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FOXE1 mutations in Thai patients with oral clefts

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Summary

Non-syndromic oral clefts comprising cleft lip with and without cleft palate (CL/P) and cleft palate only (CPO) are common birth defects worldwide. Their aetiology involves both environmental and genetic factors. *FOXE1* has previously been reported to be associated with oral clefts in some populations. Here, we investigate whether mutations in *FOXE1* play a part in the formation of oral cleft in a Thai population. We first performed PCR–RFLP to genotype a previously reported associated polymorphism, c.-1204C>G (rs111846096), in our cohort. No association was found. In addition, two unrelated unaffected controls were found to be homozygous GG, indicating that homozygous GG at this c.-1204 position was not sufficient for the development of oral clefts. We then sequenced the entire coding region of *FOXE1* in 458 unrelated individuals (146 CPOs, 108 CL/Ps and 204 Thai controls). Five different non-synonymous variants, c.274G>T (p.D92Y), c.569C>G (p.P190R), c.569C>T (p.P190L), c.664C>T (p.R222C) and c.1090G>A (p.G364S), were identified in CPOs and one, c.572C>G (p.P191R), in CL/P. All these six variants were in heterozygous state, each identified in one patient, and absent in 204 controls. Except the p.P190R, which was previously reported, the other five variants were novel. Our study identifies probable susceptibility variants of *FOXE1* for oral clefts in the Thai population.

1. Introduction

Oral clefts including cleft lip with and without cleft palate (CL/P) and cleft palate only (CPO) are common complex birth defects. The frequency of oral clefts is about 1 in 700 live births worldwide. Both genetic and environmental factors play a major etiological role (Vieira, 2008). Our previous studies in the Thai population, similar to many others in various ethnic groups, showed associations between the birth defects and variants in several genes including *MTHFR*, *p63*, *MSX1*, *TBX22*, *PVRL1*, *IRF6* and *PDGFRa* (Shotelersuk *et al.*, 2003; Leoyklang *et al.*, 2006; Tongkobpetch *et al.*, 2006, 2008; Suphapeetiporn *et al.*, 2007; Yeetong *et al.*, 2009; Rattanasopha *et al.*, 2012). Recently, *Forkhead box E1* (*FOXE1*) was reported to be associated with non-syndromic oral clefts in various populations such as *FOXE1* rs1443434 and patients with CL/P from six countries (Philippines, Colombia, China, India, Turkey and USA) (Marazita *et al.*, 2009) and *FOXE1* rs7860144 and patients with CL/P in the Estonian, Latvian and Lithuanian populations (Nikopensius *et al.*, 2011).

FOXE1 or *thyroid transcription factor 2* (*TTF2*) is located on chromosome 9q22. It consists of one exon encoding a 367-amino-acid protein with a molecular weight of 42 kD (Castanet & Polak, 2010). This protein contains the important forkhead domain with a highly conserved 100-amino acid DNA-binding motif (Castanet & Polak, 2010). Some *FOXE1* mutations located in the forkhead domain were associated with congenital hypothyroidism and CL/P phenotype in Bamforth syndrome (Bamforth *et al.*, 1989;

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Table 1. *Characteristics of the six non-synonymous variants in the FOXE1 gene (NC_000009.11) identified in patients with oral clefts*

	1	2	3	4	5	6
Nucleotide change	c.274G>T	c.569C>T	569C>G	c.572C>G	c.664C>T	c.1090G>A
Disease	CPO	CPO	CPO	CL/P	CPO	CPO
State	Heterozygous	Heterozygous	Heterozygous	Heterozygous	Heterozygous	Heterozygous
Amino acid change	D92Y	P190L	P190R	P191R	R222C	G364S
Functional domain*	Forkhead	N/A	N/A	N/A	N/A	N/A
Polarity change	Polar>polar	Non-polar>non-polar	Non-polar>polar	Non-polar>polar	Polar>polar	Non-polar>polar
Evolutionary conservation	Yes	No	No	No	Yes	No
SIFT (scoring)	Affect protein function (0·01)	Tolerated (0·21)	Tolerated (0·3)	Tolerated (0·58)	Affect protein function (<0·01)	Tolerated (0·18)
PolyPhen-2 (scoring)	Probably damaging (1·000)	Benign (0·295)	Possibly damaging (0·745)	Benign (0·232)	Probably damaging (0·990)	Probably damaging (0·996)

* Data from Alamut program v.2.2; N/A, not applicable.

Castanet *et al.*, 2002; Baris *et al.*, 2006). In addition, a mutation, c.-1204C>G (rs111846096), at the 5′ untranslated region of *FOXE1* reducing *FOXE1* mRNA expression via decreasing the binding ability of MYF-5 protein to this putative promoter region, was found to be associated with CPO in an Italian population (Venza *et al.*, 2009).

In this study, we sequenced the entire coding region of *FOXE1* in 146 CPOs, 108 CL/Ps and 204 Thai controls. Five different non-synonymous variants were identified in CPOs and one in CL/P. None of them were found in controls. Our study suggests that *FOXE1* is associated with oral clefts in the Thai population.

2. Materials and methods

(i) *Subjects*

We recruited 146 unrelated patients with non-syndromic CPO and 108 unrelated patients with non-syndromic CL/P from the Smart Smile and Speech Project. This project aimed to treat patients with oral clefts and other birth defects in under-served areas of Thailand. Healthy controls were blood donors with no oral clefts and no history of oral clefts in their families. DNA was extracted from either leukocytes or dried blood spots. This study was approved by the Institutional Review Board of the Faculty of Medicine, Chulalongkorn University. Informed consent was obtained from each participant. None of the parents of patients with non-synonymous variants were available for studies.

(ii) *Mutation analysis*

A total of 77 patients with CPO and 90 controls were initially genotyped for a variant, c.-1204C>G (rs111846096), at the 5′ non-coding region of *FOXE1* by PCR–RFLP as described (Venza *et al.*, 2009).

Subsequently, we performed PCR-sequencing of the entire coding region of *FOXE1* (NC_000009.11) in 146 CPOs, 108 CL/Ps and 204 healthy controls. Primers FOXE1E1F 5′-AGA AGG GCC GAG CGT CCG TT-3′ and FOXE1E1R 5′-GGT CCC AGT TGA GTC CTC TC-3′were used to amplify the coding exon of *FOXE1*. The 20 μl of PCR reaction contained 50–100 ng of genomic DNA, 200 μM of each dNTP, 150 nM of each primer, 1·5 mM $MgCl_2$, 0·5 unit of *Taq* DNA polymerase (Fermentas Inc., Glen Burnie, MD) and 10% of DMSO. The PCR condition was started with 95 °C for 5 min for pre-denaturation followed by 35 cycles of 94 °C for 30 s, 60 °C for 30 s and 72 °C for 90 s. The size of the amplified product was 1316 bp. For sequencing, PCR products were treated with ExoSAP-IT (USP Corporation, Cleveland, OH), and sent for direct sequencing at

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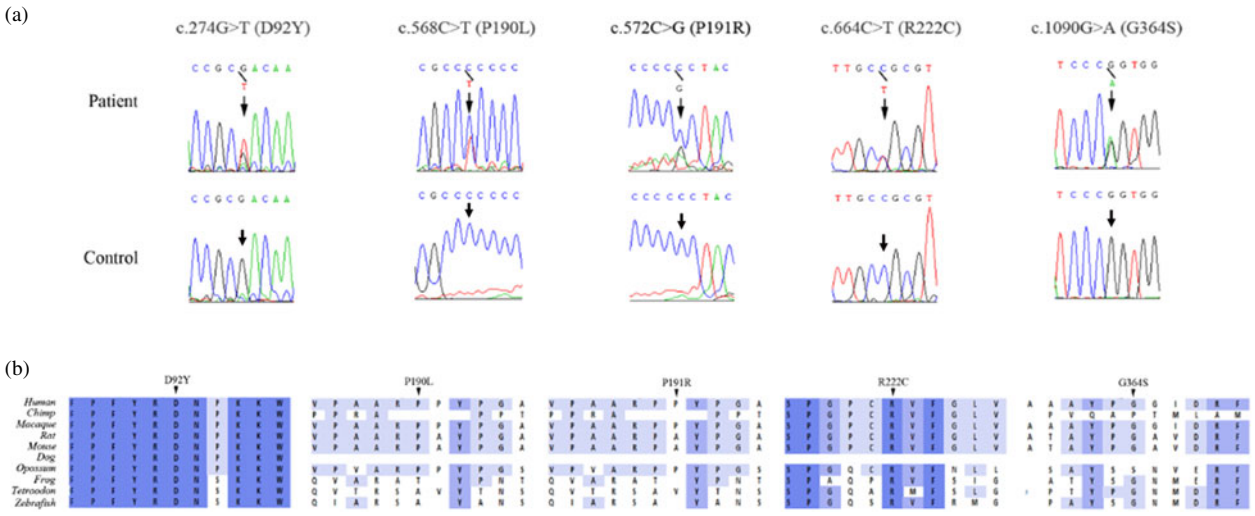

Fig. 1. (A) Electropherograms of the five novel non-synonymous variants, c.274G>T (p.D92Y), c.569C>T (p.P190L), c.572C>G (p.P191R), c.664C>T (p.R222C) and c.1090G>A (p.G364S), identified in patients with oral clefts (upper), compared with those of controls (lower). The positions of the heterozygous variants were indicated by arrows. (B) Sequence alignment of FOXE1 from different species. The site of the amino acid variant found in this study is indicated by arrow heads above the human FOXE1 sequence. Sites that are conserved are highlighted.

Macrogen Inc. (Seoul, Korea). Sequencing was done bi-directionally by using FOXE1E1F and FOXE1E1R primers and two internal primers (FOXE1F2 5′-GCA ACT ACT GGG CGC TTG AC-3′ and FOXE1F3 5′-ATC TTC CCA GGC GCG GTG-3′). Analyses were performed by Sequencher 4.2 (Gene Codes Corporation, Ann Arbor, MI). SIFT (Sorting Intolerant From Tolerant; http://sift.bii.a-star.edu.sg/www/SIFT_seq_submit2.html) and PolyPhen (http://genetics.bwh.harvard.edu/pph2/) were used for prediction of the possible impact of amino acid substitutions on the stability and function of the mutant proteins. Alamut program v.2.2 (trial version) was used to determine evolutionary conservation of the mutated codons.

3. Results

Genotyping the c.-1204C>G (rs111846096) showed that, of the 77 CPOs and 90 controls, 16 and 23 were heterozygous C/G, whereas 1 and 2 were homozygous G/G, respectively. This gave the frequencies of the G allele of 0·12 (18 out of 154) and 0·15 (27 out of 180) in CPOs and controls, respectively. Chi-square *P* value was used to compare allele frequencies between CPOs and controls. No statistically significant difference was found ($P=0{\cdot}397$ and $P=0{\cdot}654$ for the dominant and recessive models, respectively).

The entire coding exon of *FOXE1* was sequenced in 146 CPOs, 108 CL/Ps and 204 controls. A total of six different non-synonymous variants were identified in these 254 patients with oral clefts. Each was found in one patient and in heterozygous state. In CPOs, we found five different non-synonymous changes, c.274G>T (p.D92Y), c.569C>G (p.P190R), c.569C>T (p.P190L), c.664C>T (p.R222C) and c.1090G>A (p.G364S). Of these, only one, c.569C>G (p.P190R) (rs182535331), was previously reported, while the other four were novel (Table 1, Fig. 1). None of them were identified in the 204 controls. Two of these, c.274G>T (p.D92Y) and c.664C>T (p.R222C), are evolutionarily conserved and predicted to be pathogenic by SIFT. The c.274G>T changes 'aspartic acid' at the amino acid residue 92, which is in the FOX head domain, to 'tyrosine' (p.D92Y). The c.664C>T changes 'arginine' to 'cysteine' at the amino acid residue 222 (p.R222C).

Of the 108 patients with CL/P, one mutation, c.572C>G (p.P191R) was found (Table 1, Fig. 1). It was found in the heterozygous state in one patient but not identified in the 204 controls. It has never been previously reported.

One non-synonymous variant of unknown clinical significance, c.841T>C (p.Y281H), was found in one healthy control. Besides these non-synonymous variants, six synonymous variants were identified in these groups of patients and controls (Supplementary Table 1).

4. Discussion

Oral clefts are complex disorders. Groups of genes involved in the disease pathogenesis in various populations may not be the same. The c.-1204C>G variant in the promoter of *FOXE1* was previously found to be associated with CPO in Italy (Venza *et al.*, 2009). We therefore sought to study this association in our Thai

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population. With similar frequencies of the G allele in CPOs and controls, no statistically significant difference was found. Of note, 44% (11/25) of CPOs in the Italian population were found to be homozygous GG (Venza *et al.*, 2009) but this was found in only 1·3% (1/77) of our Thai CPOs. Moreover, while the homozygous GG was not found in any of the unaffected parents, unaffected sibs and controls in the Italian population (Venza *et al.*, 2009), we identified two of our Thai controls who were homozygous GG. Therefore, homozygous GG at the c.-1204 position of *FOXE1* is not sufficient for the development of CPO.

We then sequenced the entire coding region of the *FOXE1* gene. Non-synonymous variants were found in 3·4% (5/146), 0·9% (1/108) and 0·5% (1/204) of CPOs, CL/Ps and controls, respectively. Although there were no statistical differences, there was a positive trend for an association between non-synonymous variants in *FOXE1* and CPOs. Many lines of evidence have suggested that some of the variants might be pathogenic. First, none of them were found in 204 ethnic matched controls; secondly, one was in a functional domain; thirdly, three changed amino acid polarity; fourthly, two were evolutionarily conserved; and finally, four were predicted by PolyPhen-2 to be damaging (see details in Table 1). Therefore, the identified association could be resulted from a direct causation, i.e. some of these variants could be susceptibility alleles themselves, not just in linkage disequilibrium with the bona fide mutations.

Of these six non-synonymous variants identified in our patients with oral clefts, five have not been previously reported (checked with the NHLBI Exome Sequencing Project (ESP) (http://evs.gs.washington.edu.EVS/) on 14 August 2013). The heterozygous c.274G>T (p.D92Y) mutation, found in one CPO affecting an amino acid residue 92, resides in the DNA-binding Forkhead domain (amino acid residues 53–152), is evolutionarily conserved, and is predicted to affect protein function by SIFT and PolyPhen program (Table 1). The c.664C>T (p.R222C) is the other mutation that is evolutionarily conserved and predicted to affect protein function by SIFT and PolyPhen program.

Three variants, c.569C>T (p.P190L), c.569C>G (p.P190R) and c.572C>G (p.P191R), were found very close together at the two consecutive proline residues 190 and 191. Although the p.P190R and p.P191R change polarity of the amino acids from non-polar to polar, they are not evolutionarily conserved and predicted to be tolerated by SIFT. The non-synonymous variant at the most 3′ end of the gene, c.1090G>A (p.G364S), changes polarity of the amino acid residue from non-polar to polar, and is predicted to be probably damaging by PolyPhen but is not evolutionarily conserved and predicted to be tolerated by SIFT. Whether these four variants play any roles in oral clefts needs further studies.

Similar to studies in patients of European and Middle–South American descents (Nikopensius *et al.*, 2011; Lennon *et al.*, 2012), our results support a role for the *FOXE1* in the development of CPO in the Thai population.

5. Declaration of interest

There are no conflicts of interest.

Supplementary material

To view supplementary material for this article, please visit http://dx.doi.org/10.1017/S0016672313000177.

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