily of secretory signaling molecules that show essential roles in embryonic development. It is reported that orofacial cleft-11(OMIM: 600625) is mostly caused by heterozygous mutation in *BMP4* gene (OMIM: 112262) on chromosome 14q22. Various heterozygous mutations (**C1037T, A271T, and C592T**) in *BMP4* were reported in patients with cleft lip and palate (S. Suzuki et al., 2009).

In support with animal models, various studies analyzed the risk of *BMP4* genetic variants causing NSCL ± P in humans with functional SNP rs17563 being most commonly examined. Different meta-analysis studies also support the association of rs17563 with NSCL ± P (Assis Machado et al., 2018). One meta-analysis study available with only six studies, showing focused in populations of Brazil and China. For Chinese population, rs17563 show increased risk for NSCL ± P while a protective effect was observed in Brazilian population (Wu et al., 2015). Second meta-analysis with five additional case-control studies also show risk of C variant allele in Chinese population and shielding effect in Brazilians. This meta-analysis described strong linkage of rs17563 variant with a higher risk among Caucasians that was further supported by significant results of meta-analysis studies in populations from India and Iran (Li et al., 2017).

2.12. Locus 8q24.3 (OFC12)

First validation of 8q24.3 linkage with NSCL ± P developed after genome-wide association study (GWAS). A genome wide association study was conducted including 224 unrelated patients with non-syndromic CL ± P (NSCL ± P) and a 640kb region on chromosome 8q24.21 including 3 markers was observed to reach genome wide significance. The calculated population risk for this locus indicate that this signifies a major susceptibility locus for NSCL ± P (Nikopensius et al., 2009). This locus rs987525 was confirmed as a risk locus for NSCL ± P by relevant GWAS studies (Leslie et al., 2016) as well as in meta-analysis studies including previous GWAS (Leslie, Carlson, Shaffer, Butali, et al., 2017). Linkage between rs987525 and non-syndromic cleft lip and palate reviewed in independent Estonian and Lithuanian samples also show highly significant outcomes in both groups (p = 5.97 × 10^{-5} and p = 1.6 × 10^{-5}) respectively (Nikopensius et al., 2009).

First meta-analyses for NSCL ± P was performed using information from two already reported largest genome wide association studies. The highest risk was linked with A allele for rs987525 on chromosome 8q24 (p value = 5.12 × 10^{-35}) (Leslie, Carlson, Shaffer, Butali, et al., 2017). A series of *in vivo* and *in vitro* studies was also performed and reported that 8q24 region being found in an intergenic region encompasses cis-acting enhancers that regulates *Myc* expression during facial development. Loss of 8q24 region results in deformed facial structures i.e. cleft lip and cleft palate (Uslu et al., 2014).

2.13. Locus 1p33 (OFC13)

A family in which affected father and son was reported with Pierre-Robin syndrome, presented by triad of cleft palate only, glossoptosis and micrognathia, showing karyotype 46,XY,t (1; 2) (p34; q33). The breakpoint at 1p was observed to disrupt the function of *FAF1* gene (OMIM; 604460) in its first intron and decrease expression was found in proband's lymphocytes compared to controls. While analysis of breakpoint at 2q indicated that region was unlikely to show any role in phenotype (Ghassibe-Sabbagh et al., 2011).

Previous study (reported by Ghassibe-Sabbagh) was reviewed and it was found that 2q33 breakpoint in this family was nearby 896kb to *SATB2* (OMIM; 608148) and probably disturbed cis-regulatory elements of *SATB2*. Phenotype in these patients was observed to be consistent with Glass syndrome (OMIM; 612313), which is affected by functional haploinsufficiency of *SATB2* (Rainger et al., 2014). *SATB2*, is located on chromosome 2q33.1 and codes for AT-rich sequence binding protein with 733 amino acids. *SATB2* is reported as first cell-type-specific transcription factor which plays an important role as a key regulator of transcription of large chromatin domains. It directly interacts with activity of those transcriptional factors that control craniofacial development (Leoyklang et al., 2007).

2.14. Locus 1p31 (OFC14)

Linkage analysis of a large consanguineous Turkish family with isolated midline clefting syndrome showed linkage to chromosome 1p31. Evidence of shared homozygosity for a 15.3Mb region on 1p31 consistent with autosomal recessive inheritance was observed in affected family by haplotype analysis, however two subclinical relatives did not show this haplotype. Further analysis of candidate region found a homozygous deletion of 273kb that segregated with full phenotype. Deleted sequences did not contain any known genes but covered some small regions that were conserved across different species. Deletion was not identified in 121 individuals from control group. Knockdown of *LHX8* gene (OMIM; 604425) in mice was observed to cause clefting syndromes. It was proposed that human *LHX8* gene located 49bp upstream of deletion region, is a candidate target of a regulatory element within deletion region (Yildirim et al., 2014).

2.15. DLX4 (OFC15)

DLX genes form a homeodomain containing transcription factor family that contributes a crucial role in craniofacial development. During mouse embryogenesis, *DLX* genes are found to differentially express in mesenchymal tissues of first pharyngeal arch of jaw. Homozygous deletion of *DLX* (specially *DLX4*) genes in mice cause severe craniofacial abnormalities including cleft palate, more significantly showing importance of these genes in craniofacial morphogenesis (Talbot et al., 2010). It is observed that mutation in *DLX4* (OMIM; 601911) located on chromosome 17q21 is responsible for causing orofacial cleft-15 (OFC15).

Whole exome sequencing for a Hispanic woman with bilateral CL ± P and minor facial deformity was performed, however she was negative for mutation in all genes reported to be associated with CL ± P. Heterozygosity of 1bp deletion in *DLX4* that was identified in her affected son was also observed but not observed in public variant databases. Further study of *DLX4* in 3 patients with blepharocheilodontic syndrome (OMIM; 119580) and in 155 patients with nonsyndromic CL, CL ± P, or CP did not show any mutations (Wu et al., 2015). Recently, a study also identified Distal-less 4 (*DLX4*) as a causative gene for syndromic form of cleft lip with or without cleft palate. Biological analyses also supported the importance of *DLX4* in craniofacial morphogenesis which promoted *DLX4* as a promising candidate gene to further examine any possible relationship between non-syndromic orofacial clefts and *DLX4* polymorphisms (He and Bian, 2018).

2.16. MTHFR (locus 1q36)

Methylenetetrahydrofolate reductase (*MTHFR*) located on 1q36 is a major enzyme of folic acid metabolism. The genetic variations in *MTHFR* particularly rs1801133 (**C677T**) codes for a thermolabile enzyme with low activity. **C677T** polymorphism was examined in a study of NSCL ± P patients and it was observed that fetal homozygosity (TT) was three times more common in patients than in controls (Sozen et al., 2009). Homozygosity pattern for rs1801133 polymorphism in *MTHFR* was considerably more common in both sporadic CPI and CL ± P. Study observed that homozygosity for either C or T allele of **C677T** polymorphism in females was found to be an important risk factor for causing OFC. It was reported that maternal hyperhomocysteinemia may be a susceptibility factor for having CL ± P offspring. A considerably higher mutation frequency of *MTHFR* reported in hyperhomocysteinemic mothers of CL ± P patients in comparison to controls. These consequences support the association between folate pathway and CL ± P as well as specify the effect of maternal genotype instead of embryo's genotype (Martinelli et al., 2001).

Additional support for the significant role of folate pathway and related genes in etiology of CL ± P has been described to analyze linkage disequilibrium. Different meta-analysis studies reported the association of *MTHFR* polymorphism (rs1801133) as a risk factor for NSCL ± P (de Aguiar et al., 2015). On the basis of previous meta-analysis studies, a

recent study pooled all results and proposed that associated risk of rs1801133 in *MTHFR* appears to be ancestry dependent (Assis Machado et al., 2018).

2.17. CRISPLD2 (locus 16q24.1)

CRISPLD2 gene located within a region of chromosome 16 was observed to be linked with nonsyndromic CL ± P. *CRISPLD2* mapped to chromosome 16q24.1 via genome sequence analysis and it was found that *CRISPLD2* contains 15 exons spanning about 110 kb (Chiquet et al., 2007). A study was reported to find relationship between *CRISPLD2* and NSCL ± P in Xinjiang Uyghur population. Based on previous studies, it was proposed that single nucleotide polymorphisms of *CRISPLD2* have been reported to be a risk factor in the etiology of NSCL ± P. Results found in a study determine that genetic polymorphism of *CRISPLD2* was strongly linked with an increased risk of NSCL ± P in a Xinjiang Uyghur population. The SNP rs1546124 found in exon 2 of *CRISPLD* is one of most investigated variant located upstream of start codon. Any sequence alteration in promoter region of *CRISPLD2* could disrupt the binding of regulatory elements such as polymerase binding or transcription factor activator/inhibitor which in turn disrupt protein expression and consequently affect the facial developmental processes (Mijiti et al., 2015).

2.18. CLPTM1 (locus 19q13.3)

CLPTM1 (OMIM; 119530) located on chromosome 19q13.3 has been reported to linked in a family where cleft lip and palate segregated in 2 out of 3 generations with a stable chromosomal translocation t (2; 19) (q11.2; q13.3). Positional cloning method was used to identify a novel gene 'cleft lip- and palate-associated transmembrane protein-1' (*CLPTM1*) by translocation disruption on chromosome 19. By using single strand confirmation polymorphism (SSCP) in sporadic cases of clefting and direct sequencing of coding exons of *CLPTM1* in familial cases, no specific pathogenic mutations were observed but findings still support role of *CLPTM1* in the development of a CL ± P malformative phenotype (Yoshiura et al., 1998).

3. Discussion

Non-syndromic CL ± P is a complex genetic disorder caused by interaction of multiple genetic and environmental risk factors. Alteration in one or more candidate genes could disrupt orofacial development and cause cleft lip with or without cleft palate. Though, NSCL ± P are amongst the most common and distressing congenital birth defects, biological mechanisms behind this multifactorial disease are still not completely known. Currently, genetic

and environmental factors are subject of intense research because their knowledge is essential in order to create translational opportunities for genetic counselling and prevention of disease. A wide range of different genetic studies usually based in linkage or genome wide association analysis provide new tools to identify genomic susceptibility regions including multiple causative genes and chromosomal loci that are responsible for pathogenesis of orofacial clefts (NSCL ± P) (Figure 4).

The risk for CL ± P in first degree relatives is approximately 32 times the risk for individuals without a family history of CL ± P which indicated that non-syndromic CL ± P has a higher rate of family recurrence (Sivertsen et al., 2008). The contributing genetic factors for nonsyndromic CL ± P has been investigated by linkage analysis, genomic rearrangements, microarray analysis, meta-analysis and genome-wide association studies. Several candidate genes including *IRF6*, *MSX1*, *SPRY1*, *SPRY2*, *CHD7*, *GABRB3*, *NOG*, *NTN1*, *MMP16*, *KRT18*, *DICER1*, *RAD54B*, *CREBBP*, *GADD45G*, *TFAP2A*, *VAX1*, *GSC*, *PTCH1*, *MYC*, *TAF1B*, *MAFB*, *OFCC1*, *ARHGAP29*, *WNT9B*, *FGFR*1, *FGF10* (Yu et al., 2017), *PAX9*, *PAX7*, *ABCA4*, *THADA*, *FOXE1*, *GREM1_FMN1* (Ludwig et al., 2017) has been reported in different studies for NSCL ± P. Different genome wide association studies of rare low frequency coding variants i.e.*N4BP2*, *CDSN*, *PRTG*, *AHRR* (Leslie, Carlson, Shaffer, Buxo, et al., 2017), *ACSS2* and *PHYH* (Aylward et al., 2016) and microarray techniques for CNV analysis in search of potential causative genes i.e. *COL11A1*, *TERT*, *MIR4457*, *CLPTM1*, *ESR1*, *GLI3*, *FGFR*, *OFD*, *TBX1*, *PHF8* and *FLNA* (da Silva et al., 2018) has also been found to be associated with NSCL ± P.

Genome scan and association studies have successfully reported almost 37 risk genes/loci for NSCL ± P to date, which could elucidate a small fraction of heritability. Such studies have collected enough data for all genes/loci to be listed as a putative and contributing risk factor for NSCL ± P. Still all these loci together explain only a small percentage of NSCL ± P's heritability, so problem to find missing risk factors provokes a strong discussion. It is hypothesized that part of missing heritability could be clarified by rare coding variants that are not analyzed by GWAS's. Such variants can be frequently found in population with a medium to high penetrance. Furthermore, there is high variable expressivity in most single gene oral cleft syndromes supporting the fact that there is influence of genetic modifiers. Imputation studies using combined reference panels such as Haplotype Reference Consortium will increase accuracy of low frequency variants and future opportunity to find underlying genetic variants for NSCL ± P comparable to recent progresses in other disorders.
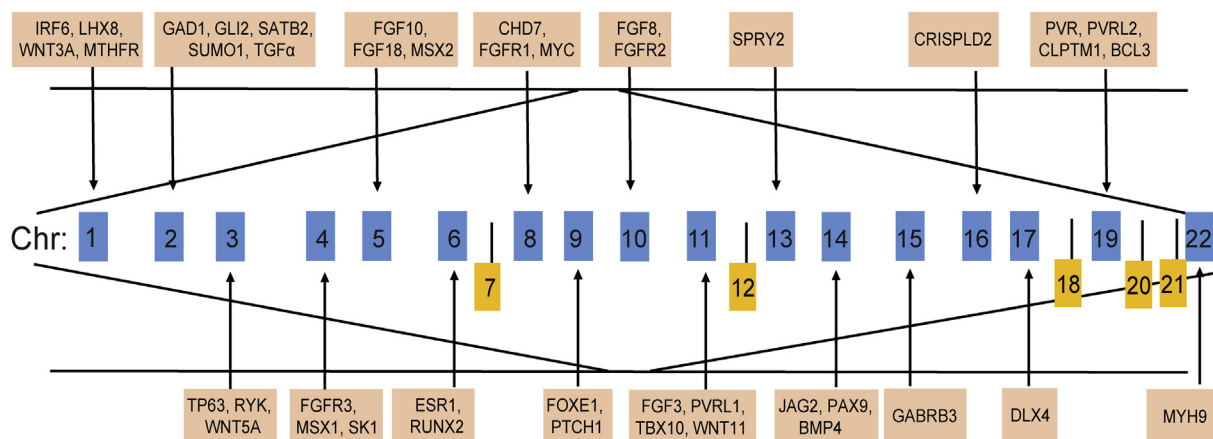

Figure 4. Potential candidate genes located on different chromosomes involved in NSCL ± P as suggested by different human genetics studies, mouse models, and expression analyses.

4. Conclusion

In conclusion, several studies yield insights into etiology of nonsyndromic orofacial clefting by enlightening novel common variants and biological information for NSCL ± P. The major cause in development of non-syndromic cleft lip and palate has not been fully elucidated due to complex interaction between genetic and environmental risk factors. In future, further research will be needed to understand multifactorial threshold concept (gene-environment interaction) of complex etiology and variable penetrance behind OFC and biological mechanisms by which related common variants affect normal craniofacial development.

Author contribution statement

All authors listed have significantly contributed to the development and the writing of this article.

Funding statement

Competing interest statement

The authors declare no conflict of interest.

Additional information

No additional information is available for this paper.

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