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ABSTRACT

Non-syndromic cleft lip, with or without cleft palate, is a heterogeneous, complex disease with a high incidence in the Asian population. Several association studies have been done on cleft candidate genes, but no reports have been published thus far on the Orofacial Cleft 1 (OFC1) genomic region in an Asian population. This study investigated the association between the OFC1 genomic region and non-syndromic cleft lip with or without cleft palate in 90 Malay father–mother–offspring trios. Results showed a preferential over-transmission of a 101-bp allele of marker D6S470 in the allele- and haplotype-based transmission disequilibrium test (TDT), as well as an excess of maternal transmission. However, no significant p-value was found for a maternal genotype effect in a log-linear model, although single and double doses of the 101-bp allele showed a slightly increased cleft risk (RR = 1.37, 95% CI, 0.527-3.4, p-value = 0.516). Carrying two copies of the 101-bp allele was significantly associated with an increased cleft risk (RR = 2.53, 95% CI, 1.06-6.12, p-value = 0.035). In conclusion, we report evidence of the contribution of the OFC1 genomic region to the etiology of clefts in a Malay population.

KEY WORDS: non-syndromic cleft lip and palate, transmission disequilibrium test (TDT), microsatellite, OFC1 genomic region.

Contribution of 6p24 to Non-syndromic Cleft Lip and Palate in a Malay Population: Association of Variants in OFC1

INTRODUCTION

Non-syndromic cleft lip with or without cleft palate (NSCL/P) is the most common congenital anomaly, with various prevalence rates among races and regions; rates are highest in Asian and lowest in African populations (Vanderas, 1987; Murray, 2002). The higher prevalence in Asia has provided beneficial resources for genetic investigations on clefts. The prevalence in the Malay population has been estimated at 1 in 600 (Boo and Arshad, 1990). Orofacial clefts are genetically heterogeneous, and this has resulted in inconsistencies in genetic mapping among various populations. Therefore, it has been suggested that between 2 and 14 genes may be involved in the etiology of oral clefts (Schliekelman and Slatkin, 2002). The OFC1 genomic region (Orofacial Cleft 1; MIM #119530) is one of the candidates that has been mapped to the 6p24.3 genomic region. Evidence for an etiologic role of 6p24.3 has been shown through several case reports of orofacial cleft with chromosomal abnormalities (Donnai *et al.*, 1992; Davies *et al.*, 1995, 1998; Balci *et al.*, 2004; Chen *et al.*, 2009) and further supported by linkage studies (Eiberg *et al.*, 1987; Carinci *et al.*, 1995; Scapoli *et al.*, 1997). This candidate region harbors 2 known candidate genes for oral clefts (*TFAP2A* and *F13A*), plus a novel gene named orofacial cleft 1 candidate 1 gene (*OFCC1*; NM_153003) (Davies *et al.*, 2004).

Here, we designed a family-based study to investigate the association between the OFC1 genomic region and non-syndromic cleft lip (with or without cleft palate) in 90 Malay father–mother–offspring trios. To date, no association study for the OFC1 genomic region has been published in an Asian population, and therefore, the present report is the first association study on this candidate region.

MATERIALS & METHODS

Study Population

We included a total of 270 individuals (90 father–mother–offspring trios) from unrelated families with non-syndromic cleft lip, with or without cleft palate. Of those families, 17 had only a cleft lip, and 73 had cleft lip and palate. All patients having cleft palate only, a known syndrome, or other major or multiple minor defects were excluded from this study. The families were recruited from the Reconstructive Sciences Unit of the Hospital Universiti Sains Malaysia, Kelantan. Kelantan is bordered by Thailand's Narathiwat

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Table 1. Primers Used for Amplification of Selected STRs

Primer	Primer Sequence and Label Dye	Tm	No. of Alleles	Het	Allele Size (bp)	Position (bp)
D6S940-F	5'-(6FAM)-GGCTCTTGCCACTGACTTTTTCT-3'	63	8	0.66	213-245	9842513-9842894
D6S940-R	5'-GCTTTGTCGAGTTTTAGCCATTCT-3'	63				
D6S470-F	5'-(6FAM)ACACATCCATCACCACACTTGCT-3'	64	11	0.79	93-123	10025715-10025955
D6S470-R	5'-GGTATCTCTTAAGCGATCTCACCATA-3'	61				
D6S296-F	5'(HEX)- TTCTATCCCAGGTTTCTCCAATAG-3'	61	19	0.78	241-296	8853649-8853997
D6S296-R	5'- GCTTCATCTATAATCAAGACACTCATC-3'	61				
D6S410-F	5'(HEX)-TCCCATCTCACCACCCAGTC-3'	63	8	0.63	179-191	8995335-8995707
D6S410-R	5'-CTGTGAGTTGTCAGCATGTGTGT-3'	61				

Tm, melting temperature; Het, heterozygosity; bp, base pairs; position, physical position of markers (UCSC Genome Browser). The 5' side of forward primers was labeled with fluorescent dyes.

Table 2. Multi-allelic Transmission-TDT with the TDTEX Program, Ignoring Parent of Origin, between Markers in and around the OFC1 Genomic and Non-syndromic Cleft Lip and Palate in 90 Malay Trios

	D6S410	D6S470 ^b	D6S296	D6S940
Multi-allelic Transmission TDT (<i>Exact test statistic</i>)	P-value	P-value	P-value	P-value
Informative count ^a	87	89	88	88
Exact McNemar test	0.37	0.02	-	0.7
Monte Carlo McNemar test	0.37	0.02	0.5	0.7
Monte Carlo Marginal Homogeneity	0.1	0.078	0.76	0.65

^aThe TDTEX program also provides the informative count for included families in analysis.

^bIn multi-allelic TDT with three different statistical tests, only marker D6S470 showed significant results (bold face), and 89 informative families for TDT are included in the analysis.

Province to the north, and therefore, there is some population admixture. However, 95% of Kelantanese and the majority of the Narathiwat province population have a Malay ethnic background. This study was approved by the Research Ethics Committee (Human) of the Universiti Sains Malaysia, and informed consent was obtained from all participants.

STR Selection and PCR Amplification

We selected 4 highly polymorphic short tandem repeat (STR) markers in and near the OFC1 genomic region, including D6S470, D6S940, D6S410, and D6S296; these markers have a heterozygosity over 0.63 (Table 1). Specific primers were designed for each marker with Primer3 software (Rozen and Skaletsky, 2000), and forward primers were labeled with fluorescent dyes (Table 1).

Genomic DNA was extracted from 200 μ L of peripheral blood with the use of a GeneAII^R ExgeneTM Genomic DNA isolation kit (GeneAll Biotechnology, Seoul, Korea). Multiplex PCR amplifications were performed with the Type-It Microsatellite Amplification Kit (QIAGEN, GmbH, Hilden, Germany) with a total volume of 25 μ L including the following, *per* reaction: 150-200 ng DNA, 12.5 μ L Type-It MasterMix, and 0.2 mM of each primer. The amplicons were subjected to electrophoresis on an ABI Prism 3100 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA) and analyzed by GeneScan v3.0 and Genotype v2.1 software (Applied Biosystems).

Statistical Analysis

The standard transmission disequilibrium test (TDT) was carried out in several ways (Spielman *et al.*, 1993). First, the TDTEX program, which is included in the S.A.G.E. package, was performed. The TDTEX program performs two different statistical tests. We used the exact statistical tests, including the Exact McNemar test, the Monte Carlo McNemar test, and Monte Carlo Marginal Homogeneity, which has been recommended by the program for studies with small sample sizes and markers with multiple alleles (Table 2). In addition, in re-analysis of the data, allele-wise TDT based on a logistic regression model and genotype-wise TDT were performed with the ETDT program (Sham and Curtis, 1995).

We used the FAMHAP package (version 19 beta) to estimate haplotype frequencies, to test for excess transmission of haplotypes, and to detect imprinting (Becker and Knapp, 2004a). A haplotype-based TDT was carried out with the HAP-TDT and HAP-TDTmax programs included in the FAMHAP package. The HAP-TDT program is based on Monte-Carlo simulations, in which the set of transmitted and non-transmitted genotypes is randomly permuted in each permutation replicate, while in HAP-TDTmax, the chi-square statistic of the marker combination is replaced with the maximum of the chi-squares of the single haplotypes (maxTDT statistic) (FAMHAP documentation, <http://famhap.meb.uni-bonn.de>). Although the Bonferroni correction is a suitable correction method for independent tests,

Table 3. Results of Allele- and Haplotype-based TDT

Marker Combination	Alleles/ Haplotypes	Frequency		TDT			
		AFF	NOTAFF	T (%)	NT	χ^2 -TDT (P-value)	$^aOR_{\text{Transmission}}$
D6S410	179	0.005	0.036	1 (12.5)	7	4.5 (0.034)*	0.143
D6S470-D6S296	93-264	0.017	0.052	2 (18)	9	4.5 (0.034)*	0.22
D6S470	101	0.46	0.33	56 (64)	32	6.54 (0.01)	1.75
D6S940-D6S296	237-276	0.08	0.03	11 (79)	3	4.57 (0.03)*	3.67
D6S470- D6S410	101-183	0.19	0.076	31 (70)	13	7.36 (0.007)	2.4
D6S470-D6S940- D6S410	101-229-183	0.04	0.005	7 (87)	1	4.5 (0.0234)*	7.0
D6S470-D6S410-D6S296	101-183-264	0.038	0.00	6 (100)	0	6 (0.014)*	-
D6S470-D6S410-D6S296	93-183-264	0.00	0.034	0 (0)	6	6 (0.014)*	-
D6S470-D6S940-D6S296	93-233-264	0.00	0.034	0 (0)	6	6 (0.014)*	-

AFF, Affected (frequency in affected case); NOTAFF, Not Affected (frequency of non-transmitted haplotypes as a pseudo-control); T, Transmitted; NT, Non-transmitted. The FAMHAP program was used with an expectation-maximization (EM) algorithm to calculate the haplotype frequencies. aOR , odds ratio for transmission of risk haplotype.

The p-values presented here are uncorrected; however, those indicated with "*" provide a corrected $p > 0.05$ with simulation procedures, whereas the bold-face values produced significant results after correction with simulation.

in the presence of linkage disequilibrium in the region, this may lead to the loss of power of the study (Becker and Knapp, 2004b); therefore, the FAMHAP program will correct the p-values *via* simulation procedures. The LD measures are computed from the frequency of each haplotype of these markers.

The HAP-PAT program, which is implemented in the FAMHAP package, considers maternal *vs.* paternal transmission to yield direct evidence for imprinting and also association *via* Monte-Carlo simulations. This program applies the Parental Asymmetry Test (PAT) that uses the preferential transmission of reference alleles to heterozygous children (Weinberg, 1999). Furthermore, the HAP-TDT-pat and HAP-TDT-mat programs, which consider only either paternal or maternal haplotype transmission, respectively, are used to detect the parent of origin and also association.

Subsequently, we performed a log-linear model to estimate single- and double-dose effects of risk alleles using the HAPLIN program, version 3.5 (Gjessing and Lie, 2006). This software uses the model that was introduced by Weinberg *et al.* (1998) and computes both relative risk and likelihood ratios. We used reciprocal reference and set a threshold of 0.01 (1%) for haplotype frequency that included 81 families in the HAPLIN analysis.

RESULTS

In total, 270 individuals (90 father–mother–offspring trios) were analyzed. The offspring were composed of 42 females and 48 males with non-syndromic cleft lip with or without cleft palate. Due to a high heterozygosity of markers, most of the families were informative and remained in the TDT analysis (Table 2). All markers except D6S410 were in Hardy-Weinberg equilibrium ($p > 0.05$); however, we did include D6S410 in the haplotype analysis. Results of the multi-allelic transmission-TDT showed a significant association for marker D6S470 by the McNemar test (Table 2). In re-analysis with the ETDT program, which is based on a logistic regression model, the allele-wise

($\chi^2 = 6.9$, df 4, $p = 0.14$; χ^2 for goodness of fit = 5.9, df 3, $p = 0.11$) and genotype-wise ($\chi^2 = 12.9$, df 7, $p = 0.07$) results were not significant, and only genotype-wise TDT showed a trend toward transmission distortion. The transmission of individual alleles, in contrast, showed preferential over-transmission of the 101-bp allele of D6S470 with high significance ($\chi^2 = 6.45$, p -value = 0.01, corrected p -value = 0.045, $OR_{\text{Transmission}} = 1.75$). The 101-bp was the most common allele, with a frequency of 0.46 among the affected offspring (Table 3).

We also conducted haplotype-based TDT using the FAMHAP package with combinations of 2, 3, and 4 markers. The results of haplotype analysis were significant, and the best haplotype was formed by the combination of the 101-bp allele and the 183-bp allele of markers D6S470 and D6S410, respectively ($\chi^2 = 7.36$, p -value = 0.007, corrected p -value = 0.06, $OR_{\text{Transmission}} = 2.4$) (Table 3). In pair-wise linkage disequilibrium (LD) analysis, markers D6S410 and D6S470 were in weak LD ($D' = 0.197$).

In addition, an imprinting trend was found for marker D6S470 (p -value = 0.067) when the data were analyzed with the HAP-PAT program. When the analysis was restricted to only maternal or paternal transmission according to the TDT approach, no evidence for paternal transmission was found. However, the results showed excess maternal transmission (corrected global p -value for all markers = 0.046), and highly significant results were obtained from the combination of D6S410, D6S470, and D6S940 marker alleles (best p -value, 0.0026). Of these alleles, only the 101-bp allele of D6S470 showed significant excess maternal transmission ($\chi^2 = 4.78$, $p = 0.028$, $OR_{\text{Transmission}} = 1.93$). Even when we used a transmission asymmetry test (TAT) that ignores parents who are both heterozygous, this allele showed a trend toward preferential maternal transmission ($\chi^2 = 3.57$, $p = 0.059$, $OR_{\text{Transmission}} = 2.1$).

The relative risk for the 101-bp allele of D6S470 was then analyzed by the likelihood ratio test (LRT), and, subsequently, we applied a log-linear model to estimate the increase in cleft risk associated with carrying single or double copies of the risk

Figure. The relative dose risk for D6S470 alleles with frequency > 1% for maternal and child genotypes. X indicates a single dose, whereas O indicates a double dose. Reciprocal reference has been used. Having one copy of the 101-bp allele showed a trend toward increasing the risk (RR = 1.92, 95% CI, 0.964-3.91, p-value = 0.067), while having 2 copies of the 101-bp allele was significantly associated with increased cleft risk (RR = 2.53, 95% CI, 1.06-6.12, p-value = 0.035), suggesting a recessive effect of this allele. Maternal single or double dose of the 101-bp allele showed slightly increased risk with a dominant effect (RR = 1.37, 95% CI, 0.527-3.4, p-value = 0.516 for double-dose effect), but the p-value is not statistically significant. There seemed to be a protective effect for mothers who are carrying either 2 copies of the 93-bp or 99-bp allele or a single copy of the 103-bp, 112-bp, or 106-bp allele. The alleles without a double-dose effect indicate that homozygotes are rare.

allele. The results of relative risk analysis showed that mothers who are carrying either single or double copies of the 101-bp allele have a slightly higher relative risk compared with those carrying other alleles with a deleterious dominant effect (RR = 1.37, 95% CI, 0.527-3.4, p-value = 0.516 for double-dose effect), but this did not produce a significant p-value (Fig.). However, carrying 2 copies of the 101-bp allele was associated with a significantly increased cleft risk for a child (RR = 2.53, 95% CI, 1.06-6.12, p-value = 0.035), suggesting a recessive effect of this allele.

DISCUSSION

Several unrelated cases have been described in which a chromosomal abnormality at 6p24 caused an orofacial cleft (Donnai *et al.*, 1992; Davies *et al.*, 1995, 1998; Balci *et al.*, 2004; Chen *et al.*, 2009), and subsequent linkage studies supported these findings (Eiberg *et al.*, 1987; Carinci *et al.*, 1995; Scapoli *et al.*, 1997). To our knowledge, this report is the first association study on the OFC1 genomic region in an Asian population. Allele- and haplotype-based TDT showed that a common allele of marker D6S470 was more frequently transmitted than expected according to Mendelian inheritance, and a range of predisposing alleles/haplotypes (> 50% transmission) and a protective allele (< 50% transmission) were observed (Table 3).

However, significant results were diminished after adjustment for those that were not highly significant. A positive association was observed for the 101-bp allele of D6S470 alone, and the combination of this allele with other marker alleles increased the significance of the results as well. The best haplotype was obtained in pair-wise combination of the 101-bp and 183-bp allele markers of D6S470 and D6S410, respectively. However, a positive but weak LD shows that the 101-bp and 183-bp alleles tend to segregate on the same chromosome more often than expected by chance. D6S470 is an intragenic marker located in intron 1 of the OFCC1 candidate gene. Translocation breakpoints in the OFCC1 gene have been reported in cases with orofacial clefts (Davies *et al.*, 2004). This gene is also known as *MRDS1* and encodes a 231-amino-acid protein (NP_694548). The function of this gene is unknown, but it is expressed in the hard palate (Davies *et al.*, 2004).

Our results suggest that the risk of oral cleft is associated with the OFC1 genomic region in the population studied and are in agreement with results from linkage studies (Eiberg *et al.*, 1987; Carinci *et al.*, 1995; Scapoli *et al.*, 1997). However, although cytogenetic and linkage studies have strongly supported the role of the OFC1 genomic region in the etiology of oral clefts, these data have not been replicated in various genome-wide association studies (Grant *et al.*, 2009; Mangold *et al.*, 2009; Beaty *et al.*, 2010). More recently, in a large family-based genome-wide association study, no significant results were found for genomic region 6p24 in five non-Malay Asian populations (Grant *et al.*, 2009; Beaty *et al.*, 2010).

In the present study, marker D6S470 showed excess maternal transmission. This result most likely reflects the maternal genotype effect rather than imprinting, because several unrelated cases of maternal UPD6 have been described with no clinical impact, except for unmasking single gene disorders (Eggermann *et al.*, 2001). However, a maternal genotype effect has been reported for isolated oral cleft and other disorders (Reutter *et al.*, 2008; Sull *et al.*, 2008, 2009). Our relative risk analyses revealed that both maternal and child genotypes for the 101-bp allele are in agreement, showing an increased cleft risk. Whereas the child genotype effect resulted in statistically significant results, the maternal genotype effect has insufficient power to produce a significant p-value.

Taken together, the results of the present study provide evidence that the OFC1 genomic region may play a role in the development of oral cleft in the Malay population. However, small sample sizes may limit our findings.

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