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

Jieni Zhang a, Huaxiang Zhao a,c, Wenbin Huang a, Fengqi Song a, Wenjie Zhong a, Mengqi Zhang a, Yunfan Zhang a, Zhibo Zhou d,***, Jiuxiang Lin a,**, Feng Chen b,*

a Department of Orthodontics, Peking University School and Hospital of Stomatology, Beijing, China
b Central Laboratory, Peking University School and Hospital of Stomatology, Beijing, China
c Department of Orthodontics, College of Stomatology, Xi'an Jiaotong University, Xi'an, Shaanxi, China
d Department of Oral and Maxillofacial Surgery, Peking University School and Hospital of Stomatology, Beijing, China

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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>G at 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>G in the FZD6 (NM_001164616) might be the genetic etiology for non-syndromic
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.5,6 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.13 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.14 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.15-17 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 QIAGEN 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'- AGCACTACCTCACTCCA-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 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

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**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 poly-nucleotides, 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 \(\geq 0.05\) 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 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. 

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

| Subject | Effective output (Gb) | Average sequencing depth | Q20% | Q30% | Exome coverage (%) | Target acquisition specificity (%) | Target coverage \(\geq 4x\) (%) | Target coverage \(\geq 20x\) (%) |
|---------|-----------------------|--------------------------|------|------|--------------------|-------------------------------------|-------------------------------|-------------------------------|
| D1      | 25.51                 | 256.79                   | 98.01| 92.31| 99.96              | 99.76                               | 202.1                        |                              |
| C1      | 23.43                 | 206.41                   | 97.89| 91.52| 99.93              | 99.77                               | 196.2                        |                              |
| C2      | 23.32                 | 206.50                   | 97.91| 91.70| 99.93              | 99.94                               | 202.2                        |                              |

| Subject | Hetero-zygous | Homo-zygous | Exon | Intron | Inter-gene | Splicing | Synonymous | Missense | Stop-gain | Stop-loss |
|---------|---------------|-------------|------|--------|------------|----------|------------|----------|-----------|-----------|
| 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        |

**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 submucous CL/P. As a multigenetic disease, NSCL/P could present various genetic patterns including dominant and recessive inheritance models. 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:
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 wingless-type (WNT) signaling pathway.\textsuperscript{21,22} FZD6 encodes three mRNA subtypes, which have been detected in adult and fetal tissues, respectively.\textsuperscript{23,24} FZD6 codes for a 706 amino-acid transmembrane protein with seven-pass.\textsuperscript{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 alignment showed evolutionary conservation of the FZD6 residue M affected by the variant. The amino acid is highly conserved among vertebrates.

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

**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.
atypical planar cell polarity (WNT/PCP) pathway. During normal craniofacial development, NCCs migrate to build the facial prominences. Any interference in NCC formation, differentiation, and migration could lead to craniofacial abnormalities. 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. In zebrafish, FZD6 is expressed in pectoral fin buds, pharyngeal arches and the head between 2 and 4 dpf in zebrafish. FZD6 mutants presented underdeveloped Meckel’s cartilage and ceratohyals and a smaller palate. Moreover, some relevant studies suggested that alteration of FZD6 expression could result in abnormal craniofacial development. 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 fz6 in zebrafish led to the craniofacial anomalies. 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>Gttt 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; Mengzi Zhang and Yunfan Zhang: assistance for samples collection.

Conflict of interests

The authors declare no conflict of interests.

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References

1. Mossey PA, Little J, Munger RG, Dixon MJ, Shaw WC. Cleft lip and palate. Lancet. 2009;374(9703):1773–1785.
2. Worley ML, Patel KG, Kilpatrick LA. Cleft lip and palate. Clin Perinatol. 2018;45(4):661–678.
3. Cox TC, Luquetti DV, Cunningham ML. Perspectives and challenges in advancing research into craniofacial anomalies. Am J Med Genet C Semin Med Genet. 2013;163C(4):213–217.
4. Dixon MJ, Marazita ML, Beatty TH, Murray JC. Cleft lip and palate: understanding genetic and environmental influences. Nat Rev Genet. 2011;12(3):167–178.
5. Spritz RA. The genetics and epigenetics of orofacial clefts. Curr Opin Pediatr. 2001;13(6):556–560.
6. Wantia N, Rettinger G. The current understanding of cleft lip cases in Denmark: support for the multifactorial threshold model of inheritance. J Med Genet. 2010;47(3):162–168.
7. Grosen D, Chevrier C, Skythe A, et al. A cohort study of recurrence patterns among more than 54,000 relatives of orofacial cleft cases in Denmark: support for the multifactorial threshold model of inheritance. J Med Genet. 2010;47(3):162–168.
8. Haaland OA, Lie RT, Romanowska J, Gjervik M, Gjessing HK, Jugessur A. A genome-wide search for gene-environment effects in isolated cleft lip with or without cleft palate triads points to an interaction between maternal periconceptional vitamin use and variants in ESRRG. Front Genet. 2018;9:60.
9. Sun Y, Huang Y, Yin A, et al. Genome-wide association study identifies a new susceptibility locus for orofacial clefts with or without cleft palate. Nat Commun. 2015;6:6414.
10. Yu Y, Zuo X, He M, et al. Genome-wide analyses of non-syndromic cleft lip with or without palate identify 14 novel loci and genetic heterogeneity. Nat Commun. 2017;8:14364.
11. Nirnbäum S, Ludwig KU, Reutter H, et al. Key susceptibility locus for nonsyndromic cleft lip with or without cleft palate on chromosome 8q24. Nat Genet. 2009;41(4):473–477.
12. Vieira AR. Unraveling human cleft lip and palate research. J Dent Res. 2008;87(2):119–125.
13. Tian H, Feng J, Li J, et al. Intraflagellar transport 88 (IFT88) is crucial for craniofacial development in mice and is a candidate gene for human cleft lip and palate. Hum Mol Genet. 2017;26(5):860–872.
14. Zhao H, Zhang M, Zhong W, et al. A novel IRF6 mutation causing non-syndromic cleft lip with or without cleft palate in a pedigree. Mutagenesis. 2018;33(3):195–202.
15. Zhao H, Zhong W, Leng C, et al. A novel PTC1H1 mutation underlies nonsyndromic cleft lip and/or palate in a Han Chinese family. Oral Dis. 2018;24(7):1318–1325.
16. Cox LL, Cox TC, Moreno Uribe LM, et al. Mutations in the epithelial cadherin-p120-catenin complex cause mendelian non-syndromic cleft lip with or without cleft palate. Am J Hum Genet. 2018 Jun 7;102(6):1143–1157. https://doi.org/10.1016/j.ajhg.2018.04.009.
17. Bash M, Demeer B, Revencu N, et al. Whole exome sequencing identifies mutations in 10% of patients with familial nonsyndromic cleft lip and/or palate in genes mutated in well-known syndromes. J Med Genet. 2018;55(7):449–458.
18. Manolio TA, Collins FS, Cox NJ, et al. Finding the missing heritability of complex diseases. Nature. 2009;461(7265):747–753.
19. Birnbaum S, Reutter H, Lauster C, et al. Mutation screening in the IRF6-gene in patients with apparently nonsyndromic orofacial clefts and a positive family history suggestive of autosomal-dominant inheritance. Am J Med Genet A. 2008;146A(6):787–790.
20. Marazita ML, Field LL, Tuncbilek G, Cooper ME, Goldstein T, Gursu KG. Genome-scan for loci involved in cleft lip with or without cleft palate in consanguineous families from Turkey. Am J Med Genet A. 2004;126A(2):111–122.
21. Stuebner S, Faus-Kessler T, Fischer T, Wurst W, Prakash N. Fzd3 and Fzd6 deficiency results in a severe midbrain morphogenesis defect. Dev Dyn. 2010;239(1):246–260.
22. MacDonald BT, Tamai K, He X. Wnt/beta-catenin signaling: components, mechanisms, and diseases. Dev Cell. 2009;17(1):9–26.
23. Tokuhara M, Hirai M, Atomi Y, Terada M, Katao M. Molecular cloning of human Frizzled-6. Biochem Biophys Res Commun. 1998;243(2):622–627.
24. Golan T, Yaniv A, Bafico A, Liu G, Gazit A. The human Frizzled 6 (HFz6) acts as a negative regulator of the canonical Wnt-beta-catenin signaling cascade. J Biol Chem. 2004;279(15):14879–14888.
25. Heiseberg CP, Tada M, Rauch GJ, et al. Silberblick/Wnt11 mediates convergent extension movements during zebrafish gastrulation. Nature. 2000;405(682):76–81.
26. Juriloff DM, Harris MJ, McMahon AP, Carroll TJ, Lidral AC. Wnt9b is the mutated gene involved in multifactorial nonsyndromic cleft lip with or without cleft palate in A/WySn mice, as confirmed by a genetic complementation test. Birth Defects Res A Clin Mol Teratol. 2006;76(8):574–579.
27. Chiquet BT, Banton SH, Burt A, et al. Variation in WNT genes is associated with non-syndromic cleft lip with or without cleft palate. Hum Mol Genet. 2008;17(14):2212–2218.
28. Yao T, Yang L, Li PQ, et al. Association of Wnt3A gene variants with nonsyndromic cleft lip with or without cleft palate in Chinese population. Arch Oral Biol. 2011;56(1):73–78.
29. Devriendt D, Fuchs E. Planar polarization in embryonic epidermis orchestrates global asymmetric morphogenesis of hair follicles. Nat Cell Biol. 2008;10(11):1257–1268.
30. Chai Y, Maxson Jr RE. Recent advances in craniofacial morphogenesis. Dev Dyn. 2006;235(9):2353–2375.
31. Dixon J, Jones NC, Sandell LL, et al. Tcof1/Treacle is required for neural crest cell formation and proliferation deficiencies that cause craniofacial abnormalities. Proc Natl Acad Sci USA. 2006;103(36):13403–13408.
32. Geetha-Loganathan P, Nimmagadda S, Antoni L, et al. Expression of WNT signalling pathway genes during chicken craniofacial development. Dev Dyn. 2009;238(5):1150–1165.
33. Sisson BE, Topczewski J. Expression of five frizzleds during zebrafish craniofacial development. Gene Expr Patterns. 2009;9(7):520–527.
34. Duncan KM, Mukherjee K, Cornell RA, Liao EC. Zebrafish models of orofacial clefts. Dev Dyn. 2017;246(11):897–914.
35. Mukhopadhyay M, Shtrom S, Rodríguez-Esteban C, et al. Dickkopf1 is required for embryonic head induction and limb morphogenesis in the mouse. *Dev Cell*. 2001;1(3):423–434.

36. Cvjetkovic N, Malli L, Weymouth KS, et al. Regulatory variant in FZD6 gene contributes to nonsyndromic cleft lip and palate in an African-American family. *Mol Genet Genomic Med*. 2015;3(5):440–451.