CHAPTER III

MATERIALS AND METHODS

3.1 MATERIALS

3.1.1 Patients

Our study was approved by the Human Ethics Committee of the Faculty of Dentistry, Chiang Mai University. Informed consent was given by the affected or their parents. Our study samples comprised of 63 patients with orofacial clefts from Nan and Tak provinces of Thailand, 23 patients with isolated ankyloglossia, 6 patients with isolated hypodontia, and 4 family members of the proband who carried the *TBX22* mutation.

Inclusion criteria for the study samples were orofacial clefts, ankyloglossia without other associated anomalies and hypodontia without other associated anomalies. Patients with orofacial clefts were those with cleft lip with or without cleft palate, isolated cleft palate and other facial clefts. These also included those who had surgery to repair the clefts. Patients with isolated ankyloglossia included (1) those whose free tongue, or the length of the tongue from the insertion of the lingual frenum at the base of the tongue to its tip, was less than 10 mm, and (2) those incapable of lifting the tip of the tongue more than half the height of the oral cavity during maximum opening. Patients with hypodontia included those with one or more congenitally missing deciduous or permanent teeth. (See details in Appendix A)

One hundred normal individuals were examined for mutation analysis as normal controls. Inclusion criteria for the controls were being healthy, absence of the phenotype of any kinds of orofacial clefts, or of ankyloglossia or hypodontia.

Table 3.1 The list of phenotypes of patients in this study.

	Total
Orofacial clefts	63
Cleft palate	10
Cleft palate with ankyloglossia	6
Cleft lip and palate	31
Cleft lip	5
Cleft lip with ankyloglossia	1
Cleft lip and palate with ankyloglossia	4
Cleft lip and palate with hypodontia	1
Cleft lip and palate with ankyloglossia and hypodontia	1
Cleft lip and palate with ankyloglossia, hypodontia and upper limb anomalies	1
Cleft lip and palate with hemifacial microsomia	1
Cleft lip and palate with oblique facial cleft	1
Cleft lip and palate with syndactyly	1
Isolated ankyloglossia	23
Isolated hypodontia	6

3.1.2 PCR materials

3.1.2.1 Genomic DNA 100 ng

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

3.1.2.3 Magnesium chloride ($MgCl_2$) 2.5 mM

3.1.2.4 Deoxynucleotide triphosphate (dNTP) 2.5 mM

3.1.2.5 AmpliTaq Gold DNA polymerase from Appliedbiosystems, Cal., USA

3.1.2.6 Oligonucleotide primers (20-22 bases in length)

Table 3.2 Oligonucleotides and PCR conditions for *TBX22* analysis (adapted from Braybrook *et al*., 2001).

Gene/exon	Primer sequence (5' to 3')	Annealing Temp. (^{o}C)
*TBX22*e1-F	CTC CCT AAC CCA GTT CAG GTT	59
*TBX22*e1-R	TAA TGT GGC TGT CTG GCT GCG T	
*TBX22*e2-F	AGC GAG AAG TGG GCA TGT GAA	59
*TBX22*e2-R	CTA CTG TAT GTC AGG GAG TTG	
*TBX22*e3-F	ACT GGA GTC AGC ATT TGT CCA	59
*TBX22*e3-R	GTC TGA AGG TCC AAA TCC CT	
*TBX22*e4-F	CTG GAG TGA AGT CCT CAG GA	59
*TBX22*e4-R	TGC AGG GCT TGA ACA GTT CCT	
*TBX22*e5-F	ACA TGG TGG AGG TGG TCA GGA	62
*TBX22*e6-R	AGA TGT CAT TGC TAT GCT GC	
*TBX22*e7-F	CTG GGG ATG CTG AAA GTT GAC T	59
*TBX22*e7-R	ACA TAT CCC CTT GTG TAG TA	
*TBX22*e8-F	CTA AGG ATG AAG CAC AGA TAG T	59
*TBX22*e8-R	TGA AGC TCA AGG CCA CTG TA	

3.2 METHODS

3.2.1 DNA extraction

In order to extract DNA from white blood cells (Figure 3.1), five millilitres of whole blood was collected from each study sample and placed into centrifugation

tubes. Red blood cells were lysed with buffer composed of 1 mM NH_4HCO_3, 115 mM NH_4Cl and diH_2O. Ten millilitres of red cell lysis buffer was added into the tubes, which were then centrifuged at 3,000 rpm for 5 minutes. Twelve millilitres of the clear solution were discharged. Ten millilitres of red cell lysis buffer was added to the remaining concentrate, which was then mixed and centrifuged at 3,000 rpm for 5 minutes. The remainder of the clear solution was then discharged. Then the remaining white blood cells were lysed by two millilitres of hypotonic solution consisting of 100 mM Tris-Cl, 40 mM EDTA, 50 mM NaCl and 2% SDS. Next, cellular and histone proteins bound to DNA were removed by adding 10µl of 20 mg/ml proteinase K and the remainder was incubated at 50-56^{o}C for 2 hours. After that, protein was precipitated by inorganic salting out. Three hundred microlitres of 6 M NaCl was added to the tubes, which were spun at 12,000 rpm for 5 minutes. After the protein precipitated, equal volumes of the remaining clear solution were poured into two tubes. The two tubes were spun at 12,000 rpm for 5 minutes and the clear solution was discharged. Subsequently, cold ethanol was added and the tubes were agitated to precipitate DNA. The DNA is insoluble in alcohol and, therefore, adhered together. The DNA was cleaned with 70% alcohol. After that, the DNA was diluted in an alkaline buffer (Lewis, 2007).

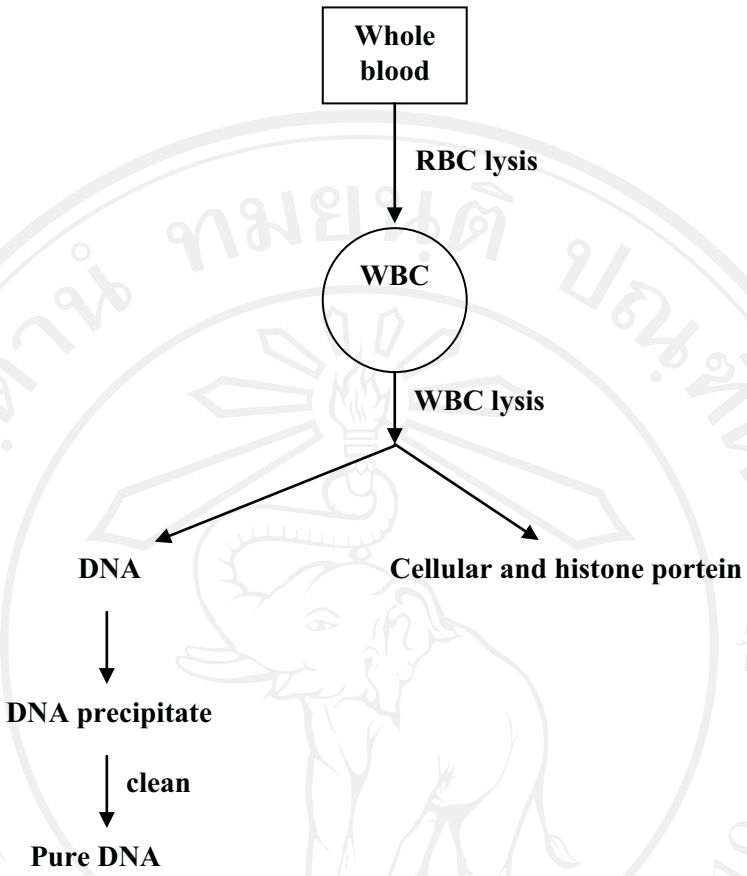

Figure 3.1 Diagram of DNA extraction from blood samples.

3.2.2 Polymerase chain reaction (PCR)

Polymerase chain reactions (PCRs) allow specific DNA sequences to be copied or amplified over a million-fold in a simple enzyme reaction (Figure 3.2).

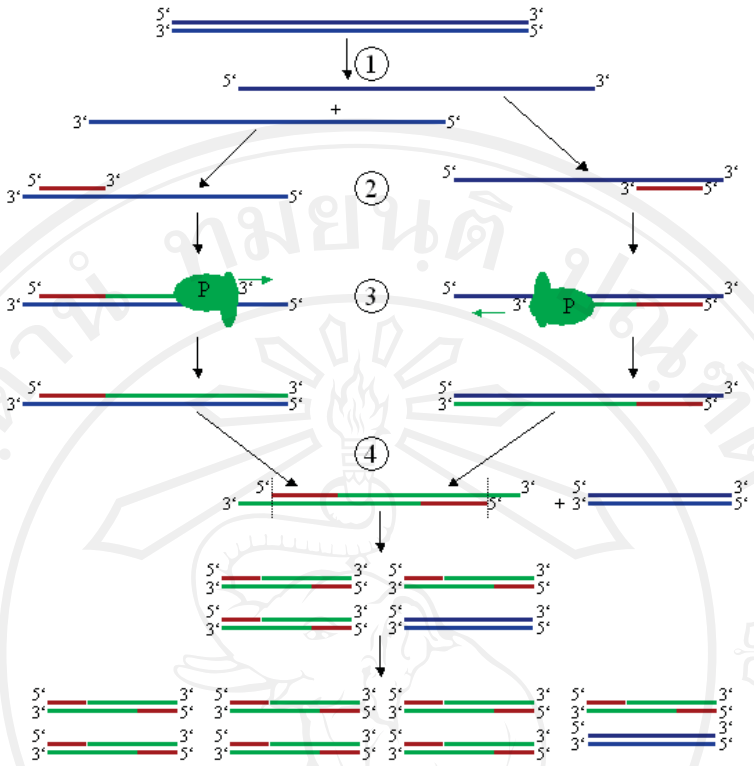

Figure 3.2 Schematic drawing of the PCR cycle. (1) Denaturing at 95°C. (2) Annealing at 59°C. (3) Elongation at 72°C (P=Polymerase). (4) The first cycle is complete. The two resulting DNA strands make up the template DNA for the next cycle, thus doubling the amount of DNA duplicated for each new cycle (adapted from Erlich and Arnheim, 1992).

We used 100 ng of genomic DNA, 1x polymerase chain reaction (PCR) buffer (Fermentas, Burlington, Ont., Canada), 1.75 mM $MgCl_2$, 0.25 mM dNTPs, 0.1 µM of each primer, and 0.8 unit of AmpliTaq Gold DNA polymerase (Applied Biosystems, Cal., USA) in a total volume of 20µl Each cycle (of 30) of the PCR had three steps with differences in temperature. The first step was denaturation, where the temperature was up to 94°C for 30 seconds. In this step DNA templates were denatured. The second step was annealing, with a temperature of 59-62°C for 60 seconds during which the primers attached to the single-stranded DNA template. The

last step was extension, with a temperature of 72°C for 60 seconds, in which the DNA polymerase synthesized new DNA strands complementary to the DNA template strands. For every cycle except the final one, a temperature of 72°C must be maintained for 60 seconds during the third step. In last cycle, a temperature of 72°C should be extended to 7 minutes to ensure complete extension (Erlich and Arnheim, 1992).

3.2.3 Gel electrophoresis

PCR products were comfirmed by size, using gel electrophoresis. Three hundred and fifty milligrams of agarose powder was dissolved in 35 ml of 1xTBE buffer. Next, 4 µl SYBR® Safe DNA gel strain was added to the solution, blended and poured into a molding containing gel box formers, to produce agarose gel. The molding containing the gel was placed in a Sub-Cell® GT Agarose Gel Electrophoresis System (Bio-Rad Laboratories, Inc., Hercules, Cal., USA), attached to a power supply. The gel box formers were then removed, revealing the gel boxes. Four µl of PCR products were mixed with 1 µl of 6x loading dye and loaded into the gel boxes in 1xTBE medium. One hundred and fifteen mA of electric current was run through the gel for 25 minutes. The electric current forced the PCR product fragments to migrate through the agarose gel. DNA fragments migrated from negative to positive potential due to the net negative charge of the phosphate backbone of the DNA chain. Fragment size determination was done by comparison to commercially available DNA ladders containing linear DNA fragments of known length (Winter *et al.*, 2002). After that, the intensity of absorbance of DNA solution was measured at wavelengths of 260 nm and 280 nm (Figure 3.3).

Figure 3.3 Gel electrophoresis of exon 1-8 of *TBX22*.

3.2.4 DNA sequencing

The order of nucleotide bases in the PCR products were revealed by dideoxy chain termination. Random termination of DNA synthesis produced a ladder of DNA molecules. These were separated by gel electrophoresis and the sequence was read from the shortest to the longest fragments (Winter *et al.*, 2002). The DNA obtained using PCR is called a DNA template. The DNA template was sequenced by reactions which contained DNA template, DNA polymerase, primers, deoxynucleotide triphosphates (dNTPs), and dideoxynucleotide triphosphates (ddNTPs). The difference between deoxynucleotide triphosphate and dideoxynucleotide triphosphates is the lack of a hydroxyl group on the 3' carbon of the ribose sugar in the latter. The dNTPs and the ddNTPs were incorporated into growing DNA polynucleotide by polymerase, but the lack of a 3' hydroxyl group in the ddNTPs prevent formation of a phosphodiester bond with the next nucleotide to be added. The elongation of DNA

polymerase was prevented. Each reaction generated a series of DNA molecules of different lengths, terminating at positions corresponding to the presence of one of the bases in the template. The DNA molecules were separated by size on a polyacrylamide gel and visualized by autoradiography. A DNA ladder was obtained and the sequence was read from the wavelength of the terminal base in successively longer fragments (Winter *et al.*, 2002). Direct sequencing was performed at Macrogen Inc, Seoul, Korea.

3.2.5 DNA sequence analysis

DNA sequences were analysed by a software program (Sequencer, version 4.8; GeneCodes Corp., Ann Arbor, Mich., USA). The program allowed sequences to be compared with other sequences in the GENBANK database and with the sequences in the control patients, to look for similarity and mutation (Winter *et al.*, 2002).

3.2.6 Protein sequence comparison

TBX22 orthologues were identified from database accession NP_001103348.1 as a reference sequence. The protein sequence in FASTA format was aligned using ClustalX (version 2.0, Bioinformatics, Oxford, England). The human TBX22 sequence was aligned with the mouse (NP_660259.1), rat (XP_228489.2), monkey (XP_001102902.1), chimpanzee (XP_529056.2), horse (XP_001502582.2), and chicken (NP_989437.1) in Clustal alignment. The multiple protein sequence alignment of TBX22 protein from different species was colored by the variation in polarity and size of amino acid chains. Small hydrophobic amino acids were colored green, large aromatic amino acids were green or blue, large polar/basic amino acids

were purple, and small polar/acidic amino acids were orange or red. The amino acid homology quality score is represented by vertical bars under the sequence alignment of the TBX22 putative orthologues in the ClustalX output. This score can estimate the significance of a given amino acid substitution within the alignment. It measures both amino acid class conservation as well as evolutionary conservation at any given site (Jezewski *et al*., 2003).

3.2.7 PolyPhen

PolyPhen or Polymorphism Phenotyping (http://genetics.bwh.harvard.edu/pph/) is an automatic tool for prediction of the possible impact of an amino acid substitution on the structure and function of a human protein. The amino acid sequence of TBX22 protein in FASTA format, the position of amino acid variants and the specific variants were input into PolyPhen. Then PolyPhen starts a fully automated pipeline of several programs. The prediction output is divided into four categories as follows (Ramensky *et al*., 2002):

- "Probably damaging" means it is predicted with high confidence to affect protein function or structure
- "Possibly damaging" means it may affect protein function or structure
- "Benign" means it is most likely not to casue any phenotypic effect
- "Unknown" means that, in some rare cases, the lack of data does not allow PolyPhen to make a prediction