Original

Associations of ABCA4 and MAFB with Nonsyndromic Cleft Lip with or without Cleft Palate in a Northeastern Chinese Population
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Abstract: Nonsyndromic cleft lip with or without cleft palate (NSCL/P) is a common congenital deformity worldwide with multifaceted etiology. The interactions of genes and environmental factors may be related to NSCL/P susceptibility. In the present study, we aimed to identify the relationship between ABCA4 (rs481931, rs560426) and MAFB (rs17820943) polymorphisms and NSCL/P in a northeastern Chinese population. A total of 248 patients, including 98 nuclear families, and 280 healthy controls were recruited for this study. The three SNPs were genotyped using the polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) method. Differences in the allele and genotype frequency between cases and controls were evaluated by chi-square test with OR values and 95% confidence intervals. The family-based association test 5.5 (FBAT 5.5) software package for genetic analysis of pedigrees with complex diseases was used to evaluate the patients and their parents’ core families. In MAFB, statistical evidence of an association between rs17820943 (p=0.022) and NSCL/P was observed in case-control analyses. FBATs showed over-transmission of the C allele at the rs481931 polymorphism (p=0.039). However, no statistically significant difference was observed at rs560426. In addition, the C (rs481931)-G (rs560426) haplotype (P=0.004) was associated with the occurrence of NSCL/P. This study shows that rs481931 in ABCA4 and rs17820943 in MAFB are involved in the occurrence of NSCL/P in a northeastern Chinese population.

Key words: NSCL/P, ABCA4, MAFB, SNPs, Associations

Introduction
Cleft lip with or without cleft palate (CL/P) is the most common birth defect, affecting 135,000 newborns every year worldwide. The incidence of CL/P varies among different populations. Asian populations exhibit the highest incidence at almost 1/500, while African populations exhibit the lowest incidence at 1/2,500. The incidence rate is 1/1,000 in Europe, and the prevalence of CL/P in China is 1.42/1,000, which is a considerable burden on both public health services and the affected families.

Nonsyndromic cleft lip with or without cleft palate (NSCL/P) refers to cleft lip, cleft palate or cleft lip with cleft palate, excluding other systematic deformities and syndromes; NSCL/P accounts for more than 70% of facial cleft deformities. NSCL/P is associated with a higher morbidity rate and longer treatment period than syndromic cleft lip and palate. The etiology of NSCL/P is complex, as it is known to be associated with multiple genetic loci and environmental factors. Twin and familial clustering studies have provided strong evidence for a genetic component of NSCL/P. Following advances in molecular biology technology, NSCL/P has been subjected to wide-coverage association studies (GWAS), and a number of gene loci that are closely related to the occurrence of cleft lip and palate have been identified.

Based on the studies by Mangold and Beatty et al., rs481931 and rs560426 in ABCA4 and rs17820943 in MAFB are risk factors of NSCL/P. Bagordakis showed that rs560426 is a risk marker in Brazilian populations; however, Borges showed opposite results despite studying almost the same population. In the study of South Indian populations by Babu et al., rs481931 and rs560426 in ABCA4 were not related to NSCL/P. A study by Duan et al. also did not reveal an association between these three SNPs and NSCL/P in Chinese populations. Altogether, rs481931 (1p22), rs560426 (1p22) and rs17820943 (2q12) are three newly reported gene loci that are closely related to the occurrence of NSCL/P. However, the conclusions are inconsistent in different populations. Therefore, in this study, correlations between the three loci rs481931 (1p22), rs560426 (1p22) and rs17820943 (2q12) and NSCL/P were investigated in a northeastern Chinese population.

Materials and Methods
This study was approved (No. 2013 [17]) by the Ethics Committee of the Affiliated Stomatological Hospital of China Medical University. Informed consent was signed by all subjects before the initiation of the study. The guardians of the children signed the informed consent on their behalf.

Patients
A total of 248 patients with NSCL/P were recruited between 2013 and 2015 from the Department of Oral and Maxillofacial Surgery of the Affiliated Stomatological Hospital of China Medical University (Shenyang, Liaoning, the People’s Republic of China), including 98 core families (patients and their parents). All patients underwent preoperative examinations to exclude palatal heart syndrome, Vander Woude Syndrome, Kabuki Made-Up Syndrome, Meckel, etc. Additionally, 280 control subjects without congenital deformities were recruited from the Liaoning Province Research Institute of Family Planning. A five-milliliter peripheral blood sample was collected. DNA was obtained from the blood samples with a TIANamp Blood DNA kit (TianGen, Beijing, China) and used as the template for PCR amplification.
Genotyping

Genotyping of the variants was performed using the polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) method. The primers sequences, restriction enzymes and amplicon sizes are shown in Table 1. PCR was performed using 2×Taq reagent (TianGen, Beijing, China) according to the manufacturer's protocol. Each 20-μl PCR reaction system contained 1 μl of genomic DNA (50 ng/μl), 1 μl of each primer (10 μM), 10 μl of 2X Taq reagent and 7 μl of ddH₂O.

The PCR conditions included an initial denaturation at 95°C for 5 min followed by 30 cycles of 95°C for 30s and 58°C for 30s, extension at 72°C for 30s, and a final extension step at 72°C for 10 min. Restriction enzymes (NEB, Beijing, China) were used to digest the PCR products. Polyacrylamide gel electrophoresis (PAGE) and staining were performed for genotyping. For quality control, 20% of these samples were randomly repeated to verify the results.

Statistical analyses

SPSS for Windows version 17.0 (SPSS Inc., Chicago, IL) was used for statistical analysis. Hardy-Weinberg equilibrium (HWE) was assessed online using http://bioinfo/iconcologia.net/snpstats, a web-based association study program. Differences in the allele and genotype frequency between cases and controls were evaluated by chi-square test with OR values and 95% confidence intervals. A family-based association test (FBAT 5.5) software package (Harvard University, USA) for genetic analysis of pedigrees with complex diseases was used to evaluate the patients and their parents' core families.

Results

The genotype and allele distributions of rs481931, rs560426 and rs17820943

All three SNPs [rs481931 (1p22), rs560426 (1p22) and rs17820943 (20q12)] were in HWE (P>0.05), which indicated that the subjects were from the same Mendelian population. There was no evidence supporting an association of rs481931 and rs560426 with the risk of NSCL/P in the northeastern Chinese population (Table 2). However, a statistically significant increase in NSCL/P risk was observed for rs17820943. The CT genotype of rs17820943 was identified in 160 individuals in the

Table 1. PCR primer sequences

| SNPs          | Forward primer 5’-3’ | Reverse primer 5’-3’ | Product (bp) | Restriction enzyme |
|---------------|----------------------|----------------------|--------------|------------------|
| rs481931      | CTAGCTGCAACATAAATTCCG| GCATCCCACTCTCTCCTCTC| 143          | Cac8I            |
| rs560426      | TAGAGGACAGGGAAGTGT   | AGGTGTCTGCTATTGTGCC  | 295          | Hpy188III        |
| rs17820943    | CCAACTCCTCAACACATAC| AACCTTACCAGGGTCCTTTA | 277          | BciT             |

Table 2. The genotype and allele distributions of rs481931, rs560426 and rs17820943

| SNP          | Genotype/allele | NSCL/P       | Controls     | OR (95% CI) | X²    | P      |
|--------------|-----------------|--------------|--------------|-------------|-------|--------|
| rs481931     | CC              | 92(0.37)     | 91(0.32)     | reference   | —     | —      |
|              | AC              | 126(0.51)    | 154(0.55)    | 0.809(0.557-1.176) | 1.235 | 0.266  |
|              | AA              | 30(0.12)     | 35(0.13)     | 0.848(0.481-1.495) | 0.326 | 0.568  |
|              | AC+AA           | 156(0.63)    | 189(0.68)    | 0.816(0.570-1.169) | 1.227 | 0.268  |
|              | C               | 310(0.63)    | 336(0.60)    | reference   | —     | —      |
|              | A               | 186(0.37)    | 224(0.40)    | 0.900(0.702-1.154) | 0.692 | 0.405  |
| rs560426     | AA              | 103(0.42)    | 111(0.39)    | reference   | —     | —      |
|              | AG              | 116(0.46)    | 145(0.52)    | 0.862(0.600-1.239) | 0.643 | 0.423  |
|              | GG              | 29(0.12)     | 24(0.09)     | 1.302(0.712-2.381) | 0.737 | 0.391  |
|              | AG+GG           | 145(0.58)    | 169(0.60)    | 0.925(0.653-1.310) | 0.195 | 0.659  |
|              | A               | 322(0.65)    | 367(0.66)    | reference   | —     | —      |
|              | G               | 174(0.35)    | 192(0.34)    | 1.028(0.797-1.324) | 0.044 | 0.834  |
| rs17820943   | TT              | 45(0.18)     | 73(0.26)     | reference   | —     | —      |
|              | CT              | 160(0.65)    | 157(0.56)    | 1.653(1.073-2.546) | 5.253 | 0.022* |
|              | CC              | 43(0.17)     | 50(0.18)     | 1.395(0.804-2.422) | 1.404 | 0.236  |
|              | CT+CC           | 203(0.82)    | 207(0.74)    | 1.591(1.046-2.419) | 4.761 | 0.029* |
|              | T               | 250(0.50)    | 303(0.54)    | reference   | —     | —      |
|              | C               | 246(0.50)    | 257(0.46)    | 1.160(0.911-1.478) | 1.447 | 0.229  |

*P<0.05

Table 3. The FBAT results for rs481931, rs560426 and rs17820943

| Gene   | SNPs          | Allele | Frequency | Fam# | Z         | P     |
|--------|---------------|--------|-----------|------|-----------|-------|
| ABCA4  | rs481931      | A      | 0.382     | 68   | -2.063    | 0.039*|
|        |               | C      | 0.618     | 68   | 2.063     | 0.039*|
| rs560426| A             | 0.659  | 74        | 0.784| -0.784    | 0.432 |
|        | G             | 0.341  | 74        | 0.275| 0.275     | 0.432 |
| MAFB   | rs17820943    | C      | 0.550     | 87   | 0.275     | 0.783 |
|        | T             | 0.450  | 87        | -0.275| 0.275     | 0.783 |

Frequency: gene frequency. Fam#: number of families. Z: vector of the FBAT statistic. *P<0.05
NSCL/P group and 157 individuals in the control group, while the TT genotype was observed in 45 individuals in the NSCL/P group and 73 individuals in the control group. The OR of the CT genotype was 1.653 (95% CI: 1.073-2.546, P=0.022) compared with the TT genotype, while the OR of the CT+CC genotype was 1.591 (95% CI: 1.046-1.324, P=0.044) compared with the TT genotype.

**Family-based association test (FBAT)**

The FBAT results (Table 3) showed that the C allele (P=0.039) of rs481931 was over-transmitted from parents to children, indicating that the C allele might be associated with NSCL/P. No allele over-transmission was detected at the rs560426 and rs17820943 loci.

**Haplotype-based association test (HBAT)**

Haplotype analysis with HBAT was performed at rs481931 and rs560426, both of which are located in the ABCA4 gene (Table 4). The P value was 0.004 (<0.05) for the C (rs481931)-G (rs560426) haplotype, which indicated that this haplotype combination was significantly associated with the occurrence of NSCL/P.

**Discussion**

The ABCA4 gene is located on 1p22. A previous GWAS performed by Beaty and Fontoura supported an association of ABCA4 (rs481931 and rs560426) with NSCL/P: rs481931 of the ABCA4 gene was also shown to be related to NSCL/P in Polish populations. Subsequent correlation studies to validate candidate genes also showed a statistically significant association. However, this protein has not been detected in the palate of E13.3-E14.5 mouse embryos, and there is little biological evidence to support a role for ABCA4 in craniofacial morphogenesis. In addition, in this study, we genotyped 248 NSCL/P samples and 280 normal controls and found no significant difference in genotype and allele frequency at rs481931 or rs560426 (P=0.05), which conformed with the results reported by Babu et al. in a south Indian population. Some researchers, such as Pan et al., believe that the strong correlation between ABCA4 and cleft lip and palate may be attributed to the impact of nearby genes, not the impact of single loci. FBAT transmission analysis using case-parent core pedigrees showed that the C allele of rs481931 was over-transmitted from parents to children (P=0.039), indicating that the C allele is associated with NSCL/P. However, this transmission phenomenon was not observed in rs560426. This result was similar to that of Pan’s study, which showed that rs560426 was not associated with NSCL/P in an eastern Chinese population, but was opposite of that from a study by Mi et al. The different conclusions could be explained by selecting the different population in this study. Pan also noted that the occurrence of NSCL/P is related to the effect of mutation accumulation in six SNPs, including the rs481931 and rs560426 loci, and that greater numbers of mutated alleles correlated with the development of cleft lip and palate. Thus, although rs481931 and rs560426 were not shown to be correlated with the occurrence of cleft lip and palate in this study, whether they interact with other SNPs to cause NSCL/P cannot be excluded. Studies of HBAT using FBAT 5.5 suggested that the C (rs481931)-G (rs560426) haplotype (P=0.044) was involved in the occurrence of NSCL/P, which supports Pan’s conclusion. The inconsistent findings from previous studies may be attributed to analyzing each SNP separately and overlooking the impact of SNP interactions on the incidence of NSCL/P.

| Haplotype | Frequency | Fam# | Z    | P    |
|-----------|-----------|------|------|------|
| C A       | 0.378     | 43   | -0.649 | 0.516 |
| A A       | 0.272     | 44   | -0.928 | 0.354 |
| C G       | 0.211     | 39   | 2.882  | 0.004* |
| A G       | 0.139     | 29   | -1.142 | 0.254 |

Frequency: gene frequency. Fam#: number of families. Z: vector of the HBAT statistic. *P<0.05

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**Competing interests**

The authors declare that they have no competing interests.

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