SHORT COMMUNICATION

ORAL DISEASES   WILEY

A novel *VEGFA* mutation as a candidate for causing non-syndromic cleft lip and/or cleft palate

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Cleft lip with or without cleft palate (CL/P) is the most common craniofacial congenital birth defect (Mossey et al., 2009). CL/P affects about 1 in 700 newborns worldwide (Dixon et al., 2011). About 70% of CL/P cases are non-syndromic cleft lip and palate (NSCL/P). With the advent of genomics tools, it is possible to explore the etiology of NSCL/P. Genome-wide association studies (GWAS), candidate genes, and animal models (Hill et al., 2015) have provided deeper insights into the etiology of NSCL/P (Dixon et al., 2011).

VEGFA is known to be an important mediator of angiogenesis and also plays a pivotal role in growth plate development (Murata et al., 2008; Zelzer et al., 2002). Deletion of *VEGFA* in cranial neural crest cells caused cleft palate in mice and was associated with reduced proliferation of cells within the palatal shelves, abnormal palatal shelf elongation and elevation, and the inability to undergo fusion. These mice also displayed abnormal vascular development and less ossification of the maxillary and palatine bones. VEGFA participates in cell proliferation, vascular development, and ossification throughout the stages of palatogenesis (Deshpande & Goudy, 2019; Hill et al., 2015).

In our study, we recruited a two-generation Han Chinese NSCL/P family with unknown etiology (Figure 1b). The male proband (D1) had a left cleft lip and palate, and his brother (D2) and father (D3) both had left cleft lip (Figure 1a). These three patients were diagnosed with NSCL/P. The mother (C1) of the proband was not affected by CL/P. All four subjects in the family underwent detailed physical examination, including external ear morphology and hearing, eyes and vision, long bone morphology, skull bone development, neuromuscular and motor system function, cardiovascular system function, external genital system morphology, which ruled out the possibility of systemic malformations. We also recruited an independent cohort of 100 unaffected individuals with Han Chinese descent as controls.

2–4 ml peripheral blood samples were collected from each participant of the proband's family and buccal swabs from 100 unaffected individuals. Then, we extract genomic DNA and preformed Whole-exome sequencing (WES) (Meng et al., 2019; Zhao, Zhang, et al., 2018; Zhao, Zhong, et al., 2018). The results of WES show that there are an average of 159,144 variants, and the quantity and
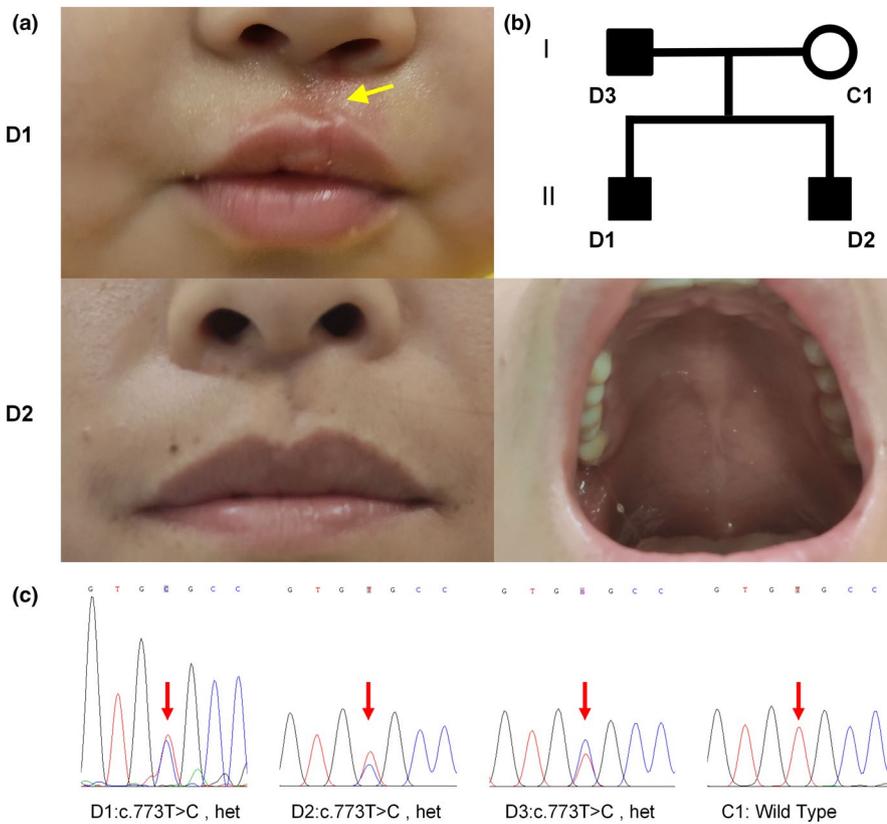

Bohui Sun and Yulin Xi contributed equally to this work.

FIGURE 1 The pedigree information, phenotypes, and Sanger sequencing of the Han Chinese NSCLP family. (a) Filled symbols indicate the patients, while blank symbols indicate the unaffected member. The proband (D1) is a male patient with left cleft lip and palate. His brother (D2) and father (D3) are also NSCLP patients with the same phenotype of left cleft lip. The mother (C1) is healthy. (b) Phenotype of D1 and D2. Unfortunately, we did not got photographs showing the palate of D1 and the lip of D2 and D3. The yellow arrow of D1 indicates the scar from left cleft lip repair. (c) Sanger sequencing validations of the potential causative mutation in four subjects in this family. The mutations in D1, D2, D3 were heterozygous, while C1 was the wild type

quality of sequencing meet the requirements of further analysis (Tables S1 and S2).

We applied a filtration process to identify the candidate variant (Figure 2a). First, we excluded variants with a minor allele frequency (MAF) ≥ 0.05 in the ExAC Browser and the 1,000 Genomes Project database and variants located in the coding region. 2,132, 2,175, 2,154, and 2,270 variants were left in D1, D2, D3, and C1, respectively. Considering that the family probably had a dominant inheritance pattern, we included variants that were heterozygous in D1, D2, and D3, but did not exist in C1, and identified 144 variants. In total, nine high impact variants, which were predicted to be deleterious by all three bioinformatic tools, PolyPhen-2, SIFT and MutationAssessor, were included (Table S4). In addition, we have also used the Combined Annotation-Dependent Depletion (CADD) to score the mutations (Table S4). Eventually, through a review of the literature, we examined the relationship between these genes
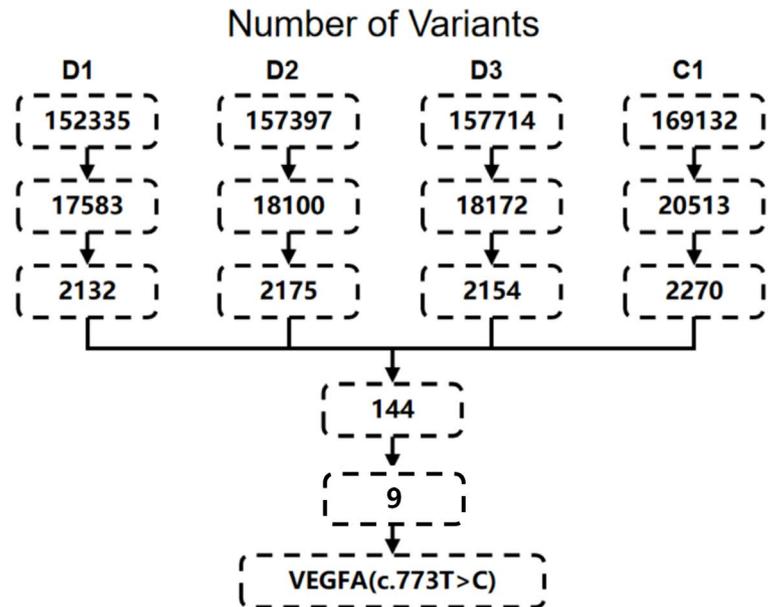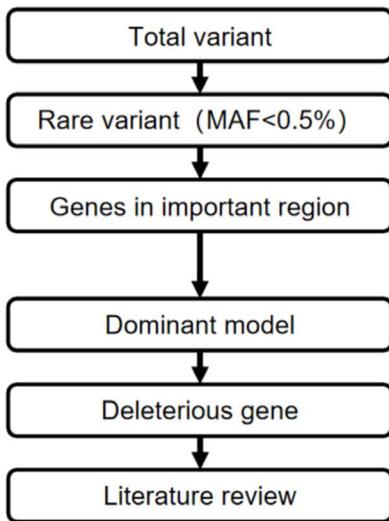
and CLP and identified a potentially pathogenic missense mutation in *VEGFA* (NM_001025366.2 c.773T > C p.Val258Ala).

We performed Sanger sequencing for validation of the minor allele frequency (MAF) on the four family members and 100 unaffected subjects. None of the 100 healthy people had c.773T > C variant (Figure S1, Table S3). All three patients were heterozygous for the mutant allele, while the mother had the WT allele (Figure 1c), which was consistent with WES results and the autosomal dominant inheritance model.

Three in silico tools predicted the c.773T > C p.Val258Ala variant would cause disease (Figure 2b). A multiple sequence alignment of VEGFA revealed that Val258 is evolutionarily conserved in vertebrate species (Figure 2d), suggesting that a genetic variant at this position is likely deleterious. Alternative splicing of the human *VEGFA* gene gives rise to at least six different transcripts, including VEGFA$_{121}$, VEGFA$_{145}$, VEGFA$_{165}$, VEGFA$_{183}$, VEGFA$_{189}$, and

FIGURE 2 Screening process of causative gene mutations, potential deleterious effects of the variant indicated by conservative analysis and protein structure analysis. (a) An overview of the flowchart of the pathogenic variants screening process (left) and the number of remaining variants after each corresponding step (right). (b) The predicted impact scores of the mutation using three in silico functional prediction tools, SIFT, PolyPhen-2, and MutationAssessor. (c) Multiple sequence alignment showing conservation among different VEGFA isoforms. (d) Multiple sequence alignment showing evolutionary conservation of the VEGFA residue Val78 affected by the variant. (e) Val78-mediated hydrophobic interaction contributed for VEGFA homodimerization. Two VEGFA monomers are in green and cyan colors. The hydrophobic core was indicated with red frame. The hydrophobic residues are shown in sticks. The hydrophobic interaction of Val78 Val46 and Phe122 are highlighted with purple frame and shown in sphere. (f) Val78-mediated hydrophobic interaction with Val48 and Phe122 stabilized the VEGFA dimer and is critical for recognition by its receptor. Overall structure of VEGFA receptor is shown in carton. The interaction region of VEGFA and VEGFR are highlighted in red frame. The receptor is shown in yellow and VEGFAs are shown in green and cyan. Val46, Val78, and Phe122 are shown in sticks and sphere, respectively

(a)

Variant screening flowchart

Number of Variants

D1	D2	D3	C1
152335	157397	157714	169132
17583	18100	18172	20513
2132	2175	2154	2270

Flowchart boxes: Total variant → Rare variant (MAF<0.5%) → Genes in important region → Dominant model → Deleterious gene → Literature review

144 → 9 → VEGFA(c.773T>C)

(b)

Software	SIFT	PolyPhen2	MutationAssessor
Deleterious threshold	≤0.05	≥0.909	≥1.9
Score	0.001	0.994	3.13

(c)

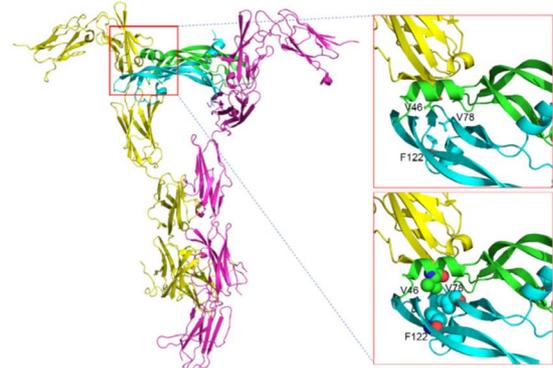|	
VEGFA$_{121}$	EIEYIFKPSCVPLMRCGGCCN
VEGFA$_{145}$	EIEYIFKPSCVPLMRCGGCCN
VEGFA$_{165}$	EIEYIFKPSCVPLMRCGGCCN
VEGFA$_{183}$	EIEYIFKPSCVPLMRCGGCCN
VEGFA$_{189}$	EIEYIFKPSCVPLMRCGGCCN
VEGFA$_{206}$	EIEYIFKPSCVPLMRCGGCCN

(d)

Mutant VEGFA	EIEYIFKPSCAPLMRCGGCCN
H. sapiens	EIEYIFKPSCVPLMRCGGCCN
D. rerio	EIEHTYIPSCVVLMRCAGCCN
M. musculus	EIEYIFKPSCVPLMRCAGCCN
R. norvegicus	EIEYIFKPSCVPLMRCAGCCN
G. gallus	EVEYIFRPSCVPLMRCAGCCG

(e)

(f)

VEGFA$_{206}$ (Apte et al., 2019; Holmes & Zachary, 2005). VEGFA$_{165}$ is the most frequently expressed isoform in tissues and Val258 is conserved among different isoforms (Figure 2c). So we use VEGFA$_{165}$ as an example to construct the crystal structure. Val258 is equivalent to Val78 in VEGFA$_{165}$, and Val78 is used to represent the mutation site in the crystal structure.

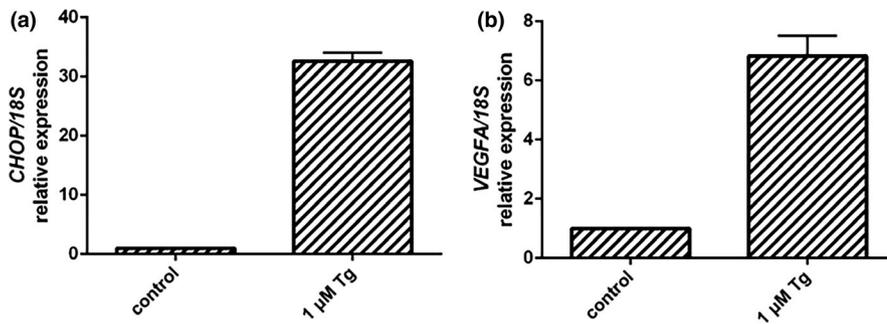Crystal structure of VEGFA in complex with VEGFR-1 domains D1-6 (https://doi.org/10.2210/pdb5T89/pdb) was be used for

visualization through PyMOL software. VEGFA is a homodimeric protein and belongs to the family of cysteine-knot growth factors (Holmes & Zachary, 2005). The structure of VEGFA revealed that the dimerization is mediated by loop regions of the central four strands and an N-terminal α-helix of the other subunit (Figure 2e). The loop regions and N-terminal α-helix have a small hydrophobic core to stabilize the homodimeric conformation. Val78 of VEGFA in the hydrophobic core plays a critical role for dimerization, which allows for hydrophobic interactions with both Phe122 in the second sheet and Val46 in the N-terminal α-helix of the other subunit to stabilize the homodimer (Figure 2e). If the Val78 was mutated to Ala78, it would compromise the hydrophobic interaction, leading to a structural conformational change. Based on the VEGFA receptor complex structure, the loop regions, together with the N-terminal α-helix, interact with the VEGFA receptor (Figure 2f). The conformational change in VEGFA caused by the Val78Ala mutation is very likely to affect recognition by the VEGFA receptor. Thus, the Val78Ala mutation may attenuate hydrophobic interactions of VEGFA to decrease its binding affinity to VEGFA receptors. Palatogenesis is take place in the background of the extracellular matrix (ECM) (Blavier et al., 2001; Brown et al., 2002). VEGFA works as an important interaction factor with the ECM, and the mutation in VEGFA may influence ECM-mediated palatogenesis.

VEGFA is induced by an array of stress factors in chondrocytes, such as mechanical stress, hypoxia, ER stress, reactive oxygen species (ROS), and inflammatory cytokines, which may affect chondrocyte viability and the cartilage metabolism (Murata et al., 2008; Pereira et al., 2014). ER stress is associated with increased ECM accumulation and plays a regulatory role in ECM degradation (Bai et al., 2016; Kasetti et al., 2017). In our study, *VEGFA* gene expression was detected after exposure to the ER stress inducer thapsigargin (Tg) in human embryonic palatal mesenchyme (HEPM) cells. HEPM cells were cultured with the vehicle or Tg (Figure 3), and real-time polymerase chain reaction (PCR) was used to assess *VEGFA* expression. Tg significantly increased the *VEGFA* mRNA levels (Figure 3b). The increased *CHOP* gene expression indicated that the ER stress signaling pathway was activated (Figure 3a).

In conclusion, we performed WES in a NSCL/P Han Chinese family with three patients. After screening and verification of the variants, we obtained a potentially pathogenic missense mutation in *VEGFA* (NM_001025366.2 c.773T > C p.Val258Ala). This means that this mutation is more likely to be a pathogenic mutation in this family than other mutations. However, it may also be caused by multiple factors, which still need to be verified by subsequent functional

experiments, which is also the focus of our follow-up studies. Besides, we also found ER stress can promote the expression of *VEGFA*, suggesting that the expression of VEGFA may be affected by environmental factors. The mutation of *VEGFA* may lead to the incorrect response of the VEGFA expression to the environmental factors during the development process and thus may cause developmental disorders, such as cleft lip and palate.

CONFLICT OF INTEREST

None to declare.

AUTHORS' CONTRIBUTION

Bohui Sun: Data curation; Formal analysis; Writing – original draft; Writing – review & editing. **Yulin Xi:** Formal analysis; Writing – original draft. **Wenbin Huang:** Data curation; Formal analysis; Software. **Wei Liang:** Resources. **Zhibo Zhou:** Resources. **Weiran Li:** Resources. **Huizhe Huang:** Funding acquisition; Resources. **Jiuxiang LIN:** Resources; Supervision. **Hsiang-Ying Lee:** Conceptualization; Project administration; Supervision. **Feng Chen:** Conceptualization; Funding acquisition; Project administration; Resources; Supervision.

PEER REVIEW

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.**How to cite this article:** Sun B, Xi Y, Huang W, et al. A novel *VEGFA* mutation as a candidate for causing non-syndromic cleft lip and/or cleft palate. *Oral Dis*. 2020;00:1–5. https://doi.org/10.1111/odi.13719