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Identification of RESP18 Gene Mutations Linked to Hereditary Non-Syndromic Cleft Lip and Palate in a Southern Chinese Family

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Statistical Analysis C
Data Interpretation D
Manuscript Preparation E
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Background: Non-syndromic cleft lip with cleft palate (NSCLP) is one of the most common congenital birth defects worldwide; it causes lifelong problems and imposes burdens on patients and their families. This study aimed to describe the genomic analysis and identification of de novo regulated endocrine-specific protein 18 (RESP18) rs2385404 and rs2385405 gene polymorphisms associated with NSCLP in a southern Chinese family and to improve prevention, treatment, and prognosis of NSCLP.

Material/Methods: We performed a genome-wide association study (GWAS) to investigate the association of NSCLP phenotype with gene mutation. We investigated a 5-persons NSCLP family to screen the genetic variation of Han nationality in southern Chinese. Whole-genome sequencing (WGS) was used to detect all candidate genetic variants, and whole-exome sequencing (WES) was implemented to further verify mutations. The Clinical Variation Data Base (ClinVar) was employed for screening gene mutations. Finally, Sanger sequencing was applied to verify gene variations.

Results: The combined analysis of WGS, WES, and ClinVar showed that a total of 9 variation positions overlapped among the 3 study cohorts. Sanger sequencing verified Glu amino acid variation in 2 mutation sites (rs2385404, rs2385405) from the *RESP18* gene, which caused abnormal RESP18 function and was associated with hereditary NSCLP.

Conclusions: The combined genomic results showed that 2 mutations (rs2385404 and rs2385405) of the *RESP18* gene were related to NSCLP in the family. The *RESP18* gene may play an important role in the etiology and pathogenesis of cleft lip and palate.

Keywords: **Whole Genome Sequencing • Regulated Endocrine Secretory Protein 18 • Whole Exome Sequencing • Cleft Lip with or without Cleft Palate, Nonsyndromic, 7 • Genetic Variation**

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Introduction

Non-syndromic cleft lip with cleft palate (NSCLP) is one of the most common congenital birth defects worldwide, and occurs in approximately 1 in 1000 live births [1-3]. NSCLP can be classified into 5 types – (A) Cleft lip and alveolus, (B) Cleft palate, (C) Incomplete unilateral cleft lip and palate, (D) Complete unilateral cleft lip and palate, and (E) Complete bilateral cleft lip and palate – all of which are caused by mesenchymal tissues disorders in cell migration, growth, differentiation, and apoptosis at 4-10 weeks of human embryonic development [4]. Although it is not a main cause of death, this serious malformation has a lifelong impact on health and causes substantial financial and social burdens to patients and their families, and is challenging to treat [2,3,5,6]. Previous studies have shown that the etiology of NSCLP is heterogeneous and closely related to genetic and environmental factors [1,3,4,7]. However, a single-gene mutation may contribute more significance in a hereditary NSCLP family [8]. There have been few studies on shared related mutant genes in NSCLP families. Thus, identification of susceptible variants plays an important role in prevention, treatment, and prognosis of people with NSCLP.

Whole-genome sequencing (WGS) and whole-exome sequencing (WES) are the most extensively used and effective genomics methods in medical genetics, which can more comprehensively identify single-nucleotide variation (SNV), single-nucleotide polymorphism (SNP), insertion deletion mutation (INDEL), copy number mutation (CNV), and structural variation (SV) in the whole genome of hereditary diseases [9,10]. Whole-genome sequencing sequences all genes, detected exons, introns, and regulatory sequences, and screens the genetic variation in the genome, allowing genotype diversity analysis, genetic evolution analysis, disease pathogenesis, and susceptibility gene screening [2,9,11,12]. WGS is increasingly utilized in the research, screening, and monitoring of various diseases. Turro et al used WGS to identify the associations between genes and diseases and Wong et al discovered an actionable target for high-risk childhood cancer by using WGS [11,12]. Whole-exome sequencing (WES) is a more efficient, complete, specific, and inexpensive sequencing technology for studying the etiology of hereditary diseases [13-16]. Basha et al used whole-exome sequencing (WES) to identify variant genes in NSCLP, and found that the susceptibility genes are not only helpful in revealing the etiology of NSCLP, but also help in diagnosis and treatment of patients with NSCLP [17-19]. Many studies have explored the causes of NSCLP [18,20-22]. However, these observations suggested that different populations have different causal variants due to different genetic backgrounds., Leslie and Marazita reviewed orofacial clefts with the many error genes that contributed to the etiology of birth defects [23]. Few studies have explored genomic loci mutation related to NSCLP among Chinese people [24].

In this study, we performed genomic analysis through sequencing technique and identification of de novo RESP18 rs2385404 and rs2385405 gene polymorphisms associated with NSCLP in a southern Chinese family. Our findings provide important insights into the etiology of NSCLP.

Material and Methods

Participants

A 5-member family with hereditary NSCLP was recruited by the Cleft Lip and Palate Treatment Center of Shantou University Medical College. The adults and the parents/guardians of the children provided informed consent for genetic testing. Three plastic surgeons with attending physician qualification were invited for clinical examination and diagnosis. Complete physical examinations were performed to exclude other organ malformations, deformities, and to identify CL/P. Considering that 5 subjects from the 1 hereditary family lived in the same conditions, variables were deemed to be identical. This study has been checked by the second affiliated hospital of Shantou university medical ethics examination department (2020-24).

We obtained clinical information and peripheral blood samples from each recruited family member. Five members participated in the study (**Figure 1**, black dotted rectangle). Three members had complete cleft lip and palate and 2 were normal controls (Normal 1 and Normal 2). The parents were II3 (Cleft 1) and II4 (Cleft 2) and both had unilateral complete cleft lip and palate, while III2 (Cleft 3) was the son with bilateral complete cleft lip and palate. This study was approved by the ethics committees of the Shantou University Medical College.

Genomic Analysis

Whole-genome sequencing and whole-exome sequencing were performed by LC-Bio Co., Ltd. (Hangzhou, China, www.lc-bio.com). Genomic DNA was extracted from patients' peripheral blood. We used RNA 6000 Nano LabChip Kits (Agilent, CA, USA) to ensure the genomic DNA samples were of high quality, with OD 260/280 ratio 1.8-2.0. The DNA library constructed from human samples using the High Sensitivity DNA Chip Kit (Agilent, CA, USA) and Agilent SureSelect Human All Exon V6 (Agilent, CA, USA) was sequenced and run with the Illumina HiSeq2500/4000 sequence platform.

For the alignment step, sequenced reads were aligned to the reference genome (UCSC Genome Browser hg19) by Burrows-Wheeler Aligner (BWA, Oxford, UK), and then sorted (Samtools v1.3.1) and repeated markers (Picard v1.141) [25]. In the second post-alignment processing step, a local read realignment is performed to correct potential alignment errors around

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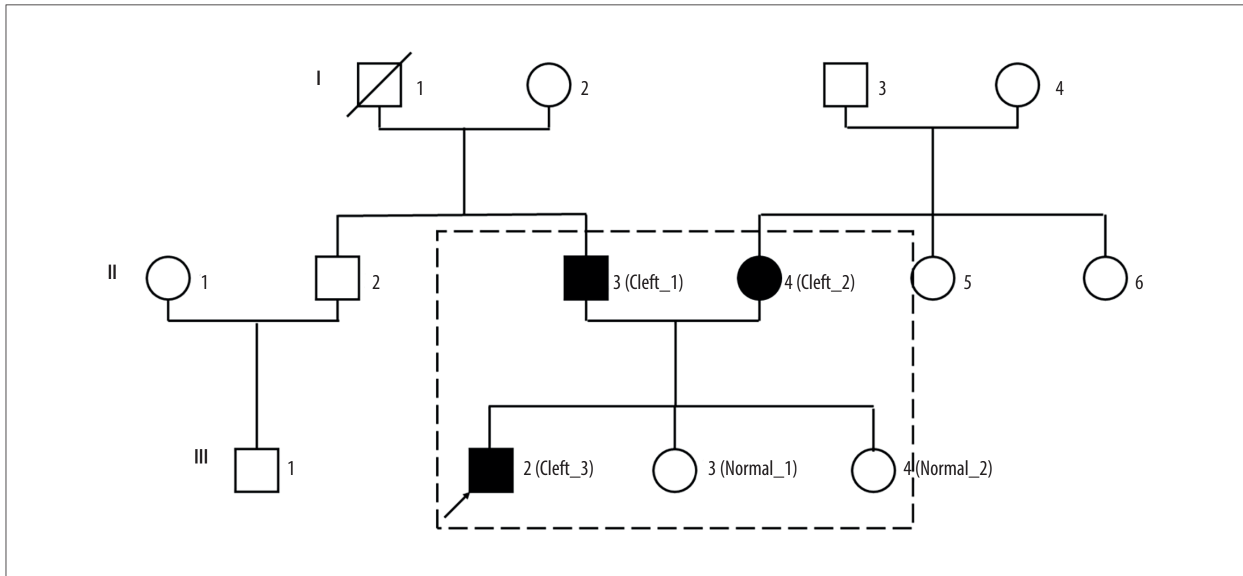

Figure 1. Pedigree of the non-syndromic cleft lip with cleft palate (NSCLP) family. II3, II4, and III2: the proband with the phenotype of NSCLP. I1, I2, I3, I4, II1, II2, II5, II6, III1, III3, and III4: members of the pedigree with no NSCLP phenotype. II3, II4, III2, III3, and III4 represent Cleft_1, Cleft_2, Cleft_3, Normal_1, and Normal_2, respectively. The black symbols represent an affected member, and the arrow indicates the proband. Five members participated in the study (Figure 1, black dotted rectangle).

indel. Mapping of reads around indel edge usually results in misaligned bases, creating false-positive SNP calls. Local realignment uses these mismatched bases to determine whether sites should be realigned, and applies a computation-intensive algorithm to determine the most consistent position of reads relative indel and to remove misalignment artifacts. More details are available from LC-Bio Co., Ltd. (Hangzhou, China, www.lc-bio.com).

Variant Calling

Each cardinality read has an associated quality score corresponding to the probability of sequencing error. Due to systematic biases, the reported quality scores are known to be inaccurate, so it must be recalibrated prior to genotyping. After recalibration, the recalibrated quality score in the output BAM will be closer to the probability of a sequencing error [25].

Variant calls can be generated with the Genome Analysis Toolkit (GATK, https://www.broadinstitute.org/gatk/guide/best-practices), which examine the evidence for variation from reference via Bayesian inference. The Gaussian mixture model is suitable for assigning an accurate confidence score to each putative mutation call and for evaluating new potential variants.

Variant Annotation

Biological functional annotation is a crucial step in finding the links between genetic variation and disease. SnpEff is utilized to add biological information to a set of variants. ANNOVAR software tool and in-house codes were used to annotate all of the mutations with Clinical Variation Data Base (https://www.ncbi.nlm.nih.gov/clinvar/). For further screening, the 1000 Genomes Project (http://www.1000genomes.org/), the Exome Aggregation Consortium (ExAC) Browser, SIFT (http://sift.jcvi.org), and PolyPhen-2 (http://genetics.bwh.harvard.edu/pph2/) were used to predict mutation function.

Sanger Sequencing

Sanger sequencing was performed by SJ-Bio Co., Ltd. (Guangzhou, China, www.saikabio.com). Premier 3.0 was used for primer design; the primer sequences are listed in **Supplementary Table 1**. Sanger sequencing products were separated using an ABI 3730 genetic analyzer, and the data were analyzed using Chromas V1.0.0.1.

Results

Clinical Characteristic

The subjects were a southern Han Chinese family with familial non-syndromic cleft lip and palate (**Figure 1**). In this family, II3, II4, and III2 were the probands with NSCLP phenotype. I1, I2, I3, I4, II1, II2, II5, II6, III1, III3, and III4 were members of the pedigree without NSCLP phenotype. In this study, 5 members were enrolled. Three had complete cleft lip and palate and 2 were normal controls. All of them were diagnosed and treated at the Cleft Lip and Palate Treatment Center of Second Affiliated

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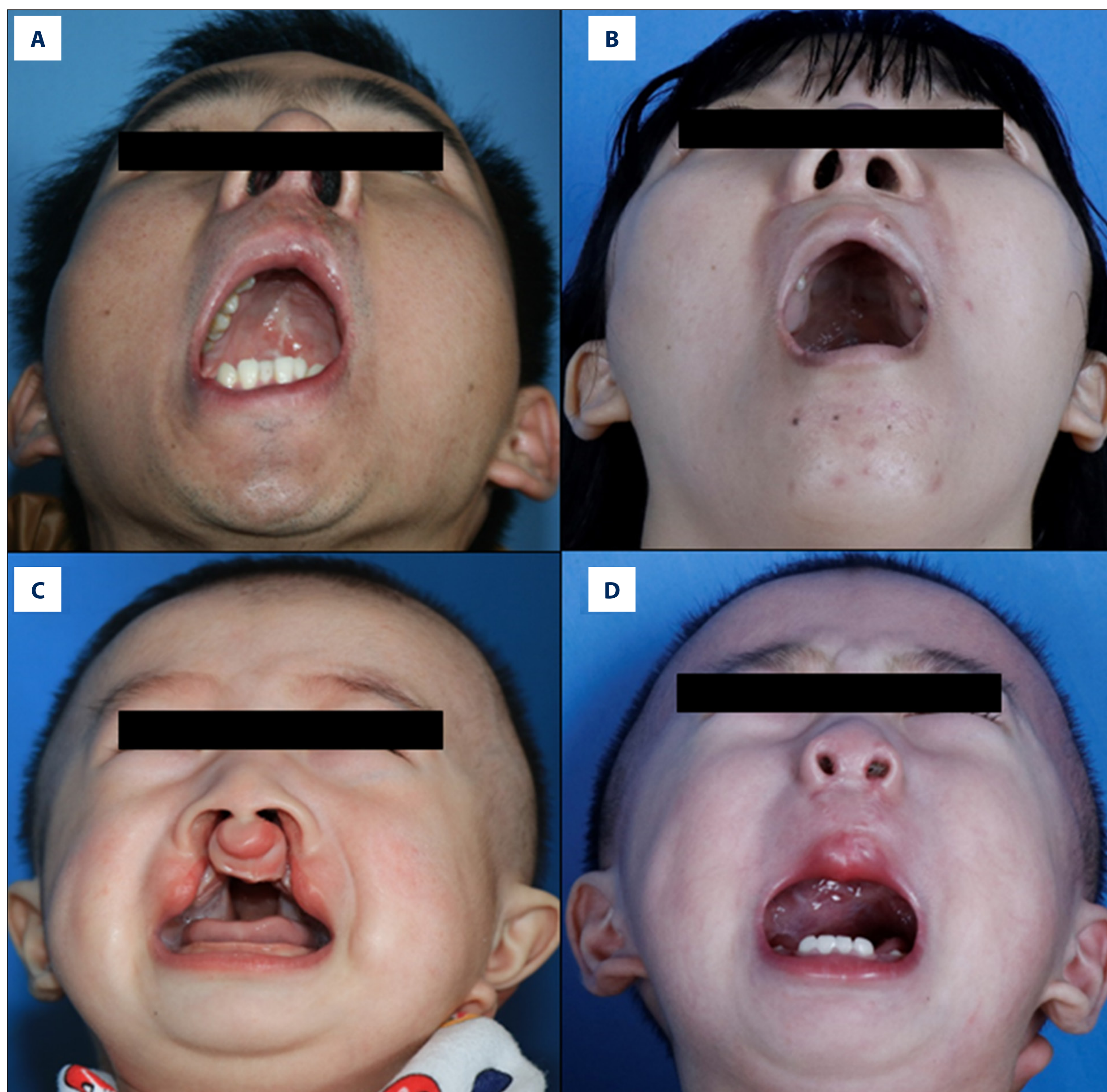

Figure 2. Clinical manifestation of the NSCLP pedigree. (**A**) (II3) and (**B**) (II4) were parents with unilateral complete cleft lip and palate after surgery, (**C**) (III2) was the son with bilateral complete cleft lip and palate before surgery. (**D**) shows the clinical features of the proband 1 year after surgery.

Hospital of Shantou University Medical College. Among these patients, II3 (**Figure 2A**) and II4 (**Figure 2B**) were the parents, with unilateral complete cleft lip and palate after surgery, III2 (**Figure 2C**) was the son, with bilateral complete cleft lip and palate before surgery. **Figure 2D** shows the clinical feature of the proband 1 year after surgery. According to these clinical features, whole-genomic sequencing and whole-exome sequencing was used to detect possible genetic lesions to explain these abnormal symptoms.

Genomic Sequencing

Whole-genome sequencing (WGS) and whole-exome sequencing (WES) were employed to evaluate 5 people of the 2 generations in the NSCLP family. WGS quality summary for the raw data is summarized in **Table 1 and Figure 3**. The Phred quality score (Q), which is the logarithm of the base error rate P, describes how reliable each sequencing base is. For example, if a base is 99% correct, the mass value is 20 (Q20), and if a base is 99.9% correct, the mass value is 30 (Q30). In the WGS result, the total effective data ranged from 92.56 billion

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base pairs (Gb) with 31.98 depth (x) to 108.5 billion base pairs (Gb) with 37.49 depth (x). On average, the ratio of valid data was 99.9%. The average ratio of Q20%, Q30%, and GC% were 97.64%, 93.70%, and 41.31%, respectively. WES quality for the raw data is summarized in **Table 2**. The average total effective data were 12.60 billion base pairs (Gb) with 217.24 depth (x). On average, the ratio of valid data was 98.2%. The average ratio of Q20%, Q30%, and GC% were 98.35%, 95.47%, and 53.44%, respectively. When measured at a minimum depth of 2×, 97.61% of the target region was covered. Likewise, when measured at 10×, 20×, and 30×, 96.04%, 92.15%, and 85.00% of the intended target was covered, respectively (**Figure 4**). Thus, the depth and coverage should be adequate to reliably detect DNA variants within the majority of the targeted regions.

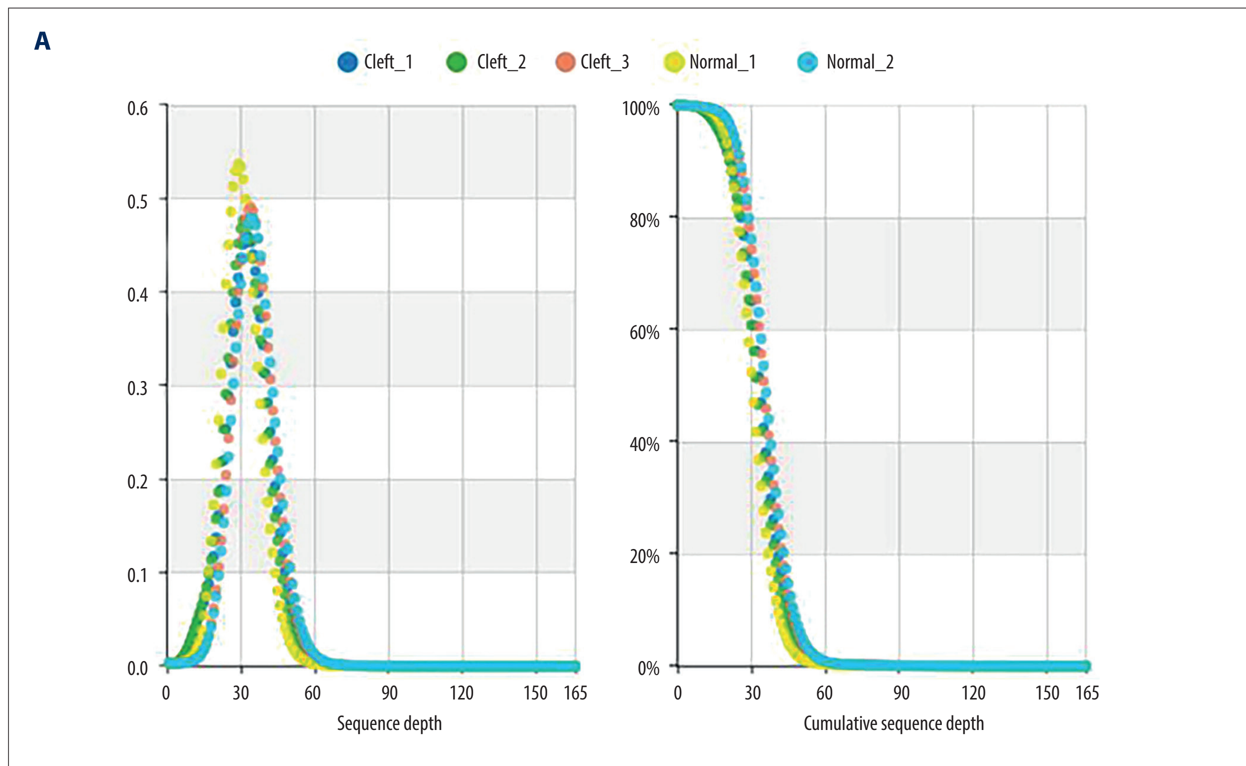
Genetic Findings

The 1000 Genomes Project, the Exome Aggregation Consortium (ExAC) Browser, SIFT, and PolyPhen-2 were used to annotate the structure mutation function. As a result, a total of 7624 mutations were screened, including 1754 mutations in the WGS cohort (23.0%) and 123 mutations in the WES cohort (1.6%). The combination analysis of WGS, WES, and Clinical Variation Data Base showed that a total of 9 variant positions (*RESP18, LSR, SPATS2L, ZNF277, IHH, C2orf69, KIR3DL1, MUC3A, and PRKAG3*) was overlap between the 3 study cohorts (**Figure 5**). Among these variant positions, 2 mutation sites (rs2385404 and rs2385405) related to cleft lip and palate in the *RESP18* gene were found, and the homozygous mutant was companied with more severe symptoms in NSCLP patients, which were

Table 1. WGS quality summary for the raw data.

Sample name	Raw reads	Raw (G)	DataRaw depth (x)	Effective (%)	Error (%)	Q20 %	Q30 %	GC %
Cleft_1	339126746	101.74	35.15	99.93	0.03	97.53	93.47	41.41
Cleft_2	327225957	98.17	33.92	99.9	0.03	97.53	93.43	41.23
Cleft_3	356214413	106.86	36.92	99.92	0.03	97.73	93.88	41.26
Normal_1	308542082	92.56	31.98	99.91	0.03	97.69	93.82	41.39
Normal_2	361657108	108.5	37.49	99.91	0.03	97.73	93.91	41.27

WGS – whole-genomic sequencing. II3, II4, III2, III3 and III4 represent Cleft_1, Cleft_2, Cleft_3, Normal_1 and Normal_2, respectively.



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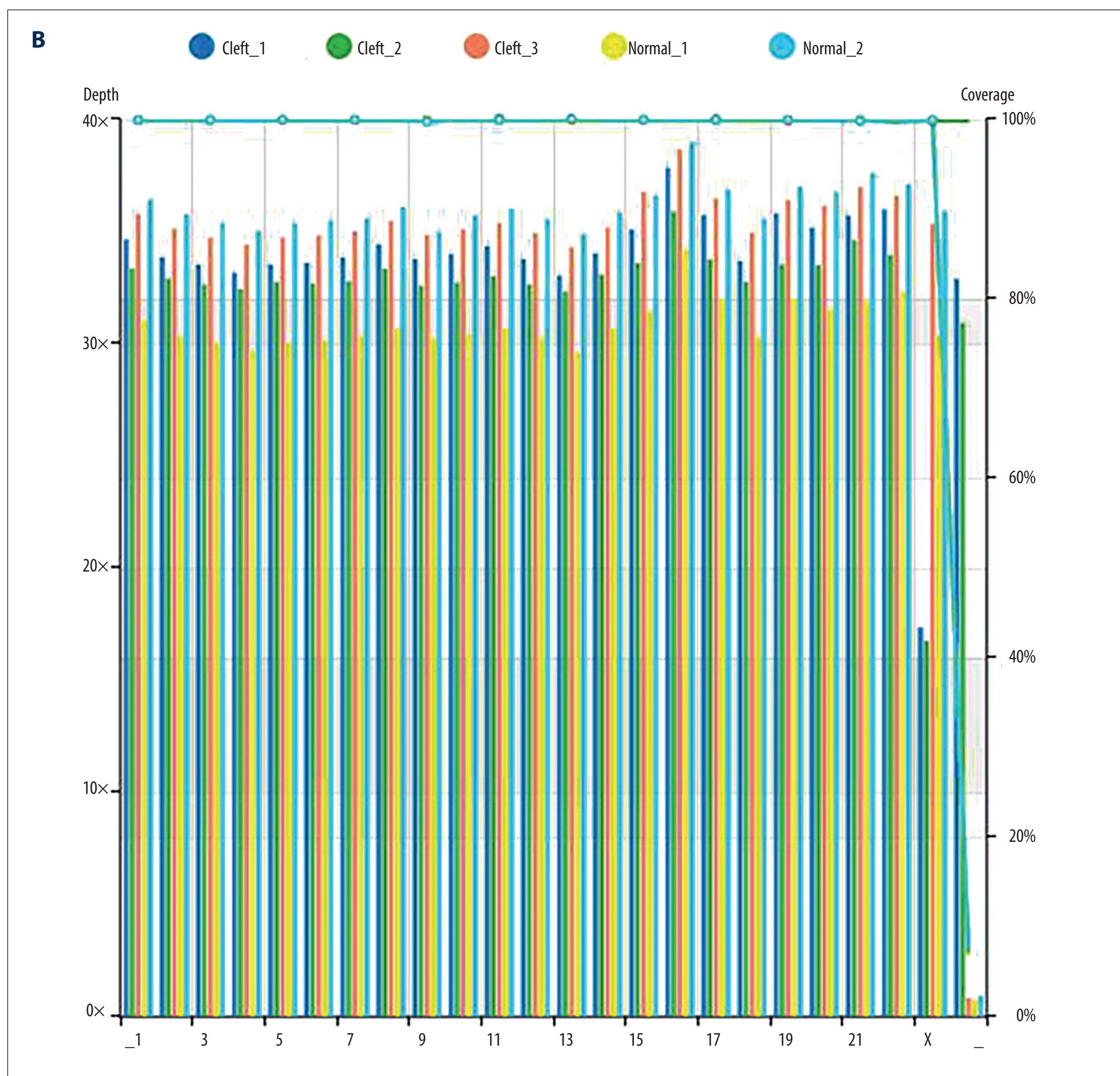

Figure 3. Depth and coverage of whole-genomic sequencing (WGS). (**A**) The sequence depth and cumulative sequence depth of WGS. (**B**) The coverage and coverage of depth of WGS. II3, II4, III2, III3, and III4 represent Cleft_1, Cleft_2, Cleft_3, Normal_1, and Normal_2, respectively.

Table 2. WES quality summary for the raw data.

Sample	Raw data		Valid data		Raw depth (x)	Valid %	Q20 %	Q30 %	GC %
	Read	Base	Read	Base					
Cleft_1	84000000	12.60G	82475296	12.37G	217.24	98.18	98.24	95.11	53.52
Cleft_2	84000000	12.60G	82560022	12.38G	217.24	98.29	98.31	95.39	53.52
Cleft_3	84000000	12.60G	82419604	12.36G	217.24	98.1	98.40	95.62	53.36
Normal_4	84000000	12.60G	82266828	12.34G	217.24	97.94	98.35	95.54	53.19
Normal_5	84000000	12.60G	82695176	12.40G	217.24	98.45	98.45	95.69	53.63

WES – whole-exome sequencing. II3, II4, III2, III3, and III4 represent Cleft_1, Cleft_2, Cleft_3, Normal_1, and Normal_2, respectively.

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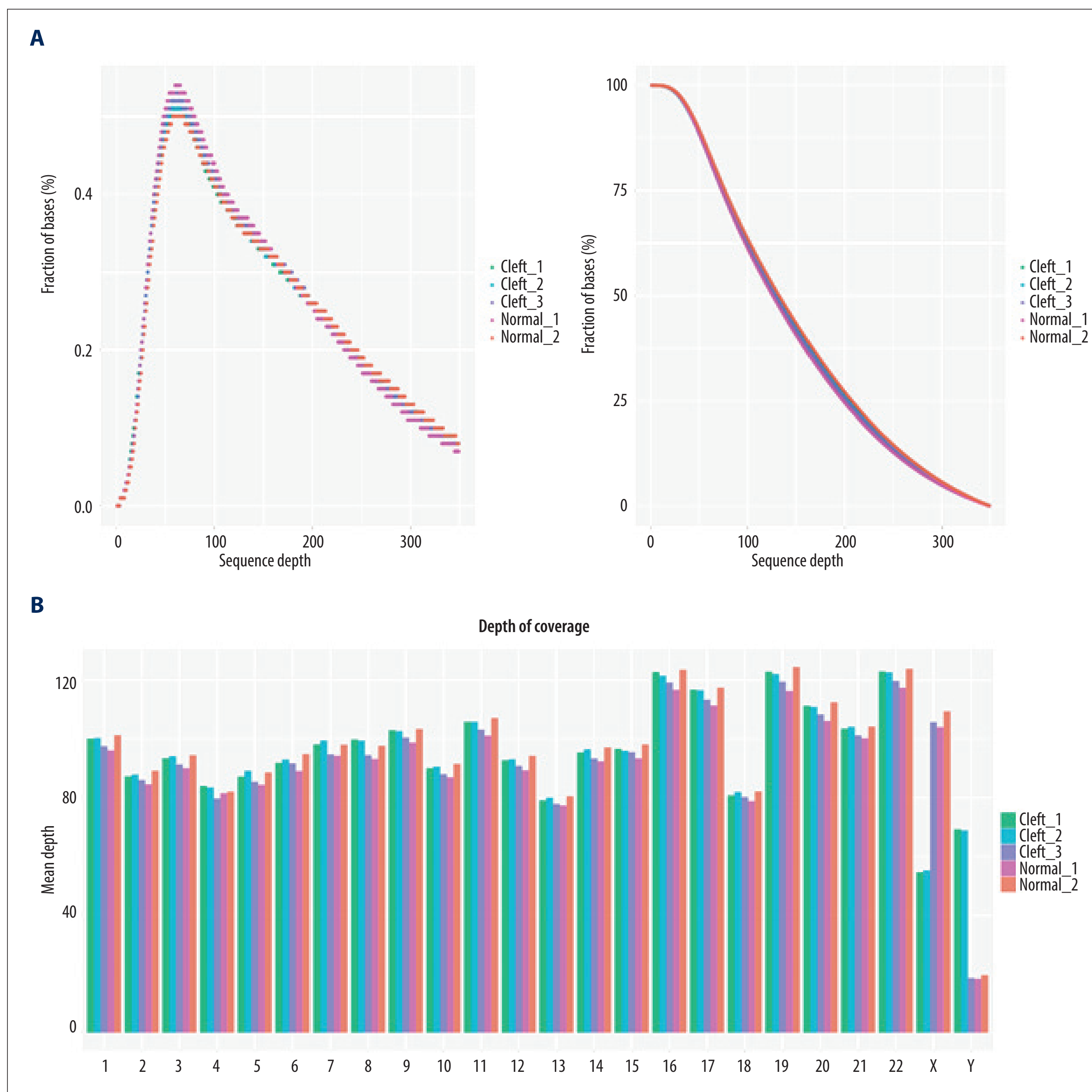
non-synonymous single-nucleotide variations (SNVs) and resulted in functional changes. Eight mutations were excluded because no similar missense variants were found; for example, some are synonymous variants or are less related to lateral or bilateral compared with regulated endocrine-specific protein 18 (RESP18). To verify the DNA sequence variation detected by WES, the criterion standard method Sanger sequencing was employed.

Sanger Sequencing

Using Sanger sequencing, 2 variant positions (rs2385404 and rs2385405) of the *RESP18* gene (NM_001007089.4) were detected in all 5 family members. Sanger sequencing validation showed that both rs2385404 and rs2385405 had mutations consistent with the WGS and WES results (**Figure 6**). Sanger sequencing results indicated that the mutation of *RESP18* (rs2385404, c.90G>T, p. Glu30Asp) resulted in the transformation of Glu into Asp in the amino acid sequence of *RESP18* protein. Sanger sequencing results indicated that the *RESP18* variant (rs2385405, c.157G>A, p. Glu53Lys) resulted in the transformation of Glu into Lys in the amino acid sequence of the *RESP18* protein. Both rs2385404 and rs2385405 were missense variants. Therefore, this result confirmed that the *RESP18* gene variant was the causal variation in this pedigree.



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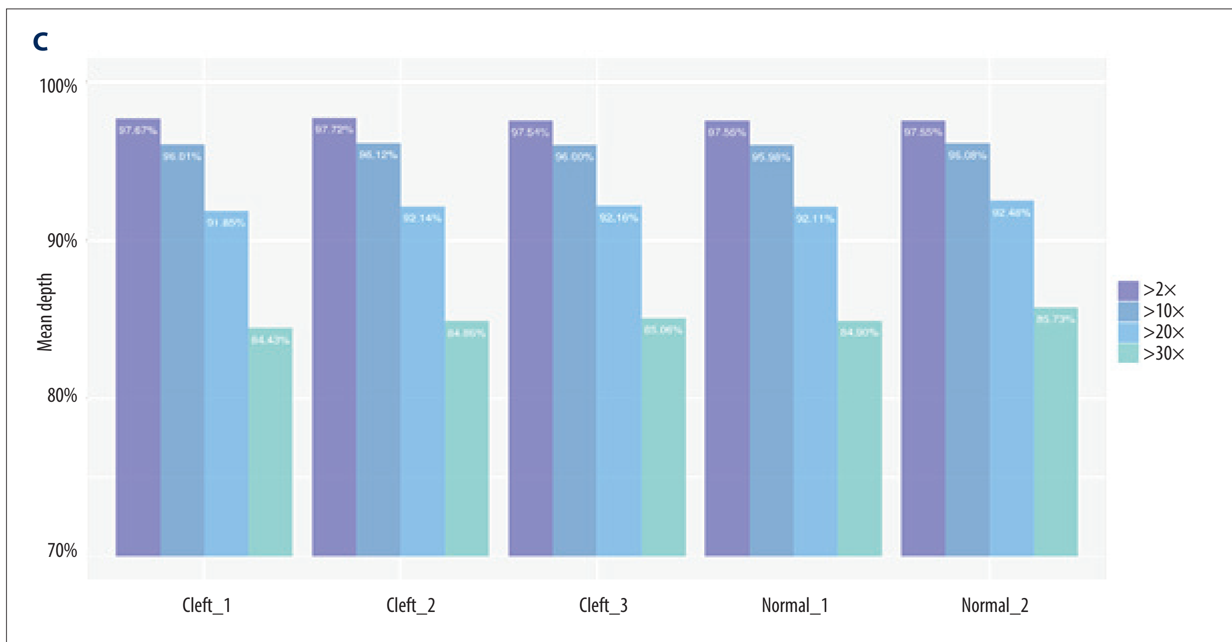

Figure 4. Depth and coverage of whole-exome sequencing (WES). (**A**) The sequence depth and cumulative sequence depth of WES. (**B**) The depth of coverage in WES. (**C**) The sequence coverage when depth at 2×, 10×, 20×, and 30×. II3, II4, III2, III3 and III4 represent Cleft_1, Cleft_2, Cleft_3, Normal_1, and Normal_2, respectively.

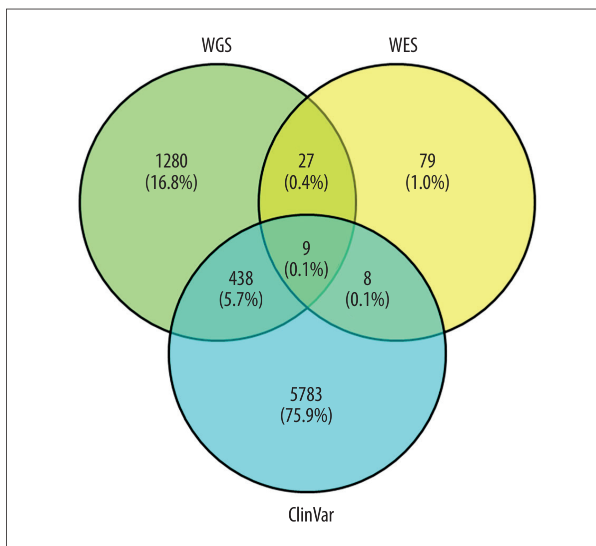

Figure 5. Venn diagram of variant position between WGS cohort (filled in green), WES cohort (filled in yellow) and ClinVar cohort. A total of 7624 mutations were screened, including 1754 mutations in the WGS cohort (23.0%) and 123 mutations in the WES cohort (1.6%). Nine variant positions overlapped between the 3 study cohorts (*RESP18, LSR, SPATS2L, ZNF277, IHH, C2orf69, KIR3DL1, MUC3A, PRKAG3*), and 2 of them were rs2385405 and rs2385404 in *RESP18* gene. WGS, whole-gonome sequencing. WES – whole-exome sequencing. ChinVar cohort – Clinical Variation Data Base. NCBI showed that these 2 mutation sites could affect the synthesis and function of *RESP18* protein. NCBI, National Center for Biotechnology Information.

Discussion

Non-syndromic cleft lip with cleft palate (NSCLP) is one of the most common congenital malformations in China, almost 20% of which are familial cases [21,26]. NSCLP is associated with various factors, and the pathogenesis of NSCLP is not completely clear. It is believed that susceptibility genes are closely related to NSCLP caused by abnormal palatal morphogenesis and tissue fusion during embryonic development [24,27-29]. A variety of approaches have been used to identify key genes contributing to NSCLP through linkage analysis, genomic rearrangements, candidate genes, and genome-wide association studies (GWAS) [23]. Researchers found several variants on *SATB2* and *IRF6* genes, which are closely related to the causes of NSCLP [17,30,31]. Yu et al performed GWAS on NSCLP and identified 41 SNPs of achieve genome-wide significance within 26 loci on 14 genes (*RAD54B, TMEM19, KRT18, WNT9B, GSC/DICER1, PTCH1, RPS26, OFCC1/TFAP2A, TAF1B, FGF10, MSX1, LINC00640, FGFR1, and SPRY1*) [2]. Awotoye et al conducted whole-genome sequencing (WGS) analyses in Africa to identify 162 high-confidence protein-altering de novo mutations (DNMs) that were predicted to be pathogenic [22]. The study of Hoebel et al processed whole-exome sequencing (WES) in individuals from independent families and suggested rare variants of 5 candidate genes (*ACACB, PTPRS, MIB1, GRHL3, CREBBP*), which might contribute to NSCLP risk [13]. Polymerase chain reaction and Sanger sequencing were used to genotype rs7078160 in *Vax1*, shown by

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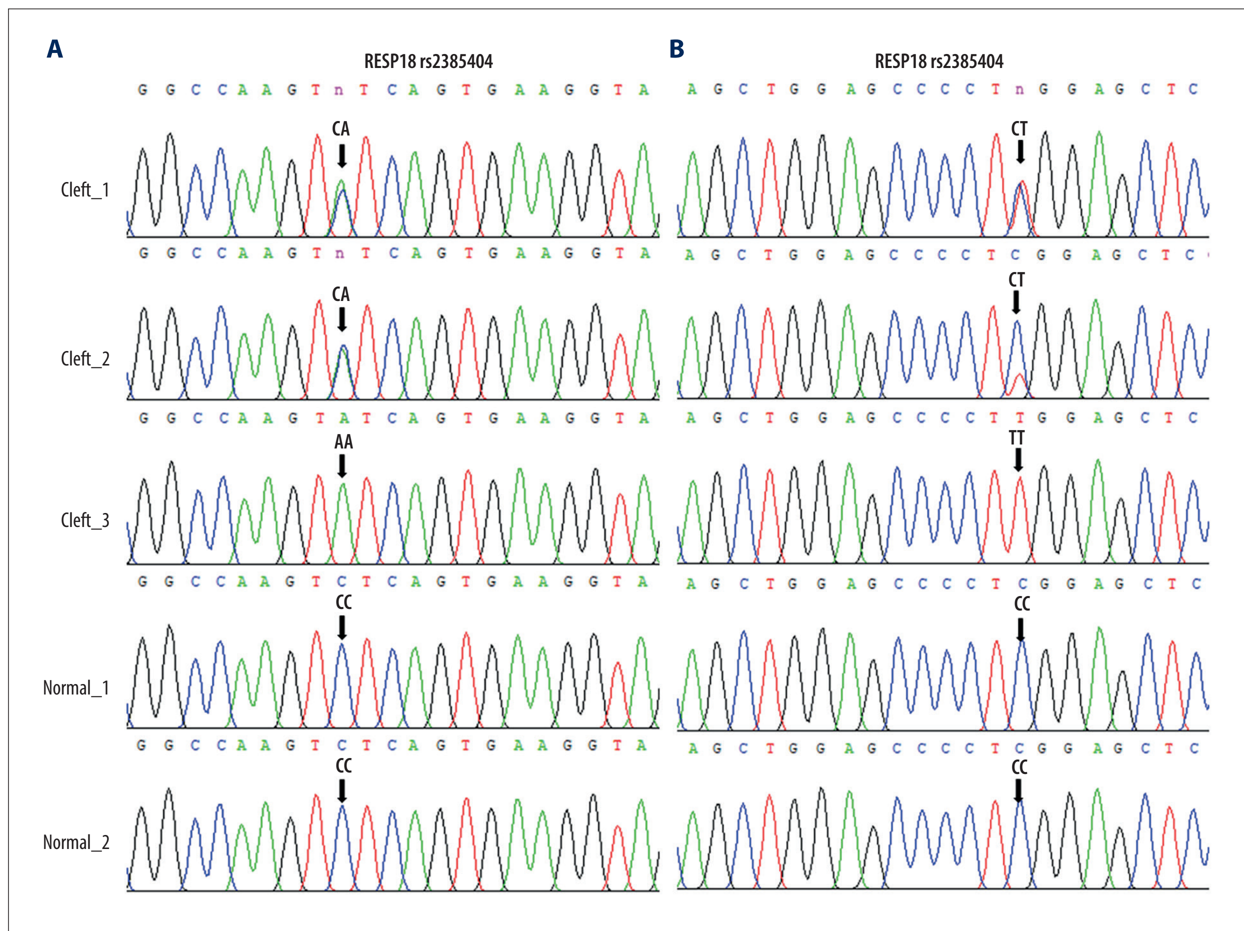

Figure 6. Sanger sequencing confirmed the variants (rs2385404 and rs2385405) in the *RESP18* gene sequence (GRCh38.p14 chr2). (**A**) Sanger sequencing showed that the mutation of *RESP18* (rs2385404, c.90G>T, p. Glu30Asp) resulted in the transformation of Glu into Asp in the amino acid sequence of *RESP18* protein. (**B**) Sanger sequencing results indicated that the *RESP18* variant (rs2385405, c.157G>A, p. Glu53Lys) resulted in the transformation of Glu into Lys in the amino acid sequence of the *RESP18* protein. Glu – glutamic acid, Asp – aspartic acid, Lys – lysine.

GWAS to be closely related to NSCLP in European populations, and compared mutation through case–control and family-based associations [32]. Therefore, NSCLP may be associated with the mutation of candidate genes.

In the present study, we used whole-genome sequencing (WGS) and whole-exome sequencing (WES) to identify susceptible variants in a south-eastern Han Chinese NSCLP family. In this family, the parents (II3 and II4) had unilateral complete cleft lip and palate, while III2 had bilateral complete cleft lip and palate. Genomic analysis showed that there are 2 mutations in *RESP18* gene in the NSCLP family. Coincidentally, patient II3 and II4 had heterozygous mutations and III2 had homozygous mutations. Our results suggest that the NSCLP phenotypic differences and severity were associated with *RESP18* gene mutations. The same result was obtained from further Sanger sequencing. As a result, we consider *RESP18* is an important gene in NSCLP development and is related to the severity of cleft palate.

Regulated endocrine-specific protein 18(*RESP18*) is an 18-kilodalton protein which mainly exists in the endoplasmic reticulum (ER) [33,34]. As an important glucocorticoid-reactive protein in the adrenocorticotropic hormone secretion pathway, *RESP18* is regulated by physiological and pharmacological stimuli such as dopamine, glucocorticoids, and insulin [35-37]. Furthermore, *RESP18* participates in regulation of the limbic system and autonomic function, regulates the growth of cytoskeleton and neurite, and then affects organ development through post-transcriptional O-glycosylation [38-41]. Therefore, *RESP18* may be an important link in development of the maxillofacial region, and its abnormal expression has a great impact on the occurrence of cleft lip and palate [42-44].

In conclusion, the incidence and condition of NSCLP are related to *RESP18* gene mutation. We speculate that the *RESP18* gene plays an important role in the pathogenesis of cleft lip and palate, and this gene may be relevant to etiology prediction for NSCLP families.

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A limitation of the present study is that we only investigated 1 NSCLP southern Han Chinese family. We need to expand the sample to verify this results in further study to eliminate sampling errors. In addition, although *RESP18* gene mutation has been found in NSCLP patients, it is still necessary to verify the function and pathogenesis of the gene in future studies.

Conclusions

WGS, WES, and Clinical Variation Data Base (ClinVar) were used to detect the gene variant in a southern Han Chinese family with non-syndromic cleft lip with cleft palate. The combined genomic results showed that 2 mutations (rs2385404 and rs2385405) of the *RESP18* gene were found in patients in this NSCLP family. Sanger sequencing validation were consistent with the former results. Moreover, we found that *RESP18* gene mutation resulting in amino acid and function changes in proteins. As a result, we speculate the *RESP18* gene plays an important role in the etiology and pathogenesis of cleft lip and palate.

Declaration of Figures' Authenticity

All figures submitted have been created by the authors, who confirm that the images are original with no duplication and have not been previously published in whole or in part.

Supplementary Table

Supplementary Table 1. Primer sequence of RESP18 mutations. RESP18, regulated endocrine-specific protein 18.

Gene	SNP	Chr	Pos	Primer sequence
RESP18	rs2385404	2q35	220197321	F: 5-ACCCCCACTTCTCAGGATCG R: 5-AAACAGATTCGGAGTCGCGG
RESP18	rs2385405	2q35	220197388	F: 5-TCTTCTCACGTCCTTAAGCA R: 5-TTCCAAGAGATGAGGGTGGG

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