CHAPTER III

METHODOLOGY

Research Instruments

1. Automatic adjustable micropipette : P2(0.1-2 µl), P10(0.5-10 µl), P20(5-20 µl), P100(20-100 µl), P200(50-200 µl), P1,000 (0.1-1ml) (Gilson, France)
2. Pipette tip : 10µl, 100µl, 200µl, 1,000µl (Elkay, USA)
3. Microcentrifuge tube : 0.2ml, 0.5ml, 1.5ml (Bio-RAD, Elkay, USA)
4. Polypropylene conical tube : 16 ml (Elkay, USA)
5. Beaker : 50ml, 100ml, 200ml, 500ml, 1,000ml (Pyrex)
6. Reagent bottle : 100ml, 250ml, 500ml, 1,000ml (Duran, USA)
7. Flask : 250ml, 500ml, 1,000ml (Pyrex)
8. Cylinder : 25ml, 50ml, 100ml, 250ml, 500ml, 1,000ml (Witeg, Germany)
9. Pipette rack (Autopack, USA)
10. Thermometer (Precision, Germany)
11. Parafilm (American National Can, USA)
12. Stirring magnetic bar
13. Plastic wrap
14. Comb
15. Vortex (Scientific Industry, USA)
16. pH meter (Eutech Cybernatics)
17. Stirring hot plate (Bamstead/Thermolyne, USA)
18. Balance (Precisa, Switzerland)
19. Centrifuge (J.P.Selecta, Spain)
20. Microcentrifuge (Eppendorf, Germany)
21. Mastercycler personal (Eppendorf, Germany)
22. Thermal cycler (Touch Down, Hybraid USA)
23. Power supply model 250 (Gibco BRL, Scotland)
24. Power poc 3000 (Bio-RAD)
25. Horizon 11-14(Gibco BRL, Scotland)

26. Sequi-gen sequencing cell (Bio-RAD)
27. Heat block (Bockel)
28. Incubator (Memmert)
29. Thermostat shking-water bath (Heto, Denmark)
30. Spectronic spectrophotometers (Genesys5, Milon Roy, USA)
31. UV Trasilluminator (Fotodyne, USA)
32. UV absorbing face shield (Spectronic, USA)
33. Gel doc 1000 (Bio-RAD)
34. Refrigerator 4°C (Misubishi, Japan)
35. Deep freeze -20 and -80°C (Revco)
36. Water purification equipment (Water pro Ps, Labconco, USA)
37. Water bath (J.P.Selecta, Spain)
38. Sequencher software 4.2 Demo (Gene Codes Corporation, MI)
39. Oligo software
40. Autoclave
41. Automatic adjustable multichanal micropipette : P100(20-100 µl) (Eppendorf, Germany)
42. Microwell strip reader capable of reading at 450 nm (620 nm as optional reference wave length)

Reagent

1. General reagents

1.1 Absolute ethanol (Merck)

1.2 Agarose, molecular grade (Promega)

1.3 Ammonium acetate (Merck)

1.4 Boric acid (Merck)

1.5 Tri-base (USB)

1.6 Disodium ethylenediamine tetraacetic acid : EDTA(Merck)

1.7 Bromphenol blue (Pharmacia)

1.8 Ethidium bromide (Gibco BRL)

1.9 Hydrochloric acid (Merck)

1.10 Phenol (Sigma)

1.11 Chloroform (Merck)

1.12 Sodium chloride (Merck)

1.13 Sodium hydroxide (Merck)

1.14 Triton X-100(Pharmacia)

1.15 100 base pair DNA ladder (Biolabs)

1.16 1 Kb pair DNA ladder (Biolabs)

1.17 Water Distillation

2. PCR reagents

2.1 10X PCR buffer with KCl (500mM KCl, 100 mM Tris-HCl pH 8.8, 0.8% Nonidet P40) (Fermentas)

2.2 10X PCR buffer with $(NH_4)SO_4$ (200mM $(NH_4)SO_4$, 750 mM Tris-HCl pH 8.8, 0.1%Tween20) (Fermentas)

2.3 Magnesium chloride (Fermentas)

2.4 Deoxynucleotide triphosphates (dNTPs) (Fermentas)

2.5 Oligonucleotide primer (Operon)

2.6 Oligonucleotide primer (BioDesign)

2.7 *Taq* DNA polymerase (Fermentas)

2.8 100% DMSO

2.9 Genomic DNA sample

2.10 cDNA sample

3. Reverse Transcriptase–Polymerase Chain Reaction (RT-PCR) (Promega)

3.1 0.5µg/µl Oligo dT Primer

3.2 0.5ng/µl RNA template sample

3.3 5X ImProm II™ bufferbuffer

3.4 25mM Magnesium chloride for RNA

3.5 10mM Deoxynucleotide triphosphates (dNTPs) for RNA

3.6 40U/µl RNAsin RNase Inhibitor

3.7 ImProm II Reverse Transcriptase 40U/ml

4. Restriction enzymes

4.1 *Tag*I

4.3 *BsaH*I

4.4 *Rsa*I

5. ELISA reagents and materials

5.1. Biotin-Conjugate anti-human DcR3 monoclonal antibody

5.2. Streptavidin-HRP

5.3. Sample Diluent

5.4. Assay Buffer Concentrate (1%PBS, Tween 20, 10% BSA)

5.5. Wash Buffer Concentrate (1%PBS, Tween 20)

5.6. Substrate Solution (tetramethyl-benzidine)

5.7. Stop Solution (1M Phosphoric acid)

5.8. Microwell Plate coated with monoclonal antibody to human DcR3

6. Commercial Kits

6.1 QIAamp®RNA Blood Mini Kit (QIAGEN)

6.2 QIAamp®DNA Blood Mini Kit (QIAGEN)

6.3 QIAamp®Gel Extraction Kit (QIAGEN)

6.4 ELISA Kit (Bender Medsystem, Austria)

Experimental Procedure

1. Subjects and sample collection

1.1 Blood collection

After informed consent was received, approximate 5 ml of peripheral blood from each individual was collected in a polypropylene tube with EDTA for RNA and DNA extraction.

Approximate 5 ml of peripheral blood was obtained in a polypropylene tube for serum collection.

1.2 Subjects

Patients from unrelated families were clinically diagnosed with ALD, Pompe, HIE, HOS and SLE at Pediatric Clinic of the King Chulalongkorn Memorial Hospital and were included in the study. Selection criteria were based on clinical presentation.

1.3 Controls

Controls were healthy volunteers unaffected with ALD, Pompe, HIE, HOS and SLE, had no family history of ALD, Pompe, HIE, HOS and SLE. DNA and RNA from controls was used for mutation screening in *ABCD1* (ALD), *GAA* (Pompe), *DcR3* (SLE). Serum from controls was used for ELISA in *DcR3* (SLE). DNA from the patients' family members who were at risk was also investigated.

2. Genetic analysis

2.1 DNA extraction and collected plasma

After informed consent, genomic DNA was isolated from peripheral blood leukocytes. This procedure was performed as:

1. 3 ml. of whole blood was centrifuged for 10 minutes at 3,300 rpm.
2. Remove plasma (supernatant) to microcentrifuge tube 1.5 ml for ELISA and transfer buffy coat to a new polypropylene tube. Then add 10 volumes of cold lysis buffer 1 (or 10ml), mix with vortex and incubate at 20°C for 5 minutes.
3. Centrifuge for 8 minutes at 13,400 rpm, and remove supernatant.
4. Add 3 ml of cold lysis buffer 1,mix thoroughly and centrifuge for 8 minutes at 13,400 rpm.
5. Discard supernatant and add 900 µl of lysis buffer 2, 10 µl of proteinase K solution (20 mg of proteinase K in 1.0 ml of 1% SDS-2 mM EDTA, and 50 µl of 10% SDS). Mix vigorously for 15 seconds.
6. Incubate the tube(S) in a 37°C shaking waterbath overnight for complete digestion.
7. Add 1 ml of phenol-chloroform-isoamyl alcohol and shake vigorously for 15 seconds and centrifuge at 6,000 rpm for 5 minutes
8. Transfer the supernatant from each tube (containing DNA) to a new microcentrifuge tube.
9. Add 0.5 volumes of 7.5 M CH_3COONH_4 and 1 volume of 100% ethanol and mix by inversion. The DNA should immediately form a stringy precipitate. Recover the DNA by centrifugation at 13,400 rpm for 15 minutes. Then remove supernatant.

10. Rinse the pellet with 70% ethanol. Decant the ethanol and air-dry the pellet. (It is important to rinse well to remove any residual salt and phenol.)
11. Resuspend the DNA in 20-300 µl of the double distilled water at 37°C until dissolved.

Calculation of DNA concentration

The reading at 260 nm is used for calculating the DNA concentration. An OD of 1 concentration to approximately 50 µg/ml for double-strand DNA. So, DNA concentration can be calculated from the following

DNA concentration = OD X 50 X dilution ration (µg/ml)

2.2 RNA extraction

Total RNA was isolated from white blood cells using QIAamp®RNA Blood Mini Kit (Qiagen, Valencia, CA). Reverse transcription was perform using Improm-II™ reverse transcriptase (Promega, Madison, WI) according to the manufacturer's instructions.

1. Mix 1 volume of human whole blood with 5 volumes of Buffer EL in an appropriately sized tube.
2. Incubate for 10-15 minutes on ice. Mix by vortexing briefly 2 times during incubation.
3. Centrifuge at 4,000 rpm for 10 minutes at 4°C, and completely remove and discard supernatant.
4. Add Buffer EL to the cell pellet (use 2 volumes of Buffer EL per volume of whole blood used in step 1). Resuspend cells by vortexing briefly.
5. Centrifuge at 4,000 rpm for 10 minutes at 4°C, and completely remove and discard supernatant.
6. Add Buffer RLT to the pellet and vortex or pipet to mix.

7. Pipet lysate directly into QIAshredder spin column in a 2 ml collection tube and centrifuge for 2 minutes at maximum speed to homogenize. Discard QIAshredder spin column and save homogenized lysate.
8. Add 1 volumn (350 µl or 600 µl) of 70% ethanol to the homogenized lysate and mix by pipetting. Do not centrifuge.
9. Carefully pipet sample, including any precipitate which may have formed, into a new QIAamp spin column in a 2 ml collection tube without moistening the rim. Centrifuge for 15 seconds at 13,400 rpm. Maximum loading aliquots onto the QIAamp spin column and centrifuge as above.
10. Transfer the QIAamp spin column into a new 2 ml collection tube. Apply 700 µl of Buffer RW1 to the QIAamp spin column and centrifuge for 15 seconds at 13,400 rpm to wash.
11. Place the QIAamp spin column in a new 2 ml collection tube (provided). Pipet 500 µl of Buffer RPE into the QIAamp spin column and centrifuge for 15 seconds at 13,400 rpm.
12. Carefully open the QIAamp spin column and add 500 µl of Buffer RPE and centrifuge at 13,400 rpm for 3 minutes.
13. Recommended: place QIAamp spin column in a new 2 ml collection tube and discard the old collection tube with the filtrate. Centrifuge at 13,400 rpm for 1 minute.
14. Transfer the QIAamp spin column into a 1.5 ml microcentrifuge tube (provided) and pipet 30-50 µl of RNase-free water (provided) directly onto the QIAamp membrane. Centrifuge for 1 min at 13,400 rpm to elute. Repeat if >0.5 ml whole blood (or $>2x10^6$ leukocytes) has been processed.

2.3 DNA amplification by Polymerase Chain Reaction (PCR)

Primer design guidelines:

- Primer length between 18-25 bp.
- Keep G-C content in the 30-80% range.

- The T_m should be 55-60°C.
- G or C at the 3'-end of primers will increase priming efficiency.
- Avoid runs of an identical nucleotide, especially guanine.
- Avoid secondary structure (hairpin, self-complementary and primer dimer)
- The five nucleotides at the 3'-end should have no more than two G and/or C bases.
- Primer sequence should be searched using BLAST and checked for cross-homology
- Primers should be specific with the target gene and not anneal with other genes

2.3.1 Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR)

For mutation screening, RT-PCR was initially performed.

RT-PCR Protocol

1. Mixture for cDNA preparation(reverse transcription)

Components	Amount
0.5 µg/µl Oligo dT Primer	1.0 µl (0.5 µg)
0.5 ng/µl RNA template	10.1 µl (5.05 ng)
Conditions	70°C, 5 minutes
	4°C, 5 minutes

2. Add the following components to the product of step 1

Component	Amount
5X Buffer	4.0 µl (1X)
25mM $MgCl_2$	2.4 µl (3.0 mM)
10mM dNTPs	2.0 µl (0.5 mM)
40U/µl RNasin® RNase Inhibitor	0.5 µl (20.0 U)
Reverse transcriptase	1.0 µl
Total Volume	20.0 µl

Condition	25°C, 5 minutes
	40°C, 60 minutes
	70°C, 15 minutes

3. cDNA can be frozen for later use or used immediately for PCR.
4. cDNA can amplify by PCR with forward and reverse primers

3. Mutation analysis

3.1. Mutation analysis with *ABCD1*

3.1.1 Polymerase Chain Reaction (PCR)

The first, primer were designed within introns to allow genomic amplification and sequencing of exons 1-10 including exon-intron boundaries as shown in Table2, 2.1, 2.2.

Table2. Primer sequences for *ABCD1* mutation analysis

Name	Primer sequence 5' to 3'	Product size (bp)
ALDe1A-F	ACA ACA GGC CCA GGG TCA GA	458
ALDe1A-R	AGG AAG GTG CGG CTC ACC A	
ALDe1B-F	AAC CGG GTA TTC CTG CAG CG	421
ALDe1B-R	ACT GGT CAG GGT TGC GAA GC	
ALDe1C-F	CCA CGC CTA CCG CCT CTA CTT	512
ALDe1C-R	AGA CTG TCC CCA CCG CTC	
ALDe2-F	GGC ACT GGG AGA CCC TG	368
ALDe2-R	TCA GCA CCC AGV GGT ATG G	
ALDe3-F	TTG CAG AAG AGC CTC GCC TT	304
ALDe3-R	TTG CAG GGA GAG AAG CAT GG	
ALDe4-F	GTC GTC GTA CAA GGA GGT AC	385
ALDe4-R	ACA GGA CAC TGC CCA GAG GC	
ALDe5-F	CTG CCA GGG ATG GGA ATG AG	373
ALDe5-R	TCT CAC CTT GAC CTT GGC CC	
ALDe6-F	GCC ATA GGG TAC GGG AAG GG	312
ALDe6-R	GCC TCT GCA GGA AGC CAT GT	
ALDe-7F	CGA TCC ACT GCC CTG TTT TGG	497
ALDe-7R	CTT CCC TAG AGC ACC TGG	

ALDE8/9-F	CTG AGC CAA GAC CAT TGC CCC CG	471
ALDe8/9-R	TGC TGC TGC CGG GCC CGC	
ALDe10-F	GAG GGG AGG AGG TGG CCT GGC	463
ALDe10-R	GCG GGG TGC GTG CAT GGG TGG	

Table2.1. PCR reaction of *ABCD1*

Component	Exon1A	Exon1B	Exon1C	Exon2	Exon3	Exon4
Distilled water (µl)	13.52 (1X)	13.52 (1X)	13.52 (1X)	13.52 (1X)	12.52 (1X)	13.52 (1X)
10XPCR buffer	2.0	2.0	2.0	2.0	2.0	2.0
25mM $MgCl_2$	1.2 (1.2mM)	1.2 (1.2mM)	1.2 (1.2mM)	1.2 (1.2mM)	1.2 (1.2mM)	1.2 (1.2mM)
10 mM dNTP	0.4 (0.2mM)	0.4 (0.2mM)	0.4 (0.2mM)	0.4 (0.2mM)	0.4 (0.2mM)	0.4 (0.2mM)
10µM ALD-F	0.4 (0.13µM)	0.4 (0.13µM)	0.4 (0.13µM)	0.4 (0.13µM)	0.4 (0.13µM)	0.4 (0.13µM)
10µM ALD-R	0.4 (0.13µM)	0.4 (0.13µM)	0.4 (0.13µM)	0.4 (0.13µM)	0.4 (0.13µM)	0.4 (0.13µM)
5U/µl Taq polymerase	0.08 (0.02U)	0.08 (0.02U)	0.08 (0.02U)	0.08 (0.02U)	0.08 (0.02U)	0.08 (0.02U)
50ng/µl Genomic DNA	2	2	2	2	3	2
Total volume (µl)	20	20	20	20	20	20

Component	Exon5	Exon6	Exon7	Exon8/9	Exon10
Distilled water (µl)	13.52 (1X)	13.52 (1X)	13.2 (1X)	13.32 (1X)	13.5 (1X)
10XPCR buffer	2.0	2.0	2.0	2.0	2.0
25mM $MgCl_2$	1.2 (1.2mM)	1.2 (1.2mM)	1.5 (1.5mM)	1.2 (1.2mM)	1.2 (1.2mM)
10 mM dNTP	0.4 (0.2mM)	0.4 (0.2mM)	0.4 (0.2mM)	0.4 (0.2mM)	0.4 (0.2mM)
10µM ALD-F	0.4 (0.13µM)	0.4 (0.13µM)	0.4 (0.13µM)	0.5 (0.16µM)	0.4 (0.13µM)
10µM ALD-R	0.4 (0.13µM)	0.4 (0.13µM)	0.4 (0.13µM)	0.5 (0.16µM)	0.4 (0.13µM)
5U/µl Taq polymerase	0.08 (0.02U)	0.08 (0.02U)	0.1 (0.025U)	0.08 (0.02U)	0.1 (0.025U)
50ng/µl Genomic DNA	2	2	2	2	2
Total volume (µl)	20	20	20	20	20

Table2.2. PCR cycle and condition of *ABCD1*

Step	Exon1A	Exon1B	Exon1C	Exon2	Exon3	Exon4
Initial	94°C/ 5	94°C/ 5	94°C/ 5	94°C/ 5	94°C/ 5	94°C/ 5
PCR cycle	35 cycles	35 cycles	35 cycles	35 cycles	35 cycles	35 cycles
Denature	94°C/	94°C/	94°C/ 1min	94°C/	94°C/ 1min	94°C/ 1min
Annealing	56°C/	60°C/	66°C/ 1min	62°C/	64°C/ 1min	64°C/ 1min
Extension	72°C/	72°C/	72°C/	72°C/	72°C/	72°C/
Final extension	72°C/ 5min	72°C/ 5min	72°C/ 5min	72°C/ 5min	72°C/ 5min	72°C/ 5min

Step	Exon5	Exon6	Exon7	Exon8/9	Exon10
Initial denaturation	94°C/ 5 min	94°C/ 5 min	94°C/ 5 min	94°C/ 5 min	94°C/ 5 min
PCR cycle	35 cycles	35 cycles	35 cycles	35 cycles	35 cycles
Denature	94°C/ 30sec	94°C/ 30sec	94°C/ 1min	94°C/ 1min	94°C/ 30sec
Annealing	60°C/ 30sec	62°C/ 30sec	60°C/ 1min	64°C/ 45sec	62°C/ 30sec
Extension	72°C/ 45sec	72°C/ 45sec	72°C/ 45sec	72°C/ 45sec	72°C/ 45sec
Final extension	72°C/ 5min	72°C/ 5min	72°C/ 5min	72°C/ 5min	72°C/ 5min

3.1.2 Agarose gel electrophoresis and DNA sequencing

The PCR product were verified for correct size on an ethidium bromide-stained 1.5-2% agarose gel. The PCR product were then treated with ExoSAP-IT (USP Corporation, Cleveland, OH) according to the manufacturer's recommendation, and sent for direct sequencing at Macrogen Inc., Seoul, Korea. The sequence was analyzed by sequencher.

3.2 Mutation analysis with *GAA*

3.2.1. Polymerase Chain Reaction (PCR)

The primers were designed within cDNA. The forward primers were designed before ATG and the reverse primers were designed after TAA, TAG or TGA for allow amplification and sequencing as shown in Table 3, 3.1, 3.2.

Table3. Primer sequences for *GAA* mutation analysis

Name	Primer sequence 5' to 3'	Product size (bp)
GAA-F1	CAC CTC TAG GTT CTC CTC GT	1,331
GAA-R2	TCG TTC CAT TGG ACG TCC AG	
GAA-F2	ACC TGG ACG TTG TGG GAT AC	1909
GAA-R1	TCC AGG TGA CAC ATG CAA CC	
GAA-F3	TCT CTC CAC ACA CTA CAA CC	For sequencing

Table3.1. PCR reaction of *GAA*

Component	GAA-F1/R1	Nest GAA F1/R2	Nest GAA F2/R1
Distilled water (µl)	13.82 (1X)	15.02 (1X)	14.82 (1X)
10XPCR buffer	2.0	2.0	2.0
25mM $MgCl_2$	1.2 (1.2mM)	1.2 (1.2mM)	1.2 (1.2mM)
10 mM dNTP	0.3 (0.15mM)	0.3 (0.15mM)	0.3 (0.15mM)
10µM GAA-F	0.3 (0.1µM)	0.2 (0.067µM)	0.3 (0.1µM)
10µM GAA-R	0.3 (0.1µM)	0.2 (0.067µM)	0.3 (0.1µM)
5U/µl Taq polymerase	0.08 (0.02U)	0.08 (0.02U)	0.08 (0.02U)
50ng/µl cDNA	2	1	1
Total volume (µl)	20	20	20

Table3.2. PCR cycle and condition of *GAA*

Step	GAA-F1/R1	Nest GAA F1/R2	Nest GAA F2/R1
Initial denaturation	94°C/ 5 min	94°C/ 5 min	94°C/ 5 min
PCR cycle	35 cycles	35 cycles	35 cycles
Denature	94°C/ 30sec	94°C/ 45sec	94°C/ 45sec
Annealing	57°C/ 30sec	64°C/ 45sec	66°C/ 45sec
Extension	72°C/ 3min 30 sec	72°C/ 2min 10 sec	72°C/ 2min 10 sec
Final extension	72°C/ 5 min	72°C/ 5 min	72°C/ 5 min

3.2.2 PCR-Restriction Fragment Length Polymorphism (PCR-RFLP)

Add enzyme *BsaH I*, 10xNEB, and BSA buffer to the PCR product as shown in Table 4.

Table 4. PCR-RFLP reaction of *GAA*

Component	solution
Distilled water (µl)	4.8 (1X)
10XNEB buffer	2.0
BSA	0.2 (0.2mM)
BsaH I	1 (10U)
PCR product	12
Total volume (µl)	20

The enzyme cleaved at position (c.1935C>A) (p.D645E) on cDNA. This mutation is a common mutation in Chinese, Taiwan, Thai patients. After enzyme digestion, the expected result on gel electrophoresis was shown in figure 9

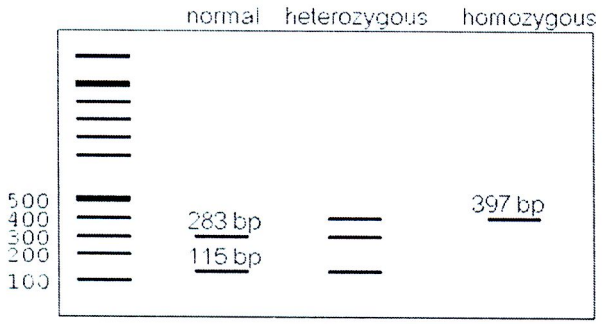

Figure 8. PCR-RFLP of *GAA* hotspot mutatoin

3.2.3 Agarose gel electrophoresis and DNA sequencing

The RFLP reaction was verified for correct size on an ethidium bromide-stained 1.5-2% agarose gel. The samples without p.D645E were sent for sequencing for mutation analysis.

3.3 Mutation analysis with *STAT3*

3.3.1 Polymerase Chain Reaction (PCR)

The primers were designed between DNA binding domain and SH2 domains in cDNA. The forward primer was designed before DNA binding domain and the reverse primer was designed after the SH2 domain domain for allow amplification and sequencing as shown in Table 5, 5.1, 5.2.

Table5. Primer sequences for *STAT3* mutation analysis

Name	Primer sequence 5' to 3'	Product size (bp)
STAT3-mRNA-F1	TCA CTG CGC TGG ACC AGA TG	1,780
STAT3-mRNA-R1.2	GAT GAT CTG GGG TTT GGC TG	
STAT3-mRNA-F2	AGA CTC TGG GGA CGT TGC AG	For sequencing
STAT3-mRNA-F3	CCT TCT GGG TCT GGC TGG AC	For sequencing

Table 5.1. PCR reaction of *STAT3*

Component	STAT3 F1/R1.2
Distilled water (µl)	14.5 (1X)
10XPCR buffer	2.0
25mM $MgCl_2$	1.2 (1.2mM)
10 mM dNTP	0.4 (0.2mM)
10µM STAT3-F	0.3 (0.1µM)
10µM STAT3-R	0.3 (0.1µM)
5U/µl Taq polymerase	0.1(0.025U)
50ng/µl cDNA	1
Total volume (µl)	20

Table5.2. PCR cycle and condition of *STAT3*

Step	STAT3 F1/R1.2
Initial denaturation	94°C/ 5 min
PCR cycle	35 cycles
Denature	94°C/ 45sec
Annealing	60°C/ 30sec
Extension	72°C/ 1min 50 sec
Final extension	72°C/ 5 min

3.3.2 Agarose gel electrophoresis and DNA sequencing

The PCR products were verified for correct size on an ethidium bromide-stained 1.5-2% agarose gel. The PCR products were then treated with ExoSAP-IT (USP Corporation, Cleveland, OH) according to the manufacturer's recommendation, and sent for direct sequencing at Macrogen Inc., Seoul, Korea. The sequence was analyzed by sequencher.

3.4 Mutation analysis with *TBX5*

3.4.1 Polymerase Chain Reaction (PCR)

The first primers was designed within introns to allow genomic amplification and sequencing of exons 2-9 including exon-intron boundaries as shown in Table 6, 6.1, 6.2.

Table 6. Primer sequences for *TBX5* mutation analysis

Name	Primer sequence 5' to 3'	Product size (bp)
TBX5-exon2-F	CCC TCC CTG TCA CTA GAA TTG	346
TBX5-exon2-R	AAG CCG AGC AGG AAA GCC AG	
TBX5-exon3-F	CTC TCT GAG ACC ACA GGC TC	387
TBX5-exon3-R	CCA GGA TCT ATC TTT CGC TC	
TBX5-exon4-F	GAT CTT GCG GAG AGC GGA AC	385
TBX5-exon4-R	CGC CTT TAG CAC ACA GTA GG	
TBX5-exon5-F	GGA GAG CCT CCA GAT TAT TC	452
TBX5-exon5-R	GGA AGT CCA GAT CAA GAA GG	
TBX5-exon6-F	CGA GAG CCG ATA TAA CAA GG	421

TBX5-exon6-R	ACT CTT AGG CTG CAG CTT TG	
TBX5-exon7-F	GAC GTG ACT GGC TTA ATT TG	352
TBX5-exon7-R	CCA TGT GCC TGG CAT TCT AC	
TBX5-exon8-F	TTC TGT GAC TTT TCT GGT GG	505
TBX5-exon8-R	GGA ACT TTT TGT TTT AGC TG	
TBX5-exon9-F	CGG TTA GGG CTA ACA GTC TC	840
TBX5-exon9-R	CGA CCT TGA GTG CAG ATG TG	

Table6.1. PCR reaction of *TBX5*

Component	Exon 2	Exon 3	Exon 4	Exon 5	Exon 6
Distilled water (µl)	13.5 (1X)	13.5 (1X)	13.5 (1X)	13.5 (1X)	13.5 (1X)
10XPCR buffer	2.0	2.0	2.0	2.0	2.0
25mM $MgCl_2$	1.2 (1.2mM)	1.2 (1.2mM)	1.2 (1.2mM)	1.2 (1.2mM)	1.2 (1.2mM)
10 mM dNTP	0.4 (0.2mM)	0.4 (0.2mM)	0.4 (0.2mM)	0.4 (0.2mM)	0.4 (0.2mM)
10µM TBX5-F	0.4	0.4	0.4	0.4	0.4
10µM TBX5-R	0.4	0.4	0.4	0.4	0.4
5U/µl Taq	0.1 (0.025U)	0.1 (0.025U)	0.1 (0.025U)	0.1 (0.025U)	0.1 (0.025U)
50ng/µl Genomic	2	2	2	2	2
Total volume (µl)	20	20	20	20	20

Component	Exon 7	Exon 8	Exon 9
Distilled water (µl)	13.5 (1X)	13.5 (1X)	13.5 (1X)
10XPCR buffer	2.0	2.0	2.0
25mM $MgCl_2$	1.2 (1.2mM)	1.2 (1.2mM)	1.2 (1.2mM)
10 mM dNTP	0.4 (0.2mM)	0.4 (0.2mM)	0.4 (0.2mM)
10µM TBX5-F	0.4 (0.13µM)	0.4 (0.13µM)	0.4 (0.13µM)
10µM TBX5-R	0.4 (0.13µM)	0.4 (0.13µM)	0.4 (0.13µM)
5U/µl Taq polymerase	0.1 (0.025U)	0.1 (0.025U)	0.1 (0.025U)
50ng/µl Genomic DNA	2	2	2
Total volume (µl)	20	20	20

Table6.2. PCR cycle and condition of *TBX5*

Step	Exon 2	Exon 3	Exon 4	Exon 5	Exon 6
Initial denaturation	94°C/ 5 min	94°C/ 5 min	94°C/ 5 min	94°C/ 5 min	94°C/ 5 min
PCR cycle	35 cycles	35 cycles	35 cycles	35 cycles	35 cycles
Denature	94°C/ 45sec	94°C/ 45sec	94°C/ 45sec	94°C/ 45sec	94°C/ 45sec
Annealing	64°C/ 45sec	64°C/ 45sec	64°C/ 45sec	64°C/ 45sec	64°C/ 45sec
Extension	72°C/ 35sec	72°C/ 35sec	72°C/ 35sec	72°C/ 35sec	72°C/ 35sec
Final extension	72°C/ 10min	72°C/ 10min	72°C/ 10min	72°C/ 10min	72°C/ 10min

Step	Exon7	Exon8	Exon9
Initial denaturation	94°C/ 5 min	94°C/ 5 min	94°C/ 5 min
PCR cycle	35 cycles	35 cycles	35 cycles
Denature	94°C/ 45sec	94°C/ 45sec	94°C/ 45sec
Annealing	64°C/ 45sec	58°C/ 45sec	64°C/ 45sec
Extension	72°C/ 35sec	72°C/ 35sec	72°C/ 1 min
Final extension	72°C/ 10min	72°C/ 10min	72°C/ 10min

3.4.2 Agarose gel electrophoresis and DNA sequencing

The PCR products were verified for correct size on an ethidium bromide-stained 1.5-2% agarose gel. The PCR products were then treated with ExoSAP-IT (USP Corporation, Cleveland, OH) according to the manufacturer's recommendation, and sent for direct sequencing at Macrogen Inc., Seoul, Korea. The sequence was analyzed by sequencher.

A previous report has recommended if *TBX5* mutation analysis fails to show a mutation, *SALL4* analysis should be considered.

3.5 Mutation analysis with *SALL4*

3.5.1 Polymerase Chain Reaction (PCR)

The primers were designed within introns to allow genomic amplification and sequencing of exons 1-4 including exon-intron boundaries as shown in Table 7, 7.1, 7.2.

Table 7. Primer sequences for *SALL4* mutation analysis

Name	Primer sequence 5' to 3'	Product size (bp)
Sall4-ex1-F1	CAG GAA TTT GTG GCG GAG AG	301
Sall4-ex1-R2	CTC CTG AAT TTG CGC TGG AC	
Sall4-ex2-F1	CGA GAG ACT TCC AGG CAT CA	2,937
Sall4-ex2-R1	GGC TGC TTC AAG TCA TAC TC	
Sall4-ex2-F2	TTC CCA ACC TCA GGT GAT CT	For sequencing
Sall4-ex2-F3	TGA ATC AGC GGA GCG CGG AT	For sequencing
Sall4-ex2-F4	GGA CTG ATA GCT CCT TGC AG	For sequencing
Sall4-ex3-F1	CCA GCT CCA GAC TCT CAA AC	624
Sall4-ex3-R1	GTG AGC TTG AGC TTG AGA TG	
Sall4-ex4-F1	CGC TGT AAG TCA AGG ATC ATC	693
Sall4-ex4-R1	GGT TGT GGT CAC AAC CAA CG	

Table7.1. PCR reaction of *SALL4*

Component	Exon1	Exon2	Exon3	Exon4
Distilled water (µl)	12.4 (1X)	13.4 (1X)	13.4 (1X)	13.4 (1X)
10XPCR buffer	2.0	2.0	2.0	2.0
25mM $MgCl_2$	1.5 (1.5mM)	1.5 (1.5mM)	1.5 (1.5mM)	1.5 (1.5mM)
10 mM dNTP	0.4 (0.2mM)	0.4 (0.2mM)	0.4 (0.2mM)	0.4 (0.2mM)
10µM SALL4-F	0.3 (0.1µM)	0.3 (0.1µM)	0.3 (0.1µM)	0.3 (0.1µM)
10µM SALL4-R	0.3 (0.1µM)	0.3 (0.1µM)	0.3 (0.1µM)	0.3 (0.1µM)
100% DMSO	1.0 (5%)	-	-	-
5U/µl Taq polymerase	0.1 (0.025U)	0.1 (0.025U)	0.1 (0.025U)	0.1 (0.025U)
50ng/µl Genomic DNA	2	2	2	2
Total volume (µl)	20	20	20	20

Table 7.2. PCR cycle and condition of *SALL4*

Step	Exon1	Exon2	Exon3	Exon4
Initial denaturation	94°C/ 5 min	94°C/ 5 min	94°C/ 5 min	94°C/ 5 min
PCR cycle	35 cycles	35 cycles	35 cycles	35 cycles
Denature	94°C/ 45sec	94°C/ 45sec	94°C/ 45sec	94°C/ 45sec
Annealing	64°C/ 45sec	64°C/ 45sec	68°C/ 45sec	60°C/ 45sec
Extension	72°C/ 20sec	72°C/ 3min	72°C/ 45sec	72°C/ 45sec
Final extension	72°C/ 5min	72°C/ 5min	72°C/ 5min	72°C/ 5min

3.5.2 Agarose gel electrophoresis and DNA sequencing

The PCR products were verified for correct size on an ethidium bromide-stained 1.5-2% agarose gel. The PCR products were then treated with ExoSAP-IT (USP Corporation, Cleveland, OH) according to the manufacturer's recommendation, and sent for direct sequencing at Macrogen Inc., Seoul, Korea. The sequence was analyzed by sequencher.

3.6 Mutation analysis with *DcR3*

3.6.1 Polymerase Chain Reaction (PCR)

The primers were designed within introns to allow genomic amplification and sequencing of all coding regions including exon-intron boundaries as shown in Table 8, 8.1, 8.2.

Table 8. Primer sequences for *DcR3* mutation analysis

Name	Primer sequence 5' to 3'	Product size (bp)
DcR3-gDNA-F1	CAC CCT TGG ACT GAG CTC TG	1,112
DcR3-gDNA-R1	GGC ATG CCT CAG GCT AGA TG	
DcR3-gDNA-F1.2	GAG TGG CAG AAA CAC CCA CC	For sequencing
DcR3-gDNA-R1.2	AAC TGG TGT CCT AGC TCA GG	
DcR3-gDNA-F2	AGC TCT CTG ACC GAA GGC TC	536
DcR3-gDNA-R2	CCT CTT TCA GTG CAA GTG GG	

Table 8.1. PCR reaction of *DcR3*

Component	DcR3-gDNA-F1/R1	DcR3-gDNA-F2/R2
Distilled water (μl)	12.4 (1X)	13.4 (1X)
10XPCR buffer	2.0	2.0
25mM $MgCl_2$	1.5 (1.5mM)	1.5 (1.5mM)
10 mM dNTP	0.4 (0.2mM)	0.4 (0.2mM)
10μM DcR3-gDNA-F	0.3 (0.1μM)	0.3 (0.1μM)
10μM DcR3-gDNA-R	0.3 (0.1μM)	0.3 (0.1μM)
100% DMSO	1.0 (5%)	1.0 (5%)
5U/μl Taq polymerase	0.1 (0.025U)	0.1 (0.025U)
50ng/μl Genomic DNA	2	1
Total volume (μl)	20	20

Table 8.2. PCR cycle and condition of *DcR3*

Step	DcR3-gDNA-F1/R1	DcR3-gDNA-F2/R2
Initial denaturation	94°C/ 5 min	94°C/ 5 min
PCR cycle	35 cycles	35 cycles
Denature	94°C/ 45sec	94°C/ 45sec
Annealing	64°C/ 45sec	66°C/ 45sec
Extension	72°C/ 1min 20 sec	72°C/ 45sec
Final extension	72°C/ 5 min	72°C/ 5 min

3.6.2 Agarose gel electrophoresis and DNA sequencing

The PCR products were verified for correct size on an ethidium bromide-stained 1.5-2% agarose gel. The PCR products were then treated with ExoSAP-IT (USP Corporation, Cleveland, OH) according to the manufacturer's recommendation, and sent for direct sequencing at Macrogen Inc., Seoul, Korea. The sequence was analyzed by sequencher.

3.6.3 SLE Disease Activity Index (SLEDAI)

This method reports disease activity, damage from disease, and health status in score form by filling clinical data into SLEDAI form (Table 21.) and

calculation of score. This method is useful for classification of severity as active and inactive SLE.

Table 9. SLEDAI form

Has the patient had the recent onset of seizures, unexplained by metabolic, infectious or drug causes?	Yes No
Has the patient shown psychotic behavior?	Yes No
Does the patient show altered mental function, with impaired orientation, memory or other intellectual functions?	Yes No
Does the patient have a visual disturbance, with retinal changes associated with lupus?	Yes No
Does the patient have a new onset of sensory or motor neuropathy involving cranial nerves?	Yes No
Does the patient have a severe, persistent headache, unrelieved by narcotic analgesics?	Yes No
Has the patient had a recent cerebrovascular accident (CVA) or stroke, not due to arteriosclerosis?	Yes No
Does the patient have skin ulcerations or areas of gangrene?	Yes No
Does the patient have tender finger nodules, periungual infarctions, or splinter hemorrhages?	Yes No
Does the patient have biopsy or angiographic evidence of vasculitis?	Yes No
Number of joints with pain, tenderness, swelling and/or effusion	joints
Does the patient have proximal muscle aching or weakness?	Yes No
Does the patient have elevated serum creatine phosphokinase or aldolase?	Yes No
Does the patient have changes in an electromyogram or a biopsy consistent with myositis?	Yes No
Does the patient have a new onset or recurrence of inflammatory type rash?	Yes No
Has the patient had a new onset or recurrence of abnormal, patchy or diffuse loss of hair?	Yes No

Has the patient had a new onset or recurrence of oral or nasal ulcerations? ○ Yes ○ No

Has the patient had pleuritic chest pain with pleural rub or effusion, or pleural thickening? ○ Yes ○ No

Does the patient have pericardial pain ? ○ Yes ○ No

Does the patient have a pericardial rub or effusion? ○ Yes ○ No

Does the patient have electrocardiogram or echocardiogram evidence of a pericardial effusion? ○ Yes ○ No

Body temperature [ ] ○ °C ○ °F

Platelet count [ ] ○ per µL ○ 10^9/L

WBC count [ ] ○ per µL ○ 10^9/L

Are the decrease in blood cell counts due to drugs or toxins? ○ Yes ○ No

Current urine protein output [ ] grams per day

Previous urine protein output [ ] grams per day

Does the patient have heme-granular or red blood cell casts in the urine sediment? ○ Yes ○ No

Number of red blood cells in the urine [ ] RBCs per high power field

Number of white blood cells in the urine [ ] WBCs per high power field

Can the urine findings be explained by stone, infection or other cause? ○ Yes ○ No

Does the patient show a decrease in CH50, C3 or C4? ○ Yes ○ No

Does the patient show evidence of increased DNA binding by the Farr or other assay? ○ Yes ○ No

3.6.4 Enzyme-linked immunosorbent assay (ELISA)

1. Prepare solution and standard dilution for plotting standard curve

2. Determine the number of microwell strips required to test the desired number of samples plus appropriate number of wells needed for running blanks and standards. Each sample, standard, blank and optional control samples should be assayed in duplicate.

3. Wash the microwell strips twice with approximately 400 µl Wash Buffer per well with thorough aspiration of microwell contents between washes. Allow the Wash Buffer to sit in the wells for about 10 – 15 seconds before aspiration. Take care not to scratch the surface of the microwells. After the last wash step, empty wells and tap microwell strips on absorbent pad or paper towel to remove excess Wash Buffer.

4. Add 100 µl of Sample Diluent in duplicate to all standard wells. Pipette 100 µl of prepared standard in duplicate into well. Mix the contents of wells by repeated aspiration and ejection and creating two rows of human DcR3 standard dilutions ranging from 5000 to 78 pg/ml

5. Add 100 µl of Sample Diluent in duplicate to the blank wells.

6. Add 50 µl of Sample Diluent to the sample wells.

7. Add 50 µl of each sample in duplicate to the sample wells.

8. Prepare Biotin-Conjugate

9. Add 50 µl of Biotin-Conjugate to all wells.

10. Cover with an adhesive film and incubate at room temperature (18 to 25°C) for 2 hours, if available on a microplate shaker set at 100 rpm.

11. Prepare Streptavidin-HRP

12. Remove adhesive film and empty wells. Wash microwell strips 6 times according to point b. of the test protocol. Proceed immediately to the next step.

13. Add 100 µl of diluted Streptavidin-HRP to all wells, including the blank wells.

14. Cover with an adhesive film and incubate at room temperature (18° to 25°C) for 1 hour, if available on a microplate shaker set at 100 rpm.

15. Remove adhesive film and empty wells. Wash microwell strips 6 times according to point b. of the test protocol. Proceed immediately to the next step.

16. Pipette 100 µl of TMB Substrate Solution to all wells.

17. Incubate the microwell strips at room temperature (18° to 25°C) for about 10 min. Avoid direct exposure to intense light.

18. Stop the enzyme reaction when the highest standard has developed a dark blue colour by quickly pipetting 100 µl of Stop Solution into each well. It is important that the Stop Solution is spreaded quickly and uniformly throughout the microwells to completely inactivate the enzyme.

19. Read absorbance of each microwell on a spectro-photometer using 450 nm as the primary wave length (optionally 620 nm as the reference wave length; 610 nm to 650 nm is acceptable). Blank the plate reader according to the manufacturer's instructions by using the blank wells. Determine the absorbance of both the samples and the standards.

20. Plot standard curve and analyze concentration of DcR3 in each sample from standard curve.

3.7 Gel extraction

1. Excise the DNA fragment from the agarose gel with a clean, sharp scalpel.

2. Weigh the gel slice in a colorless tube. Add 3 volumes of Buffer QG to 1 volume of gel (100 mg ~ 100 µl).

3. Incubate at 50°C for 10 min (or until the gel slice has completely dissolved). To help dissolve gel, mix by vortexing the tube every 2–3 min during the incubation.

4. After the gel slice has dissolved completely, check that the color of the mixture is yellow (similar to Buffer QG without dissolved agarose).

5. Add 1 gel volume of isopropanol to the sample and mix. (This step increases the yield of DNA fragments <500 bp and >4 kb.)

6. Place a QIAquick spin column in a provided 2 ml collection tube.

7. To bind DNA, apply the sample to the QIAquick column, and centrifuge for 1 min.

8. Discard flow-through and place QIAquick column back in the same collection tube.

9. (Optional): Add 0.5 ml of Buffer QG to QIAquick column and centrifuge for 1 min.

10. To wash, add 0.75 ml of Buffer PE to QIAquick column and centrifuge for 1 min.

11. Discard the flow-through and centrifuge the QIAquick column for an additional 1 min at >10,000 x *g* (~13,000 rpm).

12. Place QIAquick column into a clean 1.5 ml microcentrifuge tube.

13. To elute DNA, add 50 µl of Buffer EB (10 mM Tris·Cl, pH 8.5) or H_2O to the center of the QIAquick membrane and centrifuge the column for 1 min at maximum speed. Alternatively, for increased DNA concentration, add 30 µl elution buffer to the center of the QIAquick membrane, let the column stand for 1 min, and then centrifuge for 1 min.