rs2033806 at PAX3 Gene Associated with Non-syndromic Oral Cleft among the Chinese Population

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Objective: *To explore the association of PAX3 gene variants and non-syndromic orofacial cleft lip/palate (NSCL/P) among the Han Chinese population in Western China.*

Methods: *A total of 1,626 NSCL/P patients, 886 non-syndromic cleft palate only (NSCPO) patients and 2,255 controls were recruited for association analysis. A total of 8 NSCPO patients, 7 non-syndromic cleft lip only (NSCLO) patients, and 3 normal controls were recruited for RNA sequencing. Blood samples were collected, genotype data for single nucleotide polymorphisms (SNPs) on the PAX3 gene were obtained and multi-level association analyses were conducted to explore their relationship with NSCL/P, including functional predictions of disease-related SNPs. Additionally, RNA sequencing was performed to investigate differences in PAX3 gene expression between patients with NSCL/P and controls. The study was conducted in PLINK analysis.*

Results: *rs2033806 was significantly associated with NSCL/P, whereas rs1430651 was marginally associated with NSCL/P. None were significantly associated with NSCPO. In addition, rs2033806 was also associated with the subtypes NSCLP and NSCLO.*

Conclusion: *rs2033806 at PAX3 showed different frequency distributions between non-syndromic oral cleft (NSOC) patients and controls, providing new insights into the aetiologies of NSOC.*

Keywords: *non-syndromic orofacial cleft lip with cleft palate, non-syndromic cleft lip only, PAX3, singular nucleotide polymorphism*

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Non-syndromic oral cleft (NSOC) is one of the most common craniofacial congenital diseases,[1] which includes non-syndromic cleft lip with or without cleft palate (NSCL/P) and non-syndromic cleft palate only (NSCPO). NSCL/P can be further divided into two subtypes: non-syndromic cleft lip only (NSCLO) and non-syndromic cleft lip with cleft palate (NSCLP). Due to their similar developmental origin and epidemiological characteristics, they were typically grouped together for analysis.[2] In China, the overall incidence rate of NSCL/P is around 1.67‰, with 0.56‰ for NSCLO and 0.82‰ for NSCLP, and the overall incidence rate of NSCPO is around 0.26‰.[3] Besides affecting physical appearance, NSOC can impair speech and eating and pose a significant burden on families and society. Only by clarifying the causes of NSOC can effective prevention and control strategies be formulated to solve this social problem.

The aetiology of NSOC is multifactorial, involving genetic and environmental factors and their interactions. Candidate gene association studies and genome-wide association studies (GWAS) have identified a number of NSOC susceptibility genes. To date, more than

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Table 1 Samples used in the study

Phenotype		Number
NSCLO	NSCLO	1,047
	Other	138
	BCLO	98
	UCLO	811 (292 RCLO, 519 LCLO)
NSCLP		579
NSCL/P		1,626
NSCPO		886
Controls		2,255

BCLO, bilateral cleft lip only; LCLO, left cleft lip only; NSCPO, non-syndromic cleft palate only; RCLO, right cleft lip only; UCLO, unilateral cleft lip only.

60 susceptibility sites have been reported for NSCL/P[4-15] and over 10 for NSCPO.[16-19] However, these findings only explain a small part of the aetiology of NSOC, indicating that many disease-related genes remain to be discovered.

The paired box (PAX) family has been suggested to be crucial for embryogenesis.[20] Previous work on the association between *PAX7* and *PAX9* genes and NSOC in the Chinese Han population led to a focus on the *PAX3* gene, another member of the *PAX* gene family. *PAX3* encodes a transcription factor that not only contributes to cell proliferation, migration, apoptosis, differentiation and maturation during development of the neural crest, but also plays an important part in maintaining the pluripotency of stem cell populations during embryogenesis.[21] Waardenburg syndrome (WS), a genetic disorder characterised by varying degrees of deafness and pigmentation anomalies, is mostly caused by variants in the *PAX3* gene.[22-24] It has been reported that WS patients may have higher risk of cleft lip and palate,[24] suggesting that *PAX3* could be a genetic risk factor for NSCL/P.

Previous studies provided convincing evidence that the *PAX3* gene is significantly related to facial morphology. A GWAS of facial features reported by Paternoster et al[25] found an association between *PAX3* and nasion position. Furthermore, Liu et al[26] identified the role of *PAX3* in facial shape on a genome-wide scale. Indeed, some researchers have suggested the *PAX* gene families were associated with CL/P with animal experiments.[27,28] *PAX3* gene deficiency had been reported as a disease-causing gene associated with cleft palate in a case report.[29] This study identified a specific phenotype including cleft palate caused by interstitial deletion with 2q36 and 1q36.3 encompassing *PAX3* and *EPHA4* genes. In mice, persistent expression of *Pax3* in cranial neural crest cells resulted in cleft palate, ocular defects, malformation of the sphenoid bone and perinatal lethality.[30] However, it is important to highlight that there are no studies regarding the genetic association of *PAX3* genes and NSOC.

Thus, in this study, the present authors adopted a case-control design among the Han Chinese population in Western China to explore the role of *PAX3* in NSCL/P, aiming to broaden knowledge of the aetiology of NSCL/P.

Materials and methods

Samples recruited

A total of 1,626 patients with NSCL/P, 886 patients with non-syndromic cleft palate and 2,255 controls were recruited (Table 1). The genotyping data were extracted from the present authors' two previous Genome-wide association studies.[14,19] All the subjects were unrelated and self-reported as being of the Han Chinese population. Written informed consent was obtained from all study participants or their legal guardians before enrolment. The study was approved by the Ethics Committee of the State Key Laboratory of Oral Diseases, West China Hospital of Stomatology, Sichuan University (SIRBWCH-D-2022-237-R1]).We further divided NSCL/P group into several subtypes, including non-syndromic cleft lip with cleft palate (NSCLP), non-syndromic cleft lip (NSCLO), details were shown in Table 1.

The present authors gathered the lip tissues from six NSCLO patients, the palatal tissues from eight NSCPO patients, and the traumatised lip tissues from two patients who did not have any other congenital or systemic disorders. Transcriptome sequencing was applied to the evaluation of samples taken.

Genotyping

In this study, the present authors focused on the single nucleotide polymorphisms (SNPs) among *PAX3* (chr2:223,064,606-223,163,715, GRCh37/hg19). The genotype data of 27 polymorphisms from *PAX3* were obtained from our previous genome-wide association study. Briefly, genomic DNA were extracted after collecting a peripheral blood sample using the Lahiri and Nurnberger method,[31] and genotyped in the 12K Flex instrument (Thermo Fisher Scientific, Waltham, MA, USA) following the manufacturer's instructions.

RNA sequencing

RNA-Seq was performed by the Beijing Genomics Institution (BGI, Shenzhen, China).

Transcriptome library construction and sequencing

Briefly, total RNA was extracted from tissue samples, and mRNA was enriched by oligodT. Paired-end sequencing (2 × 100 bp) was carried out with a BGI-500 instrument (BGI) to obtain at least 20 million reads for each sample. The sequence data were processed and mapped to the human reference genome (hg19) using Bowtie2. Gene expression was quantified to Fragments-Per-Kilobase per Million mapped fragments (RPKM) using RNA-Seq by Expectation-Maximization (RSEM). Through a wide range of processes, DNA nanospheres were fabricated from authorised library products. Using the DNBSEQ platform, the sequencing process was finally finished.

Data analysis

At first, the present authors retrieved the PAX3 mRNA from the whole RNA-Seq results to detect PAX3 expressions in Han Chinese. According to each sample amount of gene expression by the DEGseq method to detect differentially expressed genes between cases and control groups. The analyses were performed on BGI's Dr. Tom System.

Statistical analysis

For each SNP, the minor allele frequency (MAF) was calculated and a Hardy-Weinberg equilibrium (HWE) test was performed using vLINK software.[32] The quality control of the genotyping included filtering of genotyping rate less than 95%, tri-allelic or tetra-allelic (di-allelic variants only), MAF above 0.01, and HWE P value < 0.05.

The SNPs passed the quality control were further enrolled into association analysis using PLINK.[32] Chi-square tests, odds ratios (OR) and its 95% confidence interval (95% CI) were calculated to compare the allele frequency of each SNP between cases and healthy controls. The Bonferroni threshold was set at $P < 0.00185 = 0.05/27$.

Linkage disequilibrium (LD) analysis was performed using Haploview software (http://www.broad.mit.edu/mpg/haploview/index.php) to evaluate LD patterns among SNP loci, with results presented as D' and r^2 values. Haplotype blocks were constructed accordingly. A generalised linear model-based haplotype association analysis was subsequently conducted using a sliding window approach.[33]

Functional annotation

For those statistically significant SNPs, the authors performed a comprehensive functional annotation using three websites: HaploReg v4 (https://pubs.broadinstitute.org/mammals/haploreg/haploreg_v4.php),[34] RegulomeDB (http://www.mulinlab.org/vportal)[35] and GTEx(https://www.gtexportal.org/home/).[36] Chromatin-state annotations and composite RegulomeDB scores were retrieved from RegulomeDB and used to identify variants with evidence of transcriptional regulatory activity. RegulomeDB is a database that provides functional annotations of SNPs, including data on chromatin structure, methylation, protein motif and binding. As the DB rank suggested that some SNPs may be located within regulatory elements, the authors further explored whether they affect transcription factor binding affinities using HaploReg. HaploReg was employed to explore noncoding genomic annotations for variants and identify their potential causal relationship with the pathogenesis of the disease. In addition, the GTEx database was also employed to study whether the genotypes of these SNPs affect gene expression.

Results

Association analysis identified disease-associated SNPs

A total of 262 SNPs at the *PAX3* gene were recruited. 27 of them were biallelic SNPs with MAF above 0.01 (Table S1, provided on request) and passed the HWE test (Table S2 and S3, provided on request), and then were recruited for association analysis in this study.

Compared to the multiple-corrected significance threshold 0.00185, only rs2033806 was significantly associated with NSCL/P (P = 4.98E-07, odds ratio [OR] 0.28, 95% confidence interval [CI] 0.17 to 0.46), whereas rs1430651 was marginally associated with NSCL/P (P = 7.76E-03, OR 0.59, 95% CI 0.39 to 0.87) (Fig 1a). None were significantly associated with NSCPO (Fig 1b).

Further analysis revealed subtype specific associations

When the phenotypes of NSCL/P were further subdivided, the authors noticed that rs2033806 was also associated with the two subtypes NSCLP (P = 8.21E-05, OR 0.29, 95% CI 0.15 to 0.53) (Fig 2a and c) and NSCLO (P = 8.46E-04, OR = 0.28, 95% CI 0.14 to 0.60) (Fig 2b and c). Furthermore, they analysed the association between rs2033806 and four subtypes of NSCLO. The results showed a weak association between rs2033806 and UCLO (P = 5.03E-03);

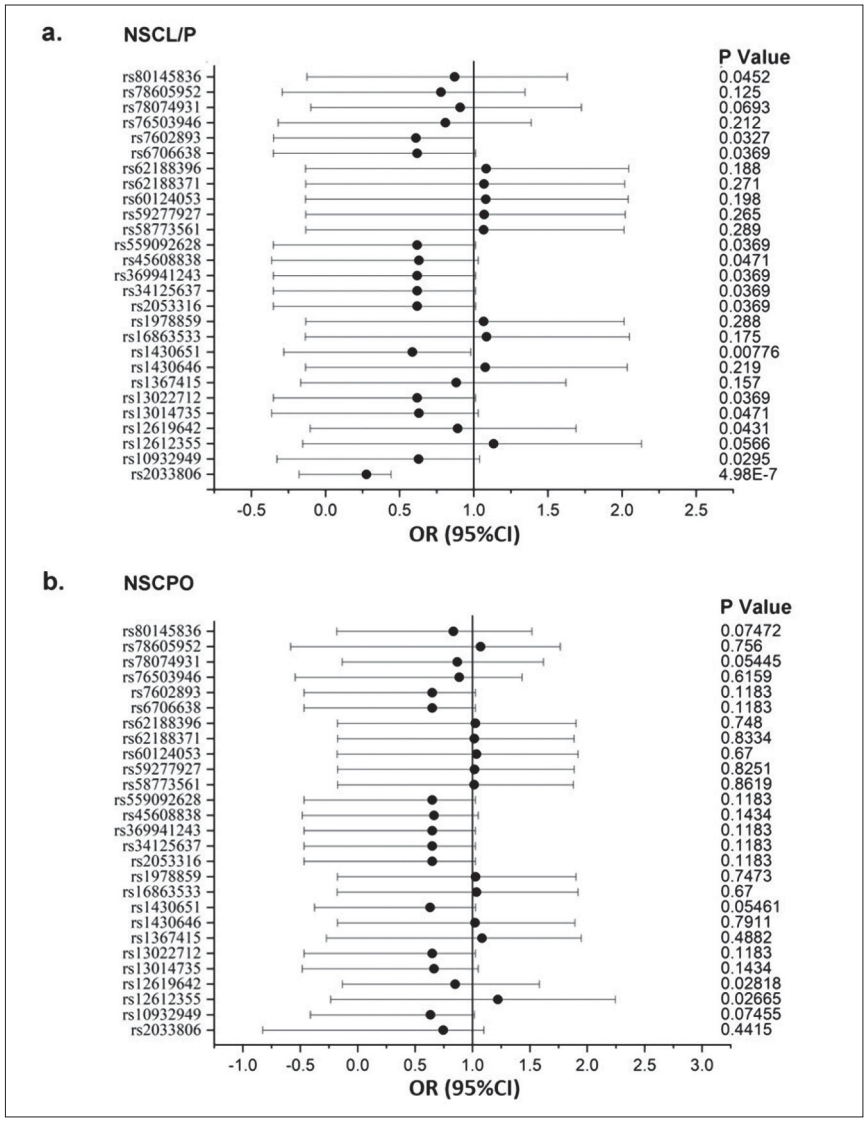

Fig 1a and b Association between 27 biallelic SNPs and NSCL/P (a) and 27 biallelic SNPs and NSCPO (b).

however, none of them reached the threshold of statistical significance (Fig 3).

Genotypic association analysis revealed that rs2033806 showed significant genotypic association across all subtypes of NSOC, including NSCL/P ($P = 1.20\times10^{-7}$), NSCLP ($P = 1.80\times10^{-4}$), NSCLO ($P = 1.50\times10^{-8}$), and NSCPO ($P = 3.20\times10^{-6}$) (Table S4, provided on request).

LD analysis

To evaluate whether the SNPs located on the same chromosome reside within the same LD block and to investigate the co-transmission of adjacent SNP loci, LD analysis was performed on these SNPs using Haploview software. The results were presented using both D' and r^2 statistics. The findings indicate that the loci rs2217466, rs35373048, rs10498134, rs16863558 and rs1438617 exhibit strong linkage across the four groups: NSCL/P, NSCLP, NSCLO and NSCPO (D' > 0.94, $r^2 > 0.88$, Fig S1, provided on request). Haplotype association analysis identifies multiple strongly associated haplotypes within the PAX3 gene region with NSOC in the Han Chinese population. The most significant associations are concentrated in haplotypes encompassing the SNP rs2033806 (Table S5, provided on request).

Functional prediction showed potential regulatory role of rs2033806

rs2033806 is an intron-variant at *PAX3*, and the present authors speculated that their influence on NSCL/P pathogenesis may be mediated through transcriptional regulation. Therefore, the Haploreg and RegulomeDB were used to detect whether the SNP was located in

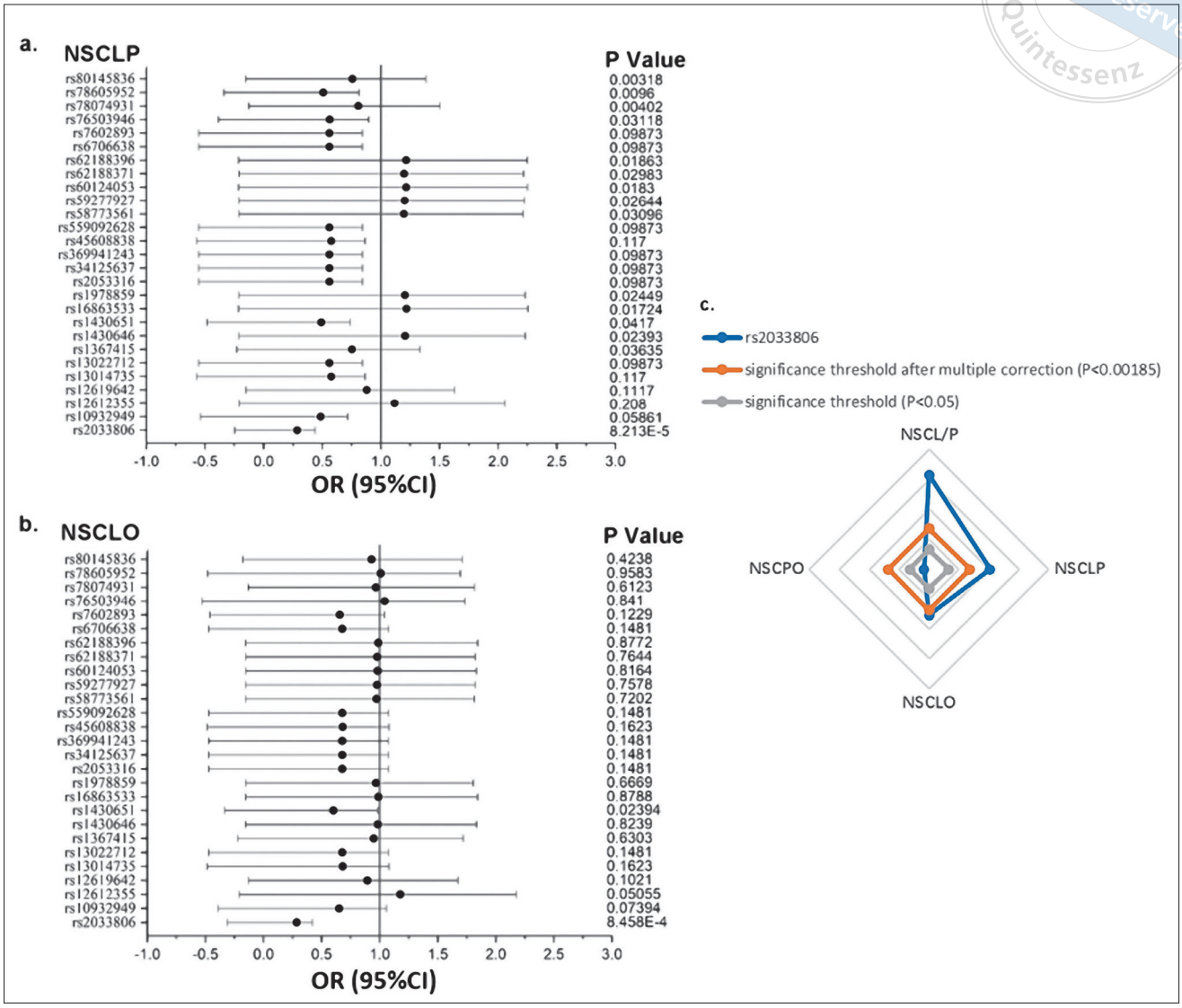

Fig 2a to c rs2033806 was also associated with the two NSCLP subtypes (a); rs2033806 was also associated with the two NSCLO subtypes (b); the association between rs2033806 and NSCL/P was the most significant (c).

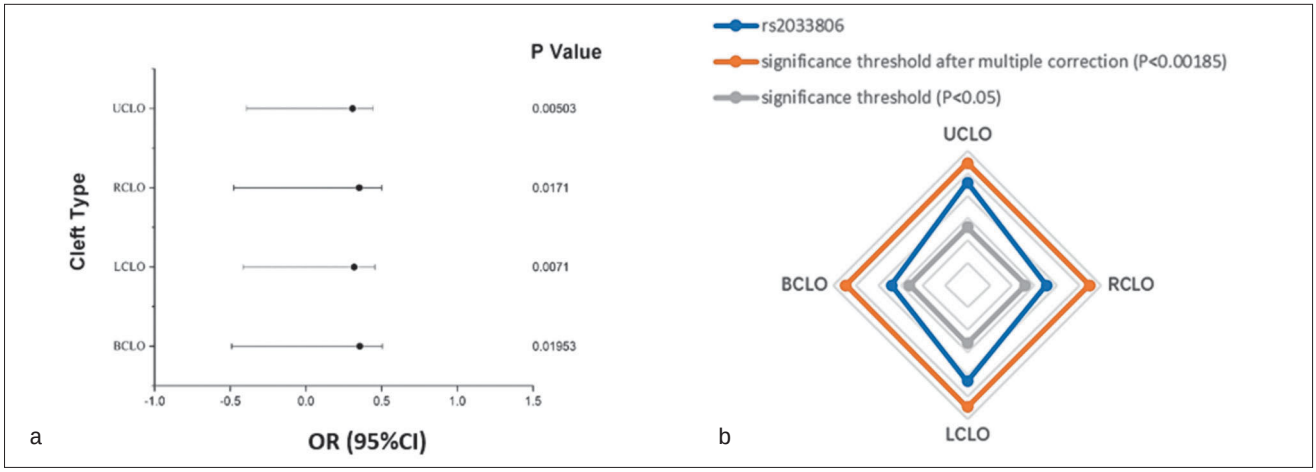

Fig 3 The association between rs2033806 and four subtypes of NSCLO.

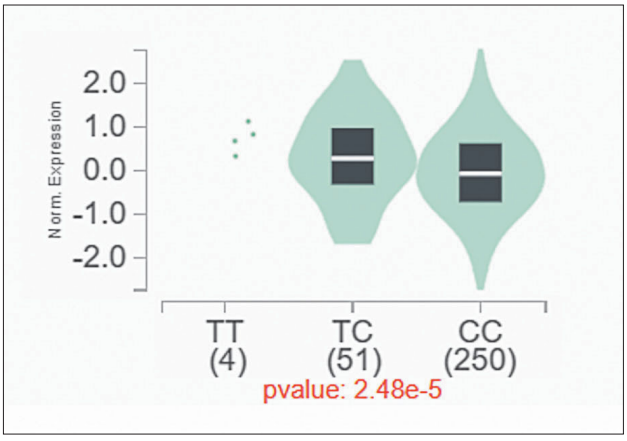

Fig 4 eQTL result from the GTEx database: the violin plot illustrates the expression levels of FARSB in skeletal muscle stratified by rs2033806 genotype using data from the GTEx v8 database. Individuals carrying the T allele (TT and TC) show higher FARSB expression compared to those with the CC genotype. The difference is statistically significant ($P = 2.48\times10^{-5}$), suggesting a strong expression quantitative trait locus effect of rs2033806 on FARSB in this tissue type.

regulation elements. The results showed that rs2033806 was not in any regulation elements. Notably, however, rs2033806 was identified to influence the expression level of FARS beta subunits (*FARSB*) in cerebellum and temporal cortex tissue using GTEx (Fig 4).

RNA-seq showed different expression level between NSOC patients and healthy control

The present authors detected the expression of *PAX3* by RNA-Seq and found *PAX3* mRNA expressed in both lip and palatal tissues, implying its essential role in craniofacial development.

In addition, the authors focused on the difference between NSOC patients and healthy controls. RNA-Seq results showed that the expression of *PAX3* was increased in NSCLO cases, compared with those in the control group ($P < 0.01$, $|\log2| > 1$, Fig 5); however, *PAX3* was not differentially expressed between the NSCPO group and control group.

Discussion

In the present study, through an open-array platform, the authors studied the association between 27 polymorphisms at *PAX3* and NSOC in the Han Chinese population. A total of 1,626 NSCL/P patients, 886 NSCPO patients and 2,255 controls were recruited, all from the Han Chinese population. The patients were divided into eight sub-phenotypes according to clinical characteristics to investigate their associations with *PAX3*. Based on such a large sample cohort, the power of locating susceptible sites can be increased, and it would be liable to find disease associated loci and reduce the occurrence of bias via sub-phenotype analysis. Furthermore, with strict statistical thresholds, reliable results had been obtained. Different frequency distributions of Rs2033806 were observed at *PAX3* when comparing NSOC patients and controls.

Limited by the sample size, research into the subtype is always insufficient. Thus, the present authors explored subtype specific associations in this study and found that rs2033806 was identified to be the most significant susceptible SNP in NSCL/P and two sub-phenotypes (NSCLP and NSCLO); however, the *P* value of rs2033806 did not reach the threshold of statistical significance in other sub-phenotypes, perhaps due to the insufficient sample size of each sub-phenotype and the presence of allelic heterogeneity at this locus in different populations. To test multiple corrections and correct for spurious associations, a stringent significance threshold was set after multiple correction ($P < 0.00185 = 0.05/27$). As a case-control design, the study is susceptible to selection bias, and the included samples may not be fully representative of the general population. Moreover, the present research was a case-control study and selection bias was objective, with the selected samples not being fully representative of the general population.

It is important to mention that the SNP associated with NSOC is functional. The functional annotations suggested that rs2033806, located in an intron of PAX3, may influence the expression of *FARSB*, thereby potentially participating in the formation of cleft lip and palate. Autosomal recessive inheritance of pathogenic variants of *FARSA* or *FARSB* can result in defective *FARS1* which are characterised by interstitial lung disease, liver disease, brain abnormalities, facial dysmorphism and growth restriction.[37] A previous study demonstrated that intronic SNPs frequently act as expression quantitative trait loci regulating gene expression in complex diseases,[38] supporting the plausibility of rs2033806 affecting *FARSB* expression. Therefore, rs2033806 may be a functional locus in facial development regulated by *FARSB*.

Nasal dysmorphology and muscle defects are the most common complications experienced by NSCL/P patients, and also a source of annoyance for surgeons even after surgical correction. Variants in *PAX3* gene affects the nasion position,[25] and it is also involved in skeletal muscle development.[39] Thus, *PAX3* has been considered as a casual factor CL/P aetiology. In

vertebrates, Pax3 plays a predominant role in somite formation as well as activating the expression of myogenic determination factor family genes that induce myogenesis in particular regions of the somites.[40] It has been elucidated that the expression of *Pax3* marked the skeletal muscle stem cell, which is important during muscle growth and later regeneration. Screening of genes regulated by *Pax3* identified that *FGFR4* is the direct target of *Pax3* in mouse embryo somites.[41] Fgf signalling was considered the key signalling pathway controlling lip and palate formation.[42] Despite the existence of indirect human genetic evidence and animal studies suggesting the association between the Pax3 gene and NSCL/P, we are the first to investigate its correlation with NSCL/P through genetic association analysis and providing novel insights into the pathogenic mechanisms of NSOC.

Although RNA-seq analysis suggested differential expression of PAX3 associated with NSCLO, the present authors acknowledge that qPCR validation of these findings was not performed due to sample limitations at the time of the study. This has now been highlighted as a limitation and the authors have planned follow-up experiments, including qPCR and protein-level assays, to further confirm the observed differences. These important findings should be considered in the near to establish the functional role of these genetic variants and their possible effects in the pathogenesis of NSOC.

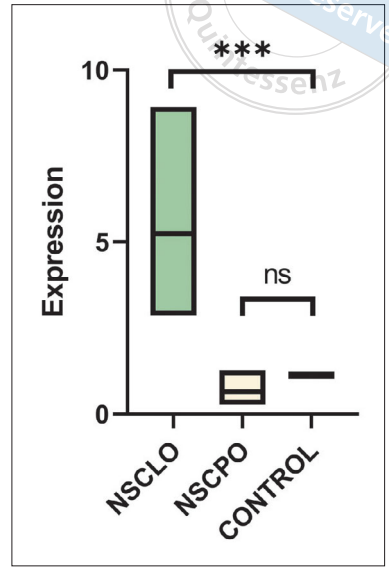

Fig 5 PAX3 mRNA expression in RNA-seq results.

Conclusion

In conclusion, this study provides the first genetic evidence linking *PAX3* to NSCL/P in the Han Chinese population. The rs2033806 variant highlights PAX3 as a susceptibility locus and underscores the need for further functional validation.

Conflicts of interest

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

Author contribution

Drs Yue YOU and Cheng Wei YANG conceived the study, performed the data analysis, collected samples and drafted the manuscript; Drs Si Di ZHANG and Si Xuan JIA carried out the statistical analysis and laboratory experiments; Drs Mu Jia LI and Shi Jun DUAN contributed to acquisition of the clinical data, quality control and bioinformatics analysis; Dr Jia Lin SUN assisted in the figure preparation and manuscript editing; Dr Bing SHI supervised the study and critically revised the manuscript; Dr Zhong Lin JIA designed and supervised the study and finally revised the manuscript.

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