Exploration of Genetic Variants of Non-syndromic Cleft Lip with or without Palate and Underlying Mechanisms

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Non-syndromic cleft lip with/without cleft palate (NSCL/P) is one of the most common birth defects in humans with an overall prevalence of one per 1000 live births. Due to genetic and environmental influences, the fusion of the lips or palate may be interrupted at any stage and cause a cleft. Over decades, dozens of susceptible genes and loci have been identified using multiple genetic approaches. Our group has collected samples of NSCL/P patients since 2008 and established the biobank. We discovered numerous susceptible loci related to the occurrence of NSCL/P in the Chinese population, such as 16p13.3, 1q32.2, 10q25.3 and 17p13.1. In addition, we performed functional studies on related loci and genes by using molecular biology, cell biology, animal models and other methods to provide a basis for the construction of the NSCL/P genetic map in the Chinese population and help to implement individualised prophylaxis and treatment. Future efforts will focus on identifying functional variants, investigating pathways and other interactions, and including phenotypic and ethnic diversity in research.

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Non-syndromic cleft lip with or without cleft palate (NSCL/P) is a common human birth defect characterised by craniofacial abnormality due to incomplete separation between the nasal and oral cavities[1]. Its prevalence ranges from 1/700 to 1/1000, depending on ethnicity and geographical area[2,3]. Common risk factors for NSCL/P include genetic risk factors, environmental exposure and their interaction[4-7]. As only a few people exposed to the same risk factors suffer from NSCL/P, genetic susceptibility is considered to play a crucial role.

Most genomic variation can be attributed to single nucleotide polymorphisms (SNPs), which are useful markers for genetic association studies of disease susceptibility or adverse drug reactions[8]. To date, various genetic approaches have been applied to identify genetic factors that put individuals at risk of NSCL/P. Initially, candidate gene association studies were performed to test genetic variants in genes relevant to NSCL/P9. Later, the associations of SNPs in the pathway with the risk of NSCL/P were investigated using a candidate pathway association study approach[10]. Genome-wide association studies (GWASs) have since successfully identified numerous loci associated with NSCL/P[11]. A possible polygenic threshold model of inheritance is supported by the identification of common genetic risk variants for NSCL/P from GWASs and SNP heritability estimates of around 30%[12]. All these studies have facilitated understanding of the pathogenic mechanisms of NSCL/P and improved clinical management of patients.

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The present review summarises the approaches to, advances in and future prospects for genetic variant discovery and functional interpretation. We then complement our description with examples from susceptibility loci identified in our study where the use of these approaches has advanced our biological understanding of NSCL/P. In addition, we assess the extensive genetic, molecular and cell biological evidence that have implications for studies on NSCL/P.

Candidate gene association studies in NSCL/P

Candidate gene association studies have proven to be an effective approach in genetic association studies based on case-control populations to identify risk variants involved in specific diseases, which have the advantages of being cheap and easy to implement quickly[13]. These studies on NSCL/P always begin with selection of a putative candidate gene, which could play a critical role in the development of cleft lip and palate under investigation[14]. To date, there is a large amount of literature and experimental and sequencing data that can be used to can be used to identify candidate genes for NSCL/P. For example, p63 as a Wnt signalling target was found to be involved in midfacial development in mice[15]. FOXE1 mutations were detected to be associated with Bamforth-Lazarus syndrome, characterised by thyroid dysgenesis and cleft lip[16]. To explore the functional significance and potential association trait of the candidate genes of NSCL/P further, selection of a putative candidate gene was followed by evaluating and screening polymorphisms, usually the representative SNPs called tagging SNPs[17] or/and functional SNPs, which affect gene transcription. Finally, the selected SNPs were genotyped in the experimental population, including cases and controls, to make an association analysis between SNPs and the risk of NSCL/P.

Thus far, candidate gene association studies have successfully identified a group of specific variants and genes that may lead to the development of NSCL/P[18-21]. In recent years, our group has also carried out candidate gene association studies to identify additional SNPs that pose risks and evaluated their potential as biomarkers in the future.

IRF6

The first genetic variant associated with NSCL/P was either valine or isoleucine at amino acid position 274 (V274I) located in IRF69. IRF6 has been reported to be involved in Van der Woude syndrome, accompanied by the occurrence of CLP or deformity of the lower lip[22]. Zucchero et al[9] carried out transmission-disequilibrium testing (TDT) and case-control analyses in 8003 individuals from 1968 multiethnic families and detected that V274I in IRF6 was the risk genetic variation related to NSCL/P. In 2010, our group also genotyped polymorphisms in IRF6 and evaluated their associations with NSCL/P in a Chinese Han population[23]. We determined that rs2235371 and rs642961, which regulated levels of IRF6 mRNA and protein, significantly affect the susceptibility of NSCL/P.

MSX1

MSX1 is regionally expressed in the early critical stage of craniofacial development and participates in craniofacial and nervous system development as a transcriptional suppressor[24]. In addition, Msx1-deficient transgenic mice have been found to show general craniofacial deformity, including cleft palate, alveolar bone abnormalities and dental dysplasia[25]. Ma et al[26] selected four functional SNPs in MSX1, which were located in 3′UTR, exon and 5′UTR regions, and evaluated their susceptibility to NSCL/P among 602 cases and 605 healthy controls from a Chinese Han population. rs12532 located in 3′UTR of MSX1 was detected to be related to the development of NSCL/P by affecting the binding of miR-3649 to MSX1[26].

MYH9

MYH9 has been reported to play an important role in the development of palatal fusion[27]. MYH9 is a candidate gene and it is therefore worth exploring which SNPs on it are associated with the risk of NSCL/P and how these sites regulate gene expression to cause the disease; thus, we selected independent functional SNPs located in 3′UTR, exon and 5′UTR regions based on the SNP database and HapMap Project database. We made a further biological functional prediction for these sites and four SNPs were included. Through the two-stage population sample verification, including 1275 cases and 1295 controls, followed by a series of functional experiments, rs12107 in the 3’UTR and rs2269529 in the exon region were identified to be related to NSCL/P by upregulating expression of MYH9[28].

Candidate pathway association studies in NSCL/P

The biological processes that occur during the development of human embryos are carried out by several pathways in a tightly regulatory manner. At the phenotypic level, dysregulation of these processes could lead

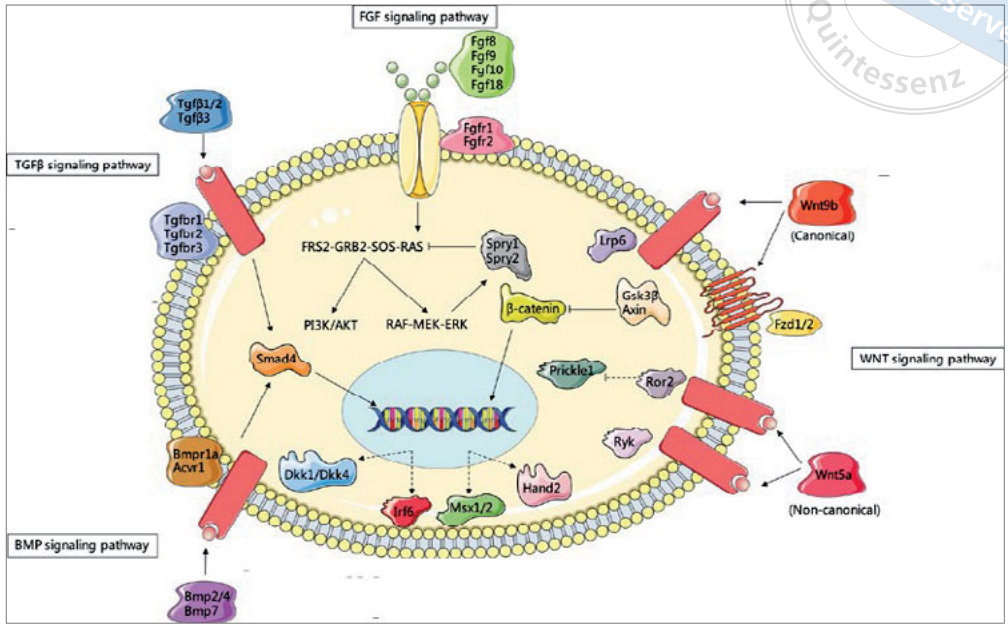

Fig 1 Main molecular pathways involved in NSCL/P. Susceptibility genes in NSCL/P affect different signalling pathways, including WNT, TGFβ, BMP, and FGF signalling pathway.

to malformations during the early embryonic development, such as NSCL/P[6,29]. Diverse signalling cues and attendant proteins work together during closure of the lip and growth of the palatal shelves across embryogenesis, including BMP, FGF, TGFβ and WNT signalling pathways (Fig 1)[10,30-32]. Pathway studies have been based on the association analysis between tag SNPs and the risk of NSCL/P defining SNPs related to NSCL/P on pathway genes.

WNT pathway

Vijayan et al[33] performed an association analysis based on 20 SNPs on WNT pathway genes in 471 individuals with NSCL/P and 504 unrelated control individuals of Caucasian ethnicity, and a significant association was found between GSK3B rs13314595 genotypes and NSCL/P. This study was the first to show the association between GSK3B and NSCL/P and confirmed the role of additional WNT pathway genes as candidates for NSCL/P[33].

Epidermal growth factor receptor (EGFR) pathway

EGFR was reported to regulate cell migration in the embryonic developmental phase[34,35], which was closely related to the development of craniofacial structure[36]. Our group has conducted an in-depth exploration of genetic variation in biological pathways[37]. Li et al[37] selected a superpathway of endocytic trafficking of EGFR and investigated the associations of SNPs in the pathway with the risk of NSCL/P. The study suggested that the genetic variants of SHTN1, AP2B1 and NTRK1 in the investigated superpathway showed statistical evidence for association with the risk of NSCL/P[37].

Autophagy pathway

In addition to selecting pathways that have been reported to be significantly related to lip and palate development, we also selected pathways related to other diseases and that are involved in early embryonic development for in-depth research. As a conserved lysosomal degradation process in eukaryotes, autophagy protects cells from different kinds of stress, such as starvation, hypoxia or exposure to toxic molecules[38]. In the early development stage, autophagy has been shown to be essential in the transition of oocytes to embryos, postpartum survival, development, differentiation and ageing in mouse models[39]. Lou et al[40] conducted a two-stage case-control study with 2027 NSCL/P cases and 1843 controls to explore associations between genetic variants in the autophagy pathway and the risk of NSCL/P, and found that rs2301104 in the autophagy pathway gene HIF1A was associated with susceptibility to NSCL/P. Moreover, the authors explored the functional roles of the SNP and the gene through in vivo and in vitro experiments and found that the risk allele of rs2301104 reduced the enhancer activity and expression of HIF1A, and also that knockdown of HIF1A affected cell functions, which may increase susceptibility to NSCL/P[40].

Table 1 GWASs identified newly discovered SNPs associated with NSCL/P.

PMID	Newly discovered SNPs with $P < 5 \times 10^{-8}$				Population	Study
19270707	rs987525				European	Birnbaum et al[44]
19656524	rs17085106				European	Grant et al[45]
20023658	rs227731	rs7078160			European	Mangold et al[46]
20436469	rs10863790				European	Beaty et al[47]
21618603	rs2294426				European	Beaty et al[48]
22863734	rs560426	rs8001641	rs7632427	rs861020	European	Ludwig et al[49]
	rs13041247	rs742071	rs7590268	rs12543318		
25775280	rs2235371	rs8049367	rs4791774		Chinese	Sun et al[50]
28054174	rs9439714	rs72728734	rs12944377	rs1588366	Asian European Latino or African	Leslie et al[51]
	rs66515264	rs6540559	rs9911652	rs6029258		
	rs3789432	rs12070337	rs55658222	rs75477785		
	rs9439713	rs6072081	rs10886040	rs11841646		
	rs7566780	rs76479869	rs11072494	rs1109430		
	rs17242358	rs1234719				
28087736	rs6740960	rs4901118	rs3746101		European Asian	Ludwig et al[12]
28232668	rs7552	rs2064163	rs12229654	rs11066150	Chinese European Asian	Yu et al[52]
	rs481931	rs6585429	rs2304269	rs957448		
	rs10512248	rs2872615	rs287982	rs9381107		
	rs12681366	rs12229892	rs6495117	rs2283487		
	rs1907989	rs13317	rs908822	rs7871395		
	rs3741442	rs705704	rs9545308	rs7148069		
	rs1243572	rs2289187	rs1838105	rs6129653		
	rs2006771	rs78212183	rs10462065	rs7017252		
30067744	rs255877	rs2522825			European	Howe et al[53]
30277614	rs72804706				African Asian Latin American North American	Carlson et al[54]
30452639	rs80004662	rs113691307			African	Butali et al[55]
31609978	rs12405750	rs17820943	rs730570	rs765366	Chinese	Huang et al[56]
	rs4752028	rs57700751	rs625882	rs116910459		
	rs730643	rs698406	rs1009136	rs3468		
	rs4646211	rs8061677	rs78669990	rs72741048		
	rs72688980	rs6791526				
32373937	rs8071332	rs8076457	rs1215465	rs3138512	European	Dardani et al[57]

GWAS of NSCL/P

GWASs are dedicated to detecting the associations between SNPs and complex traits and diseases in samples among populations[41]. An increasing number of SNPs have been reported to participate in the development of traits and diseases since the first GWAS for age-related macular degeneration (AMD) was published in 2005[42].

To date, the National Human Genome Research Institute (NHGRI) Catalog[43] of published GWASs has identified 15 studies (Table 1)[12,44-57] including 101 newly discovered SNPs relevant to NSCL/P with $P < 5 \times 10^{-8}$. In 2009, Birnbaum et al[44] conducted the first NSCL/P GWAS on a cohort of the European population and provided evidence that 8q24.21 (rs987525), which lay in a gene desert, was a major susceptibility locus for NSCL/P. Several other GWAS around this time also identified important loci[45-49]. In 2015, our group conducted the first NSCL/P GWAS in a Chinese population, followed by two stages of replication. There were 2577 cases and 3193 controls in total. We identified 16p13.3 (rs8049367 between CREBBP and ADCY9) as a new susceptible locus for NSCL/P and confirmed that the reported loci at 1q32.2, 10q25.3, 17p13.1 and 20q12 were effectual[50]. Then, a 2017 GWAS and meta-analysis on the Chinese population linked both previously known and novel SNPs and genes with NSCL/P[52].

As the lip formation processes differ from those for the palate, as do their respective causes and risk factors, Huang et al[56] aimed to dissect the risk factors underlying the pathogenesis of cleft lip only (CLO) and cleft palate only (CPO) using 6986 cases and 10,165 controls. A total of 18 genes/loci were responsible for subtypes, including nine for CPO, seven for CLO and two for both conditions. Interestingly, an opposite effect of the genetic variants was observed in the IRF6 gene for CPO and CLO. The latest GWAS of NSCL/P not only performed a meta-analysis, but also sought to evaluate the causal effects of genetic liability to NSCL/P on educational attainment and intelligence[57].

GWAS offers great advantages in identifying novel variant–trait associations which lead to the discovery of novel biological mechanisms and provide insight into ethnic variation of complex traits[58]; however, GWAS cannot necessarily specify which variant at a locus is the 'causal variant' and identify all genetic determinants of complex traits[59]. Thus, post-GWAS strategies have been proposed to identify the causal variants and understand their biological consequences.

Hah et al[60] conducted a targeted sequencing study of 13 NSCL/P GWAS loci in 1409 trios from European and Asian ancestries and found that rs227727 near the NOG gene disrupted enhancer activity, a mutation in PAX7 disrupted the DNA binding of the encoded TF in vitro and another mutation disrupted the activity of a neural crest enhancer downstream of FGFR2 both in vitro and in vivo. In our study, rs2262251 (G>C) in lncRNA RP11-462G12.2 was in high linkage disequilibrium (LD) with rs8049367, which was identified in our previous GWAS on NSCL/P. Through a series of experiments, we found rs2262251 was involved in the RP11-462G12.2-miR-744-5p-IQSEC2 regulatory axis to affect NSCL/P development[61]. The functional consequences illustrated an SNP in lncRNA leading to NSCL/P and also proved that lncRNA, miRNA and genes constituted a complicated and coordinative regulatory network.

Conclusion and future perspectives

The past decades have seen a series of remarkable discoveries in human genetic variants related to NSCL/P through genes, pathways and GWAS strategies. The future of NSCL/P research is likely to be characterised by three aspects. The first challenge is to understand the functional consequences of these SNPs and to accurately elucidate the biological mechanism in the 'post-GWAS' era[62]. Second, next-generation sequencing (NGS) efforts are necessary to uncover rare variants that play an important role in NSCL/P[41]. Third, the combination of whole-genome surveys of genetic variation and multiomics data will show significant value for making new fundamental discoveries in human genetics[58].

Conflicts of interest

The authors declare no conflicts of interest related to this study.

Author contribution

Prof Yong Chu PAN and Dr Lan MA drafted the manuscript; Dr Shu LOU made the figure; Drs Gui Rong ZHU and Xin YU made the table. Profs Lin WANG and Yong Chu PAN designed the manuscript; Prof Lin WANG critically revised the manuscript. All authors read and approved the final manuscript.

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