Association of single-nucleotide polymorphisms, rs2235371 and rs2013162, in the *IRF6* gene with non-syndromic cleft palate in northeast China

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ABSTRACT. The aim of this study was to determine the association between two SNPs (rs2235371 and rs2013162) in the interferon regulatory factor 6 (*IRF6*) gene and non-syndromic cleft palate (NSCP) in northeast China. We genotyped these two SNPs in 104 NSCP cases, as well as in 178 parents and 300 controls. Case-control and case-parent analyses were performed using χ^2 tests and family-based association tests (FBAT). Results indicated that there were significant differences in both genotypic and allelic distributions between patients and controls at rs2235371 and rs2013162 in the *IRF6* gene. Case-parent analysis revealed over-transmission of the C allele in rs2235371 and the A allele in rs2013162. Lastly, FBAT showed over-transmission of the CA haplotype. This study demonstrated that the two SNPs, rs2235371 and

rs2013162, are strongly associated with NSCP in the northeast Chinese population.

Key words: Non-syndromic cleft palate; Interferon regulatory factor 6; Single nucleotide polymorphism

INTRODUCTION

Non-syndromic cleft lip and/or palate (NSCL/P) is usually categorized into non-syndromic cleft palate (NSCP) and non-syndromic cleft lip with or without cleft palate (NSCL ± P). It is known that NSCL/P arises as a multi-factorial trait, which includes a strong genetic component, as well as environmental overlays and stochastic factors (Dixon et al., 2011). Recent epidemiologic data suggest that cleft lip may have unique etiologic features, including strong genetic associations, while some individuals with cleft palate show evidence of sub-clinical cleft lip. (Grosen et al., 2010). Furthermore, separate cellular and genetic etiologies for cleft lip and/or palate are consistent with the general observations (Figueiredo et al., 2014).

Previous data have indicated that the interferon regulatory factor 6 (*IRF6*) gene is significantly associated with NSCL/P (Birnbaum et al., 2009; Beaty et al., 2010; Figueiredo et al., 2014). The *IRF6* gene is located at 1q32.3-q41, and codes for IRF6, which plays a vital role during craniofacial development. Scapoli et al. (2005) examined four SNPs in *IRF6* of 219 Italian families via transmission disequilibrium tests, and found transmission disequilibrium at rs2013162 and rs2235375 in NSCL/P nuclear families. Blanton et al. (2005) also confirmed these results in American populations. Studies among different populations showed that the *IRF6* gene contributes to NSCL/P risk (Jugessur et al., 2008; Wu et al., 2010; Li et al., 2012).

This result is further supported by experiments using animal models. Recent studies have found that *IRF6* mutant mice exhibit a hyper-proliferative epidermis that fails to undergo terminal differentiation, which leads to multiple epithelial adhesions that can occlude the oral cavity and result in NSCL/P (Ingraham et al., 2006; Richardson et al., 2006). These results demonstrated that the *IRF6* gene plays a critical role during the keratinocyte proliferation/differentiation switch. Subsequent studies indicated that this gene also plays a key role in oral periderm formation and spatio-temporal regulation, which is essential in ensuring appropriate palatal adhesion (Thomason and Dixon, 2008).

However, there are few studies examining the association between the *IRF6* gene and risk to NSCP. Therefore, we here investigated the association between rs2013162 and rs2235371 in the *IRF6* gene and NSCP in a northeast Chinese population.

MATERIAL AND METHODS

Subjects

We recruited 104 NSCP patients who were diagnosed by surgeons in the Affiliated Stomatological Hospital of China Medical University. In addition, the parents of these patients (86 fathers, 92 mothers, and 78 nuclear families), as well as 300 healthy volunteers, were also included in the study. All participants were from northeast China, and written informed consents were obtained from all subjects prior to enrolment into the study. We also obtained approval for all study protocols from the Ethics Committee of Liaoning Province.

PCR, extension, hybridization, and scanning of microarrays

The 20-μL PCR mixture contained 1X PCR buffer, 0.125 mM dNTPs, 0.4 μM primer (Table 1), 0.05 U Ex Taq (Takara, Dalian, China) and 2.5 ng genomic DNA. The thermal cycling parameters were as follows: 10 min at 94°C, followed by 35 cycles of 94°C for 30 s, 57°C for 30 s, 72°C for 30 s, with a final 3-min incubation at 72°C. The specific primer extension system (15 μL) contained 5 μL PCR product, 0.375 μL dATP + 0.375 μL dTTP + 0.375 μL dGTP + 0.375 μL Cy3-dCTP (Amersham, Buckinghamshire, UK), 0.02 U VentR (exo-) DNA polymerase (New England Biolabs, Ipswich, MA, USA), and 0.025 μM extension primer (Table 1). The reaction conditions were as follows: 94°C for 3 min; 35 cycles of 94°C for 30 s, 55°C for 30 s, 72°C for 45 s, with a final 3-min incubation at 72°C.

Table 1. Sequences of PCR primers, probes, and extension primers for IRF6 SNPs.

SNP	PCR primers	Probes (5'-3')[a]	Extension primer (5'-3')
rs2235371	GAGTCACAGGGATGAACAGG GCTTCTGCTTCTCATTGGTA	CGTTCAGATAGAGCCACTGATGAGG ATGAGGTACACCAAGCGATTCATCC	CCTCATCAGTGGCTCTATCTGAACGTC CCGTCAGCCTGGAGCAGC GGATGAATCGCTTGGTGTACCTCATTC CCGTCAGCCTGGAGCAGT
rs2013162	CCCTGGGATGAGAAGGATAA	GCTTATCGGAAGTGAACGAATACTT GATAGGATTAGAAGGTCGAACCGTT	AAGTATTCGTTCACTTCCGATAAGCA AGATGAGCTGGATCAGTCC AACGGTTCGACCTTCTAATCCTATCAA GATGAGCTGGATCAGTCA
Positive control	ACCTCTGACTCCCACTTGCT	TGATAAAGTTGGGGATCTGTAGAGGCAGCC	GGCTGCCTCTACAGATCCCCAACTTTA TCA-Cy3

[a]Probes were modified with amino at the 3' end. SNP = single-nucleotide polymorphism.

The microarray probes were bound to the aldehyde-modified slides. These chips were hybridized with extension reaction products, which were mixed with positive control markers. Hybridization was performed at 60°C for 1 h in the hybridization oven.

Microarrays were scanned by the GenePix 4000B scanner (Axon Instruments, USA), and the fluorescent intensity of each probe was read. Genotypes were identified by calculating the allelic fractions (AF) of each SNP. AF was calculated according to the following formula:

$$AF = B\ allele / (A\ allele + B\ allele) \quad \text{(Equation 1)}$$

If $AF < 0.4$, the genotype was AA; if $0.4 \leq AF \leq 0.6$, the genotype was AB; if $AF > 0.6$, the genotype was BB.

Statistical methods

Hardy-Weinberg equilibrium (HWE) was used to assess the SNPs in control subjects. Case-control analysis was performed using χ^2 statistics. Case-parent analysis was performed by family-based association test (FBAT).

RESULTS

There was no evidence of deviation from HWE for any of the SNPs in the controls ($P > 0.05$). To ensure accuracy of the SNP genotypes, 10% of the samples was selected at random to be sequenced directly. Both methods produced identical results.

Distributions of alleles and genotypes among cases were compared with that of the controls (Table 2). For rs2235371 and rs2013162 in the *IRF6* gene, case-control analysis revealed significant differences in both genotypic (P = 0.001, P = 0.001) and allelic (P = 0.001, P = 0.002) distributions.

Table 2. Genotypic and allelic distributions for rs2235371 and rs2013162 in case-control analysis.

SNPs			Controls	χ^2	P value
rs2235371	Genotypes				
	CC	54 (0.52)	114 (0.38)	10.891	0.001
	CT	44 (0.42)	134 (0.45)		
	TT	6 (0.06)	52 (0.17)		
	Alleles				
	C	152 (0.73)	362 (0.60)	10.837	0.001
	T	56 (0.27)	238 (0.40)		
rs2013162	Genotypes				
	CC	24 (0.23)	116 (0.39)	10.543	0.001
	CA	60 (0.58)	152 (0.50)		
	AA	20 (0.19)	32 (0.11)		
	Alleles				
	C	108 (0.52)	384 (0.64)	9.460	0.002
	A	100 (0.48)	216 (0.36)		

FBAT revealed strong associations between both SNPs (rs2235371 and rs2013162) and NSCP. The C allele (Z = 4.417, P = 0.001) at rs2235371 and the A allele (Z = 4.472, P = 0.001) at rs2013162 showed over-transmission from parent to child (Table 3).

Table 3. FBAT for rs2235371 and rs2013162 in case-parent trios.

SNP	Allele	Frequency	Fam No.	Z	P value
rs2235371	C	0.609	56	4.417	0.001
	T	0.391	56	-4.417	0.001
rs2013162	C	0.641	56	-4.472	0.001
	A	0.359	56	4.472	0.001

The CA haplotype (order: rs2235371-rs2013162) showed over-transmission (P = 0.001, Z = 4.463) in case-parent trios. This significant evidence indicated that the CA haplotype (order: rs2235371-rs2013162) attributes to NSCP. Lastly, the TC haplotype showed under-transmission (P = 0.001, Z = -5.146) in case-parent trios (Table 4).

Table 4. Haplotypes showing transmission distortion.

Haplotype	Frequency	Fam No.	Z	P value
CC	0.356	66	0.479	0.632
TC	0.285	60	-5.146	0.001
CA	0.253	50	4.463	0.001
TA	0.106	38	1.155	0.248

Order of SNPs: rs2235371- rs2013162.

DISCUSSION

Developmental genetic studies have shown that the mechanisms of disease onset are different between NSCL ± P and NSCP. Moreover, the incidences of these diseases also vary with gender: NSCL ± P is more common in men, whereas NSCP is more common in women. In

a previous study, it was found that two SNPs in the transforming growth factor alpha (TGFα) gene are associated with NSCL ± P, but not NSCP, in a northern Chinese population (Xu et al., 2014). In recent years, various investigations have shown the *IRF6* gene is involved in NSCL/P (Birnbaum et al., 2009; Beaty et al., 2010), but studies on the association between the *IRF6* gene and risk to NSCP are very limited. In this study, we investigated the association between the SNPs rs2013162 and rs2235371 in *IRF6* and NSCP in a northeast Chinese population using case-control and case-parent analysis.

We found that the frequency of the C allele at rs2235371 was 60% in controls. Similar results were also reported in studies by Zucchero et al. (2004) and Srichomthong et al. (2005) (58 to 66% in East Asia and Southeast populations, and 61% in Thailand populations). However, these frequencies may differ among populations (90-100% in European populations and 100% in African populations) (Zucchero et al., 2004). Significant differences in genotype ($P = 0.001$) and allele ($P = 0.001$) frequencies at rs2235371 between cases and controls were found in this study. We detected significantly higher frequency of the C allele in the NSCP patients (73%) as compared with the controls (60%). This suggested that the C allele may be associated with an increased risk for NSCP. Such risk effect was further supported by significant over-transmission in case-parent trios ($P = 0.001$, $Z = 4.417$) at the C allele of rs2235371. In contrast, Jugessur et al. (2008) suggested that rs2235371 in IRF6 is not related to NSCP in Norwegian populations ($P = 0.717$), but some other SNPs in IRF6 are related to NSCP.

A study by Jugessur et al. (2008) found a significant association between rs2013162 and NSCP ($P = 0.025$). In the present study, this association was confirmed via case-control analysis ($P < 0.05$). Furthermore, FBAT analysis showed over-transmission of the A allele at rs2013162 ($P = 4.472$, $Z = 0.001$). These results suggested that rs2013162 is a "risk allele" for NSCP in the northeast Chinese population.

Based on haplotype analysis, we found over- and under-transmission of the CA (order: rs2235371-rs2013162) and TC haplotypes, respectively. Therefore, the TC haplotype may have protective properties against NSCP. In addition, these results further supported the hypothesis that the C allele at rs2235371 and the A allele at rs2013162 are significantly associated with NSCP in the northeast Chinese population.

Lu et al. (2013) have previously reported that rs2235371 and rs2013162 in the *IRF6* gene are significantly associated with NSCL/P in a northeast Chinese population. Here, we found that those two SNPs are strongly associated with NSCP in the same population. Jugessur et al. (2008) observed that rs2013162, but not rs2235371, is associated with NSCP in a Norwegian population. These contradicting results may be attributed to genetic heterogeneity of different populations.

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