CHAPTER III

MATERIALS AND METHODS

3.1 Materials

3.1.1 Sample collection

Twenty-eight Thai unrelated patients with non-syndromic orofacial clefts and two Thai unrelated patients with syndromic orofacial cleft, and 30 Thai unrelated patients with non-syndromic hypodontia from dental clinics and hospitals in several parts of Thailand were studied (Table 3.1 and 3.2). The conditions were verified by physical and radiographic examination. Medical and dental histories were obtained. The inclusion criteria for non-syndromic hypodontia were congenital tooth agenesis of any form of either dentition and otherwise normal systemic health. The inclusion criteria for non-syndromic orofacial clefts were any type of orofacial cleft, such as cleft lip, cleft palate or cleft lip with cleft palate and otherwise normal systemic health. The inclusion criteria for syndromic orofacial cleft were any types of orofacial clefts with other organ abnormalities. All 100 control samples were Thai volunteers (staff members of the Faculty of Dentistry, Chiang Mai University) without hypodontia or orofacial clefts upon clinical examination, who donated blood for this study. Informed consent was obtained from the individuals, or from their parents if they were under 18 years old. The study was approved by the Human Experimentation Committee of the Faculty of Dentistry, Chiang Mai University.

Table 3.1 The phenotypes of patients with orofacial clefts in this study.

Phenotypes	Numbers of patients
Cleft lip and palate	23
Cleft lip	2
Cleft palate	3
Cleft lip and palate with hypodontia	1
Cleft lip and palate with hypodontia and limb anomalies	1
Total	30

Table 3.2 The phenotypes of patients with hypodontia in this study.

Phenotypes	Numbers of patients
Missing teeth in incisor region	9
Missing teeth in canine region	2
Missing teeth in premolar region	6
Missing teeth in molar region	4
Missing more than 6 teeth	8
Missing primary teeth	1
Total	30

3.1.2 PCR materials

3.1.2.1 Genomic DNA 50 nanograms

3.1.2.2 1x polymerase chain reaction (PCR) buffer (Fermentas, Burlington, Ont., Canada)

3.1.2.3 Magnesium chloride ($MgCl_2$) 2.5 millimolars

3.1.2.4 Deoxynucleotide triphosphates (dNTPs) 2.5 millimolars

3.1.2.5 0.5 Unit Taq polymerase from Fermentas, Foster City, Cal., USA (for exon 1)

3.1.2.6 AmpliTaq Gold DNA polymerase from Applied biosystems, Foster City, Cal., USA (for exon 2)

3.1.2.7 Oligonucleotide primers (Table 3.3)

Table 3.3 Oligonucleotides and polymerase chain reaction (PCR) conditions for *MSX1* mutation analysis (Modified from Tongkobpetch et al., 2006).

Name	Primer sequences for PCR 5′–3′	Product size (bp)	Annealing temperature (°C)
1F	CCAGTGCTGCGGCAGAAGG	848	62
1R	ATTCATCCGCTGGGGTGAA		
2F	GGCTGATCATGCTCCAATGC	556	58
2R	CACCAGGGCTGGAGGAAT		
1RS	TGGAACCTTCTTCCTGGGTG	–	–

3.2 Methods

3.2.1 Mutation analysis

Genomic DNA was isolated from five milliliters of peripheral blood samples using the standard inorganic salting-out method (Seielstad et al., 1999). Two exons of *MSX1* were amplified with the use of four primer pairs, as previously published (Tongkobpetch et al., 2006). Polymerase chain reactions (PCRs) were performed in a 20 microliter volume containing 50 nanograms genomic DNA, 1x PCR buffer

(Fermentas, Burlington, Ont., Canada), 1.8–2.0 millimolars $MgCl_2$, 0.2 millimolar dNTPs, 0.15-0.2 micromolar of each primer, and 0.5 Unit Taq polymerase (Fermentas, Foster City, Cal., USA) using the parameters identified in (Tables 3.4, 3.5, 3.6 and 3.7). Then 4 microliters of the PCR products were verified by electrophoresis (Figure 3.1), using 1% agarose gel in TBE buffer in a Sub-Cell® GT Agarose Gel Electrophoresis System (Bio-Rad Laboratories, Inc., Hercules, Cal., USA) at 115 Volts for 30 minutes. PCR products were sent for direct sequencing to Macrogen Inc., Seoul, Korea. Primers used for sequencing were the same as those used for the PCR reactions, except that the primer 1RS (Table 3.3) was used for sequencing exon 1 in the 3′–5′ direction. Analyses were performed using Sequence analysis software: Sequencher 4.8 (Genecodes, Corp., Ann Arbor, Mich., USA). When the results indicated a possible new variant, the sample was resequenced.

Table 3.4 Reagents and concentration used in PCRs of exon 1.

Reagent	**Concentration**
	MSX1 **Exon 1**
10X PCR buffer	1X
25 mM Magnesium chloride	2.0 mM
10 mM Deoxynucleotide triphosphates	0.2 mM
10 μM Oligonucleotide primers	0.2 μM
5U/μL *Taq* DNA polymerase	0.5 U
100% DMSO	5%
50 ng/μL Genomic DNA sample	2 μl

Table 3.5 PCR conditions of exon 1.

Process	**Temperature/Time**
	MSX1 Exon 1
Initiation	94°C/5min
Denaturing	94°C/30sec (35 cycles)
Annealing	62°C/45sec (35 cycles)
Extension	72°C/1min (35 cycles)
Final extension	72°C/5min

Table 3.6 Reagents and concentration used in PCRs of exon 2.

Reagent	**Concentration**
	MSX1 **Exon 2**
10X PCR buffer	1X
25 mM Magnesium chloride	1.875 mM
10 mM Deoxynucleotide triphosphates	0.2 mM
10 μM Oligonucleotide primers	0.15 μM
5U/μL Taq DNA polymerase	0.5 U
100% DMSO	5%
50 ng/μL Genomic DNA sample	2 μl

Table 3.7 PCR conditions of exon 2

Process	Temperature/Time
	MSX1
	Exon 1
Initiation	95°C/5min
Denaturing	94°C/1min (35 cycles)
Annealing	58°C/1min (35 cycles)
Extension	72°C/1min (35 cycles)
Final extension	72°C/7min

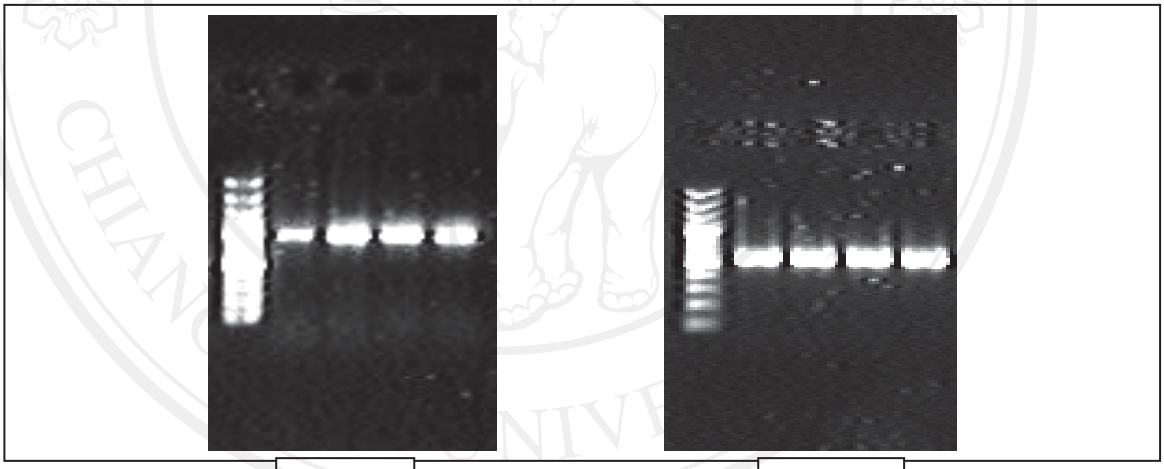

Figure 3.1 Gel electrophoresis of exon 1 and exon 2.

3.2.2 Data analysis

The data from sequencing were compared with the coding sequence for the nucleotide position within the Genbank entry AF426432. The changed nucleotides and amino acids were analyzed to the levels of conservation of nucleotides and amino acids. For protein sequence comparisons, MSX1 orthologs were first recognized

through a BLAST search of nonredundant databases, using Homo sapiens MSX1, accession NP_002439 as the reference sequence, and the variations in the nucleotides were confirmed using the UCSC (University of California Santa Cruz) web site (http://genome.ucsc.edu). All known and complete MSX1 sequences were compared to those of species in the vertebrate lineage. All of these sequences in FASTA file format were then analyzed by the ClustalX program (version 2.0, Bioinformatics, Oxford, England). The program classified amino acids by their variation in polarity and evaluated both evolutionary conservation and amino acid class conservation at any given site. The human MSX1 was aligned with Chimpanzee (Pan_troglodytes), House mouse (Mus_musculus), Brown rat (Rattus_norvegicus), Cow (Bos_taurus), Dog (Canis_familiaris), Chicken (Gallus_gallus), African clawed frog (Xenopus_laevis) and Zebrafish (Danio_rerio) to investigate amino acid class conservation and evolutionary conservation at any given site (Jezewski et al., 2003). PolyPhen or Polymorphism Phenotyping (http://genetics.bwh.harvard.edu/pph/) was used to predict the effects of the mutations.