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Single-nucleotide polymorphisms (SNPs) of the *IRF6* and *TFAP2A* in non-syndromic cleft lip with or without cleft palate (NSCLP) in a northern Chinese population

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ABSTRACT

Non-syndromic cleft lip with or without cleft palate (NSCLP) is a common birth defect that is presumably caused by genetic factors alone or gene alterations in combination with environmental changes. A number of studies have shown an association between NSCLP and single-nucleotide polymorphisms (SNPs) in the interferon regulatory factor 6 (*IRF6*) gene in several populations. The transcription factor AP-2a (*TFAP2A*), which is involved in regulating mid-face development and upper lip fusion, has also be considered a candidate gene contributing to the etiology of NSCLP. The potential importance of *IRF6* and *TFAP2A* in the NSCLP is further highlighted by a study showing that the two molecules are in the same developmental pathway. To further assess the roles of the *IRF6* and *TFAP2A* in NSCLP, we investigated two identified *IRF6* SNPs (rs2235371, rs642961) and three *TFAP2A* tag SNPs (rs3798691, rs1675414, rs303050) selected from HapMap data in a northern Chinese population, a group with a high prevalence of NSCLP. These SNPs were examined for association with NSCLP in 175 patients and 160 healthy controls. We observed a significant correlation between *IRF6* rs642961 and NSCLP, and a lack of association between *IRF6* rs2235371 polymorphisms and NSCLP in this population. This investigation indicated that there is no association between the three SNPs in the *TFAP2A* and NSCLP, suggesting that *TFAP2A* may not be involved in the development of NSCLP in the northern Chinese population. Our study provides further evidence regarding the role of *IRF6* variations in NSCLP development and finds no significant association between *TFAP2A* and NSCLP in this northern Chinese population.

1. Introduction

Cleft lip with or without cleft palate is a common birth defect found in more than 300 recognizable syndromes, but more often is observed as an isolated birth defect, called non-syndromic cleft lip with or without cleft palate (NSCLP). NSCLP, which occurs with a frequency of approximately 1/700 live births and causes significant facial anomalies [1,2]; the native American Indians and Asians have a even higher prevalence of orofacial clefting [3]. NSCLP is a complex malformation believed to be caused by the alterations of multiple genes and environmental factors; it is estimated that approximately 12–25% of the genetic variations associated with NSCLP have been identified [4]. Although genetic studies have identified a number of candidate genes and chromosomal regions associated with cleft lip and palate (CLP), findings from different studies have been inconsistent.

Interferon regulatory factor 6 (*IRF6*) is among the candidate genes associated with both syndromic and non-syndromic forms of CLP. Mutations in *IRF6* are linked to Van der Woude syndrome, in which CLP occurs in an autosomal dominant pattern with lip pits disorder (OMIM; 119500), suggesting that this gene may play a role in NSCLP [5]. Additionally, several recent studies have provided evidence for an association between polymorphic variations in *IRF6* and non-syndromic CLP. In a study of 36 SNPs within and around *IRF6* in 10 populations with ancestry in Asia, Europe, and South America, Zucchero et al. detected an altered transmission of a SNP rs2235371 in the overall sample [6]. When the populations were analyzed separately, evidence for altered transmission was still present in several groups. When haplotypes were examined, several haplotypes appeared to be significantly associated

Abbreviations: NSCLP, non-syndromic cleft lip with or without cleft palate; CLP, cleft lip and palate; CLO, cleft lip only; *IRF6*, interferon regulatory factor 6; *TFAP2A*, transcription factor AP-2a; BOFS, Branchio–Oculo–Facial; LDR, ligase detection reactions; PCR, polymerase chain reactions; HWE, Hardy–Weinberg equilibrium; LD, linkage disequilibrium; MAF, minor allele frequency; OR, odds radio.

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with NSCLP. Overall, Zucchero et al. estimated that the variations of *IRF6* accounted for approximately 12% of the genetic risk for NSCLP. In an Italian population, Scapoli et al. studied four of the SNPs reported by Zucchero et al. and found evidence for altered transmission of three of the four SNPs [7]. More recently, a study identified a strong association between cleft lip and *IRF6* rs642961 polymorphism, and showed that rs2235371 polymorphism is not associated with oral clefts without the rs642961 polymorphism [8].

Previous linkage studies have implicated a possible role of the transcription factor AP-2a (*TFAP2A*) gene region in NSCLP [9–11]. *TFAP2A* has been shown to play an etiologic role in the CLP syndrome, BOFS (Branchio–Oculo–Facial) [12]. More recently, the *TFAP2A* gene has been shown to bind to a regulatory element of *IRF6* involved in van der Woude syndrome [8]. In a timely review of murine genetic models for CLP, *TFAP2A* is one of several genes whose alterations are predicted to be linked to NSCLP but has not been examined in mutant animal models [13]. To date, there have been no association studies about the role of *TFAP2A* in human NSCLP pathogenesis.

In the present study, we evaluated two *IRF6* SNPs (rs2235371, rs642961) reported by Rahimov et al. [8] and three *TFAP2A* tag SNPs selected from the CHB HapMap data to investigate the association between these markers and NSCLP risk in a northern Chinese population, which is well suited for investigation of a common genetic etiology for NSCLP since these people are stable and lack of influx from other populations with high birth defect prevalence.

2. Materials and methods

2.1. Samples

All samples were unrelated Han Chinese in origin. A hundred and seventy-five NSCLP patients (94 females and 81 males, age range 1–28 yr) and 160 phenotypically normal individuals (85 females and 75 males, age range 4–35 yr) were recruited between 2006 and 2009 in the Affiliated Stomatology Hospital of Harbin Medical University and the Second Affiliated Hospital of Harbin Medical University. Written informed consent was obtained from all subjects and the Clinical Research Ethics Committee of Harbin Medical University approved the study.

Most previous studies combined the cleft lip only (CLO) and the cleft lip and palate (CLP) into a common etiologic risk groups [14]. However, recent epidemiologic evidence suggests that CLO may present as a separate entity [15]. In the current study, the 175 cleft lip patients with or without cleft palate (CL/P, $n = 175$) were classified as one group, while the CLO patients ($n = 50$) out of the 175 patients were taken as another separate group.

2.2. Marker selection and genotyping

A previous study by Rahimov et al. [8] demonstrated a strong association between cleft lip and *IRF6* rs642961 polymorphism and no relationship between rs2235371 polymorphism and oral clefts independent of the rs642961 polymorphism. A study [16] in a Brazilian population showed a lack of involvement of *IRF6* rs642961 and rs2235371 polymorphisms in the NSCLP pathogenesis, while studies in the Filipino population [6] and the western Chinese population [17] showed that rs2235371 was associated with NSCLP. In the present investigation, we selected the two SNPs in the *IRF6* gene (rs2235371, rs642961) to evaluate their relationship with NSCLP in a northern Chinese population.

For *TFAP2A* analysis, three SNPs (rs3798691, rs1675414, and rs303050) were selected using the HapMap project for association studies with minor allele frequencies greater than 0.05 in the CHB population. The primary information of these three SNPs within the *TFAP2A* gene is shown in Table 1.

After obtaining informed consent, blood samples were collected and DNA was extracted using AxyPrep-96 DNA Isolation kit following the manufacturer's protocol. All genotyping experiments were performed using ligase detection reactions (LDR). The target DNA sequences were amplified using multiplex polymerase chain reactions (PCR), then treated with 1 μl of proteinase K (20 mg/ml), heated at 72 °C for 7 min and quenched at 94 °C for 15 min. Ligation was performed in a final volume of 20 μl containing 20 mM Tris–HCl (pH 7.6), 25 mM potassium acetate, 10 mM magnesium acetate, 10 mM dithiothreitol (DTT), 1 mM NAD, 0.1% Triton X-100, 10 μl of Multiplex PCR product, 1 pmol of each discriminating oligonucleotide, 1 pmol of each common probe and 0.5 μl of 40 U/μl Taq DNA ligase (New England Biolabs, Berverly, MA, USA). The LDR was performed using 35 cycles of 94 °C for 30 s and 60 °C for 2 min. The fluorescent products of LDR were differentiated using an ABI sequencer 377 (Applied Biosystems, Foster City, CA, USA).

2.3. Statistical analysis

Comparison of genotype and allele frequencies among case groups (CL/P and CLO cases) and the control group was analyzed by the $\chi 2$ test. Pairwise linkage disequilibrium (LD) was computed as both D' and r^2 for all the three SNPs in *TFAP2A*, by use of the haploview program (http://www.broad.mit.edu/mpg/haploview/index.php/). All genotype frequencies and Hardy–Weinberg equilibrium (HWE), pair-wised linkage disequilibrium (LD) and haplotype analysis were conducted online using http://bioinfo/iconcologia.net/snpstats, a web-based association study application program. Association studies are based on regression analyses in accordance with the single SNPs [18].

3. Results

All SNPs were in HWE. Allele frequencies of the SNPs in *IRF6* gene are listed in Table 1. There were significant differences in the allele frequencies of rs642961 ($P = 0.002$) between NSCLP and control groups, while no significant discrepancies of allele frequencies for rs2235371 were found between the two groups.

Table 1
Primary information for SNPs and allelic distribution.

Gene	SNP ID	Genomic location	Base change	MAF[a] for control	CL/P			CLO		
					MAF[a]	*P* value[b]	*P* value[c]	MAF[a]	*P* value[b]	*P* value[c]
Irf6	rs2235371	Exon – 7	C > T	0.36	0.33	0.20	0.39	0.30	0.15	0.73
Irf6	rs642961	Enhancer	G > A	0.15	0.24	**0.002**	1.00	0.27	**0.009**	0.47
Tfap2a	rs3798691	Intron – 1	G > C	0.27	0.24	0.20	0.84	0.23	0.29	1.00
Tfap2a	rs1675414	Exon – 1	A > G	0.15	0.16	0.40	1.00	0.18	0.26	1.00
Tfap2a	rs303050	Intron – 4	T > C	0.34	0.32	0.30	0.22	0.30	0.29	0.32

[a] MAF, (minor allele frequency) in HapMap database for CHB population.
[b] *P* value by χ^2 test.
[c] *P* value for HWE.