CHAPTER III
METHODOLOGY

Research Instruments

1. Pipette tip : 10 µl, 100 µl, 200 µl, 1,000 µl (Elkay, USA)
2. Microcentrifuge tube : 0.2 ml, 0.5 ml, 1.5 ml (Bio-RÁD, Elkay, USA)
3. Polypropylene conical tube : 15 ml (Elkay, USA)
4. Beaker : 50 ml, 100 ml, 200ml, 500 ml, 1,000 ml (Pyrex)
5. Flask : 250 ml, 500 ml, 1,000 ml (Pyrex)
6. Reagent bottle : 100 ml, 250 ml, 500 ml, 1,000 ml (Duran, USA)
7. Cylinder : 25 ml, 50 ml, 100 ml, 250 ml, 500 ml, 1,000 ml (Witeg, Germany)
8. Glass pipette : 2 ml, 10 ml (Witeg, Germany)
9. Pipette rack (Autopack, USA)
10. Thermometer (Precision, Germany)
11. Parafilm (American National Can, USA)
12. Plastic wrap
13. Stirring-magnetic bar
14. Combs
15. Automatic adjustable micropipette : P2 (0.1-2 µl), P10 (0.5-10 µl), P20 (5-20 µl), P100 (20-100 µl), P1000 (0.1-1 ml) (Gilson, France)
16. Pipette boy (Tecnomara, Switzerland)
17. Vortex (Scientific Industry, USA)
18. pH meter (Eutech Cybernatics)
19. Stirring hot plate (Bamstead/Thermolyne, USA)
20. Balance (Precisa, Switzerland)
21. Centrifuge (J.P.Selecta, Span)
22. Microcentrifuge (Eppendorf, Germany)
23. Mastercycler personal (Eppendorf, Germany)

24. Thermal cycler (Touch Down, Hybraid USA)
25. Power supply model 250 (Gibco BRL, Scotland)
26. Power poc 3000 (Bio-RAD)
27. Horizon 11-14 (Gibco BRL, Scotland)
28. Sequi-gen sequencing cell (Bio-RAD)
29. Heat block (Bockel)
30. Incubator (Memmert)
31. Thermostat shaking-water bath (Heto, Denmark)
32. Spectronic spectrophotometers (Genesys5, Milon Roy, USA)
33. UV Transilluminator (Fotodyne, USA)
34. UV-absorbing face shield (Spectronic, USA)
35. Gel doc 1000 (Bio-RAD)
36. Refrigerator 4 ^{0}C (Misubishi, Japan)
37. Deep freeze -20 ^{0}C, -80 ^{0}C (Revco)
38. Water purification equipment (Water pro Ps, Labconco, USA)
39. Water bath (J.P.Selecta, Spain)
40. Storm 840 and ImageQuaNT software (Molecular dynamics)
41. 24-well culture plates (Corning, New York)
42. T-25 and T-75 Flasks (Corning, New York)
43. Costar® Stirpipette® : 0.2 ml, 10 ml, 25 ml (Corning, New York)
44. Haematocytometer counting chamber
45. Cryotube vial 2.0 ml (Corning, New York)
46. Cryo 1°C Freezing container (Nalgene® Labware)
47. MicroAmp®Optical 96-Well Reaction Plate with Barcode, 0.2-mL
48. MicroAmp® Optical Adhesive Film
49. Applied Biosystems 7500 Real-Time PCR System

Reagents

1. General reagents

1.1 Absolute ethanol (Merck)

1.2 Agarose, molecular glade (Promega)

1.3 Ammonium acetate (Merck)

1.4 Boric acid (Merck)

1.5 Bromphenol blue (Pharmacia)

1.6 Disodium ethylenediamine tetracetic acid : EDTA (Merck)

1.7 Ethidium bromide (Gibco BRL)

1.8 Ficoll 400 (Pharmacia)

1.9 Hydrochloric acid (Merck)

1.10 Mineral oil (Sigma)

1.11 Phenol (Sigma)

1.12 Chloroform (Merck)

1.13 Isoamyl alcohol (Merck)

1.14 Sodium chloride (Merck)

1.15 Sodium dodecyl sulfate (Sigma)

1.16 Sodium hydroxide (Merck)

1.17 Sucrose (BDH)

1.18 Tris base (USB)

1.19 Triton X-100 (Pharmacia)

1.20 100 base pair DNA ladder (Biolabs)

1.21 40% acrylamide/bis solution 19:1 (Bio-RAD)

1.22 GelStar (Camberx)

2. RNA and DNA extraction

2.1 QIAamp® RNA blood mini kit (QIAGEN)

2.2 QIAamp® DNA blood mini kit (QIAGEN)

3. PCR reagents

3.1 10X PCR buffer (500 mM KCl, 200 mM Tris-HCl, pH 8.4) (Promega)

3.2 10X PCR buffer (500 mM KCl, 100 mM Tris-HCl, pH 8.8, 0.8% Nonidet P40) (Fermentas)

3.3 Magnesium chloride (Promega)

3.4 Magnesium chloride (Fermentas)

3.5 Deoxynucleotide triphosphates (dNTPs) (Promega)

3.6 Deoxynucleotide triphosphates (dNTPs) (Fermentas)

3.7 Oligonucleotide primers (BSU)

3.8 Oligonucleotide primers (Biogenomed)

3.9 *Taq* DNA polymerase (Promega)

3.10 *Taq* DNA polymerase (Fermentas)

3.11 100% DMSO

3.12 Genomic DNA sample

4. Reverse Transcription

4.1 Oligo (dT) primer (Promega)

4.2 Magnesium chloride (Promega)

4.3 Deoxynucleotide triphosphates (dNTPs) (Promega)

4.4 Im Prom-II^{TM} 5X reaction buffer (Promega)

4.5 Reverse transcriptase (Promega)

4.6 RNasin$^{®}$ RNase

5. Cell culture

5.1 RPMI medium 1640 (GIBCO, Invitrogen)

6.2 Fetal Bovine Serum (FBS) (GIBCO, Invitrogen)

6.3 PenStrep(GIBCO, Invitrogen)

6.4 Phosphate-buffered saline (PBS)

6.5 Tryphan blue

6.6 Phytohemagglutinins (PHA)

6.7 Ficoll

7. AMOs administration

7.1 AMOs : AntisenseIVS3 (Gene Tools, LLC)

7.2 AMOs : MismatchIVS3 (Gene Tools, LLC)

7.3 AMOs : InvertIVS3 (Gene Tools, LLC)

7.4 Endo-Porter (Gene Tools, LLC)

8. Real-time PCR reagent

8.1 TaqMan®Gene expression assay (Applied Biosystems)

8.2 TaqMan®Gene expression master mix (Applied Biosystems)

Experimental Procedure

1. Subjects and sample collection

After informed consent, 3-6 ml. of peripheral blood were obtained from each individual.

Subjects

Six patients from six unrelated families were clinically diagnosed with XLA at the Pediatric Clinic of King Chulalongkorn Memorial Hospital and the Pediatric Clinic of Children Hospital. Selection criteria were based on clinical presentation and immunological tests.

Controls

Controls were healthy volunteers unaffected with XLA and had no family history of XLA. DNA from the controls was used for mutation screening in the *BTK* gene identified in the XLA patients. DNA from the patients' family members who were at risk was also investigated.

2. Genetic analysis

2.1 RNA extraction

Equilibrate samples to room temperature (15-25°C)

2.1.1 Pipet 560 μl of prepared Buffer AVL containing Carrier RNA into a 1.5-ml microcentrifuge tube.

2.1.2 Add 140 μl of the patient' plasma to the Buffer AVL/Carrier RNA in the microcentrifuge tube. Mix by pulse-vortexing for 15 sec.

2.1.3 Incubate at room temperature (15-25°C) for 10 min.

2.1.4 Briefly centrifuge the 1.5-ml microcentrifuge tube to remove drops from the inside of the lid.

2.1.5 Add 560 μl of ethanol (96-100%) to the sample, and mix by pulse-vortexing for 15 sec. After mixing, briefly centrifuge the 1.5-ml microcentrifuge tube to remove drops from inside the lid.

2.1.6 Carefully apply 630 μl of the solution from step 5 to the QIAamp spin column (in a 2-ml collection tube) without wetting the rim. Close the cap, and centrifuge at 6000xg (8000 rpm) for 1 min. Place the QIAamp spin column into a clean 2-ml collection tube, and discard the tube containing the filtrate.

2.1.7 Carefully open the QIAamp spin column, and repeat step 6.

2.1.8 Carefully open the QIAamp spin column, and add 500 μl of Buffer AW1. Close the cap, and centrifuge at 6000xg (8000 rpm) for 1 min. Place the QIAamp spin column in a clean 2-ml collection tube (provided), and discard the tube containing the filtrate.

2.1.9 Carefully open the QIAamp spin column, and add 500 μl of Buffer AW2. Close the cap and centrifuge at full speed (20,000xg; 14,000 rpm) for 3 min. Continue directly with step 10, or to eliminate any chance of possible Buffer AW2 carryover and then continue with step 10.

2.1.10 Place the QIAamp spin column in a clean 1.5-ml microcentrifuge tube (not provided). Discard the old collection tube containing the filtrate. Carefully open the QIAamp spin column and add 60 μl of Buffer AVE equilibrated to room temperature. Close the cap, and incubate at room temperature for 1 min. Centrifuge at 6000xg (8000 rpm) for 1 min.

2.2 DNA extraction

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

2.2.1. 3 ml. of whole blood were centrifuged for 10 minutes at 3,000 rpm.

2.2.2. Remove supernatant and transfer buffy coat to a new polypropylene tube. Then add 10 volumes of cold lysis buffer 1 (or 10 ml.), mix thoroughly and incubate at –20°C for 5 minutes.

2.2.3. Centrifuge for 8 minutes at 1,000 g, and remove supernatant.

2.2.4. Add 3 ml. of cold lysis buffer 1, mix thoroughly and centrifuge for 8 minutes at 1,000g,

2.2.5. 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.

2.2.6. Incubate the tube(s) in a 37°C shaking waterbath overnight for complete digestion.

2.2.7. Add 1 ml. of phenol-chloroform-isoamyl alcohol and shake vigorously for 15 seconds and centrifuge at 6,000 rpm for 5 minutes.

2.2.8. Transfer the supernatant from each tube (containing DNA) to a new microcentrifuge tube.

2.2.9. Add 0.5 volume 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 14,000 rpm for 15 minutes. Then remove supernatant.

2.2.10. 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.)

2.2.11. Resuspend the DNA in 20-300 μl of the double distilled water at 37°C until dissolved.

2.2 RNA and DNA amplification

The *BTK* cDNA is composed of 2,560 nucleotides and has an open reading frame encoding a 659-amino acid polypeptide. For mutation screening of the entire coding region of the *BTK* gene, cDNAs were amplified using the primers as shown in table 2. The PCR reaction and condition are shown in table 2.1 and 2.2.

Table 2: Primer sequences and the product size

Primer sequences for PCR (5' to 3')		Product size (bp)
Forward	**Reverse**	
CAATGCATCTGGGAAGCTAC	AGCTTGGGATTTCCTCTGAG	2,128

Table 2.1: Mixture of PCR reactions

Components	Volume per reaction* (μl)
	Amount (ul)
1. 10X PCR buffer (+KCl,-$MgCl_2$)	2.0 (1X)
2. 25 mM $MgCl_2$	1.2 (1.5 mM)
3. 10 mM dNTP	0.4 (0.2 mM)
4. 10 μM Forward primer	0.4 (0.1 μM)
5. 10 μM Reverse primer	0.4 (0.1 μM)
6. 5 U/μl Taq polymerase	0.1 (0.5 U)
7. Distilled water	13.5
8. 50 ng/μl of genomic DNA	2.0 (100 ng)
Total volume (μl)	20

* Final concentration per reaction in each PCR reaction

Table 2.2: PCR cycle and condition

Step	Temperature and incubation time
1. Initial denaturation	94 ^{0}C / 5 min
2. PCR cycle	35 cycles
Denature	94 ^{0}C / 60 sec
Annealing	63 ^{0}C / 60 sec
Extension	72 ^{0}C / 2 min
3. Final extension	72°C/ 7 min

To PCR-amplify a part of intron 4, primer sequences, the PCR reaction and condition are shown in table 3, 3.1 and 3.2, respectively.

Table 3: Primer sequences for PCR-amplification of intron 4

Primer sequences for PCR (5' to 3')	
Forward (BTKE3-F)	**Reverse (BTKE4-R)**
CCTGGTGCCACCTCACTTTG	GATCCTGAGAGAACTGAGGG

Table 3.1: Mixture of PCR reaction

Components	Volume per reaction* (μl)
1. 10X PCR buffer(+KCl,-$MgCl_2$)	2.0 (1X)
2. 25 mM $MgCl_2$	1.2 (1.5 mM)
3. 10 mM dNTP	0.4 (0.2 mM)
4. 10 μM Forward primer	0.4 (0.1 μM)
5. 10 μM Reverse primer	0.4 (0.1 μM)
6. 5 U/μl Taq polymerase	0.1 (0.5 U)
7. Distilled water	13.5
8. 50 ng/μl of genomic DNA	2.0 (100 ng)
Total volume (μl)	20

* Final concentration per reaction in each PCR reaction

Table 3.2: PCR cycle and condition

Step	Temperature and incubation time
1. Initial denaturation	94 ^{0}C / 3 min
2. PCR cycle	35 cycles
Denature	94 ^{0}C / 30 sec
Annealing	58 ^{0}C / 30 sec
Extension	72 ^{0}C / 45 sec
3. Final extension	72°C/ 7 min

2.3 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 recommendations, and sent for direct sequencing at the Macrogen Inc., Seoul, Korea. The sequence was analyzed using Sequencher (version 4.2; Gene Codes Corporation, Ann Arbor, MI).

3. Cell culture

3.1 Purification of lymphocytes from peripheral blood

3.1.1 Add 4 ml of ficoll to a 15 ml tube.

3.1.2 Pipette 3 ml to 6 ml of fresh heparinized blood and put on the top of ficoll. Try not to mix blood and lower ficoll.

3.1.3 Centrifuge for 30 min at 1,500 rpm at room temperature, if the separation is not clear or clean, repeat centrifugation (possible cause = old or cold blood; too old = no separation)

3.1.4 After centrifugation, remove plasma carefully with a pipette up to the 0.5 cm above the opaque band which contains the cells.

3.1.5 Transfer cells in a 15 ml tube and wash with 10 ml of RPMI medium, centrifuge for 10 min at 4°C at 2,000 rpm

3.1.6 Resuspend cells in 5 ml of RPMI medium, then count the cells.

3.1.7 The peripheral blood lymphocytes obtained from a donor are cultured in tissue culture medium RPMI 1640 supplemented with 10% fetal bovine serum and PenStrep. Cells were cultured in a 24-well plate.

3.1.8 Add 50 μl of Phytohemagglutinins (PHA) per well.

3.1.9 Incubate the PBMCs overnight in a CO_2 incubator.

4. Antisense morpholino oligonucleotides (AMOs) administration

This procedure was performed to investigate an effect of AMOs in correcting a novel splicing mutation of the *BTK* gene. Basically, AMOs target pre-mRNA in a sequence-specific manner, sterically blocking the targeted splice sites and redirecting the splicing machinery to a more appropriate nearby site [15].

4.1 Construction of AMOs

A Morpholino oligo is radically different from natural nucleic acids, with morpholine rings replacing the ribose or deoxyribose sugar moieties and non-ionic phosphorodiamidate linkages replacing the anionic phosphates of DNA and RNA (Fig. 11). Each morpholine ring suitably positions one of the standard DNA bases (A, C, G, T), so that a 25-base Morpholino oligo strongly and specifically binds to its complementary 25-base target site in a strand of RNA via Watson-Crick pairing. Because the backbone of the Morpholino oligo is not recognized by any cellular enzymes or signaling proteins, it is completely stable to nucleases and does not trigger an innate immune response through the toll-like receptors. This avoids the problems of loss of oligo during the course of the experiment, and inflammation or interferon induction, which commonly plague other gene knockdown reagents.

Figure 11: Structure of AMOs

4.2 Design and target AMOs to the *BTK* mutation site in the pre-mRNA of the patient's PBMCs.

In this study, a novel intronic mutation leading to an insertion of a pseudoexon in the *BTK* mRNA of the patient with XLA was identified. The intronic mutation causing aberrantly splicing was targeted with AMOs. The normal mRNA could therefore be generated. (Fig: 12)

(A)

5' GTCGTGCAACGTGGAACACGGTAAAGTTTCCTTTAAAAATTC 3'

Target AMOs 3' CCTTGTGCCATTTCAAAGGAAATTT 5'

(B)

Pt

exon3 exon4

Morpholino

Abnormal splicing

Splicing pattern after AMOs administration

exon3 exon4

c.240+109 C>A

exon3 exon4

Figure 12: **(A)** Annealing of the AMOs to the splice site mutation of the *BTK* at intron 3 in the patient's pre-mRNA

(B) Schematic representation of the *BTK* gene containing a splice site mutation. A mutated intron 3 (thin line) of the *BTK* gene causing aberrant splicing (boxes). AMOs (heavy bar) blocking the aberrant splice site leading to restoration of correct pre-mRNA splicing.

Morpholino control oligos:
Invert of antisense: InvertIVS3
Mispaired control : MISIVS3

For a rigorous specific study, an antisense sequence with 5 mispairs appropriately distributed along the sequence provides a much more stringent and realistic assessment of sequence specificity. Table 4 shows nucleotide sequences of each oligos.

Table 4: Nucleotide sequence of each AMOs

AMOs	Nucleotide sequence 5'→ 3'
MISIVS3	TTaAAAcGAAAgTTTACCGTcTTgC
Target AMOs (25 nt)	TTTAAAGGAAACTT*ACCGTGTTCC
InvertIVS3	CCTTGTGCCATTTCAAAGGAAATTT

* **T** is an antisense nucleotide base of the c.240+109 C>A mutation
MISIVS3 contains five nucleotide mismatches.

4.3 Correction of c.240+109 C>A pre-mRNA splicing by delivering AMOs to the patient's peripheral blood mononuclear cells.

Human PBMC were isolated from peripheral blood of patient and healthy volunteers by Ficoll. PBMC were incubated overnight in CO_2-equilibrated RPMI 1640 culture medium plus 10% fetal bovine serum and PenStrep.

To be effective, AMOs should be delivered into cell nuclei in relative high concentrations. The traditional delivery is performed using physical and chemical techniques, such as using the Endo-porter.

For AMOs administration, $8*10^5$ PBMCs were grown in 24-well plates, and,after overnight culture,different concentrations of AMOs with 5 ml of EndoPorter were added to the culture medium. Cells were harvested at different times, and mRNA was isolated by resuspend cells in 5 ml of RPMI medium. Centrifuge for 5 min at 2,000 rpm at room temperature and resuspend cells with 5 ml of PBMC. Finally, Centrifuge for 5 min at 2,000 rpm at room temperature before RNA extraction from collected PBMCs

To find an optimal dose of AMOs, cells were treated with 2-60 μM of AMOs and cells were collected after 24 hour of treatment.

To test the stability of the restored, correctly spliced *BTK* mRNAs, the PBMCs were treated with AMOs and harvested from day 1 to day 30.