

FULL LENGTH ARTICLE

A novel *FZD6* mutation revealed the cause of cleft lip and/or palate in a Chinese family

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Abstract Cleft lip and/or palate (CL/P) is a most common craniofacial birth defect which has multifactorial etiology. In our study, we aimed to discover the underlying etiological gene variation in a Chinese family diagnosed as non-syndromic CL/P (NSCL/P). The blood sample of the proband and her parents were detected by whole exome sequencing. The Mendelian inheritance pattern, allele frequency, variation location, function analysis and literature search were applied to filtrate and screen the mutation. Besides, the candidates were confirmed by Sanger sequencing. We meanwhile explored the conservative analysis and protein homology simulation. As a result, a start-lost mutation c.1A > GAtg/Gtg in the Frizzled-6 (*FZD6*) gene predicting p.Met1 was detected. The variation has not been reported before and was predicted to be harmful. The alteration caused missing of two starting amino acids that are evolutionarily conserved for *FZD6* protein. Moreover, the specific structure of the mutant protein obviously changed according to the results of the homologous model. In conclusion, the results suggest c.1A > GAtg/Gtg in the *FZD6* (NM_001164616) might be the genetic etiology for non-syndromic

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CL/P in this pedigree. Furthermore, this finding provided new etiologic information, supplementing the evidence that *FZD6* is a strong potential gene for CL/P.

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Introduction

Cleft lip and/or palate (CL/P) is a most common craniofacial birth defect. CL/P patients usually require long-term and multidisciplinary therapy to solve the abnormality of swallowing, feeding, speaking, malocclusion and mental health.^{1–3} CL/P influences about 1/700 births all over the world with a reported prevalence of 1 in 500 births in Asia, higher than other regions.^{4,5}

CL/P has a complicated etiology including genetic, environmental, geographic factors and so on.^{4,6} The infected patients could be classified as syndrome type (SCL/P) which accounts for approximately 30% and non-syndrome type (NSCL/P) accounting for 70% according to whether they also have other system defects.⁷

Genome-wide association and linkage studies (GWASs) have been applied to detect the genetic factors for CL/P. Numerous researches have been performed to detect the underlying molecular causes.^{8–10} However, GWASs identified characterization of a limited proportion of the genetic variation mainly with following limitations. The GWASs usually investigated variants with relatively higher allele frequencies.¹¹ However, candidate variants occur less frequently in healthy populations. As a result, it is complicated to identify the relationship between NSCL/P and the rare variation at a low allele frequencies.¹² Besides, GWASs could easily lead to false positive and false negative data as it relied primarily on the statistical analysis. Thus, most of the genetic factors underlying NSCL/P is unexplained.¹ Recently the next-generation sequencing technology including the whole-genome and the whole exome sequencing (WES) were applied to identify candidate mutation and further explain etiologic mechanisms of NSCL/P, complementing the spectrum of NSCL/P mutations.¹³ Many candidates of causal variants of CL/P were recently reported using WES which was more efficient, complete and specific, and capable of identifying rare or de novo variants.^{14–17} This could explain a portion of the heritability of NSCL/P.¹⁸

In our study, WES was used to examine a Chinese family diagnosed with NSCL/P. The genetic variants were filtered and screened to identify the underlying causal factors. Sanger sequencing confirmed the mutation and recessive inheritance pattern in this pedigree. Besides, species conservative analysis, variation function prediction, and protein homologous simulation were meanwhile used to analyze the variant. We aimed to identify potential genetic variants that might cause NSCL/P for this pedigree and to expand the pathogenic spectrum of NSCL/P.

Materials and methods

Human subjects

In this study, a Han Chinese family with NSCL/P was recruited at Peking University Hospital of Stomatology. We collected the peripheral blood samples and performed clinical examination for the patient and her parents. This study and related studies were ethically approved by the ethics committee of Peking University Hospital of Stomatology and the ethics approval number is PKUSSIRB-20150012. In addition, each recruited subject agreed and signed a written informed consent.

The clinical examination was performed by two maxillofacial surgeons. To confirm the absence of other organ abnormalities, the patient also underwent a complete physical examination, including eye and vision, ear form, craniofacial development, cardiovascular system, skeletal development and neuromuscular system. The oral examination consisted examination of the location and degree of cleft, morphology of palate, number of teeth, enamel hypoplasia and occlusion relationship. Besides, we collected a detailed history of the exposure of the proband's mother during her pregnancy, such as smoking, alcohol consumption, medical history, drug and supplementary intake, exposure to radiation and poisons and so on. The other family members without phenotype were checked to exclude occult submucous CL/P.

After clinical examination, approximately 4 ml peripheral blood samples were obtained from the proband and her parents. Genome DNA was then obtained using the QIAamp DNA Blood Mini kit (Qiagen, Hilden, Germany) following the instructions.

Whole exome sequencing (WES)

High-throughput sequencing of the DNA library with exon sequence enrichment was performed for each participant. Then the raw sequence data were collected through BGISEQ-500 platform (BGI, Beijing, China). Adapter sequences, undetected and low quality bases were excluded. The rest clean reads were processed using the burrows-wheeler Aligner software (Oxford, UK) through variant calls mapped to the human reference genome database (GRCh37/hg19). Mutation analysis including recalibration of base quality score and local rearrangement around indels was conducted to guarantee the accurate variant calling with the Genome Analysis Toolkit (GATK v3.3.0) following the guidelines. Duplicate reads were removed using Picard Tools. Sequencing depth, capture specificity and target

coverage were calculated basing on the alignments. Subsequently, single-nucleotide polymorphisms (SNPs) and insertions/deletions (InDels) were varified and screened using HaplotypeCaller (GATK v3.3.0). SnpEff was then used for prediction and annotation.

Candidate variation screening

After annotation was completed, the filtering process was applied to identify the underlying gene variation for the CL/P. Variants were included with the minor allele frequency (MAF) < 0.05 in the ExAC Browser and the public database of 1000 Genomes Project for East Asian population. The Mendelian inheritance pattern was applied to narrow the scope of the candidates. We also selected mutant regions, including splice receptor-site or donor-site mutations, non-synonymous mutations, insertion and deletion. Besides, the literatures were reviewed to identify the potential causal variation. Besides, in silico tools SIFT and PolyPhen-2 were used to predict underlying functional impacts of the variation.

Sanger sequencing validation

We further utilized Sanger sequencing to confirm the WES results in the family members. Primers for PCR were designed (forward: 5'-TCCATACAGCACCAAC-3'; reverse: 5'-AGCACTACTCACCTCCA-3') using Primer5 v0.4.0 and BLAST of NCBI. Chromas v1.0.0.1 was used to analyze Sanger sequencing data.

Conservation analysis

The amino acid sequence and the variant mutation position were obtained and conducted for conservative analysis using ClustalX v2.1 and the UniProt Browser.

Homology protein modelling

To get further information of impacts of the variation on the molecular structure of FZD6, an online tool swiss-model was used to build a homologous model. The sequence of FZD6 was obtained from NCBI. Template 517d.1.A was used to create the model for the FZD6 protein. And then PyMOL v0.99 was applied for visualization.

Results

Description of the pedigree phenotype

We recruited a four-generation Han Chinese family with NSCL/P. The female proband (D1) showed left cleft lip and cleft palate (CLP), and her parents (C1 and C2) had no cleft (Fig. 1). Besides, her cousin uncle, the son of grandpa's sister, exhibited the same phenotype as female proband according to the oral description from the family members but was not available for examination. To eliminate the possibility of syndromic deformities, the proband underwent a general physical examination including craniofacial development, eyes and vision, ear form and hearing, neuromuscular function and cardiovascular system. Therefore, the patient was diagnosed with non-syndromic CLP. Besides, there was no exposure for C1 to smoking, alcohol abuse, diseases, radiation or chemical teratogens during the pregnancy.

A novel *FZD6* mutation is identified as potential causal variant in the NSCL/P pedigree

In this family, WES was applied for the patient and her parents (D1, C1 and C2) to identify the underlying etiologic variant. Each subject yielded more than 23 Gb raw base reads, and an average sequencing depth was more than 200 times at the target regions. The Q20 of the clean reads for

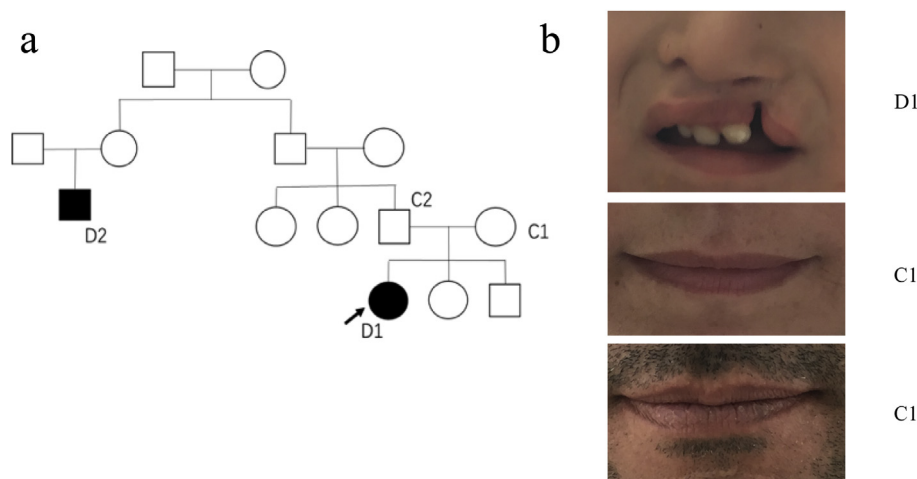


Figure 1 Pedigree and phenotype information of the Han Chinese family with NSCL/P. (a) The proband (D1) is a female patient who exhibited left cleft lip and palate. Her mother (C1) and father (C2) were unaffected as control. The patient's cousin uncle, the son of her grandpa's older sister, exhibited the same phenotype according to the description but was not available for examination. Filled symbols indicate the patients, whereas open symbols indicate unaffected members. The black arrow indicates the proband. (b) Phenotype of D1.

each subject was more than 97% and Q30 was more than 91%, indicating a high sequencing quality. In total, more than 97% of the exon regions were covered at least 20 times (Table 1a).

We respectively identified 125777, 126223 and 125519 variants, which included mononucleotides and polynucleotides, insertion and deletion variants in D1, C1 and C2. There were 74995, 74176 and 69656 heterozygous variants for in D1, C1 and C2. Besides there were 50782, 52047 and 55863 homozygous ones (Table 1b). The sequences were matched and searched to human GRCh37/hg19 reference genome. Both quantity and quality of the sequencing met the criteria for further analysis.

Following the variants annotation, the filtration and screening procedures were applied to confirm the underlying gene mutations (Fig. 2a). The variants with MAF ≥ 0.05 in the 1000 Genomes and the ExAC databases were excluded, with 25149, 24863 and 24888 variants respectively left for D1, C1 and C2. Then we mainly focused on the non-synonymous mutation (NSVs), splice acceptor-site or donor-site variants and InDels in coding DNA sequences (CDS). Thus 3147, 2938 and 3065 variants remained. Subsequently, it was considered that the family was likely to exhibit a recessive inheritance pattern, we selected homozygous variants of D1 and heterozygous variants shared by C1 and C2, resulting 32 variants at last. Then bioinformatic tools PolyPhen-2 and SIFT were used to predict the potential functional impacts, obtaining deleterious variants with the moderate and high effects. After that we examined the genes reported in literature with known roles in the pathogenesis of CL/P. Finally we identified c.1A > GAtg/Gtg in the *FZD6* (NM_001164616), predicting p.Met1, which led to a start-lost mutation that was evaluated as deleterious.

FZD6 mutation was validated by sanger sequencing

The sanger sequencing was performed to confirm the *FZD6* mutation in the proband and her unaffected parents (Fig. 2b). The patient was homozygous and her parents were heterozygous at the variation allele. And this result was consisted with the WES data and conformed to the autosomal recessive inheritance type.

Potentially etiologic impacts of the variation were analyzed by conservation analysis and protein homology modelling

Multiple-sequence alignment of *FZD6* showed the residue M was evolutionarily conserved in compared species which were showed in Fig. 2c.

The c.1A > GAtg/Gtg p.Met1 variant affected a highly conserved amino acid which is located in the extracellular region of the *FZD6* protein, indicating the variation could be deleterious. Moreover, the homology modelling of *FZD6* showed a obvious difference in the mimetic structures between wide-type (Fig. 3a) and mutant proteins (Fig. 3b). For the mutant simulation, the starting two amino acids were missing. Besides, the mutant protein presented an extra α -helix in sequence Val99-Thr102. Moreover, the structures were significantly different for the sequence from Phe105 to Asp124. The starting two amino acids might interact with the sequence from Phe105 to Asp124 in the view of electron cloud. However, the mutant *FZD6* did not show this interaction within the biological complex.

Discussion

NSCL/P is a well-known multifactorial disease including genetic and environmental etiology. In clinical examination and history collection, we collected a detailed history of the exposure for the proband's mother during pregnancy, including smoking, alcohol consumption, medical history, drug and supplementary intake, exposure to radiation, poison and chemicals. All of the above factors were excluded. Besides, Family members without phenotype were checked to exclude the possibility of occult sub-mucous CL/P. As a multigenetic disease, NSCL/P could present various genetic patterns including dominant^{13,19} and recessive inheritance models.²⁰ According to the pedigree information of the recruited family for this study, a recessive Mendelian inheritance pattern was thought to be the most suitable.

In this study, we used WES in the patient with left CLP and her unaffected parents in a Han Chinese family. Following the filtering and screening the variants, we detected a novel start-lost variation in the gene of *FZD6*:

Table 1 Summary of whole exome sequencing data and the information for identified variants.

Subject	Effective output (Gb)	Average sequencing depth	Q20%	Q30%	Genome localization (%)	Exome coverage (%)	Target acquisition specificity (%)	Target coverage $\geq 4x$ (%)	Target coverage $\geq 20x$ (%)		
a. Specific data of whole exome sequencing											
D1	25.51	256.79	98.01	92.31	99.96	99.76	59.36	99.43	97.71		
C1	23.43	206.41	97.89	91.52	99.93	99.77	51.95	99.51	97.25		
C2	23.32	206.50	97.96	91.70	99.93	99.94	52.22	99.69	97.53		
Subject	Over-all variation	Hetero-zygous	Homo-zygous	Exon	Intron	Inter-gene	Splicing	Synonymous	Missense	Stop-gain	Stop-loss
b. Summary of identified variants information											
D1	125777	74995	50782	25408	85504	3360	142	11385	10489	86	42
C1	126223	74176	52047	25048	85778	3528	144	11147	10372	86	36
C2	125519	69656	55863	24981	85257	3326	139	11159	10353	94	38

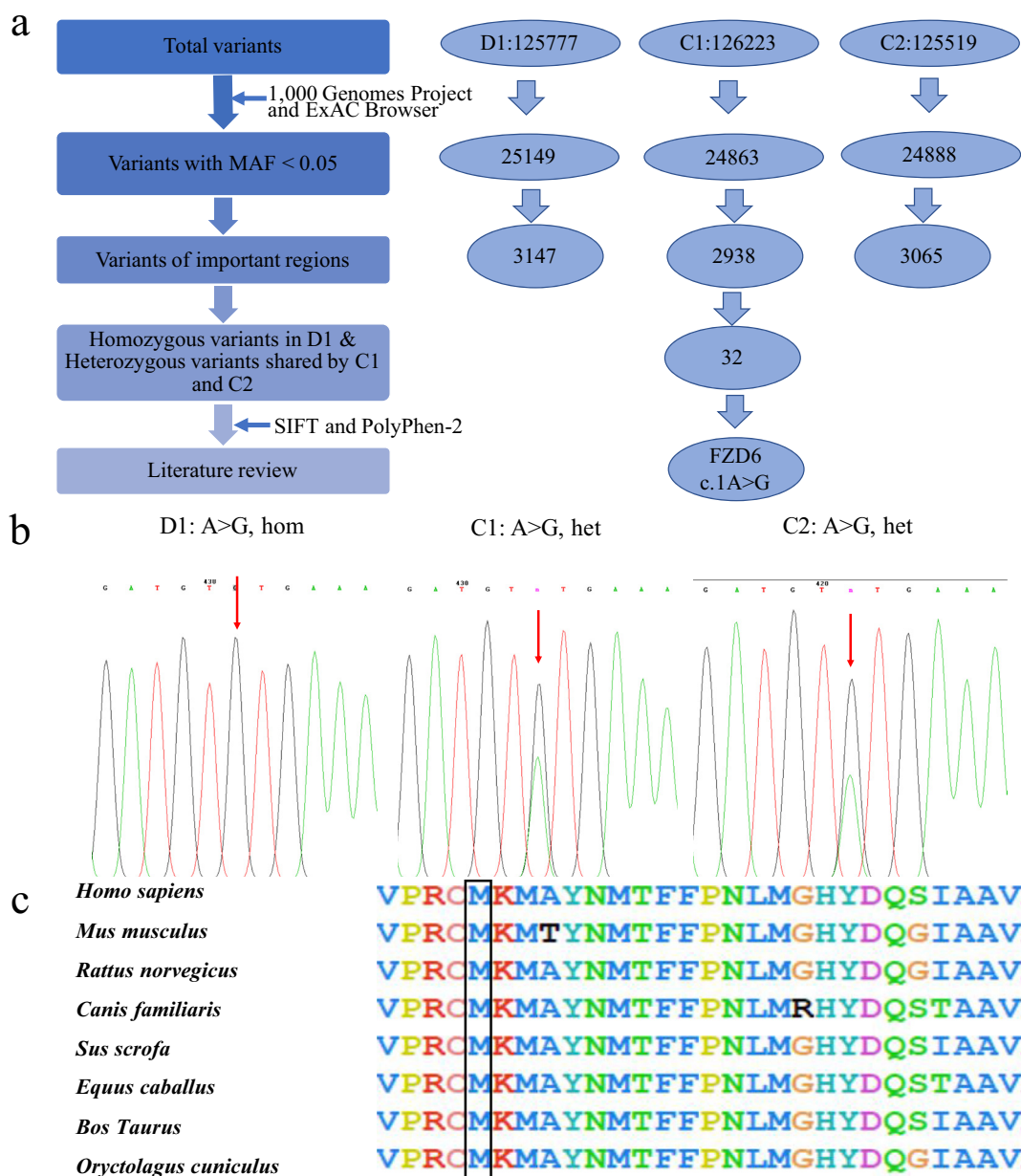


Figure 2 Bioinformatic analysis of the candidate gene mutation. (a) Causative gene mutation filtration of the NSCL/P pedigree. (b) Sanger sequencing validation of the FZD6 c.1A > GAtg/Gtg mutation. C1 and C2 carried the same heterozygous A > G mutation, while D1 was homozygous A > G. Red arrows indicate the position of FZD6 mutation. (c) Multiple sequence alignment showing evolutionary conservation of the FZD6 residue M affected by the variant. The amino acid is highly conserved among vertebrates.

c.1A > GAtg/Gtg predicting p.Met1. This variant has not been reported in current databases and is predicted to be harmful with in silico tools. Besides, sanger sequencing verified the variation and the recessive inheritance pattern of the family. Moreover, conservative analysis and homologous models suggested that the variation could change the wide-type structure and function of the protein and thus is an underlying etiologic mutation.

FZD6 is part of the Frizzled gene family, which encodes a set of receptors critical for initiating the wiggless-type (WNT) signaling pathway.^{21,22} FZD6 encodes three mRNA subtypes, which have been detected in adult and fetal tissues, respectively.^{23,24} FZD6 codes for a 706 amino-acid transmembrane protein with seven-pass.²³ Our research

identified a start-lost variation in the FZD6 coding area of the proband and predicted that this mutation might be likely harmful to the function of FZD6 protein. Besides, multiple sequence allocated the mutation to a highly conserved amino acid. This amino acid is proved to be highly conserved in the process of evolution. Furthermore, there were significant differences in the local structure of proteins between wildtype and mutant types, suggesting that the amino acid change might be detrimental and could interfere with the WNT signaling pathway.

Craniofacial dysplasia including maxillofacial clefts has been found in WNT knockout mice and zebrafish.^{25,26} An association between individual WNT genes and NSCL/P was reported in humans.^{27,28} FZD6 was reported to adjust the

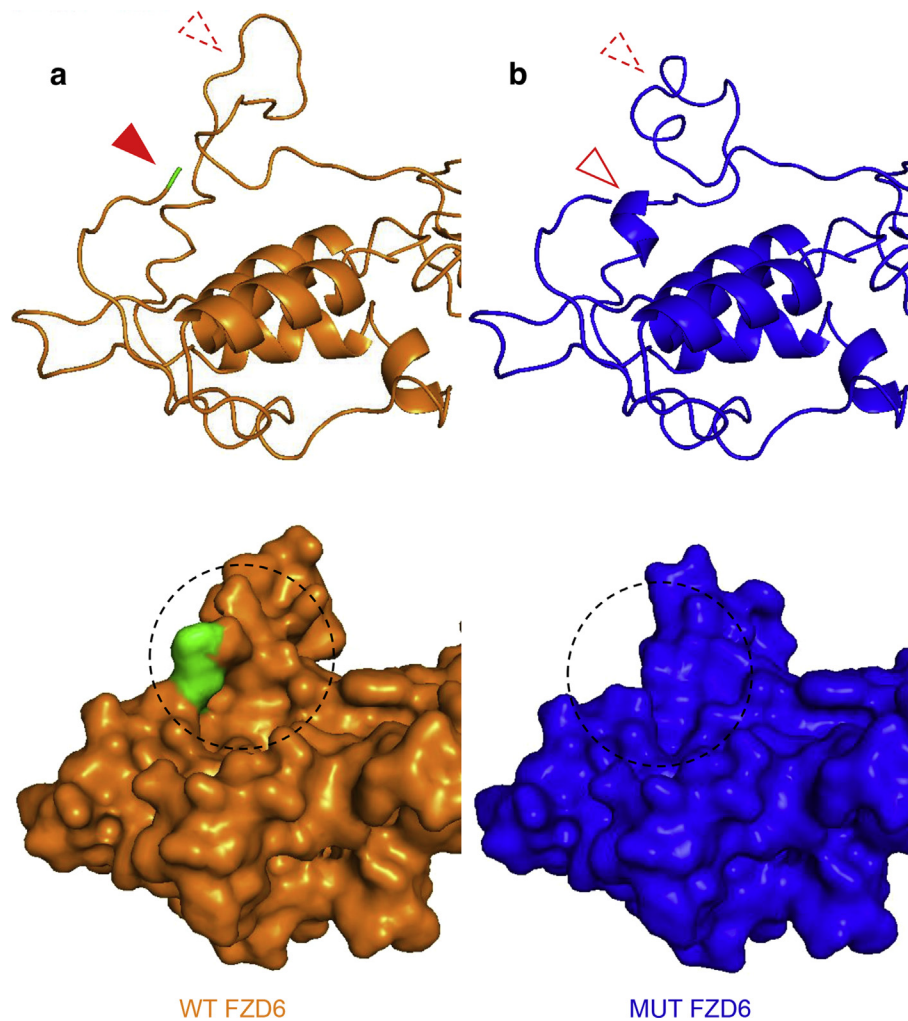


Figure 3 Homology model for the (a) WT (orange) and (b) mutant (blue) FZD6. (a). Upper panel: The short green line represented the missing two amino acids of WT FZD6, indicated with a red solid triangle. The red hollow triangle with dotted line represented the structures which were significantly different for the sequence of Phe105–Asp124. Lower panel: In the view of electron cloud, circled dotting regions showed the likely interaction between the starting two amino acids and the changed sequence of Phe105–Asp124. (b). Hollow triangle with solid line showed an extra α -helix in sequence Val99–Thr102 of mutant FZD6. The red hollow triangle with dotted line represented the changed structures.

atypical planar cell polarity (WNT/PCP) pathway.²⁹ During normal craniofacial development, NCCs migrate to build the facial prominences.³⁰ Any interference in NCC formation, differentiation, and migration could lead to craniofacial abnormalities.³¹ Thus a mutation in FZD6 expression might influence the WNT/PCP pathway and change the migration of neural cell in craniofacial structures and then potentially lead to NSCL/P.

Besides, FZD6 is widely expressed in the craniofacial mesenchyme, indicating FZD6 involves the craniofacial development for zebrafish and chick.^{32,33} In zebrafish, FZD6 is expressed in pectoral fin buds, pharyngeal arches and the head between 2 and 4 dpf in zebrafish.³⁴ FZD6 mutants presented underdeveloped Meckel's cartilage and ceratohyals and a smaller palate.³³ Moreover, some relevant studies suggested that alteration of FZD6 expression could result in abnormal craniofacial development.^{35,36} And Cvjetkovic et al reported a rare mutation in the 1st intron of FZD6 in a big NSCL/P African-American family and

demonstrated loss or excess of *fzd6* in zebrafish led to the craniofacial anomalies.³⁶

Based on the above content, the gene of *FZD6* is suggested to be a strong potentially pathogenic gene for the NSCL/P. Certain variation in *FZD6* could affect the WNT pathway and in turn result in NSCL/P.

In conclusion, this study identified a novel variation in the coding area of *FZD6* using WES for the Chinese pedigree with NSCL/P. The preliminary analysis including functional prediction, variation screening, conservative analysis and protein homology simulation indicated that the mutation c.1A > GAtg/Gtg p.Met1 potentially underlie the cleft in the family. The results further supported that *FZD6* mutation played a role in the etiology of NSCL/P.

Authorship statement

Jiuxiang Lin and Feng Chen: study design;
Zhibo Zhou: samples collection;

Huaxiang Zhao and Wenbin Huang: literature search and manuscript editing;

Jieni Zhang: experimental studies, data analysis and manuscript preparation;

Fengqi Song and Wenjie Zhong: statistical analysis;

Mengqi Zhang and Yunfan Zhang: assistance for samples collection.

Conflict of interests

The authors declare no conflict of interests.

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