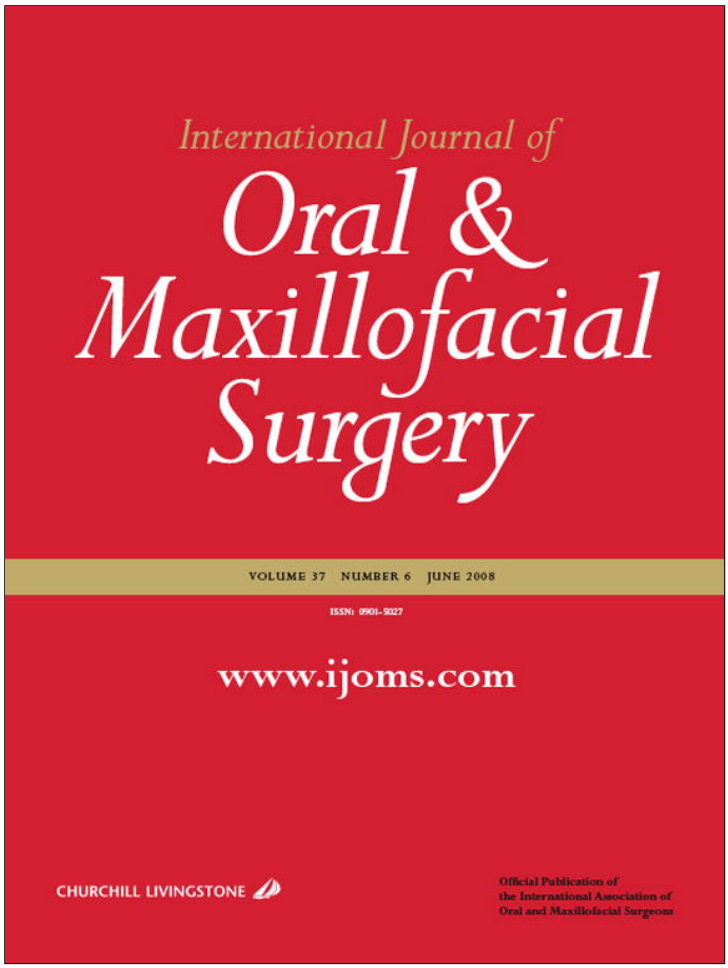

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Research Paper
Congenital Craniofacial Anomalies

Study of the *poliovirus receptor related-1* gene in Thai patients with non-syndromic cleft lip with or without cleft palate

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Abstract. Non-syndromic cleft lip with or without cleft palate (CL/P) has a complex etiology with several genetic and environmental factors playing a role. The *poliovirus receptor related-1* gene (*PVRL1*) has been shown to underlie a syndromic form of CL/P and, in some populations, contribute to non-syndromic CL/P. To investigate whether mutations in *PVRL1* play a part in the formation of non-syndromic CL/P in the Thai population, 100 CL/P patients were analyzed for mutations in *PVRL1* by polymerase chain reaction amplification and direct sequencing of all the coding regions of its alpha isoform. Of this series of patients, one was found to be heterozygous for 1183G>A in exon 6, expected to result in the substitution of a valine by a methionine at position 395 (V395M). This mutation was not found in 200 unaffected Thai control individuals. The valine position is conserved across all known mammalian PVRL1 sequences. In conclusion, a novel non-synonymous *PVRL1* mutation was found in a Thai patient with non-syndromic CL/P, suggesting a possible etiologic role of *PVRL1* in non-syndromic CL/P across different populations.

Keywords: *PVRL1*; non-syndromic cleft lip with or without cleft palate; Thai; novel mutation.

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Cleft lip with or without cleft palate (CL/P) is one of the most common major birth defects occurring in 0.4–2.0 per 1000 live-born infants in most populations[5,15]. Approximately 70% of CL/P cases are non-syndromic. The majority of non-syndromic CL/P cases are considered multifactorial in etiology, with several genes and environmental factors involved. The remaining 30% occur as part of more than 300 different malformation syndromes in which CL/P is only one manifestation[10]. In the Thai population, similar to many others, it was found that many genes responsible for Mendelian clefting syndromes have roles in non-syndromic CL/P. These include the *interferon regulatory factor-6* gene (*IRF6*) responsible for Van der Woude syndrome (MIM 119300)[8,11], *p63* for ectrodactyly-ectodermal dysplasia-clefting syndrome (MIM 129900), ankyloblepharon-ectodermal dysplasia-clefting syndrome (MIM 106260) and Rapp-Hodgkin syndrome (MIM 129400)[2,7], and *MSX1* for hypodontia with orofacial cleft (MIM 106600)[16,18]. These findings led to the hypothesis that some

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Table 1. Oligonucleotides and PCR conditions for *PVRL1* mutation analysis

Exon	Primer sequences for PCR 5′ to 3′		Annealing temperature (°C)
	Forward	Reverse	
1	TGCAGCTGACCTGGATCCTT	GACCCAACTTTTCTGGCTCC	60
2	CTGTGCCTGACACTGACCAC	AGGTCACAGGCCTCTGGATG	58
3	CCTTTTGGCCGGTGCATCTT	AAGGAGAGGAGGAGGGAGGA	58
4	TCCTGGCTGAAGTTCCCTTG	GGTCCAGTCAGCTGTCTTCC	58
5	GGAACAGCTTCTTGAGGCTG	TCCTGCAGTGGCATTGCTCA	58
6	GAGCACACGCTGATGCTGTC	AGCGGTCACAGACAGAGGCT	58

patients with non-syndromic CL/P might have mutations in other genes known to cause 'syndromic' CL/P.

The *poliovirus receptor related-1* gene (*PVRL1*) was first shown to be involved in facial development when it was found to be responsible for an autosomal recessive CL/P ectodermal dysplasia syndrome (CLPED1; MIM 225060)[13]. *PVRL1* encodes nectin1, a cell-to-cell adhesion molecule expressed in the developing face and palate[13,14]. It is a receptor-like protein containing an immunoglobulin-like extracellular domain. PVRL1 has three differently spliced isoforms: α, β and γ. *PVRL1* was subsequently shown to contribute to non-syndromic CL/P in several populations, including Venezuelan[9], Italian[3], Iowan, Danish and Filipino[1], but no *PVRL1* variants were found in Taiwanese non-syndromic CL/P patients. The aim of this study was to perform mutation analysis of all the coding regions of the *PVRL1* gene in 100 Thai patients with non-syndromic CL/P.

Methods

Patients and sample collection

The subjects of this study were 88 sporadic cases of non-syndromic CL/P and 12 additional cases with a positive family history. Details of their characteristics and recruitment were previously reported[2]. All patients were screened for the presence of associated anomalies or syndromes by a geneticist, and only those determined to have isolated cleft lip with or without cleft palate (normal growth, normal development, no other major anomalies including ectodermal abnormalities, and no apparent visual or hearing deficits) were included in this study. The study was approved by the institutional review board of the Faculty of Medicine of Chulalongkorn University, and written informed consent was obtained from each person included in the study. The control samples were Thai blood donors with no oral clefts, who reported no history of oral clefts in other family members.

Mutation analysis

Genomic DNA was extracted from peripheral leukocytes according to standard protocols. PCR amplification of the α isoform of *PVRL1* coding exons 1-6 was performed using primers as shown in Table 1, and 100 ng of genomic DNA, 1×PCR buffer (Fermentas, Burlington, ON, Canada), 1.5 mM $MgCl_2$, 0.2 mM dNTP, 0.2 μM of each primer, and 0.5 U Taq DNA polymerase (Fermentas) in a total volume of 20 μl. PCR products were treated with ExoSAP-IT (USP Corp., Cleveland, OH, USA), according to the manufacturer's recommendations, and sent for direct sequencing at Macrogen Inc. (Seoul, Korea). The GenBank accession number of human *PVRL1* mRNA is NM_002855.

The V395M mutation was confirmed in the patient and genomic DNA of unaffected controls screened for the mutation by restriction enzyme digestion of PCR products. The forward and reverse primers for amplification of *PVRL1* exon 6 (Table 1) were used to generate a 628-bp PCR product. The mutant PCR product lacked the cleavage site for the restriction endonuclease *Pml*I, which allowed detection of the mutation by agarose gel electrophoresis. The V395M mutation found in our non-syndromic CL/P case was used as a positive control in PCR-RFLP analysis of the samples from 200 unaffected control individuals.

Protein sequence comparisons

PVRL1 orthologues were first identified through a BLAST search of the non-redundant database using *Homo sapiens* PVRL1, accession NP_002846 as the reference sequence. All known and complete PVRL1 sequences were included from the vertebrate lineage. These files in FASTA format were then analyzed by the ClustalX 1.81 program. The human PVRL1 was aligned with rhesus monkey (accession XP_001098654.1), cow (accession XP_884996.1), domestic pig (accession Q9GL76), dog (accession XP_546481.2), house mouse (accession NP_067399.2) and Norway rat (accession XP_236210.3). The program classified amino acids by the variation in polarity, assessing both amino acid class conservation and evolutionary conservation at any given site.

Results

The entire coding sequences of the *PVRL1* α isoform were analyzed by PCR sequencing for all 100 non-syndromic CL/P Thai patients. One was found to be heterozygous for 1183G>A in exon 6, expected to result in the substitution of a valine by a methionine at position 395 (V395M) (Fig. 1). The mutation was confirmed by restriction enzyme digestion analysis. Using PCR-RFLP, this mutation was not found in 200 unaffected ethnic-matched control individuals. The valine of the codon 395 is conserved across all known mammalian PVRL1 sequences (Fig. 2).

Five other patients (5%) were found to be heterozygous for a previously known polymorphism, 437insE (1480insGAG), in exon 6a. This variant was also found in 5% (5/100) of unaffected Thai control individuals.

Discussion

Non-syndromic CL/P is etiologically complex with both genetic and environmental factors playing a role. Identification of genes for rare developmental syndromes has provided an important source of candidate genes for more common birth defects as similar genes may also be involved. It has been suggested that predisposition to non-syndromic CL/P involves a number of different candidate genes and loci. Mutations in candidate genes have already been identified and found to be associated with non-syndromic CL/P cases, although there have been some inconsistencies and various magnitude of significance among different populations. In the Thai population, several genes have been identified as associated with non-syndromic CL/P including

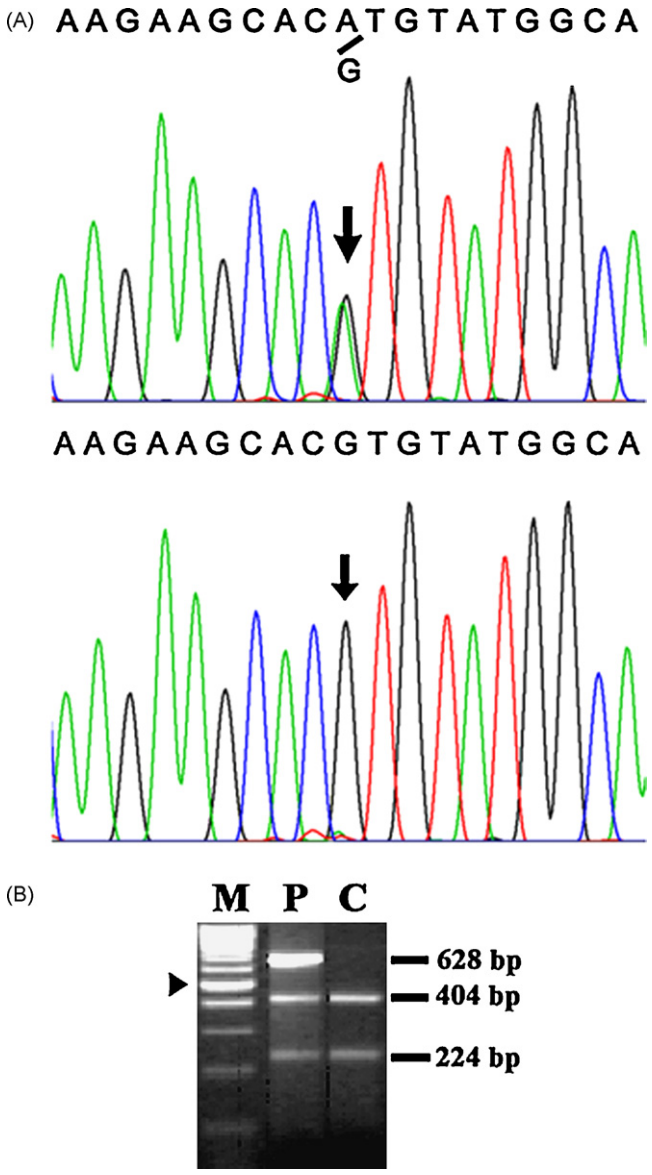

Fig. 1. **Mutation analysis**.(A) The sense sequence electropherograms of *PVRL1* exon 6 from the non-syndromic CL/P patient (upper panel) and control (lower panel). A heterozygous G → A transition (indicated by an arrow) resulting in the substitution of a valine by a methionine at position 395 (V395M) was found in the patient. (B) M: 100-bp marker. The 500-bp band is indicated by an arrow head. C: unaffected control. P: patient. Restriction enzyme analysis of PCR products showing the mutant allele lacking the cleavage site for the restriction endonuclease *Pml*I resulting in the 628-bp band, and the wild-type allele with bands of 404 and 224 bp. The 628-, 404- and 224-bp fragments were found in the patient with a heterozygous mutation.

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HsN1   LVLIVVGGIVVALRRRRHTFKGDYSTKKHVYGNGYSKAGIPQHHPPMAQNLQYPDDSDD
MmuN1  ----------------------------V----------------------------
BtN1   ----------------------------V-----------------------E----
CfN1   --A-------------------------V----------------------------
SsN1   ---F---------C--------------V----------------------------
MmN1   ---------I------------------V----------------------------
RnN1   ---------I------------------V----------------------------
```

Fig. 2. **Sequence alignment of the PVRL1 intracellular domain**.The site of the amino acid variant found in this study is indicated in bold red in all conserved mammalian species. Sites that are 100% conserved across all sequences are indicated by dashes (−). N1 represents Nectin1. Hs, *Homo sapiens*; Mmu, *Macaca mulatta*; Bt, *Bos Taurus*; Cf, *Canis familiaris*; Ss, *Sus scrofa*; Mm, *Mus musculus*; Rn, *Rattus norvegicus*.

Table 2. Prevalence of gene mutations in Thai patients with non-syndromic oral clefts

Gene	Cleft type	Prevalence
MSX1	CL/P	4/100
P63	CL/P	3/100
PVRL1	CL/P	1/100
IRF6	CL/P	NA*
MTHFR	CL/P	NA*
TBX22	CPO	4/53

CL/P, cleft lip with or without cleft palate; CPO, cleft palate only.
* NA, not applicable; association study not mutation analysis performed.

MTHFR[6], *MSX1*[16], *IRF6*[11], *p63*[2] and *TBX22*[12]. Table 2 summarizes the prevalence of potential disease-causing mutations in genes found in the Thai non-syndromic cleft population.

In this study, the presence of mutations in *PVRL1* was investigated in 100 Thai patients with non-syndromic CL/P by direct sequencing of the *PVRL1* α isoform. This isoform is the longest (517 amino acids, compared to 458 amino acids of the β isoform and 352 amino acids of the γ isoform). Heterozygosity for 1183G>A (V395M) in exon 6a was identified in one patient, but not in 200 ethnic-matched control individuals. This particular mutation has never been described nor is it included in the SNP databases. This amino acid is within the intracellular domain of the protein. Samples from the parents were unavailable, which makes it impossible to determine whether it was inherited or *de novo*. The evidence that V395M was not detected in 200 unaffected control individuals as well as that this particular residue was conserved among different organisms (chimpanzee, cow, dog, pig, mouse, rat) (Fig. 2) supports the role of V395M in non-syndromic CL/P. Since no reliable assay is available for testing the function of PVRL1, a significant effect of its mutation could not be verified.

The only other variant found in this study was 437insE (1480insGAG), a previously known non-pathogenic polymorphism. This was also found in 5% of unaffected Thai controls indicating that it is not associated with CL/P in the Thai population. Since only the α isoform of *PVRL1* was sequenced, mutations in the other isoforms would not be have been detected.

PVRL1 mutations have been demonstrated in non-syndromic CL/P cases in different ethic groups as shown in Table 3. Interestingly, the W185X mutation in *PVRL1* exon 3, a genetic risk factor for non-syndromic CL/P in northern Venezuela[9], was not present in Thai patients. This finding was similar to the previous

Table 3. Prevalence of *PVRL1* mutations in patients with non-syndromic CL/P from various populations

Population	Method used	Mutation	Prevalence
Northern Venezuela[9]	Sequencing of exon 3		
- Margarita Island		W185X	2/30
- Cumaná region		W185X	14/243
Italian[4]	Restriction enzyme digestion	W185X	0/146
Italian[3]	Sequencing of entire coding regions	R199Q	3/143
		R210H	3/143
		R212H	1/143
Taiwanese[17]	Sequencing of exons 3 and 5	None	0/66
Iowan[1]	Sequencing of entire coding regions	R199Q	2/93
		R210H	2/93
Filipino[1]	Sequencing of entire coding regions	S112T	1/87
		T131A	1/83
Thai	Sequencing of entire coding regions	V395M	1/100

investigations in Italian and Taiwanese populations[4,17]. It was not surprising as the numerous genetic and environmental factors in distinct populations would probably contribute differently to the formation of CL/P.

In conclusion, a novel non-synonymous mutation in *PVRL1* has been identified in a Thai patient with non-syndromic CL/P. Available evidence supports its involvement in the occurrence of non-syndromic CL/P although its causal role remains to be proven by functional studies. Further studies with a larger sample size are needed to verify a pathogenic role, if any, played by *PVRL1* mutations in the Thai population.

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