CHAPTER III

MATERIALS AND METHODS

3.1 Sample collection

This study was approved by the Human Experimentation Committee of the Faculty of Dentistry, Chiang Mai University. Informed consent for the use and publication of clinical information, relevant family history and DNA samples were obtained from all participants or their parents if they were under 18 years old. All 16 unrelated participants examined for the diagnosis of XLHED and non-syndromic hypodontia were recruited from various parts of Thailand. Of these, four had XLHED and the other 12 had non-syndromic hypodontia.

The inclusion criteria for XLHED were the three typical manifestations of hypohidrosis, hypotrichosis and hypodontia. In addition to physical examination, all participants with XLHED were radiographically examined for dental anomalies and confirmed to be free of any additional congenital defect, which could be a manifestation of other types of ectodermal dysplasia. The pedigree of each family included in this study showed the X-linked recessive pattern of inheritance. However, sporadic cases of HED were also included in this study.

In the case of non-syndromic hypodontia, the inclusion criteria for the pattern of inheritance were same as those for HED. Both X-linked and sporadic patterns were recruited. Patients with only a congenitally missing tooth (teeth), which occurred in either the deciduous or permanent dentition, were included in the non-syndromic hypodontia group. All were clinically and radiographically examined to

confirm the diagnosis of hypodontia. If any participant had other anomalies, they were excluded from this study.

3.2 DNA extraction

DNA from all participants was isolated from whole blood.

3.2.1 Materials

(1.1) Two-milliliter whole blood samples collected in EDTA.

(1.2) QuickGene DNA whole blood kit L (DB-L)

- Protease (EDB)
- Lysis Buffer (LDB)
- Wash Buffer (WDB)
- Elution Buffer (CDB)

(1.3) Nuclease-free ultra pure water

(1.4) >99% Ethanol

(1.5) QuickGene-610L Nucleic Acid Isolation machine (AutoGen, Inc., Holliston. Massachusetts, USA)

3.2.2 Methods

In order to isolate DNA from whole blood samples, two-milliliter peripheral blood samples from all available donors were collected in EDTA and prepared for DNA extraction. The genomic DNA was isolated from each blood sample using a QuickGene-610L Nucleic Acid Isolation machine. According to the manufacturer's protocol, 300 µl EDB were first added into a 15-milliliter centrifuge tube. Two milliliters of whole blood from each individual were placed into the EDB-containing tubes, and 2.5 ml LDB was immediately added. In order to mix all available samples

thoroughly, they were shaken 10 times vertically and vibrated by vortex mixer at maximum speed for 15 seconds. The samples were incubated in a water bath at 56°C for 10 minutes. After that, 2.5 ml absolute ethanol was added to the samples and mixed as previously described. In the lysis procedure, the whole lysate samples were transferred into the cartridges of the QuickGene-610L Nucleic Acid Isolation machine. Both WDB and CDB were automatically added into all sample cartridges for washing and eluting, respectively. At the end of process, the genomic DNA was isolated from the whole blood sample and kept in the collection tube.

3.3 Polymerase chain reaction

3.3.1 Materials

(1) Genomic DNA 100 ng

(2) 1X polymerase chain reaction (PCR) buffer (Fermentas, Burlington, Ontario, Canada)

(3) Magnesium chloride ($MgCl_2$) 1.5-2 mM

(4) Deoxynucleotide triphosphate (dNTP) 0.25 mM

(5) AmpliTaq Gold DNA polymerase (Applied Biosystems, Carlsbad, California, USA).

(6) Oligonucleotide primers (Table A)

(7) GENEAMP® PCR Instrument System 9700 (Applied Biosystems)

3.3.2 Methods

After completion of DNA isolation, DNA fragments corresponding to each of eight exons of *EDA* with intronic flanking sequences were amplified by polymerase chain reaction using a GENEAMP® PCR Instrument System 9700 with specific

primers and conditions as previously published (Table 1) (Gunadi et al., 2009; Schneider et al., 2001). PCR reactions were carried out in a 40-microliter volume containing 100-nanograms genomic DNA, 1X PCR buffer, 1.5-2.0 mM $MgCl_2$, 0.25 mM dNTPs, 0.2 μM of each primer, 0.5 Unit of AmpliTaq Gold DNA polymerase (Table 2). Samples were submitted to 38 cycles of denaturation at 94°C for 30 seconds, annealing at appropriate temperatures for each exon (Table 1) for 30 seconds, and extension at 72°C for 30 seconds. In the extension period of the final cycle, the temperature of 72°C was extended to 7 minutes to ensure complete extension (Figure 1).

Table 3.1 Primer pairs used for mutation detection in *EDA*

Exon	Primer sequence (5' to 3')	Fragment Size (bp)	Annealing temp (°C)	Reference
1A-F 1A-R	TGAACGGCTGAGGCAGACG TCCGAGCGCAACTCTAGGTA	262	66	Gunadi et al., 2009
1B-F 1B-R	GCCTGCTCTTCCTGGGTTT GCCCCTACTAGGTGACTCA	298	58	Gunadi et al., 2009
3-F 3-R	ATGTTGGCTATGACTGAGTGG CCCTACCAAGAAGGTAGTTC	248	57	Schneider et al., 2001
4-F 4-R	GATCCCTCCTAGTGACTATC CAGACAGACAATGCTGAAAGA	215	57	Schneider et al., 2001
5-F 5-R	AAAAAAGTAACACTGAATCCTATT CTCTCAGGATCACCCACTC	287	57	Schneider et al., 2001
6-F 6-R	GGAAGTCAAAAGATTATGCCC CTACCCAGGAAGAGAGCAAT	113	57	Schneider et al., 2001
7-F 7-R	CTGAGCAAGCAGCCATTACT GGGGAGAAGCTCCTCTTTG	156	57	Schneider et al., 2001
8-F 8-R	ACTGAGTGACTGCCTTCTCT GCACCGGATCTGCATTCTGG	214	57	Schneider et al., 2001
9-F 9-R	TGTCAATTCACCACAGGGAG CACAGCAGCACTTAGAGG	410	60	Schneider et al., 2001

Table 3.2 Reagents and concentrations used for polymerase chain reaction of *EDA*

Commercial concentration	Concentration used in this study
10X PCR buffer	1X
25 mM Magnesium chloride	1.5-2 mM
10 mM Deoxynucleotide triphosphates	0.25 mM
100 µM Oligonucleotide primers	0.2 µM
5U/µL AmpliTaq Gold DNA polymerase	0.5 U

Figure 3.1 Amplification conditions for polymerase chain reaction of *EDA*

3.4 Gel electrophoresis

3.4.1 Materials

(1) Agarose powder

(2) 1X TBE Buffer

(3) SYBR® Safe DNA gel stain

(4) 6X Loading dye

(5) Sub-Cell® GT Agarose Gel Electrophoresis System (Bio-Rad Laboratories, Hercules, California, USA)

(6) Molecular Imager Gel Doc XR+® System (Bio-Rad Laboratories)

3.4.2 Methods

All PCR products were verified by electrophoresis. First, three hundred and fifty milligrams of agarose powder was dissolved in 35 µl warm TBE buffer to produce 1% agarose gel. Next, 3.5 µl SYBR® Safe DNA gel stain was added and mixed with the warm agarose gel. This study used a Sub-Cell® GT Agarose Gel Electrophoresis System (Bio-Rad Laboratories) and 1X TBE medium. Four microliters of all PCR products were mixed with 1 µl of 6X loading dye before loading into the gel boxes. After that, electrophoresis was conducted on the PCR products at 115 mA for 22 minutes and visualized by UV illumination from the Gel Doc XR+® machine.

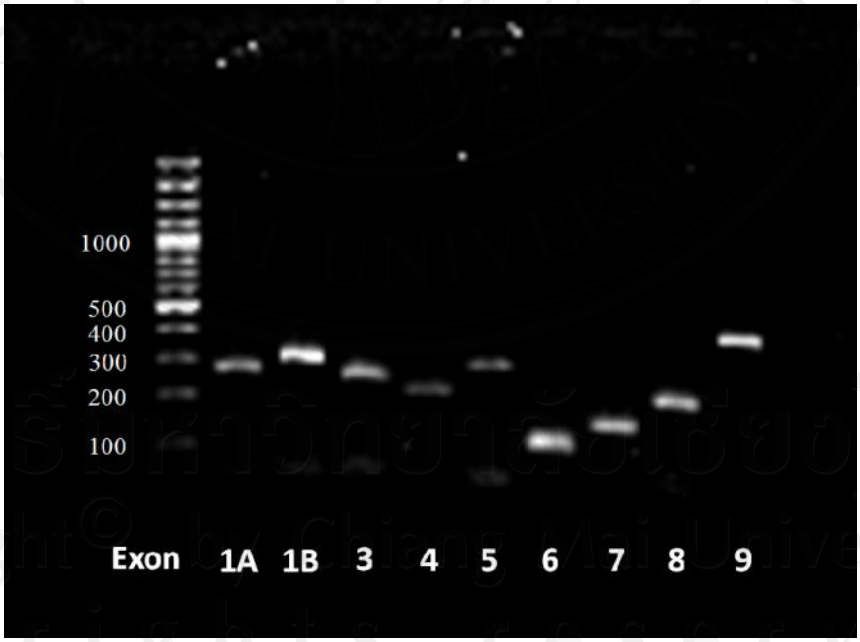

Figure 3.2 Gel electrophoresis. Aliquots of PCR-amplified products of exons 1A-9 were performed on 1% agarose gel and visualized by UV illumination. Each band comparing with the base pair-number labeled marker corresponds to it fragment size.

3.4 Data analysis

Direct sequencing (Functional Biosciences, Madison, Wisconsin, USA) was performed to detect mutations and polymorphisms in all patients and 100 normal controls (150 X-chromosomes) of Thai ethnic background. The sequencing data was compared with the coding sequence of the GenBank accession number NM_001399. The changed amino acids were analyzed to investigate the level of conservation of amino acids. In order to compare the protein sequence, Homo sapiens EDA accession NP_001390.1 was used as the reference sequence. All variations were also confirmed by using the NCBI (http://www.ncbi.nlm.nih.gov/blast) and UCSC (http://www.geniome.ucsc.edu) Blast servers. Moreover, the protein sequence in FASTA was performed in ClustalX (version 2.0, Bioinformatics, Oxford, England) and compared with those of different species.