CHAPTER III
MATERIALS AND METHODS

3.1 Materials

3.1.1 Sample selection

Six cases of amelogenesis imperfecta, hypoplastic type were studied (Table 3.1). The conditions were verified by clinical and radiographic examination. Medical and dental histories of the cases were obtained. The inclusion criteria for amelogenesis imperfecta, hypoplastic type were clinically congenital enamel malformation with rough to smooth surface, thin or deficient in amount of enamel, radiographically showed thin to absent enamel with reduction of radiodensity of enamel compared with dentine of any dentitions and otherwise the systemic health is normal. All 100 control samples were Thai volunteers without amelogenesis imperfecta 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 amelogenesis imperfecta, hypoplastic type in this study.

Patient	Sex	Phenotypes
1	Male	- Generalized hypoplastic amelogenesis imperfecta - Fusion of the primary mandibular left central and lateral incisors - Fusion of the permanent mandibular left central and lateral incisors - Unerupted supernumerary maxillary left premolar - All permanent first molars were crowned with stainless steel crowns - Additional features: Ectrodactyly in both hands and feet, ectodermal dysplasia, including small hair bulbs, brownish, slow-growing and thin hair, thin eyebrows, dry skin, hyperpigmentation of the skin, and dystrophic nails.

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Table 3.1 The phenotypes of patients with hypoplastic amelogenesis imperfecta in this study. (continued)

Patient	Sex	Phenotypes
2	Female	- Generalized hypoplastic amelogenesis imperfecta - Generalized gingival overgrowth
3	Male	- Generalized hypoplastic amelogenesis imperfecta - Generalized gingival overgrowth - Root dilaceration - Supernumerary tooth - Pericoronal radiolucency lesions associated with unerupted teeth
4	Female	- Generalized hypoplastic amelogenesis imperfecta
5	Female	- Generalized hypoplastic amelogenesis imperfecta
6	Male	- Generalized hypoplastic amelogenesis imperfecta - Suspected missing mandibular right permanent canine -Hyperpigmented gingivae
Total 6 patients		

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 AmpliTaq Gold DNA polymerase (Fermentas, Foster City, Cal., USA)

3.1.2.6 Oligonucleotide primers (Table 3.2 and 3.3)

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Table 3.2 Oligonucleotides and polymerase chain reaction (PCR) conditions for *ENAM* mutation analysis (Modified from (Gopinath et al., 2008).

Exon	Primer	Primer sequences for PCR 5´–3´	Product size (bp)	Annealing temperature (°C)
1	1F 1R	TGATTGGTTATGCAGGTCTACA GCAATAAGGAGAATTAACCA	381	57
2-3	2F 2R	TTGTCAACATCGCCCTAGA TAGTGTTTGGCCCTCTCA	420	57
4-5	3F 3R	TCCTTGACAGACAAGTAGGCTAGTA GTGTAGGATAGGACTCTGGA	373	57
6	4F 4R	CTGGGAAGTTCTAAGGTTAA AGGATAGGGGCAATAATTTT	311	57
7	5F 5R	AAGTTCCAGGACTTTTAAGC CATGTCAAAACAAACATTGG	502	57
8	6F 6R	CACAGAGTTTAATGCAAAGG GATTGGTATATGGTATTCCA	293	57
9	7F 7R	CAGAGTACTAACTGAGCTCAAT CATCAATCTTTGGACCACTG	352	57
10-I	8F 8R	AAATTCCAAACAACACCATG TTTTCTGGAATCTCCTCTTG	987	57
10-II	9F 9R	CCCCTGGAGAAACTCTCAAC TTGTAAATGTGTTGCCTGAT	1015	57
10-III	10F 10R	CCTATAGAGAGCGGCTA TGTTAGATGGTCTTTGCTGTTG	937	Unable to optimize
10-IV	11F 11R	GCTCAATGAAAGAACTGTTG TAAAGGTAAAGAGAGTGGCAGT	927	57
10-V	12F 12R	TCCACACATCAGTATAGTCC GTAAGTAATTCTGCATGGAC	905	59
10-VI	13F 13R	GAAGACATTTTGGGAAACACT CCCATGCATTTTGGTGAAAT	905	59

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Table 3.3 Oligonucleotides and polymerase chain reaction (PCR) conditions for *ENAM* mutation analysis (Modified from Kim et al., 2005a).

Exon	Primer	Primer sequences for PCR 5´–3´	Product size (bp)	Annealing temperature (°C)
10-III	14F 14R	CAAAGGAGGCCCAACAGTTA TTGTTCCCATCTCCTCCAAG	957	57
10-III	15F 15R	CCCTAACTTCATCCCACCAA TCATGGCCTTCTTGCTTTTT	1128	57

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. Ten exons of *ENAM* were amplified with the use of 13 primer pairs (Table 3.2), as previously published (Gopinath et al., 2008). Polymerase chain reactions (PCR) were performed in a 20 microliter volume containing 50 nanograms genomic DNA, 1X PCR buffer (Fermentas, Burlington, Ont., Canada), 1.75 millimolars $MgCl_2$, 0.25 millimolar dNTPs, 0.5 micromolar of each primer, and 0.8 Unit Taq polymerase (Fermentas, Foster City, Cal., USA), using the parameters identified in (Table 3.4, 3.5 and 3.6). 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.

During the experiment in this study, it is unable to optimize the appropriate condition for some of the PCR product of exon 10 with primer 10F/10R (Table 3.2). This part of exon 10 of *ENAM* was later amplified with the use of new 2 primer pairs (Table 3.3), as previously published (Kim et al., 2005a). Polymerase chain reactions (PCR) were performed in a 20 microliter volume containing reagents as described above, using the parameters identified in (Table 3.4 and 3.5). Then 4 microliters of the PCR products were verified by electrophoresis (Figure4.1), using the condition as described above.

PCR products were sent for direct sequencing to Functional Biosciences Inc., (Madison, Wis., USA). Primers used for sequencing were the same as those used for the PCR reactions. Analyses were performed using Sequence analysis software:

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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 all exons.

Reagent	Concentration
10X PCR buffer	1X
25mM Magnesium chloride	1.75 mM
10mM Deoxynucleotide triphosphates	0.25 mM
10µM Oligonucleotide primers	0.5 µM
5U/µL AmpliTaq Gold DNA polymerase	0.8 U
50ng/µL Genomic DNA sample	2 µl

Table 3.5 PCR conditions of exon 1-10 (for all primers except 10F/10R, 12F/12R and 13F/13R).

Process	Temperature/Time
Initiation	95˚C/5min
Denaturing	94˚C/30sec (40 cycles)
Annealing	57˚C/1min (40 cycles)
Extension	72˚C/1min (40 cycles)
Final extension	72˚C/7min

Table 3.6 PCR conditions of exon 10 (Primer 12F/12R and 13F/13R).

Process	Temperature/Time
Initiation	95˚C/5min
Denaturing	94˚C/30sec (40 cycles)
Annealing	59˚C/1min (40 cycles)
Extension	72˚C/1min (40 cycles)
Final extension	72˚C/7min

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3.2.2 Data Analysis

The data from sequencing were compared with the coding sequence for the nucleotide position within the Genbank entry AY167999. The changed nucleotides and amino acids were analyzed to the level of conservation of nucleotides and amino acids. For protein sequence comparisons, ENAM orthologs were first recognized through a BLAST search of nonredundant databases, using Homo sapiens ENAM, accession NP_114095.2 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).

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