Polymorphic variants in VAX1 and the risk of nonsyndromic cleft lip with or without cleft palate in a population from northern China

Dongmei Li, MD, Tingting Liu, MD, Xiangbiao Meng, MD, Qiang Guo, PhD, Jinna Shi, PhD, Yanru Hao, PhD, Xiaohui Jiao, PhD, Kewen Lv, PhD, Tao Song, MD, PhD

Abstract

**Background:** Nonsyndromic cleft lip with or without cleft palate (NSCL/P) is one of the most common craniofacial birth defects, and the etiology of NSCL/P involves both genetic and environmental factors. Genome-wide association study (GWAS) identified a novel susceptibility locus of ventral anterior homeobox 1 (*VAX1*) in patients with NSCL/P. However, the association of single nucleotide polymorphisms (SNPs) of VAX1 with NSCL/P is inconclusive due to the differences in the racial and ethnic populations. The aim of this study was to replicate the association between VAX1 and NSCL/P in a northern Chinese Han population.

**Methods:** Our study included 186 patients with NSCL/P and 223 healthy individuals from northern China. Five SNPs (rs4752028, rs10787760, rs7078160, rs6585429, and rs1871345) on VAX1 were genotyped using the SNaPshot method.

**Results:** Recessive genetic model analysis revealed that homozygous genotype CC of VAX1 rs4752028 was associated with an increased risk of NSCL/P (odds ratio = 1.89, 95% confidence interval = 1.12–3.19, *P* = 0.017), but the results were not significant after the Bonferroni correction for multiple comparisons. The allele and genotype frequencies of rs10787760, rs7078160, rs6585429, and rs1871345 and the allele frequencies of rs4752028 showed no significant differences between cases and controls. Haplotype and SNP-SNP interaction analyses did not detect any significant association of VAX1 with the occurrence of NSCL/P.

**Conclusion:** VAX1 rs4752028 was weakly associated with NSCL/P development in the studied northern Chinese Han population.

**Abbreviations:** *χ*² = Chi-square, CIs = confidence intervals, CVC = cross-validation consistency, GWAS = genome-wide association study, HWE = Hardy–Weinberg equilibrium, LD = linkage disequilibrium, MDR = multifactor dimensionality reduction, NSCL/P = nonsyndromic cleft lip with or without cleft palate, OR = odds ratio, SNPs = single nucleotide polymorphisms, TA = testing balanced accuracy, VAX1 = ventral anterior homeobox 1.

**Keywords:** association, nonsyndromic cleft lip with or without cleft palate, single nucleotide polymorphisms, VAX1 gene

1. Introduction

Nonsyndromic cleft lip with or without cleft palate (NSCL/P), which results from an impaired facial process growth and fusion during embryogenesis, is one of the most common craniofacial birth defects in humans.[1,2] The average incidence of NSCL/P varies widely among different geographical regions and ethnic groups.[1,2] Moreover, its prevalence varies widely among different geographical regions and ethnic groups.[1,2] The highest incidence of NSCL/P is observed in the Asians and Native Americans, and the lowest is observed in the Africans. The high prevalence of NSCL/P (1.42/1000) is reported in the Chinese population.[3] Newborns with NSCL/P may have speech and feeding problems, poor nutrition, psychiatric diseases, and infection of the middle ear. Although these defects can be partly corrected by a series of surgical interventions and multidisciplinary treatments, they still bring long-term burdens to the individual, family, and society.[7]

The etiology of NSCL/P is complex and is associated with both the genetic and environmental factors.[8,9] Although the specific genetic and environmental risk factors associated with NSCL/P remain unclear, the linkage and association analysis and the genome-wide scanning have provided significant evidence for the potential candidate genes in the development of NSCL/P, such as *MSX1*,[10] *IRF6*,[11] *PVRL1*,[12] *SUMO1*,[13] and *FGF*.[14]

Recently, 2 genome-wide association analyses have confirmed a susceptibility candidate gene that may be involved in NSCL/P, VAX1 at locus 10q23.3,[15,16] and it was later identified on meta-analysis by Ludwig et al.[17] These studies have revealed that single nucleotide polymorphisms (SNPs) in or near VAX1 were involved in the risk of NSCL/P, and the analysis of participants of European and Asian origin from multiple populations provided a significant evidence for the susceptibility gene.[15–17] A previous study performed using mouse model suggested that lack of functionally active VAX1 results in craniofacial deformity, including cleft palate.[18] However, several similar investigations were conducted in the Mesoamericans, Central Africans, Southeast Asians, and southern Chinese population, which...
showed inconsistent results.\(^{19-21}\) To the best of our knowledge, no GWAS examining the association between SNPs of VAX1 and the risk of NSCL/P in a northern Chinese population has been reported. Therefore, the aim of our current study was to investigate whether the 5 SNPs (rs4752028, rs10787760, rs7078160, rs6585429, and rs1871345) of VAX1 were associated with the susceptibility of NSCL/P in a northern Chinese population.

2. Methods

2.1. Subjects
The current case-control study was approved by the Institutional Ethics Committee of the Harbin Medical University and was a hospital-based study. Diagnosis of the case group (patients with NSCL/P) was done through clinical investigations by 2 experienced dentists to assess individual phenotypic features and cases were identified through medical records; the cases with other major congenital anomaly and syndromes were excluded from the study. The study consisted of 186 cases (101 female subjects and 85 male subjects), who had no history of major congenital anomaly and syndromes were excluded from the study. Further, the PCR products were purified and scanned using 3730 Genetic Analyzer (Life Technologies Corporation, Vancouver, British Columbia, Canada).

2.2. Polymorphism selection
To investigate the role of VAX1 gene polymorphisms on NSCL/P risk in a northern Chinese population. We selected VAX1 tag SNPs (rs10787760, rs6585429, and rs1871345), which were selected from CHB (Beijing Han population of China) with a minor allele frequency (MAF) >0.05 in the HapMap Project. In addition, on the basis of the genome-wide association studies (GWAS) of Mangold et al.,\(^{15}\) we selected other 2 SNPs (rs7078160 and rs4752028) of VAX1.

2.3. Genotyping
DNA was extracted from the peripheral venous blood samples from each participant using the QIAamp DNA Blood Kit (Valencia, CA), according to the manufacturer’s protocol. Genotyping for VAX1 (rs4752028, rs10787760, rs7078160, rs6585429, and rs1871345) polymorphisms was performed using SNaPshot technology. Primers were designed using the Primer 3 software (http://frodo.wi.mit.edu/).

Table 1

| Gene | SNP       | Position | HWE P   | Call rate (%) | MAF   | Alleles |
|------|-----------|----------|---------|---------------|-------|---------|
| VAX1 | rs7078160 | 118817550| 0.7738  | 100           | 0.472 | G:A     |
|      | rs4752028 | 118824981| 0.4263  | 100           | 0.401 | T:C     |
|      | rs10787760| 118880683| 0.7961  | 100           | 0.274 | C:T     |
|      | rs6585429 | 118883221| 0.372   | 100           | 0.405 | A:G     |
|      | rs1871345 | 118885368| 0.6094  | 100           | 0.467 | G:A     |

1 MAF = minor allele frequency calculated for the cases and controls.

2.4. Statistical Analyses
Hardy-Weinberg equilibrium (HWE) of the genotype distributions of cases and controls was examined by using Chi-square (\(\chi^2\)) test. The differences in genotype and allele frequencies of the tested SNPs between cases and control groups were evaluated using standard \(\chi^2\) and Fisher tests. The association between SNPs and risk of NSCL/P was evaluated by calculating the odds ratios (ORs) and 95% confidence intervals (CIs). Bonferroni correction of \(P < 0.01 (0.05/5)\) was used to note the statistical significance and solve the problem of multiple comparisons. Statistical analyses were performed using PLINK (a free open-source whole genome association analysis toolset), and R. Linkage disequilibrium (LD) was evaluated using Haploview 4.2 software\(^{22}\) depending on \(D^2\) and \(r^2\) values. SNP-SNP interactions in VAX1 were evaluated using the R package of Multifactor Dimensionality Reduction (MDR).\(^{23}\) A result with \(P\) value of <0.05 was considered as a statistically significant result.

3. Results
All the SNPs in the cases and control groups were observed to be consistent with the Hardy–Weinberg equilibrium (\(P > 0.05\)) (Table 1). The allele and genotype frequencies of rs10787760, rs7078160, rs6585429, and rs1871345 and the allele frequencies of rs4752028 in the NSCL/P cases were not significantly different from those in the controls (\(P > 0.05\)) as summarized in Table 2. Further, analyses of the dominant and recessive genetic models revealed that VAX1 rs4752028 was differently distributed between the cases and control groups (\(P=0.017\)). The results of the recessive genetic model showed that the homozygous genotype CC was associated with an increased risk of NSCL/P (OR=1.89, 95% CI=1.12–3.19), combining the TT and CT genotype of VAX1 rs4752028. However, the results did not show significance after the Bonferroni correction for multiple
comparisons was applied (corrected $P = 0.05/5 = 0.01$). The LD pattern among these 5 SNPs is depicted in Fig. 1. A haplotype block was constructed in this region with the $D^*$ and $r^2$ values (Table 3). In the haplotype analysis, the haplotype distributions between cases and control groups were compared and it was noted that no haplotype was associated with the risk of NSCL/P ($P > 0.05$; Table 4). The results of MDR analyses of SNP–SNP interactions are summarized in Table 5 and Fig. 2. The 1-locus based model of rs4752028 revealed the highest cross-validation consistency (CVC) of 10/10 and testing balanced accuracy (TA) of 0.53, but it not reached statistical significance ($P = 0.127$). However, no SNP–SNP interactions were found to be associated with the risk of NSCL/P.

4. Discussion

The current study investigated the association of VAX1 with the risk of NSCL/P in a northern Chinese Han population. In this study, we successfully genotyped 5 SNPs of VAX1, and the allele and genotype frequencies of rs10787760, rs7078160,
rs7078160, and rs1871345 and the allele frequencies of rs4752028 showed no significant differences between cases and controls. We found that the homozygous genotype CC of rs4752028 was associated with an increased risk of NSCL/P (OR = 1.89, 95% CI = 1.12–3.19, P = 0.017, using a recessive model). However, the association disappeared after the Bonferroni correction that controlling for multiple comparisons.

These results seem to be a little disappointing; however, given the complicated heterogeneous nature of NSCL/P and a number of other confounding factors, this is an expected result. Studies from animal models suggested that VAX1 has played a crucial role in the process during craniofacial development. VAX1 was widely expressed in the craniofacial structures in rats, while VAX1-knockout mice exhibit phenotype of cleft palate. In humans, the VAX1 mutation could result in an uncharacterized syndrome with bilateral lip, which was one of the clinical features from animal models. Several studies have analyzed syndrome with bilateral lip, which was one of the clinical features from animal models. Several studies have analyzed syndrome with bilateral lip, which was one of the clinical features from animal models. Some previous studies have identified that VAX1 rs7078160 was associated with the risk of NSCL/P in the Estonians and Mesoamericans. However, these studies have not found association of VAX1 rs7078160 with NSCL/P in populations from Poland, Brazil, and southern China. Consistent with results of the study conducted by Pan et al in a southern Chinese population, we did not find an association between rs7078160 and NSCL/P. Moreover, the MAF of VAX1 rs7078160 in our controls was similar to those observed in HapMap CHB populations (0.43 vs 0.35). However, the interpretation of our results has some limitations; after the Bonferroni correction for multiple comparisons, the association was found to be nonsignificant. Fundamentally, multiple markers (comparisons) were used for testing multiple corrections, correcting for spurious associations, and it may be stringent to our research and could lead to a loss of the significant finding. Moreover, our work was a hospital-based case-control study and the selection bias was unavoidable, where the subjects may not be a representative of the general population.

In the current study, we replicated the result reported in the study by Mangold et al. The association of VAX1 rs4752028 with NSCL/P risk was weak in our study, and the reasons for the discrepancy may be attributed to the existing allelic heterogeneity at this locus in various populations. The MAF for VAX1 rs4752028 in our controls was 0.43, which is distinctly different from that of 0.16 in the European population. Nevertheless, the MAF of VAX1 rs4752028 in our controls was similar to those observed in HapMap CHB populations (0.43 vs 0.35). However, the interpretation of our results has some limitations; after the Bonferroni correction for multiple comparisons, the association was found to be nonsignificant. Fundamentally, multiple markers (comparisons) were used for testing multiple corrections, correcting for spurious associations, and it may be stringent to our research and could lead to a loss of the significant finding. Moreover, our work was a hospital-based case-control study and the selection bias was unavoidable, where the subjects may not be a representative of the general population.

In conclusion, our study has demonstrated that SNP rs1871345, which was found to be associated with other genetic factors, has a modest effect on the risk of NSCL/P. The MDR approach used to explore gene–gene interactions for orofacial clefting has been confirmed. Our study failed to find SNP–SNP interactions of VAX1. The discrepancy may be impacted by the sample size, which was insufficient to detect a modest effect of the tested SNP variants.

In conclusion, our study has demonstrated that SNP rs4752028 was involved with the risk of NSCL/P in a northern Chinese Han population, although weak, which to some extent, revealed an association between VAX1 and the risk of NSCL/P. Discrepancy in results may be due to a complex genetic background and environmental exposure among different populations. Therefore, further studies are required to confirm the current data in a larger sample and with various ethnic groups.

### Table 3

| Pairwise linkage disequilibrium measures for VAX1. | SNPs | rs7078160 | rs4752028 | rs10787760 | rs4752028 | rs1871345 |
|---|---|---|---|---|---|---|
| D' values above the diagonal | 0.81 | 0.81 | 0.95 | 0.54 | 0.49 | 0.32 |
| D' values below the diagonal | 0.97 | 0.97 | 0.98 | 0.97 | 0.85 | 0.85 |
| r values above the diagonal | 0.55 | 0.33 | 0.53 | 0.96 | 0.28 | 0.48 |
| r values below the diagonal | 0.34 | 0.97 | 0.31 | 0.96 | 0.31 | 0.97 |

### Table 4

| Haplotype association between SNPs rs10787760, rs6585429, and rs1871345. | Haplotypes | Total frequency | Case frequency (%) | Control frequency (%) | P |
|---|---|---|---|---|---|
| Block1 | CAA | 0.46 | 175.7 (47.2) | 200.5 (45.0) | 0.5193 |
| Block1 | TGG | 0.27 | 94.4 (25.4) | 124.6 (27.9) | 0.4102 |
| Block1 | CAG | 0.13 | 50.2 (13.5) | 58.3 (13.1) | 0.8594 |
| Block1 | CGG | 0.13 | 48.2 (13.0) | 57.9 (13.0) | 0.9933 |

* Haplotypes present in more than 3% of the study population.
and to determine the association between VAX1 and the risk of NSCL/P.

References

[1] Kurosaka H, Iulianella A, Williams T, et al. Disrupting hedgehog and WNT signaling interactions promotes cleft lip pathogenesis. J Clin Invest 2014;124:1660–71.

[2] Ray HJ, Niswander L. Mechanisms of tissue fusion during development. Development 2012;139:1701–11.

[3] Lidral AC, Murray JC. Genetic approaches to identify disease genes for birth defects with cleft lip/palate as a model. Birth Defects Res A Clin Mol Teratol 2004;70:893–901.

[4] Birnbaum S, Ludwig KU, Reutter H, et al. Key susceptibility locus for non-syndromic cleft lip with or without cleft palate on chromosome 8q24. Nat Genet 2009;41:473–7.

[5] Dixon MJ, Marazita ML, Beaty TH, et al. Cleft lip and palate: understanding genetic and environmental influences. Nat Rev Genet 2011;12:67–78.

[6] Dai L, Zhu J, Mao M, et al. Time trends in oral clefts in Chinese newborns: data from the Chinese National Birth Defects Monitoring Network. Birth Defects Res A Clin Mol Teratol 2010;88:41–7.

[7] Stanier P, Moore GE. Genetics of cleft lip and palate: syndromic and non-syndromic genes contribute to the incidence of non-syndromic clefts. Hum Mol Genet 2004;13 Spec No 1R73–81.

[8] Carinci F, Pezzetti F, Scapoli L, et al. Recent developments in orofacial cleft genetics. J Craniofac Surg 2003;14:130–43.

[9] Carinci F, Pezzetti F, Scapoli L, et al. Genetics of nonsyndromic cleft lip and palate: a review of international studies and data regarding the Italian population. Cleft Palate Craniofac J 2000;37:33–40.

[10] Jezewska PA, Vieira AR, Nishimura C, et al. Complete sequencing shows a role for MSX1 in non-syndromic cleft lip and palate. J Med Genet 2003;40:399–407.

[11] Rahimov F, Marazita ML, Visel A, et al. Disruption of an AP-2alpha binding site in an IRF6 enhancer is associated with cleft lip. Nat Genet 2008;40:1341–7.

[12] Sozen MA, Suzuki K, Tolarova MM, et al. Mutation of PVRL1 is associated with sporadic, non-syndromic cleft lip/palate in northern Venezuela. Nat Genet 2003;29:141–2.

[13] Pauws E, Stanier P. FGF signalling and SUMO modification: new players in the aetiology of cleft lip and/or palate. Trends Genet 2007;23:631–40.

[14] Akuraya FS, Saadi I, Lund JJ, et al. SUMO1 haploinsufficiency leads to cleft lip and palate. Science 2006;313:1751.

[15] Mangold E, Ludwig KU, Birnbaum S, et al. Genome-wide association study identifies two susceptibility loci for nonsyndromic cleft lip with or without cleft palate. Nat Genet 2010;42:24–6.

[16] Beaty TH, Murray JC, Marazita ML, et al. A genome-wide association study of cleft lip with and without cleft palate identifies risk variants near MAFB and ABCA4. Nat Genet 2010;42:525–9.

[17] Ludwig KU, Mangold E, Herms S, et al. Genomewide meta-analyses of nonsyndromic cleft lip with or without cleft palate identify six new risk loci. Nat Genet 2012;44:968–71.

[18] Hallonet M, Hollemann T, Pieler T, et al. a novel homeobox-containing gene, directs development of the basal forebrain and visual system. Genes Dev 1999;15:3106–14.

[19] Rojas-Martinez A, Reutter H, Chacon-Camacho O, et al. Genetic risk factors for nonsyndromic cleft lip with or without cleft palate in a Mesoamerican population: evidence for IRF6 and variants at 8q24 and 10q25. Birth Defects Res A Clin Mol Teratol 2010;88:355–7.

[20] Figuiredo JC, Ly S, Raimondi H, et al. Genetic risk factors for orofacial clefts in Central Africans and Southeast Asians. Am J Med Genet A 2014;164A:2572–80.

[21] Pan Y, Ma J, Zhang W, et al. Replication of two novel susceptibility loci for non-syndromic orofacial clefts in a Chinese population. Oral Dis 2011;17:304–8.

[22] Barrett JC, Fry B, Maller J, et al. Haploview: analysis and visualization of LD and haplotype maps. Bioinformatics 2005;21:263–5.

[23] Hahn LW, Ritchie MD, Moore JH. Multifactor dimensionality reduction software for detecting gene-gene and gene-environment interactions. Bioinformatics 2003;19:376–82.

[24] Slavotinek AM, Chao R, Vacik T, et al. VAX1 mutation associated with microphthalmia, corpus callosum agenesis, and orofacial clefting: the first description of a VAX1 phenotype in humans. Hum Mutat 2012;33:364–8.

[25] Nikopoulopoulos T, Birnbaum S, Ludwig KU, et al. Susceptibility locus for non-syndromic cleft lip with or without cleft palate on chromosome 10q25 confers risk in Estonian patients. Eur J Oral Sci 2010;118:317–9.

[26] Zawislak A, Wozniak K, Jakubowska A, et al. Polymorphic variants in FGFR1, VCL, CX43 and VAX1 in Brazilian patients with nonsyndromic cleft lip/palate. Hum Mutat 2012;33:364–8.

[27] Bagordakis E, Paranaiba LM, Brito LA, et al. Polymorphisms at regions 1p22.1 (rs560426) and 8q24 (rs1530300) are risk markers for nonsyndromic orofacial clefts in the Brazilian population. Am J Hum Genet 2010;86:580–4.

[28] de Aquino SN, Messem AC, Bagordakis E, et al. Polymorphisms in FGF12, VCL, CX43 and VAX1 in Brazilian patients with nonsyndromic cleft lip with or without cleft palate. BMC Med Genet 2013;14:53.

[29] Mostowska A, Hozyaś KK, Wojcicki P, et al. Associations of folate and choline metabolism gene polymorphisms with orofacial clefts. J Med Genet 2010;47:809–15.