GTMB-2015-0325-ver9-Ittiwut_1P
Type: research-article

GENETIC TESTING AND MOLECULAR BIOMARKERS
Volume 00, Number 00, 2016

Pp. 1–4
DOI: 10.1089/gtmb.2015.0325

ORIGINAL ARTICLE

Variants of the *CDH1* (E-Cadherin) Gene Associated with Oral Clefts in the Thai Population

AU1 ▶

Rungnapa Ittiwut,[1,2] Chupong Ittiwut,[3] Pichit Siriwan,[4] Vichai Chichareon,[5] Kanya Suphapeetiporn,[1,2] and Vorasuk Shotelersuk[1,2]

Objective: The etiology of oral clefts in humans is complex because it is associated with several genes. *CDH1* (E-cadherin) has been found to be involved in lip and palate development, and *CDH1* mutations are associated with oral clefts in some populations. *Materials and Methods:* To determine this association in a Thai population, we sequenced the entire 6.5-kb coding region of *CDH1* in 80 oral cleft patients and compared the identified variants with those found in 138 unrelated Thai individuals who did not have oral clefts, as genotyped by exome sequencing. *Results:* Among the oral cleft patients, four nonsynonymous single nucleotide variants (SNVs), c.1235T>C (p.V412A), c.1273G>A (p.V425I), c.1565C>T (p.T522I), and c.1888C>G (p.L630V), were identified. Only one nonsynonymous variant (c.1409C>T; p.T470I) was found among the 138 noncleft exomes. The frequency of nonsynonymous SNVs on the *CDH1* gene in oral cleft patients (4/80) was significantly higher than that in the control group (1/138) ($p = 0.042$). *Conclusion:* We found that nonsynonymous variants of *CDH1* were associated with oral clefts in the Thai population.

Introduction

ORAL CLEFTS, INCLUDING CLEFT LIP with or without cleft palate (CL/P) and cleft palate only (CPO) are common complex birth defects. The frequency of oral clefts is about 1–2 in 1000 live births worldwide (Watkins *et al.*, 2014). The prevalence of oral clefts is different according to geographic area, race, and environmental factors. Several studies reported that the prevalence rates of oral clefts were the highest in Asians and the lowest in Africans (Tolarova and Cervenka, 1998; Watkins *et al.*, 2014). It has been reported that there are 1.1 – 2.4 babies born with oral cleft defects per 1000 births in Thailand (Pradubwong *et al.*, 2012).

Multiple genetic and environmental factors play major etiologic roles (Vieira, 2008). Our previous studies found potentially pathogenic variants in several genes, including *MTHFR* (Shotelersuk *et al.*, 2003), *p63* (Leoyklang *et al.*, 2006), *MSX1* (Tongkobpetch *et al.*, 2006), *TBX22* (Suphapeetiporn *et al.*, 2007), *PVRL1* (Tongkobpetch *et al.*, 2008), *IRF6* (Yeetong *et al.*, 2009), *PDGFRa* (Rattanasopha *et al.*, 2012), and *FOXE1* (Srichomthong *et al.*, 2013). However, several other underlying genes are yet to be identified.

New pathways and new genes in nonsyndromic oral clefts are continually being found, including a recent *CDH1* gene (Vogelaar *et al.*, 2013). *CDH1* is located on chromosome 16q22.1 and is composed of 16 exons spanning around 100 kb. It encodes E-cadherin, a 120-kDa glycoprotein that is a member of the classic cadherin group. This protein is involved in calcium-dependent cell–cell connections and is required for the adhesive function of epithelial cells (Vogelaar *et al.*, 2013). CDH1 is also involved in lip and palate development in hereditary gastric cancer patients (Guilford *et al.*, 1998; Frebourg *et al.*, 2006). In addition, whole exome sequencing in multiple cleft families revealed novel and damaging single nucleotide variants (SNVs) in the *CDH1* gene in an Indian family (Bureau *et al.*, 2014).

So far, no *CDH1* mutation data have been presented from a Southeast Asian population, the area with the highest prevalence of oral clefts. In this study, we determined variants in the entire coding regions of the *CDH1* gene by Sanger sequencing of 80 oral cleft patients. We compared the identified variants with those found in 138 unrelated Thai individuals who did not have oral clefts as genotyped by exome sequencing. We found an association between rare variants of *CDH1* and oral clefts.

AU2 ▶

[1]Department of Pediatrics, Faculty of Medicine, Center of Excellence for Medical Genetics, Chulalongkorn University, Bangkok, Thailand.
[2]Excellence Center for Medical Genetics, King Chulalongkorn Memorial Hospital, The Thai Red Cross Society, Bangkok, Thailand.
[3]Central Laboratory, Department of Pediatrics, Faculty of Medicine, Chulalongkorn University, Bangkok, Thailand.
[4]Deputy Director of Relief and Community Health Bureau, The Thai Red Cross Society, Bangkok, Thailand.
[5]Department of Surgery, Faculty of Medicine, Prince of Songhla University, HaadYai, Songkla, Thailand.

Table 1. Number of Oral Cleft Patients and Controls

Sex	*CL*	*CLP*	*CP*	*Controls*
Male	10	22	0	70
Female	17	24	7	68
Total	27	46	7	138

CL, cleft lip; CLP, cleft lip and palate; CP, cleft palate.

Materials and Methods

Participants

We recruited 46 patients with cleft lip and cleft palate, 27 with cleft lip only, and 7 with CPO from the Smart Smile and Speech Project (Table 1). This project aims to treat patients with oral clefts and other birth defects in underserved areas of Thailand. Of these 80 unrelated patients, 69 were sporadic, while 11 were familial cases. DNA was extracted from peripheral blood leukocytes using the phenol–chloroform conventional method. Informed consent was obtained from all patients. Controls were 138 unrelated Thai individuals with various diseases but without oral clefts. Their genomic DNA was isolated from peripheral blood leukocytes using the Puregene Blood Kit (Qiagen, Hilden, Germany).

Polymerase chain reaction amplification and Sanger sequencing

The entire 6.5-kb coding regions in 16 exons of the *CDH1* gene were amplified using the polymerase chain reaction (PCR). All primers were designed by Primer3 software (SourceForge, sfnet_ops@slashdotmedia.com). The 13 fragments of *CDH1* coding regions were amplified using various cycling conditions. PCR was carried out in 20 μL of a solution containing 100 ng of genomic DNA, 200 μM of each dNTP, 150 nM of each primer, 2.5 mM of $MgCl_2$, 1× PCR buffer, 0.5 U of *Taq* DNA polymerase (Fermentas, Inc., Glen Burnie, MD), and 5–10% of dimethyl sulfoxide when needed. The thermocycling condition consisted of predenaturation at 95°C for 5 min, 35 cycles of denaturation at 95°C for 30 s, annealing at 55–65°C for 30 s, and extension at 72°C for 30 s. ExoSAP-IT (USB, Cleveland, OH) was used to remove the excess nucleotides and primers. All purified PCRs were sent to Macrogen, Inc. (Seoul, Korea) for direct sequencing. The Mutation Surveyor (State College, PA) performed the sequencing analysis. We used Sorting Intolerant From Tolerant (SIFT; http://sift.bii.a-star.edu.sg/www/SIFT_seq_submit2.html) and PolyPhen (http://genetics.bwh.harvard.edu/pph2/) software to predict the possible impact of amino acid substitutions on the stability and function of the mutant proteins. The coordinate position of each *CDH1* variant was evaluated in the ExAC Browser (http://exac.broadinstitute.org) to determine its minor allele frequency and whether it had been previously reported.

Exome sequencing

The DNA of each of the 138 unrelated controls who were affected with other diseases but not oral clefts was sent to Macrogen, Inc., South Korea, for whole exome sequencing using the HiSeq 2000 platform. Sample quality control, sequencing, and data analysis were performed by Macrogen, Inc. Variants in the region of the *CDH1* gene were captured and exported using a tool in the Golden Helix Genome Browse software (http://goldenhelix.com/GenomeBrowse/index.html).

Results

PCR–Sanger sequencing of the entire coding regions of *CDH1* of 80 unrelated patients with nonsyndromic oral clefts identified four nonsynonymous SNVs as follows: c.1235T>C (p.V412A), c.1273G>A (p.V425I), c.1565C>T (p.T522I),

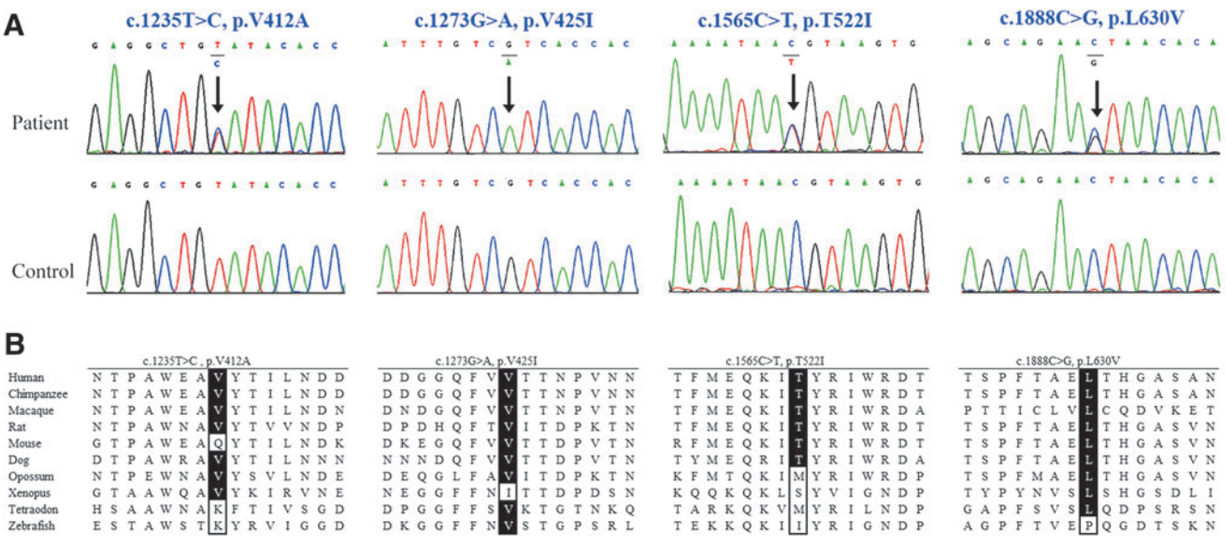

FIG. 1. **(A)** Chromatograms of four nonsynonymous single nucleotide variants—c.1235T>C (p.V412A), c.1273G>A (p.V425I), c.1565C>T (p.T522I), and c.1888C>G (p.L630V)—in patients with an oral cleft (*upper*) and controls (*lower*). Positions of nucleotide changes are indicated by *arrows*. **(B)** Homology was conserved in another 10 species. The amino acid variants found in this study are marked as *dark blocks*.

and c.1888C>G (p.L630V) (Fig. 1 and Table 2). The first SNV (c.1235T>C) and the third SNV (c.1565C>T) had not been reported previously. The second SNV (c.1273G>A) and the fourth SNV (c.1888C>G) had been previously found and are reported at the frequencies of 1.65×10^{-5} and 3.8×10^{-4}, respectively, in the ExAC database. Among these four SNVs, three were present in the heterozygous state. The exception (c.1565C>T) was homozygous AA (Fig. 1). Overall, the allele frequencies of all four nonsynonymous SNVs found in our patients were less than 0.001 in the 1000 Genome SNP and ExAC databases.

PolyPhen software predicted that three of the four SNVs (c.1235T>C, c.1565C>T, c.1888C>G) could be damaging. In addition, the third SNV (c.1565C>T) was a variant located in the last base of exon 10 and could lead to aberrant splicing. The software predicted that the second SNV (c.1273G>A) would be benign. Multiple alignments of these four nonsynonymous SNVs are compared with nine other vertebrate species in Figure 1. All four amino acids are evolutionarily conserved in at least six vertebrate species.

Only one nonsynonymous SNV (c.1409C>T; p.T470I) was found in the 138 controls. It was in a heterozygous state. This variant is reported in the ExAC browser with a minor allele frequency of 3.3×10^{-5}. The SIFT and PolyPhen software predicted it to be ''damaging'' and ''probably damaging,'' respectively (Table 2). The frequency of nonsynonymous SNVs on the *CDH1* gene in patients with an oral cleft (4/80) was significantly higher than that in the control group (1/138, $p=0.042$).

Discussion

To determine whether *CDH1* variants are associated with oral clefts in the Thai population, we first sequenced the entire coding regions of the *CDH1* gene in 80 patients and found four nonsynonymous SNVs as follows: c.1235T>C (p.V412A), c.1273G>A (p.V425I), c.1565C>T (p.T522I), and c.1888C>G (p.L630V). All of them were in the extracellular cadherin repeats 3, 4, or 5 of the CDH1 protein. The extracellular cadherin domain mediates adhesive interactions between cells (Shapiro and Weis, 2009). In addition, one of the four SNVs, c.1565C>T (p.T522I), occurred at the last nucleotide of an exon, possibly affecting the exon splicing process. Thus, these SNVs could have functional consequences.

We then compared their frequency with that found in 138 Thai individuals who did not have oral clefts. We found that the frequency of nonsynonymous *CDH1* mutations in oral cleft patients (4/80) was statistically significantly higher than that in the control group (1/138, $p=0.042$). The association in our Thai ethnic group is consistent with previous studies in other populations in various continents, including Asians (Han Chinese and Indian), Europeans (Dutch and Polish), and South Americans (Brazilian). In the Han Chinese population, a SNV in the *CDH1* promoter (rs16260) was associated with CPO (Song and Zhang, 2011). In an Indian family with oral clefts, a nonsense mutation (c.2143G>T; p.G715X) in the *CDH1* gene was identified by whole exome sequencing. It was proposed to be a potentially causal variant (Bureau *et al.*, 2014). Of the 80 Dutch patients with oral clefts, four were found to harbor nonsynonymous SNVs in the *CDH1* gene (Vogelaar *et al.*, 2013) for a frequency of 5% (4/81), which was the same as ours. In the Polish population, a *CDH1* SNV

Table 2. Position, Allele Frequency, and Software Prediction Information of All Nonsynonymous Single Nucleotide Variants

Single nucleotide variant	*Position NM_004360.3 (on assembly GRCh37)*	*Coding position*	*Protein position*	*Zygosity*	*Exon*	*Allele frequency (ExAC browser)*	*Number*		*SIFT prediction*	*PolyPhen prediction*
							Patients (n = 80)	*Controls (n = 137)*		
1	Chr16: 68847313	c.1235T>C	p.V412A	Heterozygous (TC)	9	Novel	1	0	Tolerated	Possibly damaging
2	Chr16: 68847351	c.1273G>A	p.V425I	Homozygous (AA)	9	1.65×10^{-5} (2 heterozygous alleles in 121,412 alleles)	1	0	Tolerated	Benign
3	Chr16: 68849662	c.1565C>T	p.T522I	Heterozygous (CT)	10	Novel	1	0	Tolerated	Possibly damaging
4	Chr16: 68856080 (rs2276331)	c.1888C>G	p.L630V	Heterozygous (CG)	12	3.8×10^{-4} (46 heterozygous alleles in 121,400 alleles)	1	0	Tolerated	Possibly damaging
Controls										
1	Chr16: 68849506 (rs370864592)	c.1409C>T	p.T470I	Heterozygous (CT)	10	3.3×10^{-5} (4 heterozygous alleles in 121,412 alleles)	0	1	Damaging	Probably damaging

SIFT, sorting intolerant from tolerant.

(rs1801552) was found to be associated with oral clefts (Hozyasz *et al.*, 2014). Two other *CDH1* polymorphisms, rs11642413 and rs9929218, were found to be associated with unilateral cleft lip with or without cleft palate in Brazilian patients (Letra *et al.*, 2009).

In conclusion, an association between nonsynonymous variants in the *CDH1* gene and oral clefts was found in the Thai population.

Acknowledgments

The Thailand Research Fund (RTA 56800003 to V.S.), the Birth Defects Association of Thailand (to K.S.), and the Chulalongkorn Academic Advancement into Its 2nd Century Project supported this work.

Author Disclosure Statement

No competing financial interests exist.

References

Bureau A, Parker MM, Ruczinski I, *et al.* (2014) Whole exome sequencing of distant relatives in multiplex families implicates rare variants in candidate genes for oral clefts. Genetics 197:1039–1044.

Frebourg T, Oliveira C, Hochain P, *et al.* (2006) Cleft lip/palate and CDH1/E-cadherin mutations in families with hereditary diffuse gastric cancer. J Med Genet 43:138–142.

Guilford P, Hopkins J, Harraway J, *et al.* (1998) E-cadherin germline mutations in familial gastric cancer. Nature 392: 402–405.

Hozyasz KK, Mostowska A, Wojcicki P, *et al.* (2014) Nucleotide variants of the cancer predisposing gene CDH1 and the risk of non-syndromic cleft lip with or without cleft palate. Fam Cancer 13:415–421.

Leoyklang P, Siriwan P, Shotelersuk V (2006) A mutation of the p63 gene in non-syndromic cleft lip. J Med Genet 43:e28.

Letra A, Menezes R, Granjeiro JM, *et al.* (2009) AXIN2 and CDH1 polymorphisms, tooth agenesis, and oral clefts. Birth Defects Res A Clin Mol Teratol 85:169–173.

Pradubwong S, Pongpagatip S, Prathanee B, *et al.* (2012) The treatment of 4–5 year-old patients with cleft lip and cleft palate in Tawanchai Center: follow-up. J Med Assoc Thai 95 Suppl 11:S135–S140.

Rattanasopha S, Tongkobpetch S, Srichomthong C, *et al.* (2012) PDGFRa mutations in humans with isolated cleft palate. Eur J Hum Genet 20:1058–1062.

Shapiro L, Weis WI (2009) Structure and biochemistry of cadherins and catenins. Cold Spring Harb Perspect Biol 1: a003053.

Shotelersuk V, Ittiwut C, Siriwan P, *et al.* (2003) Maternal 677CT/1298AC genotype of the MTHFR gene as a risk factor for cleft lip. J Med Genet 40:e64.

Song Y, Zhang S (2011) Association of CDH1 promoter polymorphism and the risk of non-syndromic orofacial clefts in a Chinese Han population. Arch Oral Biol 56:68–72.

Srichomthong C, Ittiwut R, Siriwan P, *et al.* (2013) FOXE1 mutations in Thai patients with oral clefts. Genet Res (Camb) 95:133–137.

Suphapeetiporn K, Tongkobpetch S, Siriwan P, *et al.* (2007) TBX22 mutations are a frequent cause of non-syndromic cleft palate in the Thai population. Clin Genet 72:478–483.

Tolarova MM, Cervenka J (1998) Classification and birth prevalence of orofacial clefts. Am J Med Genet 75:126–137.

Tongkobpetch S, Siriwan P, Shotelersuk V (2006) MSX1 mutations contribute to nonsyndromic cleft lip in a Thai population. J Hum Genet 51:671–676.

Tongkobpetch S, Suphapeetiporn K, Siriwan P, *et al.* (2008) Study of the poliovirus receptor related-1 gene in Thai patients with non-syndromic cleft lip with or without cleft palate. Int J Oral Maxillofac Surg 37:550–553.

Vieira AR (2008) Unraveling human cleft lip and palate research. J Dent Res 87:119–125.

Vogelaar IP, Figueiredo J, van Rooij IA, *et al.* (2013) Identification of germline mutations in the cancer predisposing gene CDH1 in patients with orofacial clefts. Hum Mol Genet 22:919–926.

Watkins SE, Meyer RE, Strauss RP, *et al.* (2014) Classification, epidemiology, and genetics of orofacial clefts. Clin Plast Surg 41:149–163.

Yeetong P, Mahatumarat C, Siriwan P, *et al.* (2009) Three novel mutations of the IRF6 gene with one associated with an unusual feature in Van der Woude syndrome. Am J Med Genet A 149A:2489–2492.

Address correspondence to: ◀AU3
Kanya Suphapeetiporn, MD, PhD
Division of Medical Genetics and Metabolism
Department of Pediatrics
Faculty of Medicine
Chulalongkorn University
Sor Kor Building, 11th Floor
Bangkok 10330
Thailand

E-mail: kanya.su@chula.ac.th

AUTHOR QUERY FOR GTMB-2015-0325-VER9-ITTIWUT_1P

AU1: Please review all authors' surnames for accurate indexing citations.
AU2: Please confirm the authors' affiliations.
AU3: The address of the corresponding author mismatches with the affiliation footnote. Please fix both consistently.