

Original Article

The association between the EPHA3 gene polymorphism and non-syndromic cleft lip with or without palate in the western Han Chinese population

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Abstract: Non-syndromic cleft lip with or without palate (NSCL/P) is a common craniofacial congenital disease which results from multiple susceptibility genes and adverse environmental factors. A recent study reported that the T allele of rs7650466 of the EPHA3 gene is a genetic risk factor in the etiology of NSCL/P among the northern Chinese Han population. This study aimed to evaluate the association between the EPHA3 gene variations and NSCL/P in the western Han Chinese population. Here, we conducted targeted region sequencing around rs7632427 (EPHA3) among 159 unrelated NSCL/P cases from the western Han Chinese population and performed a gene-based burden analysis on the rare variations and a single variation association analysis on the common SNVs (single nucleotide variants). Then we found 438 SNVs and 120 indels in all. A burden analysis showed no statistical significance. The association analysis results suggested that the common SNV rs13094064 was associated with NSCL/P ($P=1.86E-07$ and $OR=0.17$), and rs7632427 was most closely related to NSCL/P ($P=7.49E-05$ and $OR=0.315$). Our study primarily confirmed that the EPHA3 gene was associated with NSCL/P in the western Han Chinese population, making available scientific evidence for future research and genetic counseling.

Keywords: Non-syndromic cleft lip with or without palate, EPHA3 gene, single nucleotide variants (SNVs), single nucleotide polymorphism (SNPs)

Introduction

Cleft lip with or without palate (CL/P) is one of the most common oral and craniofacial congenital diseases, with an incidence of one in every 700 newborns worldwide. In China, the incidence rate is about 1.67%, including 2.7% for cleft palate only (CPO), 5.6% for cleft lip only (CLO) and 8.2% for cleft lip and palate (CLP) [1, 2]. According to its clinical phenotypic characteristics, it can be divided into syndromic cleft lip with or without palate (SCL/P) and non-syndromic cleft lip with or without palate (NSCL/P) [3]. The approximately 70% of the CL/P patients who have no apparent deformity of other parts of the body are collectively known as NSCL/P. Moreover, NSCL/P presents a more complicated pathogenesis due to the combined effects

of multiple genetic mutations and environmental risk factors compared with SCL/P [3, 4]. Unfortunately, once a baby is born with NSCL/P disease, the family faces lots of problems, such as feeding difficulties, speech loss, craniofacial deformity, psychological barriers, and social integration problems, etc. and also bears severe economic burden because of the need for multiple surgeries and multidisciplinary integrated sequence therapies [5]. Despite the rapid development of modern medicine, the long and painful treatment process of NSCL/P is always unsatisfactory for the patients. So, it is urgent to deeply explore the pathogenesis of this birth defect and strive to provide some scientific theoretical basis for the early diagnosis and treatment of NSCL/P at the level of molecular genetics.

EPHA3 is associated with the pathogenesis of NSCL/P

Many studies have been carried out on the etiological mechanism of NSCL/P. Genome-wide association studies (GWAS) have provided valuable guidance for researchers to capture new susceptibility loci and genes of NSCL/P [6-9], such as MAFB and ABCA4 [6], rs80493-67 between CREBBP and ADCY9 and loci at 1q32.2, 2q35, 10q25.3, 17p13.1 and 20q12 [7], rs7590268, rs7632427, rs12543318, and rs1873147 [8], 1p36, 2p21, 3p11.1, 8q21.3, 13q31.1, and 15q22 [9]. Obviously, GWAS were widely performed in studying the pathogenesis of complex genetic diseases due to the advantages of high efficiency, sensitivity, and wide range, but GWAS were also limited by racial and regional differences. So it is understood that the verified *P* values of the susceptible genes associated with NSCL/P varied among the different countries and ethnic groups, which also indicated that the correlation strength was different. Based on the previous studies, our research team also conducted a series of relevant studies among the western Han Chinese population as follows. Jiang et al. [10] once revealed a strong association between the allele G at SNP rs4791774 of the NTN1 gene and NSCLO and further enhanced the evidence in the etiology of NSCL/P. In view of the research based on the MAFB gene, a genotypic transmission-disequilibrium test (TDT) analysis confirmed that the C/C homozygote at rs17820943 and T/T homozygote at rs13041247 were over-transmitted and both the C allele at rs17820943 and the T allele at rs13041247 were over-transmitted among NSCL/P patients, suggesting that the MAFB gene was a susceptibility gene for NSCL/P [11]. Other studies also confirmed that rs12543-318 was associated with NSCLO and that the allele A at rs7078160 of the VAX1 gene was a risk factor for NSCL/P [12, 13]. However, there was no evidence associated with the EPHA3 gene among NSCL/P patients in the western Han Chinese population.

EPHA3, located on the 3p11.1 chromosome, belongs to the ephrin receptor subfamily of the protein-tyrosine kinase family. Receptor tyrosine kinase (RTK) signaling pathways are both functionally conserved and diverse, and regulate cell-cell physiological interactions such as cell migration guidance, axon pathway guidance for angiogenesis, stem cell maintenance and metastasis, neural network forma-

tion and neural tube formation, etc. [5, 14, 15]. GWAS once identified the SNP of rs763242 at EPHA3 which was associated with NSCL/P patients (Asian & European) and found that EPHA3 played an important regulatory function in the development of cranial and maxillofacial structure [8, 9]. Before this, Maunakea et al. [16] confirmed that there was a strong methylation site approximately 200 bp downstream of rs7632427 near EPHA3. This was a valuable discovery for the functional study of the EPHA3 gene and NSCL/P. Recently, Chen et al. [5] discovered that the SNP rs7650466 of EPHA3 gene was associated with NSCL/P in the northern Chinese population and also confirmed that rs7650466 was associated with NSCL/P, but not NSCPO, after a stratified analysis. But there is no other study that confirms the association between the EPHA3 gene variations and NSCL/P among the western Han Chinese population.

In this study, we performed a targeted deep sequencing based on the haplotype (hg19: chr3:89,412,794-89,552,876) around rs7632-427 (EPHA3) to elucidate the relationship between the EPHA3 gene and the occurrence of NSCL/P in the western Han Chinese population (**Figure 1**).

Materials and methods

Samples

In this study, 159 unrelated NSCL/P patients (including 79 cases of NSCLO and 80 cases of NSCLP) from the western Han Chinese population (**Table 1**), including Chongqing, Sichuan, Guizhou, and Yunnan provinces etc., were collected at the Department of Cleft Lip and Palate Surgery, West China College of Stomatology, Sichuan University. All the enrolled patients were diagnosed by a professional physician team to rule out deformities in other parts of the body. To perform the case-control burden analysis and the association analysis, we used 542 normal individuals' WGS data from the Novogene internal database (<http://www.novogene.com/>) as controls. All of the subjects or their guardians read and signed the informed consent before enrolling in this study, and the experimental study program was approved by the Hospital Ethics Committee of West China Hospital of Stomatology, Sichuan University (WCHSIRB-D-2016-012R1).

EPHA3 is associated with the pathogenesis of NSCL/P

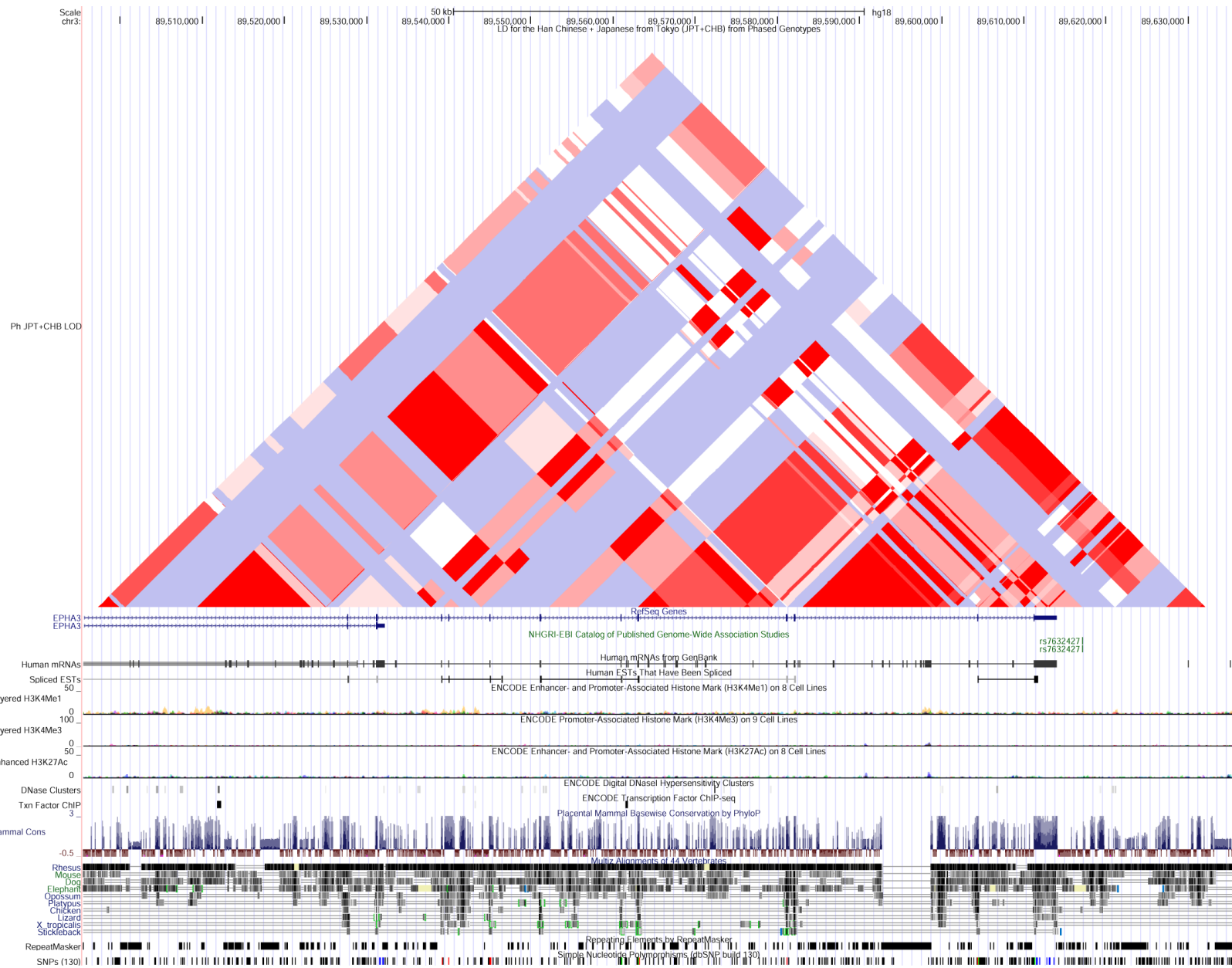


Figure 1. The targeted sequencing region of rs7632427 at EPHA3 Hg18.

EPHA3 is associated with the pathogenesis of NSCL/P

Table 1. Type of non-syndromic cleft lip with or without a palate

Cleft type	Case			Unilateral		Bilateral	Family history
	Male	Female	Unknown	Right	Left		
NSCLO	46	33	0	29	37	13	0
NSCLP	56	21	3	27	29	24	2
NSCL/P	102	54	3	56	66	37	2
Total	204	108	6	112	132	74	4

Note: NSCL/P, non-syndromic cleft lip with or without a cleft palate (NSCLO & NSCLP); NSCLP, non-syndromic cleft lip with a cleft palate; NSCLO, non-syndromic cleft lip only.

DNA extraction

Peripheral venous blood was collected from the patients, and their DNA was extracted using the salting out method. Then it was stored in a Tris-EDTA buffer in a freezer kept at -80°C . The quality of the extracted DNA was verified in the following ways: 1) run 1% agarose gel electrophoresis to confirm the DNA degradation and suspected RNA/Protein contamination; 2) use the NanoPhotometer® spectrophotometer (IMPLEN, CA, USA) (OD260/OD280) to identify the DNA purity and concentration. The OD value of a qualified sample is between 1.8 and 2.0. All the samples met the sequencing requirements.

Deep sequencing

We picked the haplotype region spanning hg19:chr3:89,412,794-89,552,876 based on the linkage imbalance (LD) structure in the CHB/JPT HapMap around rs7632427 (EPHA3) for the targeted deep sequencing (**Figure 1**). The sequence of this region was captured and enriched from the genomic DNA using the Agilent liquid capture system (Agilent Sure-SelectXT Custom Kit) according to the manufacturer's protocol, then sequenced on Illumina HiSeq 4000 for paired-end 150 bp reads.

Bioinformatics analysis

All the raw data was organized using the pipelined bioinformatics analyses procedure. The original results in the BAM format were presented using valid sequencing data according to the reference genome (GRCh37/hg19) with Burrows-Wheeler Aligner (BWA) software. And the bam files were sorted and marked with Samtools and Picard, and we generated a final bam draft. The variation calling and identification of SNP were performed using Samtools mpileup, and bcftools. The variation position,

variation type, conservative prediction and other information were recognized through various databases, such as dbSNP, 1000 Genome, HGMD, CADD, and ExAC. The gene transcript annotation databases, such as RefSeq, Consensus CDS, Ensembl and UCSC, were used for their exonic variations to determine the amino acid alternation, and the protein functional pre-

diction for the nonsynonymous SNVs was performed using PolyPhen-2, CADD, SIFT, and MutationTaster.

Statistical analysis

In this study, we performed the Hardy-Weinberg equilibrium (HWE), and the minor allele frequency (MAF) among the 542 normal controls WGS data from the Novogene internal database (<http://www.novogene.com/>) at each common variation. For the common variations (MAF ≥ 0.01) such as the HWE test and the MAF determination, association analyses were performed using PLINK. For the rare variations, we included non-synonymous variations with a database MAF threshold (MAF < 0.01 in CHB & CHS from the 1000 Genome database and the Novogene internal database, MAF < 0.001 in GnomAD) and bioinformatics predictions as harmful variations using at least two of the four software programs (SIFT, Polyphen, MutationTaster, and CADD) to do the gene based burden analysis with two-tailed Fisher's exact tests by PLINK. The significant threshold was taken as $7.86\text{E-}05$ ($P=0.05/636$ SNV) after multiple corrections.

Results

Sequencing data

Of the 159 NSCL/P patients, we identified 438 SNVs and 120 indels in all. Of these, 224 SNVs and 71 indels did not exist in the 1000 Genome database, which included one deleterious novel variation (NM_005233: c.T1634C:p.I545T). It is much conserved across several species. Sanger sequencing targeting the NM_005233: c.T1634C:p.I545T variation were performed among the NSCLP carrier and his parents, showing that the variation was inherited from his unaffected mother (**Figure 2**).

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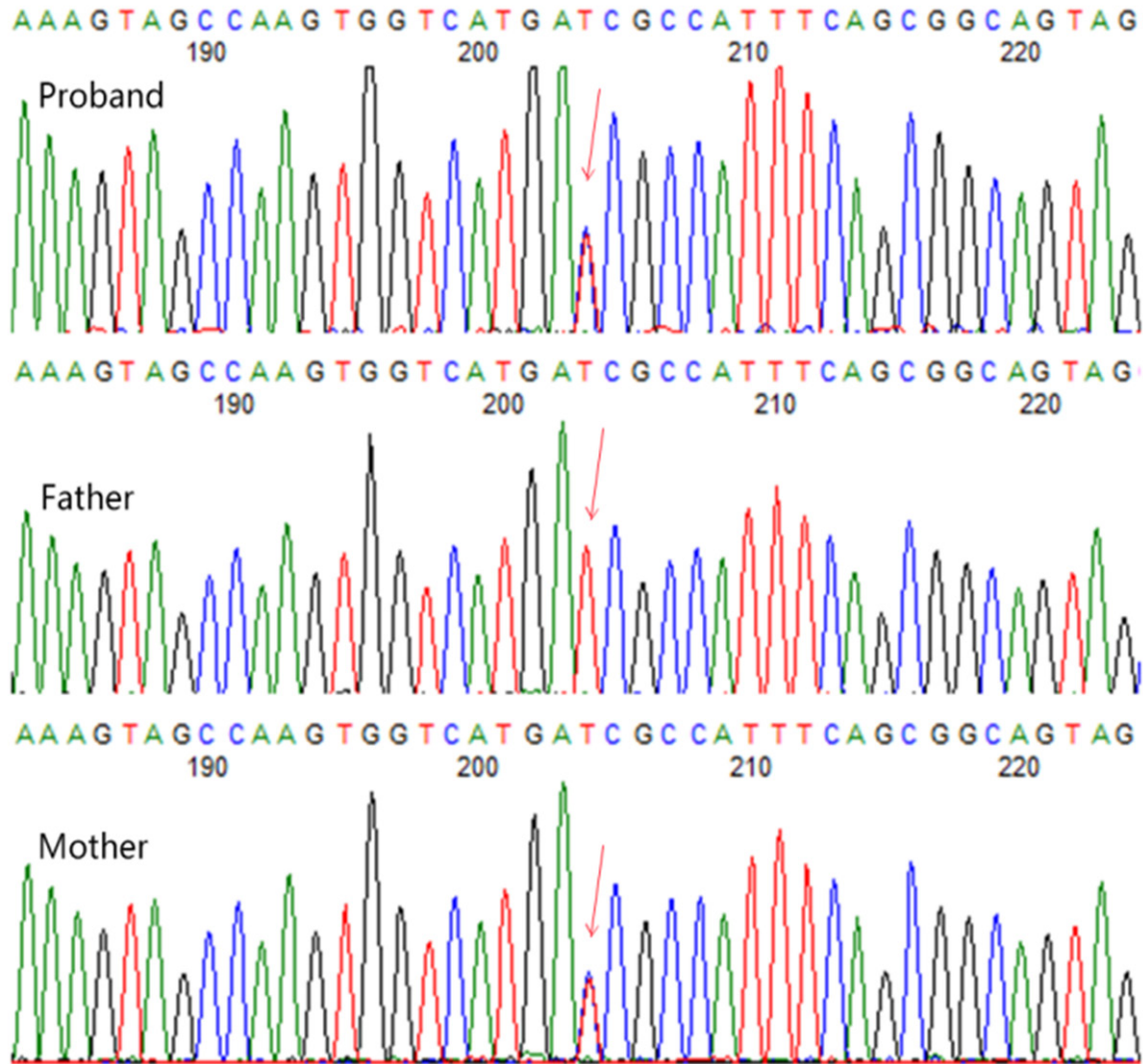


Figure 2. One variation: NM_005233: c.T1634C:p.I545T (indicated by the red arrow).

The variations with call rates > 95% were split into two groups: common variations (with minor allele frequency, MAF 0.01) and rare variations (MAF < 0.01) based on database threshold.

Gene based burden analysis

There was only one variation, NM_005233: c.T1634C:p.I545T, that conformed to the inclusion standard, and the results show no significance (data not shown).

Association analysis

To determine whether the single SNVs around the EPHA3 were associated with NSCL/P, we selected the SNVs with a call rate \geq 95% in

both the cases and the controls, and those with an HWE-p value above 0.000001 in the controls. Finally, there are 462 SNVs in NSCLO, 481 SNVs in NSCLP, and 636 SNVs in NSCL/P (NSCLO & NSCLP) that passed the threshold, and they were enrolled in the association analysis. The results showed rs7632427 is significantly associated with NSCLO ($P=7.49E-05$, $OR=0.32$) (Table 2 and Figure 3) and rs13094064 is significantly associated with NSCL/P ($P=1.86E-07$, $OR=0.17$) (Table 2) after multiple corrections.

Discussion

NSCL/P has significant genetic heterogeneity and is thought to be affected by a combination

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Table 2. The association results of the SNVs with a *p* value less than 0.005

Cleft type	SNP	Bp (hg19)	REF	ALT	AF	Case		Control		P	OR
						Alt	Ref	Alt	Ref		
NSCLO	rs7632427	89534377	T	C	6.96%	11	147	208	876	7.49E-05	0.32
	rs13094064	89418822	A	G	1.92%	3	153	111	971	0.00017	0.17
	rs11918555	89541934	C	T	7.59%	12	146	207	877	0.0002	0.35
	rs12637608	89551301	T	C	10.13%	16	142	226	858	0.0012	0.43
	rs7650184	89530057	C	A	10.76%	17	141	233	851	0.0013	0.44
	rs35124509	89521693	T	C	10.76%	17	141	231	853	0.0013	0.45
	rs12636275	89523038	C	A	10.76%	17	141	228	856	0.0018	0.45
	rs564128127	89423366	A	G	3.16%	5	153	4	1080	0.0026	8.8
	rs184256457	89462453	A	G	3.16%	5	153	4	1080	0.0026	8.8
	rs545642854	89420353	C	T	2.53%	4	154	2	1082	0.0031	14
	rs2117137	89525505	A	G	10.76%	17	141	220	854	0.0033	0.47
NSCLP	rs13094064	89418822	A	G	1.90%	3	155	111	971	0.00017	0.17
	rs1272903987	89510051	G	C	5.77%	9	147	15	1061	0.0016	4.32
NSCL/P	rs13094064	89418822	A	G	1.91%	6	308	111	971	1.86E-07	0.17
	rs112335559	89525297	A	T	2.56%	8	304	83	989	0.00064	0.31
	rs11918555	89541934	C	T	12.26%	39	279	207	877	0.0043	0.59

Note: BP, base pair position; Ref, reference allele; Alt, alternate allele; AF, allele frequency; P, *p* value; OR, odds ratio; NSCLO, non-syndromic cleft lip only; NSCLP, non-syndromic cleft lip with a cleft palate; NSCL/P, non-syndromic cleft lip with or without a cleft palate (NSCLO & NSCLP). We only listed the SNPs with *p*-values less than 0.005 in this table.

of susceptibility genes and environmental factors, especially in the first three months of pregnancy. Numerous studies have identified the susceptible loci and genes of the NSCL/P regulated important signaling pathways during the craniofacial development of the embryo, including the EPHA3 gene. The EPHA3 gene belongs to the family of receptor tyrosine kinases (RTKs), split into the A-subfamilies and the B-subfamilies [17, 18]. RTKs are involved in some biological activities, such as cancer, embryonic development, and inflammation through cellular processes [18-20]. The research suggested that the control of the EPHA3 gene expression on cell physiological activities was closely related to tumor development in some types of cancer, and could be a new target for tumor therapy [19]. Binding to the receptor tyrosine kinases (RTKs) guided two typical signaling pathways, including receptor activation in the Eph-bearing cells (forward signaling) and intracellular signaling inside the ephrin-bearing cells (reverse signaling) [15, 20]. Also, the research indicated that ephrins may locate in the downstream of TGF- β 3 (one confirmed gene associated palatogenesis) in palatal fusion, and this special reverse signaling mediated palatal fusion and epithelial-to-mesenchymal transition

(EMT) [21-23]. Significantly, the special role of the B-type ephrin in craniofacial morphogenesis has been confirmed [18]. Research has demonstrated that mutations of the EFNB1 gene (B-type Ephrin) contributed to craniofrontonasal syndrome (CFNS), an X-linked disorder, with the clinical features of cleft palate, frontonasal dysplasia, and craniosynostosis of the coronal sutures [18, 24]. Also, the EFNB1 gene located on the Xq12 and Xq13.1 chromosomes encoding for the ephrin-B1 protein [25]. Otherwise, Benson etc. [15] found that both the forward and reverse signaling of eph-B and ephrin-B were necessary for palate development, such as controlling palatal fusion, intersecting the TGF- β 3 signaling pathways, and acting on the transcriptional program in palatal epithelial mesenchymal transformation (EMT) etc. Meanwhile, the high expression patterns of the ephrin-A ligand family (EPHA3, EPHA4, and EPHA7 especially) supported a potential role in the development of the secondary palate [17]. Another study suggested that ephrin-EphA signaling regulated the development of the murine palate and tongue and many EphA genes were expressed during the process of palatogenesis except EphA6 and ephrin A3, but the EphA3 gene was strongly expressed in the lateral

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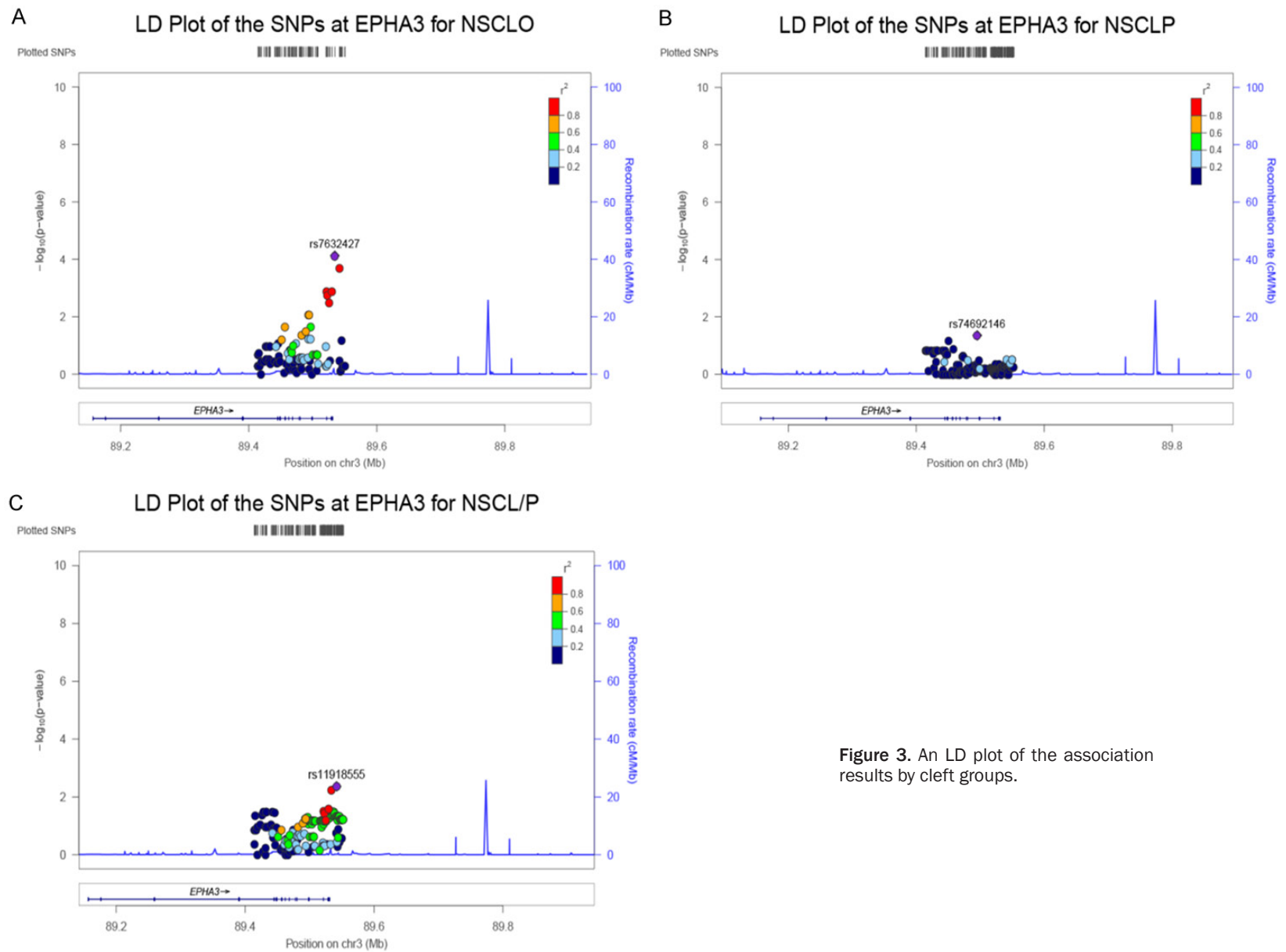


Figure 3. An LD plot of the association results by cleft groups.

tongue mesenchyme [26]. Moreover, Kousa et al. [27] indicated that the interaction of the IRF6 and RTKs signaling pathways was associated with human orofacial development and RTKs signaling components, including members of the FGF, EPHA3, and SPRY2, and might also increase a risk for isolated orofacial clefting. Based on the above studies, it is clear that the Eph and ephrin receptors are particularly important for the occurrence of NSCL/P. However, there has been no evidence to show the association between the EPHA3 gene and NSCL/P in the western Han Chinese population until now.

This study divided 159 unrelated NSCL/P patients into two groups (the NSCLO group and the NSCLP group) for a stratified analysis to explore the association (**Table 1**). The minor allele frequency (MAF) of all the significant SNPs (**Table 2**) were consistent with the CHB & CHS data from the 1000 Genome database, and the genotype frequencies were consistent with the Hardy-Weinberg equilibrium (HWE), confirming the representativeness of the data in our research. The Sanger sequencing method was also used to filter the de novo deleterious mutations among the patients and their parents. Based on the gene burden analysis, only one variation - NM_005233: c.T1634C:p.I545T - conformed to the inclusion standard. The susceptible variation in this region was inherited from an unaffected mother (**Figure 2**) and revealed that parents carrying the risky SNPs could have an increased risk of giving birth to a baby with cleft. And the function of this locus needs to be demonstrated in subsequent functional studies. Most prominently, rs7632427 of EPHA3 gene had an observably strong correlation with NSCLO in the western Han Chinese population ($P=7.49E-05$ and $OR=0.31$) (**Table 2**). However, the correlation of rs7632427 among NSCL/P ($P=0.00584$ and $OR=0.606$) was not as strong as NSCLO. We speculated that rs7632427 contributed to the NSCLO phenotype specifically. Otherwise, rs13094064 was significantly associated with NSCL/P in the western Han Chinese population ($P=1.86E-07$ and $OR=0.17$) (**Table 2**). But the association of rs13094064 was not statistically significant in the NSCLO group ($P=0.00017$ and $OR=0.17$) or in the NSCLP group ($P=0.00017$ and $OR=0.17$) (**Table 2**). So, we speculate that the rs13094064 of the EPHA3

gene contributes the most to NSCL/P. By comparing the *P* values and ORs of the NSCLO and NSCLP subtypes, we found that the susceptibility SNPs may contribute to each subtype differently due to the distinct embryological development mechanisms. Similarly, Huang etc. [28] indicated that susceptibility genes were different for the specific subtypes of NSCL/P by performing a genome-wide association study and making an interesting point that IRF6 has the opposite effect among CLO and CPO due to the gene expression dosage of IRF6. In this research, 11 genes/loci for CPO and 9 genes/loci for CLO were identified in all, such as: IRF6 associated with all three subtypes: CPO, CLO, and CLP; IRF2, NSD1, and ALX1 significantly contributed to CLO; MAFB, MYCN, and VAX1 mainly associated with CLO and CLP; PAX9, PAX4, WHSC1, FOXC2, and FOXF1 significantly contributed to CPO, etc. [28]. This view was consistent with the differences in the association between the SNPs and the different subtypes of NSCL/P in our study.

Single Nucleotide Polymorphisms (SNPs) refer to variations in a single nucleotide on a genome. The type of non-synonymous SNPs refers to that a change in the base sequence may alter the corresponding protein sequence, thus affecting the function of the protein. This change was usually the direct cause of changes in biological traits. In this study, although the SNPs rs7632427/rs13094064 are located in the intergenic/intronic region of the EPHA3 gene, these mutations might still affect the gene expression and cause a relevant dysfunction in some way. This view should be further confirmed in future studies. In conclusion, our study confirmed that EPHA3 gene variations were associated with NSCL/P, and as an important part of the etiology of NSCL/P. Moreover, current researches also confirmed the complexity of the pathogenesis of NSCL/P. The research, conducted by Fontoura et al. [29], showed a strong association between the rs1530364 of WNT9B and NSCL/P in Brazilian families, but no association for SNPs in WNT3. Cura et al. [30] suggested an important role of the SNAI1 gene in the pathogenesis of NSCL/P and also indicated that the variations in the epithelial mesenchymal transformation (EMT) in orofacial development can contribute to NSCL/P. Other studies also supplemented the evidence for revealing the susceptibility sites of

NSCL/P, including rs34246903 of MSX1, rs762642 of BMP4, rs6127973 of BMP7, and rs908822 of SPRY1, etc. [31-34].

However, there are some limitations to our study. First, the sample size was relatively small for basic genetic disease research. Second, our study only analyzed the association between the EPHA3 gene and NSCL/P, ignoring the influence of the environmental factors completely. Zhou et al. [34] found that people with a haplotype of the SPRY2 gene exposed to harmful environmental factors can have an increased risk of NSCL/P, compared to those who remain unexposed. Third, further study is still necessary to elucidate the interaction between NSCL/P and the family of receptor tyrosine kinases (RTKs), including the EPHA3 gene.

Conclusion

In aggregate, our study further confirmed that the SNPs of the EPHA3 gene can contribute to the occurrence of NSCL/P in the western Han Chinese population, making available scientific evidence for future research and genetic counseling.

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Disclosure of conflict of interest

None.

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References

[1] Massenbun BB, Jenny HE, Saluja S, Meara JG, Shrimme MG and Alonso N. Barriers to cleft lip

and palate repair around the world. *J Craniofac Surg* 2016; 27: 1741-5.

- [2] Fan D, Wu S, Liu L, Xia Q, Tian G, Wang W, Ye S, Wang L, Rao J, Yang X, Yu Z, Xin L, Li S, Duan Z, Zhang T, Wu S, Guo X and Liu Z. Prevalence of non-syndromic orofacial clefts: based on 15,094,978 Chinese perinatal infants. *Oncotarget* 2018; 9: 13981-90.
- [3] Zhao HX, Zhang MQ, Zhong WJ, Zhang JN, Huang WB, Zhang YF, Li WR, Jia PZ, Zhang TW, Liu ZH, Lin JX and Chen F. A novel IRF6 mutation causing non-syndromic cleft lip with or without cleft palate in a pedigree. *Mutagenesis* 2018; 33: 195-202.
- [4] Dixon MJ, Marazita ML, Beaty TH and Murray JC. Cleft lip and palate: understanding genetic and environmental influences. *Nat Rev Genet* 2011; 12: 167-78.
- [5] Chen R, Guo S, Wang X, Mu Y, Duan E and Xu Y. Association of EPHA3 gene polymorphisms with nonsyndromic cleft lip with or without cleft palate. *Genet Test Mol Biomarkers* 2018; 22: 420-4.
- [6] Beaty TH, Murray JC, Marazita ML, Munger RG, Ruczinski I, Hetmanski JB, Liang KY, Wu T, Murray T, Fallin MD, Redett RA, Raymond G, Schwender H, Jin SC, Cooper ME, Dunnwald M, Mansilla MA, Leslie E, Bullard S, Lidral AC, Moreno LM, Menezes R, Vieira AR, Petrin A, Wilcox AJ, Lie RT, Jabs EW, Wu-Chou YH, Chen PK, Wang H, Ye X, Huang S, Yeow V, Chong SS, Jee SH, Shi B, Christensen K, Melbye M, Doheny KF, Pugh EW, Ling H, Castilla EE, Czeizel AE, Ma L, Field LL, Brody L, Pangilinan F, Mills JL, Molloy AM, Kirke PN, Scott JM, Arcos-Burgos M and Scott AF. A genome-wide association study of cleft lip with and without cleft palate identifies risk variants near MAFB and ABCA4. *Nat Genet* 2010; 42: 525-9.
- [7] Sun Y, Huang Y, Yin A, Pan Y, Wang Y, Wang C, Du Y, Wang M, Lan F, Hu Z, Wang G, Jiang M, Ma J, Zhang X, Ma H, Ma J, Zhang W, Huang Q, Zhou Z, Ma L, Li Y, Jiang H, Xie L, Jiang Y, Shi B, Cheng J, Shen H, Wang L and Yang Y. Genome-wide association study identifies a new susceptibility locus for cleft lip with or without a cleft palate. *Nat Commun* 2015; 6: 6414.
- [8] Pan YC, Han Y, Zhang HC, Zhou L, Li DD, Cai Q, Ma JQ, Zhang WB and Wang L. Association and cumulative effects of GWAS-identified genetic variants for nonsyndromic orofacial clefts in a Chinese population. *Environ Mol Mutagen* 2013; 54: 261-7.
- [9] Ludwig KU, Mangold E, Herms S, Nowak S, Reutter H, Paul A, Becker J, Herberz R, Aichawa T, Nasser E, Bohmer AC, Mattheisen M, Alblas MA, Barth S, Kluck N, Lauster C, Braumann B, Reich RH, Hemprich A, Potzsch S, Blaumeiser B, Daratsianos N, Kreusch T, Murray JC, Marazita ML, Ruczinski I, Scott AF, Beaty TH, Kramer

EPHA3 is associated with the pathogenesis of NSCL/P

- FJ, Wienker TF, Steegers-Theunissen RP, Rubini M, Mossey PA, Hoffmann P, Lange C, Cichon S, Propping P, Knapp M and Nothen MM. Genome-wide meta-analyses of nonsyndromic cleft lip with or without cleft palate identify six new risk loci. *Nat Genet* 2012; 44: 968-71.
- [10] Jiang SY, Shi JY, Lin YS, Duan SJ, Chen XL, Jiao JJ, Shen W, Jin XJ, You M, Wang MY, Shi B and Jia ZL. NTN1 gene was risk to non-syndromic cleft lip only among Han Chinese population. *Oral Dis* 2019; 25: 535-42.
- [11] Zhang BH, Duan SJ, Shi JY, Jiang SY, Feng F, Shi B and Jia ZL. Family-based study of association between MAFB gene polymorphisms and NSCL/P among Western Han Chinese population. *Adv Clin Exp Med* 2018; 27: 1109-16.
- [12] Zhang BH, Huang N, Shi JY, Shi B and Jia ZL. Homozygote C/C at rs12543318 was risk factor for non-syndromic cleft lip only from Western Han Chinese population. *J Oral Pathol Med* 2018; 47: 620-6.
- [13] Zhang BH, Shi JY, Lin YS, Shi B and Jia ZL. VAX1 gene associated non-syndromic cleft lip with or without palate in Western Han Chinese. *Arch Oral Biol* 2018; 95: 40-3.
- [14] Klein R. Eph/ephrin signalling during development. *Development* 2012; 139: 4105-9.
- [15] Benson MD and Serrano MJ. Ephrin regulation of palate development. *Front Physiol* 2012; 3: 376.
- [16] Maunakea AK, Nagarajan RP, Bilenky M, Ballinger TJ, D'Souza C, Fouse SD, Johnson BE, Hong C, Nielsen C, Zhao Y, Turecki G, Delaney A, Varhol R, Thiessen N, Shchors K, Heine VM, Rowitch DH, Xing X, Fiore C, Schillebeeckx M, Jones SJ, Haussler D, Marra MA, Hirst M, Wang T and Costello JF. Conserved role of intragenic DNA methylation in regulating alternative promoters. *Nature* 2010; 466: 253-7.
- [17] Agrawal P, Wang M, Kim S, Lewis AE and Bush JO. Embryonic expression of EphA receptor genes in mice supports their candidacy for involvement in cleft lip and palate. *Dev Dyn* 2014; 243: 1470-6.
- [18] Bush JO and Soriano P. Ephrin-B1 forward signaling regulates craniofacial morphogenesis by controlling cell proliferation across Eph-ephrin boundaries. *Genes Dev* 2010; 24: 2068-80.
- [19] Janes PW, Slape CI, Farnsworth RH, Atapattu L, Scott AM and Vail ME. EphA3 biology and cancer. *Growth Factors* 2014; 32: 176-89.
- [20] Janes PW, Nievergall E and Lackmann M. Concepts and consequences of Eph receptor clustering. *Semin Cell Dev Biol* 2012; 23: 43-50.
- [21] Sarper SE, Kurosaka H, Inubushi T, Minagi HO, Kuremoto K, Sakai T, Taniuch I and Yamashiro T. Runx1-Stat3-Tgfb3 signaling network regulating the anterior palatal development. *Sci Rep* 2018; 8: 11208.
- [22] Nakajima A, F Shuler C, Gulka AOD and Hanai JI. TGF- β signaling and the epithelial-mesenchymal transition during palatal fusion. *Int J Mol Sci* 2018; 19: 3638.
- [23] Serrano MJ, Liu J, Svoboda KK, Nawshad A and Benson MD. Ephrin reverse signaling mediates palatal fusion and epithelial-to-mesenchymal transition independently of Tgf beta 3. *J Cell Physiol* 2015; 230: 2961-72.
- [24] Wieland I, Jakubiczka S, Muschke P, Cohen M, Thiele H, Gerlach KL, Adams RH and Wieacker P. Mutations of the ephrin-B1 gene cause craniofrontonasal syndrome. *Am J Hum Genet* 2004; 74: 1209-15.
- [25] Wieland I, Makarov R, Reardon W, Tinschert S, Goldenberg A, Thierry P and Wieacker P. Dissecting the molecular mechanisms in craniofrontonasal syndrome: differential mRNA expression of mutant EFNB1 and the cellular mosaic. *Eur J Hum Genet* 2008; 16: 184-91.
- [26] Xavier GM, Miletich I and Cobourne MT. Ephrin ligands and eph receptors show regionally restricted expression in the developing palate and tongue. *Front Physiol* 2016; 7: 60.
- [27] Kousa YA, Roushangar R, Patel N, Walter A, Marangoni P, Krumlauf R, Klein OD and Schutte BC. IRF6 and SPRY4 signaling interact in periderm development. *J Dent Res* 2017; 96: 1306-13.
- [28] Huang L, Jia Z, Shi Y, Du Q, Shi J, Wang Z, Mou Y, Wang Q, Zhang B, Wang Q, Ma S, Lin H, Duan S, Yin B, Lin Y, Wang Y, Jiang D, Hao F, Zhang L, Wang H, Jiang S, Xu H, Yang C, Li C, Li J, Shi B and Yang Z. Genetic factors define CPO and CLO subtypes of nonsyndromic orofacial cleft. *PLoS Genet* 2019; 15: e1008357.
- [29] Fontoura C, Silva RM, Granjeiro JM and Letra A. Association of WNT9B gene polymorphisms with nonsyndromic cleft lip with or without cleft palate in Brazilian nuclear families. *Cleft Palate Craniofac J* 2015; 52: 44-8.
- [30] Cura F, Palmieri A, Girardi A, Carinci F, Morselli PG, Nouri N, Pezzetti F, Scapoli L and Martinelli M. Possible effect of SNAIL family transcriptional repressor 1 polymorphisms in nonsyndromic cleft lip with or without cleft palate. *Clin Oral Invest* 2018; 22: 2535-41.
- [31] Tasanarong P, Pabalan N, Tharabenjasin P and Jarjanazi H. MSX1 gene polymorphisms and non-syndromic cleft lip with or without palate (NSCL/P): a meta-analysis. *Oral Dis* 2019; 25: 1492-501.
- [32] Hao JS, Gao RR, Wu WL, Hua L, Chen YY, Li F, Liu JY, Luo DY, Han J and Wang HT. Association between BMP4 gene polymorphisms and cleft lip with or without cleft palate in a population from South China. *Arch Oral Biol* 2018; 93: 95-9.
- [33] Yu QQ, He S, Zeng N, Ma J, Zhang BH, Shi B and Jia ZL. BMP7 gene involved in nonsyndro-

EPHA3 is associated with the pathogenesis of NSCL/P

- mic orofacial clefts in western Han Chinese. *Med Oral Patol Oral* 2015; 20: E298-E304.
- [34] Zhou R, Wang MY, Li WY, Wang SY, Zheng HC, Zhou ZB, Hu YH, Li J, Wu T, Zhu HP and Beaty TH. Haplotype and haplotype-environment interaction analysis revealed roles of SPRY2 for NSCL/P among Chinese populations. *Int J Environ Res Public Health* 2019; 16: 557.