An Association Study of Candidate Gene Variants in Chinese Nonsyndromic Cleft Lip with or without Palate Subjects

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Research article

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Abstract

**Background:** Nonsyndromic cleft lip with or without palate is a common birth defect of complex etiology involving the interaction of genetic factors and environmental exposures. Previous reports identified several candidate genes and risk variants of the disease, and their functions were verified by model animal studies.

**Methods:** In order to depict the composition of the orofacial cleft susceptibility loci in Chinese population, we genotyped ten common SNPs of six genes (VAX1, MAFB, PAX7, ABCA4, NTN1 and NOG) in 249 nonsyndromic cleft lip with or without palate individuals, 62 nonsyndromic cleft palate only individuals and 480 controls.

**Results:** Three loci: VAX1 rs7078160, MAFB rs11696257 and NTN1 rs4791774 showed significant relevance with NSCL/P. Carrying both VAX1 rs7078160 and NTN1 rs4791774 further increased the risk, compared with carrying only one of them.

**Conclusions:** This result supported that SNPs of genes VAX1, MAFB and NTN1 are associated with NSCL/P in Chinese subjects.

Background

Cleft lip with or without palate (CL/P) is among the most common congenital craniofacial defects, with an average incidence ranging from 1/500 to 1/1000 live births, based on different ethnic populations [1]. Approximately 70% of cases of CL/P occur with no other apparent structural abnormalities are defined as nonsyndromic cleft lip with or without cleft palate (NSCL/P) [2]. The prevalence of NSCL/P in China was 1.22/1000 [3]. It is considered to be a multifactorial disease with both genetic and environmental factors contributing to the etiology. Nonsyndromic cleft palate only (NSCPO) is to some extent different though not completely distinct from NSCL/P in genetic mechanism [4].

Nonsyndromic CL/P does not entirely follow Mendel's genetic characteristics, a large part of parental phenotype of cleft lip and palate patients is normal[5]. Genetic variations increase the risk of this phenotype, although they are common in population. The genome wide association studies found more than 40 candidate genes and their marker SNPs [6–11]. In addition to being a marker of susceptibility, several of these genes also have been linked to cleft lip and palate by animal model. Gene VAX1 located in chromosome 10q25 is expressed widely during development of craniofacial structures [6]. The Vax1 knockout mouse developed cleft palate [12]. A missense mutation H131Q in a strongly conserved sequence region of MAFB was found association with CL/P, and Mafb expression was upregulated during palatal fusion [11]. Mice lacking Pax7 showed malformations of the nasomaxillary complex [13]. Gene NOG is expressed in palate shelves and functions as a signaling molecule during embryonic development [14]. Inactivation of NOG showed cleft palate in a rat model [15].
Although deleterious changes in these genes can cause cleft in model animals, the contribution of polymorphisms remains to be investigated. And these variants may not play roles independently [16]. Dissimilar results of the gene contributions in the populations with different genetic backgrounds and living conditions were reported. For example, \textit{ABCA4} achieved more significant association among the Asian families compared to the European's while the 8q24 region showed an opposite result [11]. Therefore, we selected 10 SNPs of six candidate genes: \textit{VAX1}, \textit{MAFB}, \textit{PAX7}, \textit{ABCA4}, \textit{NTN1} and \textit{NOG} to conduct this case-control study.

\textbf{Results}

In total of 10 SNPs from \textit{VAX1} (rs7078160, rs4752028), \textit{MAFB} (rs13041247, rs11696257), \textit{PAX7} (rs4920520, rs766325), \textit{ABCA4} (rs560426, rs481931), \textit{NTN1} (rs4791774) and \textit{NOG} (rs17760296) were selected and genotyped in the case and the control samples. Statistical results of each SNP, distribution of alleles and genotypes were listed in Table 1 and S1. All the SNPs were in Hardy–Weinberg equilibrium among the controls.

Allele analyses showed that \textit{VAX1} rs7078160 and \textit{MAFB} rs11696257 in the NSCL/P cases were significantly different from those among the controls (Bonferroni method adjusted p-values were 0.020 and 0.00031, respectively). The risk allele frequencies in the case and the control groups were 48.6% and 40.3% for rs7078160 A and 60.1% and 48.5% for rs11696257 C, respectively. Genotype analyses under the additive model also identified the association of \textit{VAX1} rs7078160 and \textit{MAFB} rs11696257 (p-values were 0.025 and 0.00044, respectively). The highest risk of this study was found in homozygote comparing CC vs. TT for \textit{MAFB} rs11696257 (OR = 2.47, 95% CI: 1.58 to 3.87). Variants \textit{NTN1} rs4791774 passed the significant test under the dominate model (p-values were 0.030). For this SNP, the genotype GG + AG increased disease risk 1.63 times (95% CI: 1.18 to 2.25) comparing with the homozygote AA.
| Genes | Loci      | Genotype Padj   | Allele Padj | OR          |
|-------|-----------|----------------|-------------|-------------|
|       |           | AA + Aa vs. aa | AA vs. aa   | A vs. a     | (95%CI)     |
| VAX1  | rs7078160 | 0.257          | 0.025       | 0.020       | 1.41 (1.13–1.76) |
|       | rs4752028 | > 1            | > 1         | > 1         | 1.00 (0.79–1.26) |
| MAFB  | rs13041247| 0.979          | 0.487       | 0.441       | 1.26 (1.01–1.57) |
|       | rs11696257| **0.024**      | **0.00044** | **0.00031** | 1.60 (1.28–2.00) |
| PAX7  | rs4920520 | > 1            | > 1         | > 1         | 1.30 (0.94–1.80) |
|       | rs766325  | > 1            | > 1         | > 1         | 0.98 (0.73–1.31) |
| ABCA4 | rs560426  | 0.275          | 0.140       | 0.077       | 1.37 (1.09–1.73) |
|       | rs481931  | > 1            | > 1         | 0.158       | 1.27 (1.07–1.60) |
| NTN1  | rs4791774 | **0.030**      | > 1         | 0.067       | 1.44 (1.11–1.88) |
| NOG   | rs17760296| p value for AA vs. Aa | > 1 | 1.29 (0.45–3.70) |

Note: NSCL/P: nonsyndromic cleft lip and/or palate; Allele A: the risk allele of the polymorphism; Genotype AA: the homozygote of the risk allele; Padj: Bonferroni method adjusted p-value; Padj < 0.05 were showed in bold; OR: Odds Ratio.

There were no association found between the CPO group and all the SNPs. Then the CL/P (CL + CLP) and the CPO subjects were combined into one orofacial cleft case group to calculate. MAFB rs11696257 remained positive, but the significant level reduced, compared with the result when only NSCL/P subjects included (Table S1).

In order to test whether a second risk gene would further increase the disease risk, the number of subjects who carried any one or two of the three positive loci (VAX1 rs7078160 A, MAFB rs11696257 C and NTN1 rs4791774 G) were compared between NSCL/P patients and controls (Table 2). Individual, who carrying both risk genes VAX1 rs7078160 A and NTN1 rs4791774 G, has more than two times higher risk compared with these carrying only one of these two risk genes. These two genes are independent risk factors to each other.
Table 2

| Risk gene carried | CL/P | Control | Padj | OR   (95% CI) |
|-------------------|------|---------|------|------------|
| VAX1 and NTN1     | 83   | 94      |      | 2.02       |
| vs. VAX1 only     | 80   | 183     | 0.0014** | (1.36–3.00) |
| vs. NTN1 only     | 24   | 59      | 0.018*  | (1.24–3.80) |
| VAX1 and MAFB     | 140  | 233     |      | 1.22       |
| vs. VAX1 only     | 30   | 61      | >1    | (0.75–1.98) |
| vs. MAFB only     | 59   | 130     | 0.52  | (0.91–1.92) |
| MAFB and NTN1     | 82   | 117     |      | 1.64       |
| vs. MAFB only     | 96   | 225     | 0.024* | (1.13–2.38) |
| vs. NTN1 only     | 16   | 35      | 0.60  | (0.80–2.95) |

Note: CL/P: Cleft lip and/or palate; Padj: Bonferroni method adjusted p-values; Padj < 0.05 were showed in bold; *: p < 0.05, **: p < 0.01; OR: Odds Ratio.

Discussion

NSCL/P is considered as a multifactorial disease resulting from the interaction between genetic and environmental factors. We conduct an association study for 10 SNPs and support that MAFB rs11696257, VAX1 rs7078160 and NTN1 rs4791774 increased NSCL/P risk in Chinese subjects. The ABCA4 polymorphisms also showed weak association but they had not passed the multiple test adjustment. Similar results were found in MAFB rs13041247 and PAX7 rs4920520. But for VAX1 rs4752028 and PAX7 rs766325, the frequencies were nearly equal in the cases and the controls (32.21% vs. 32.29% and 16.67% vs. 16.99%). The minor allele frequency of NOG rs17760296 is only about 1.5%, so the contribution of this variant must be limited in Chinese population. This study indicates that CL/P and CPO should be considered as two phenotypes caused by different genetic reasons, at least for these positive genes.
The highest risk was found in *MAFB*, which is a transcription factor located in chromosome 20q12. This gene encodes a basic leucine zipper transcription factor and also associated with another disease with maxillary hypoplasia phenotype named Multicentric Carpotarsal Osteolysis Syndrome. Expression analysis in mouse embryos revealed its function in lip and palate morphogenesis especially palatal fusion [17], which reminded the role of gene *IRF6*. Beaty's GWAS identified the association of two *MAFB* SNPs rs13041247 and rs11696257 with NSCL/P [11], but no positive result was obtained for the first one in this study.

In the population that acquired both *VAX1* rs7078160 A and *NTN1* rs4791774 G, the risk of disease increased, higher than the risk variant obtained from either of these two genes. SNP rs7078160 A significantly associated with NSCL/P in rs4791774 G positive subgroup (AG or GG, p = 0.0014). Similar result discovered for rs4791774 G in rs7078160 A positive subgroup (AG or AA, p = 0.018). These two genes play independent roles in the disease onset.

*VAX1* encodes a conserved homeobox transcription factors and involves in the regulation of development and morphogenesis. Mice heterozygous for the *Vax1* mutation were fertile and appeared normal, although homozygous exhibited craniofacial malformations including cleft palate [12]. *NTN1* encodes a laminin-related secreted protein and plays a critical role in axon guidance, cell migration and adhesion during development. Mice that lack *Ntn1* die during the perinatal period with a cleft palate phenotype [18]. High-level NTN1 protein was observed in the mesenchyme, especially along the basement membrane of the palatal shelves [19]. Taken together, *VAX1* is involved in the development of the tissue structure of the palate, while *NTN1* may ensure the cell adhesion of the palate flaps. They may play different roles in different aspects of CL/P onset. Although both SNPs are not codon variants, the double-site detection can be developed as a better early warning marker for cleft lip and palate risk in Chinese.

**Conclusions**

The SNPs of genes *VAX1*, *MAFB* and *NTN1* are associated with NSCL/P in Chinese subjects.

**Methods**

**Sample Collection**

This study involved 311 nonsyndromic orofacial clefts cases and 480 controls. Subjects were collected from Shanghai Ninth People's Hospital affiliated to Shanghai JiaoTong University School of Medicine, which were physically screened and were carefully diagnosed by at least two physicians. Healthy controls were also recruited from Shanghai. The case group consisted of 57 cleft lip (CL), 192 cleft lip and palate (CLP), and 62 cleft palate only (CPO) patients. Gender ratio is 1.75:1.

**Genomic DNA Extraction and SNP selection**
DNA samples were extracted from peripheral blood using Flexi Gene DNA Kit (Qiagen, Germany). The DNA was measured for concentration and purity and then stored at -20 °C.

In total of 10 SNPs from VAX1 (rs7078160, rs4752028), MAFB (rs13041247, rs11696257), PAX7 (rs4920520, rs766325), ABCA4 (rs560426, rs481931), NTN1 (rs4791774) and NOG (rs17760296) were eventually selected and genotyped in case and control samples.

Primer Design and PCR

PCR primers were designed for these ten SNPs based on hg19 of the human genome. All amplicons were in the range of 200 to 300bp. For each primer pair designed, the forward and reverse primers were tagged with a common sequence 1 (CS1: 5′-CCTACACGACGCTCTTCCGATCT-3′) and common sequence 2 (CS2: 5′-AGTTCCTTGGCACCCGAGAATTCCA-3′), respectively. The primer pairs were synthesized from Shanghai Morgen Biotechnology Co., Ltd.

All the primer pairs were divided into 3 combinations, each combination of 3 or 4 primer pairs. Every DNA sample was amplified in separate multiplex PCR reactions (HotStarTaq, Qiagen, Germany) with these primer combinations and mixed after electrophoresis. PCR products were cleaned up by AMPure XP Beads (Beckman Coulter, CA).

Barcoding and Illumina Sequencing

Barcoding was performed in a 20 μL reaction mixture that contained 8 μL of the cleaned up PCR products, 10 μl of KAPA2G Robust hotstart ready mix (Kapa Biosystems, USA), 1μmol/L barcode F primers and 1 μmol/L barcode R primer. The barcoded PCR products from various samples were cleaned up by the AMPure XP Beads (Beckman Coulter, CA).

Purified PCR product library was quantified using a Qubit Fluorometer. According to library quantitation, the PCR product was pooled together with equal mole. Purified libraries were sequenced on a MiSeq Benchtop Sequencer or a NextSeq 500 sequencer (Illumina Inc., San Diego, CA) using protocol. The quality of sequence reads were checked by FastQC algorithm. SNPs were identified using Genome Analysis Toolkit (GATK) and annotated by Annovar software.

Sequence Data Analysis

Demultiplexed compressed FASTQ files were generated from BCL by bcl2fastq Conversion Software v1.8.4 (Illumina, San Diego, CA). For all successful sequencing runs, read depth was 1600x at any given position, with 2700x mean coverage across the entire targeted sequence, and 100% of bases above Q30 at 2 * 150 bp. The variant calling and the coverage of each captured region were analyzed by an in-house developed bioinformatics pipeline, based on the general analysis algorithm pipeline. Briefly, the reads
were mapped to the hg19 version of the human reference genome, filtered to remove off-target and poor-quality reads. Variants were identified and annotated. The variants and annotation results were transformed into Excel sheets.

**Statistical Analysis**

Statistical analysis was performed with the SPSS 11.0 statistical software package (SPSS Inc., Chicago, IL, USA) or R (Version 3.0.2: www.r-project.org/). Differences in genotype and allele frequencies were analyzed using Pearson $\chi^2$ test or Fisher’s exact test and with a $p<0.05$ taken as being significant after multiple testing adjustment. Odds ratio and 95% CI of alleles had also been calculated. Power analysis was estimated assuming a prevalence of NSCL/P in China of 0.0012.

**Declarations**

**Ethics approval and consent to participate**

The study was approved by the Ethical Committee of Chinese National Human Genome Center (2014-09). Written informed consent was obtained from all participants or their legal guardians.

**Consent for publication**

Not applicable

**Availability of data and materials**

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

**Competing interests**

The authors declare that they have no competing interests.

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Authors' contributions

Z.C, Z.N and W.H conceived and designed the experiments. L.P, Z.N, J.C and L.Y performed the experiments. L.P and Z.N analyzed the data. T.W, D.W, Y.Y and G.W contributed materials. L.P, Z.N, Z.C and W.H wrote the main manuscript text. All authors reviewed the manuscript.

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