CHAPTER III

MATERIALS AND METHODS

Research instruments

1. Pipette tip : 10 µl, 200 µl, 1000 µl (Axygen)
2. Fiter-tip : 10 µl, 100 µl,200 µl, 1000 µl (Axygen)
3. Microcentrifuge tube : 0.2 ml, 0.5 ml, 1.5 ml (Axygen)
4. Polypropylene tube : 15 ml (Sterilin)
5. Beaker : 50 ml, 100 ml, 500 ml, 1000 ml (Pyrex)
6. Flask : 100 ml, 200 ml (Pyrex)
7. Bottle : 100 ml, 500 ml, 1000 ml (Pyrex)
8. Cylinder : 50 ml, 100 ml, 500 ml (Pyrex)
9. Glass pipette : 5 ml, 10 ml (Pyrex)
10. Pipette rack (Axygen)
11. Cell line culture flask (Corning)
12. Stirring-magnetic bar
13. Stirring hot plate (Schott)
14. Fume hood (Captair)
15. Micropipette : 10 µl, 20 µl, 100 µl, 200 µl, 1000 µl (Gilson)

16. Pipette boy (Brand)
17. Waterbath (Memmert)
18. Centrifuge (Haraeus)
19. Vortex (Scientific industries)
20. Microcentrifuge (Hettich)
21. Electrophoretic chamber (Amersham)
22. Electrophoretic power supply (Amersham)
23. Parafilm
24. Spectrophotometer (Thermo specific)
25. Cuvette (Starna)
26. Gel doc (Syngene)
27. Thermal cycler (MJ research)
28. Refrigerator (Sanyo)
29. Freezer : -20 °C (Sanyo)
30. Freezer: -80 °C (Thermo Forma)
31. Freezer: -196 °C (Forma Scientific)
32. comb (Amersham)
33. Gel doc software (Syngene)
34. Safety cabinet (Nuaire)
35. Incubator (Forma Scientific)

36. Realtime-PCR machine (MJ research)

37. Microcentrifuge tube for realtime PCR machine (Bio-Rad)

38. Microcentrifuge for realtime PCR machine (Bio-Rad)

39. Cryogenic vial (Corning)

40. Beacon designer software for TaqMan assay

41. Biotools software (Chang Bioscience)

42. MJ Opticon Monitor Analysis software version 3.1 (Bio-Rad)

Reagents

A. General reagents

1. Double distilled water
2. Absolute ethanol (Merck)
3. Phenol/chloroform (USB)
4. Sucrose (USB)
5. Tris base (USB)
6. EDTA (Fluka)
7. Hydrochloric acid (Merck)
8. Sodium chloride (Merck)
9. Ammonium acetate (USB)
10. Ethidium Bromide (Sigma)
11. Sodium hydroxide (AnalaR)

12. Sodium dodecyl sulfate (USB)

13. Proteinase K

14. Triton X-100 (USB)

15. 100 base pair ladder (Sigma)

16. 100% DMSO

17. RPMI (Biological Industries)

B. Reagents for PCR

1. 10X PCR buffer (Qiagen)

2. Q-solution (Qiagen)

3. Magnesium chloride (Qiagen)

4. Deoxynucleotide triphosphates (dNTP) (Qiagen)

5. Oligonucleotide primers (Proligo)

6. HotstarTaq DNA polymerase (Qiagen)

C. Reagents for Reverse transcription

1. RNA blood mini kit (Qiagen)

2. Improm-II™ reverse transcription system (Promega)

3. RNase-free DNase set (Qiagen)

4. DEPC-treated water (USB)

D. Reagent for quantitative realtime-PCR

1. QuantiTect Probe PCR Kit (Qiagen)
2. Oligonucleotide primer (Proligo)
3. TaqMan probes (Operon)

Procedure

1. Subjects and sample collection

The peripheral blood samples of cases, who were already characterized, were sent from Chiang Mai to Rajanukul Insitute with informed consents.

The 10 characterized normal control samples were already collected and processed as DNA and lymphoblastoid cell line.

2. DNA extraction

The conventional phenol/chloroform extraction was performed as follow:

Day 1

1. 3 ml. of whole blood is centrifuged at 3,500 rpm for 10 minutes.
2. Remove supernatant and collect buffer coat to 1.5 ml centrifuge tube.
3. Add cold Lysis buffer I until it reach 10 ml scale, vortex for 1 minute.
4. 4 °C centrifuge at 3,500 rpm for 15 minute.
5. Remove supernatant, wash leukocyte with 5 ml Lysis buffer I.
6. 4 °C centrifuge at 3,500 rpm for 15 minute.
7. Discard supernatant. The white pellet will appear, if not, repeat step 5-6
8. Add 10 µl 20 mg/ml proteinaseK, 1 ml Lysis buffer II and 50 µl 10 % SDS. Vortex 30-60 seconds, incubate overnight at 37 °C.

Day 2

9. Add 500 µl phenol/chloroform, vortex for 1 min then centrifuge at 6,000 rpm for 5 minutes at room temperature.
10. Discard lower fraction, centrifuge at 6,000 rpm for 5 minutes.

11. Equivalent transfer upper part into two 1.5 ml tubes (~500 µl).
12. Add 500 µl cold absolute ethanol, 250 µl ammonium acetate then invert several times.
13. Incubate at -20 °C for 1 hour or more, centrifuge at 14,000 for 20 minutes.
14. Discard supernatant with 500 µl cold 70 % ethanol, tap pellet with finger, centrifuge at 14,000 rpm for 5 minutes.
15. Discard supernatant and overnight dry at room temperature.

Day 3

16. Resuspend with 150 µl 1 M Tris-HCl (pH 8.0), overnight incubate at 37 °C.

Day 4

17. Pool DNA, quantify the DNA by spectrophotomer.

3. PCR (Polymerase chain reaction)

The primer sets for conventional PCR were designed to cover coding regions and splice sites, then polymerase chain reactions were performed to screen a mutation in candidate genes consisting *LNP* (*KIAA1715*), *EVX-2*, *HOXD11*, *HOXD10*, and *HOXA11*. The primer sets, PCR components and PCR condition are shown as follow;

Table1 Candidate genes and primer sets for PCR

Gene	Region	Total exon	Name	Sequence (5'-3')	GC-rich	AT (°C)	Size (bp)
LNP	5UTR		5U1F	GCAGAGCTCTAGGCCACTTG	Y	60	452
			5U1R	CATAACTGCGACACGACACC			
			5U2F	TTCAAGGCTATAATCAAAGGAGA	N	50	306
			5U2R	TTCCATATATGTGGCATCCAA			
EVX2	CDS	4	E1-1F	TCAGAGTAGTTGCCCAGGGT	N	60	473
			E1-1R	GCCATTATTCAGAGGCGG			
			E1-2F	GTTTTCCTTAAGCTGAGCGG	N	58	477
			E1-2R	GCGCAGCCTATCATTAGGAC			
			E2F	AGTTTGGGAAGCTCTGGGTT	N	60	296
			E2R	TTTTGGGAAAGGTACCCTGG			
			E3F	AAACAATCAACCCAGATGCC	N	58	466
			E3R	TGTAAGCATGACAGACCCCA			
			E4-1F	AGTGGCCGGATGGAAGTAG	Y	58	557
			E4-1R	GAAAGGTTGGATTGGGACAA			

Gene	Region	Total exon	Name	Sequence (5'-3')	GC-rich	AT (°C)	Size (bp)
EVX2			E4-2F	GACTGACTGCTGTGGCAACT	Y	62	423
			E4-2R	CGACCCCAGCTTCTACACCT			
			E4-3F	GCTCCTTCTCCCCCAATTC	Y	62	432
			E4-3R	CAGTTGCCACAGCAGTCAGT			
	5UTR		5U1F	TCCCGAGATGTCATTCATCA	N	58	545
			5U1R	CTTATGCCCCCAGCTTGTAA			
			5U2F	CCGAGTTGGACAAATTGGAG	N	59	425
			5U2R	TGAAGCTGCCTTCATTACCC			
HOXD11	CDS	2	E1-1F	CGGTGGGGATTTTTGCTTAG	Y	62	311
			E1-1R	GTAGTCGCGGAAGGCCACT			
			E1-2F	AGTGGCCTTCCGCGACTAC	Y	58	375
			E1-2R	GCTGCCTGTAGAACTGGTC			
			E1-3F	CCGCCTCCAACTTCTACAGC	Y	65	320
			E1-3R	CAGTACCCGGTGCCTACCT			
			E2-1F	CCATATATCATCCCCCACGA	Y	56	524

Gene	Region	Total exon	Name	Sequence (5'-3')	GC-rich	AT (°C)	Size (bp)
			E2-1R	GTGGGAAGGAATCGTGAAGT			
HOXD11	5UTR		5U1F	GAAGAGAAACGAGGCAAGGA	Y	58	443
			5U1R	TCCAGGCTCCCTAGAAGCTA			
			5U2F	CGGTGAACAAGAAGCAACAA	Y	60	585
			5U2R	CTTGGGCCAGGATCAACTAA			
			5U3F	TTGGCGAGCGTTGATATAGA	Y	58	348
			5U3R	TAGTCGCGGAAGGCCACT			
HOXD10	CDS	2	E1-1F	TCTCATTGGCTTGGTTGTCA	N	60	574
			E1-1R	AAATATCCAGGGACGGGAAC			
			E1-2F	GTCTTGTCCCGTTGAGAACC	Y	60	500
			E1-2R	GGAGAAGCGGGGACTATCTC			
			E2-1F	ACCAACCAGCAATTGGCTC	N	58	
			E2-1R	GCACGAACATAATGACCCC			
			E2-2F	GAAGATGAGCCGAGAGAACC	N	60	422
			E2-2R	GTCACATTGCTGGATGGATG			

Gene	Region	Total exon	Name	Sequence (5'-3')	GC-rich	AT (°C)	Size (bp)
			E2-3F	CGCATTTTCATTTGGGTCTT	N	58	303
			E2-3R	GGCATAAATCGCCAATATCC			

Note

CDS : coding sequences

UTR : Untranslated region

AT : annealing temperature

Table 2 Reaction components for conventional PCR/ 25 µl final volume

No.	Components	Stock concentration	Final concentration
1	Sterile ddH2O	variable	-
2	Buffer(QIAGEN)	10 X	1 X
3	MgCl2(Qiagen)	-	-
4	dNTP(Biolabs)	25 mM	0.2 mM
5	Forward primer (Proligo)	10 µM	0.5 µM
6	Reverse primer(Proligo)	10 µM	0.5 µM
7	HotstarTaq(Qiagen)	5 unit/µl	0.625 unit
8	DNA	100 pg-100 ng	

Note

- MgCl2 is already supplemented with Qiagen's buffer which will result in final concentration of 1.5 mM.

Table 3 Reaction components for GC-rich PCR/ 25 µl final volume

No.	Components	Stock concentration	Final concentration
1	Sterile ddH2O	variable	-
2	Buffer(QIAGEN)	10 X	1 X
3	Q-slution (Qiagen)	5X	1X
4	dNTP(Biolabs)	25 mM	0.2 mM
5	Forward primer (Proligo)	10 µM	1 µM
6	Reverse primer(Proligo)	10 µM	1 µM
7	HotstarTaq(Qiagen)	5 unit/µl	2.5 unit
8	DNA	100 pg-100 ng	

Table 4 PCR Condition

Step	Temperature and incubation time
1. Initial denaturation	95°C/ 15 min
2. 35 cycles of Denaturation Annealing Extension	 94°C/ 1 min See table 1/ 1 min 72°C/ 1 min
3. Final extension	72°C/ 10 min

4. RNA extraction

RNA extractions were performed using QIAamp RNA miniprotocol for Isolation of total RNA from cultured cells QiAamp® RNA Blood mini Kit.

Procedure

1. Centrifuge 5-ml cultured cells at 300 x g for 5 minutes, remove supernatant.
2. Wash pellets two times with 1 x PBS by pipette up and down, centrifuge at 300 x g for 5 minutes.
3. In final wash, rest 1 ml PBS for pellet resuspension.
4. Transfer suspension into new sterile 1.5-ml centrifuge tube.
5. Disrupt cells by add 600 µl of buffer RLT, mix by pipet

6. Pipet lysate into QIAshredder spin column sitting in a 2-ml collection tube, centrifuge at 13,000 rpm. Discard QIAshredder spin column and save homogenized lysate.
7. Add 1 volume of 70% ethanol to the homogenized lysate and mix well by pipetting.
8. Pipet sample in to a new spin column sitting in a 2-ml collection tube. Centrifuge for 15 seconds at 13,000 rpm.
9. Transfer the QIAamp spin column into a new 2-ml collection tube. Add 700 µl Buffer RW1 into spin column, centrifuge for 15 second at 13,000 rpm.
10. Transfer the spin column into a new collection tube. Pipet 500 µl buffer RPE in to the spin column and centrifuge for 15 seconds at 13,000 rpm.
11. Add 500 µl Buffer RPE, centrifuge at 13,000 rpm for 3 minutes.
12. Transfer the spin column into a new 1.5-ml collection tube and pipet 50 µl of RNase-free water directly on to the membrane, centrifuge for 1 minute at 13,000 rpm for elution.
13. Use immediately or store at -80°C until use.

5. DNase-I treatment

The mRNA samples are treated with DNase-I to eliminate the residue genomic DNA which might interfere with subsequent processes.

Table 5 Genes, primer sets and probes for quantitative real-time PCR

Gene	Region	Selected position	Name	Sequence	Annealing Temperature (°C)	Size (bp)
LNP	CDS	7,9	LNPF	CCGGACTCAAAGAAAGCAAAG	56	75
			LNPR	GAATCTCTTGTCCAGGTCTTG	56	
			LNPP	TGCTCCAGCAGATGGCGGCTCACA	69	
GAPDH		5'UTR-exon3	GAPDHF	CGACAGTCAGCCGCATCTTC	56	104
			GAPDHR	CGCCCAATACGACCAAATCCG	56	
			GAPDHP	CGTCGCCAGCCGAGCCACATCG	72	

Note The 5' terminal of LNP probe was labeled with FAM, while 3' was labeled with TAMRA.

The 5' terminal of GAPDH probe was labeled with Cy5, while 3' was labeled with BHQ (Black hole quencher).

6. Reverse transcription

The DNase-I treated mRNA samples are transcribed to cDNA by reverse transcriptase using oligodT primer. To prevent RNase contamination, DEPC-treated water and RNase inhibitor are applied.

Procedure

A. Target RNA and primer combination and denaturation

1. Place sterile thin-walled dilution tubes and reaction tubes on ice.
2. Prepare the reaction as follow

Table 6 Components for RNA-Primer combination and denaturation

No.	Component	volume
1	RNA template	Up to 1 μg
2	Oligo-dT Primer	20pmol or 0.5 μg
3	Nuclease free water	Adjust to **5** μl

3. Incubate at 70 ° C for 5 minutes.
4. Chill on ice immediately at 4 ° C for 5 minutes.

B. Reverse transcription

1. Prepare reaction mix (table 7)

Table7 Components for reverse transcription/ 15 µl total volume

No.	Component	Stock concentration	Final concentration
1	Nuclease-free water	variable	-
2	ImProm-II™5X reaction buffer	5X	1X
3	$MgCl_2$	25mM	3mM(1.5-8)
4	dNTP mix	25mM	0.5mM
5	Recombinant RNasin®Ribonuclease inhibitor		20 unit
6	Vortex the mixture, then add Improm-II™Reverse Transcriptase		

2. Aliquot reaction mix to each reaction tube on ice. Add 5 µl of RNA and primer mix to each reaction.
3. Anneal by incubate at 25 ° C for 5 minutes.
4. Extend by incubate at 42 ° C for one hour.
5. Inactivate enzyme by incubate at 70 ° C for 15 minutes
5. Proceed next step or store at -20 ° C until use.

7. RT-PCR

Table 8 Components for RT-PCR

No.	Components	Stock concentration	Final concentration
1	Sterile ddH2O	variable	-
2	Buffer(QIAGEN)+15 mM MgCl2	10 X	1 X
3	MgCl2 (Qiagen)	25mM	0.5 mM
4	dNTP(Biolabs)	25 mM	0.2 mM
5	Forward primer (Proligo)	10 µM	1 µM
6	Reverse primer(Proligo)	10 µM	1 µM
7	HotstarTaq(Qiagen)	5 unit/ µl	2.5-5 unit
8	cDNA	variable	< 500 ng/ reaction

Table 9 RT-PCR condition

Step	Temperature and incubation time
1. Initial denaturation	95°C/ 15 min
2. 35 cycles of	
Denaturation	94°C/ 1 min
Annealing	See table 5/ 1 min
Extension	72°C/ 1 min
3. Final extension	72°C/ 10 min

8. Quantitative real-time RT-PCR

Since relative quantification is used for determine the expression level of interested gene compare with the house-keeping gene, the PCR efficiency must be controlled. Before proceed to a relative quantification, the validation of multiplexed PCR must be completed.

Table 10 Probe-primer mixture preparation

No.	Components	Stock concentration	Final concentration
1	RNase-free water	-	-
2	Forward primer	100 µM	8 µM
3	Reverse primer	100 µM	8 µM
4	Probe	100 µM	4 µM

Table 11 Multiplexed-PCR components/ 20 µl total volume

No.	Components	Stock concentration	Final concentration
1	RNase-free water	-	-
2	Quantitect PCR master mix	2X	1X
3	Probe-primer mix (GAPDH)	8 µM	0.4 µM
4	Probe-primer mix (LNP)	8 µM	0.4 µM
5	cDNA	variable	< 500 ng/ reaction

Table12 Multiplexed PCR condition

Step	Temperature and incubation time
1. Initial denaturation	95°C/ 15 min
2. 40 cycles of	
Denaturation	94°C/ 15 sec
Annealing/extension	60°C / 30 sec
Read plate	

9. Agarose gel electrophoresis

1% agarose gel is used for observe the PCR products from conventional PCR, GC-rich template PCR and RT-PCR. For Multiplexed PCR, 2% agarose gel is applied.