CHAPTER 2
RESEARCH METHODOLOGY

Materials

Peripheral blood samples

Peripheral blood samples (3-5 ml) were obtained from two study groups and collected in sodium-heparin anticoagulant.

1. Idiopathic mental retardation patients

Idiopathic mental retardation (IMR) patients with and without multiple congenital anomalies (MCA) were recruited by clinicians at Rajanukul Institute. Metaphase chromosomes all patients with normal G-banded karyotypes at the 450-550 band levels was studied with the FISH technique using specific-subtelomeric probes for all chromosome ends.

2. Autistic patients

Peripheral blood of autistic children recruited for the study was tested by high-resolution chromosome analysis, FISH analysis using PWS/AS region, DiGeorge/VCF region and DNA screening for fragile X syndrome. Six randomly selected were also examined with subtelomeric-specific probes.

Reagents

Acetic acid
Agarose
Ammonium sulfate
Chelex-100 resin
Chloramphenical
Colcemid
Dextran sulfate
DAPI (4,6-Diamino-2-Phenyl-indole)
EDTA
Ethanol
Ethidium bromide
Fetal calf serum
Formamide
Glucose
Glycerol
Human Cot-1 DNA (Invitrogen)
Human placenta DNA
IMMOLASE™ DNA polymerase
Kanamycin
Magnesium Chloride
MEM medium
Methotexate
Methanol
NaOH
Nick translation kit (Vysis)
phytohemagglutinin
PN buffer
Potassium chloride
Potassium acetate
p-Phenylenediamine
Rubber solution
Spectrum green-dUTP (Vysis)
Spectrum orange-dUTP (Vysis)
Sodium chloride
Sodium citrate
Sodium acetate
SDS
Tris-Cl
TWEEN 20
Trypsin
Thymidine
Tryptone
Yeast extract
7-deaza dGTP

Equipments

Applied Spectral Imaging system
Centrifuge
Cover slip 24X60 mm
Centrifuge tube 15 ml
Diamond pencil
Fluorescence microscope with light source and appropriate filter sets
Forceps
Gel electrophoresis with power supply
Gloves
Glass coplin jars
Hot plate
Humidified chamber
Incubator
Microscope slide
Microcentrifuge tube 1.5 ml
Micropipette, range 1-200 µl and tip
Phase contrast microscope
PCR Thermal cycle (MJ research PTC200)
Syringe
Timer
Vortex
Water bath

Methods

High-resolution chromosome analysis

Peripheral blood was cultured at 37°C for 72 hours in MEM medium supplement with 10% fetal calf serum and PHA (phytohemagglutinin, M-form). After incubating for 48 hours, 0.1 ml of methotexate was added to the culture and it was incubated at 37°C for a further 16-18 hours. The culture was washed with plain medium and thymidine was added before incubating at 37°C for 3 hours and 10 minutes. Ethidium bromide was added into the culture and incubated at 37°C for a further 1 hour and 45 minutes. Colchicine was added to culture to arrest cells during mitosis. Hypotonic solution (0.075 M potassium chloride) and fixative (methanol and acetic acid in a 3:1 ratio) were then applied sequentially. The cell suspension was placed overnight in a freezer at -20°C. Finally, the chromosome suspension was dropped onto glass slides on the following day and oven baked at 63-65°C overnight before the banding procedure.

The banding protocol followed the standard GTG protocol using trypsin and geimsa. The slides were immersed into a trypsin solution for 5-10 seconds (depending on the quality of the chromosomes) and immediately immersed in a serum solution for 30 seconds to stop the trypsin reaction. Then the slide was placed into geimsa stain for 5-8 minutes and washed with distilled water.

15 metaphases were counted and 5 metaphases were fully analyzed and karyotyped under a bright field microscope. The images were captured by a cool CCD camera and Applied Spectral Imaging software (Clousto, 2001).

Fluorescence in situ hybridization (FISH)
The FISH probes were performed with bacterial artificial chromosome (BAC) and/or P1-derived artificial chromosome (PAC) clones containing 41 different subtelomeric-specific sequences for all human chromosome ends according to the publicly available genome source (NCBI Map Viewer: http://www.ncbi.nlm.nih.gov/mapview/). BAC/PAC clones were purchased through the BAC/PAC website at http://www.bacpac.chori.org.

Bacterial colonies containing single BAC/PAC clones were inoculated in 5 ml of LB medium containing 25 \( \mu \text{g/ml} \) chloramphenical for the BAC clones and 50 \( \mu \text{g/ml} \) kanamycin for the PAC clones. After each clone was incubated at 37 \( ^\circ \text{C} \) overnight the cells were harvested. Plasmid DNA was extracted using the mini-preparation method of Sambrook and Russel (2001) and labeled with spectrum green-dUTP for the p-arm and spectrum orange-dUTP for the q-arm of the chromosome by nick translation.

The nick translation reaction was set up in a total volume of 50 \( \mu \text{l} \) and 1 \( \mu \text{g} \) of plasmid DNA was used. The calculated volume in \( \mu \text{l} \) of plasmid DNA, 2.5 \( \mu \text{l} \) of 0.2 mM spectrum green-dUTP or spectrum orange-dUTP, 5 \( \mu \text{l} \) of 0.1 dTTP, 10 \( \mu \text{l} \) of dNTP mix, 5 \( \mu \text{l} \) of 10X nick translation buffer and 10 \( \mu \text{l} \) nick translation enzyme were added sequentially into a microcentrifuge tube. Sterile water was added to bring the volume to 50 \( \mu \text{l} \). The reaction was carried out at 15 \( ^\circ \text{C} \) for 16 hours and then the reaction was stopped at 72 \( ^\circ \text{C} \) for 10 minutes. 5 \( \mu \text{l} \) of labeled probe was precipitated by adding 1.2 \( \mu \text{l} \) of 3 M NaOAc, 1 \( \mu \text{g} \) of Cot-1 DNA and 2 \( \mu \text{g} \) of human placental DNA. 30 \( \mu \text{l} \) of cold ethanol were added into the tube and it was placed in a freezer at -80 \( ^\circ \text{C} \) for 15 minutes, after which it was centrifuged at 13,000 rpm for 30 minutes. After centrifugation, the supernatant was discarded and the pellet allowed to dry. The pellet was then resuspended by adding 10 \( \mu \text{l} \) of hybridization solution and incubated at 37 \( ^\circ \text{C} \) for 1 hour.

For the FISH analysis, 3 slides per case were each divided into eight sections with a diamond pencil and 4 \( \mu \text{l} \) of metaphase suspension was placed onto each section. Metaphase slides were pretreated with 2XSSC/0.05% TWEEN 20 at 37 \( ^\circ \text{C} \) for 30 minutes and dehydrated with an ethanol series (70%, 80% and 100%) for 2 minutes each at room temperature. The p-arm and q-arm probes of each chromosome were denatured and hybridized to the denatured-metaphase, one chromosome per section, eight chromosomes per slide. The slides were incubated in a humidified box at 37 \( ^\circ \text{C} \) overnight. Excess probes were eliminated by washing with 0.4XSCC at 68 \( ^\circ \text{C} \) for 1 minute, 2XSCC at room temperature for 2 minutes and the chromosomes
were then counterstained with DAPI. The slides were analyzed in a fluorescence microscope and the images were captured by a cool CCD camera and Applied Spectral Imaging software. The presence or absence of p-arm and q-arm signals for each chromosome were recorded for at least five metaphases from each patient’s sample. Inverted DAPI staining was used for chromosome identification during the FISH analysis (Kearney and Buckle, 2001)

Multiplex PCR for screening FMR1, FMR2 and SRY genes

DNA extraction

50 µl peripheral blood was dropped into a microcentrifuge tube and incubated at 37 °C until dried. Dried-blood was added with 500 µl of TE buffer, and then vortexed for 2 seconds before incubating at room temperature for 30 minutes. After centrifugation at 12,000 rpm for 2 minutes, the supernatant was discarded, and 50 µl of 25% Chelex-100 resin were added to the sample and it was further incubated at 56 °C for 30 minutes to 24 hours. The sample was heated at 95 °C for 10 minutes and centrifuged at 12,000 rpm before PCR analysis.

Polymerase Chain Reaction analysis

Multiple DNA amplifications were performed; for the FMR1, FMR2 and SRY gene, a final reaction volume of 20 µl was used with multiplex buffer (50.25 mM Tris pH 8.8, 12.45 mM (NH₄)₂ SO₄; 1.0 mM Magnesium Chloride; 200 µM each dATP, dTTP, dCTP; 10% dimethylsulfoxide (DMSO); 150 µM of dGTP, 50 µM 7-deaza dGTP; 0.25 µM primer SRY; 0.25 µM primer FRAXE and 0.5 µM primer FRAXA and 1U of IMMOLASE™ DNA polymerase.

PCR was carried out following an initial hot start at 95 °C for 10 min, 10 cycles of 95 °C for 60 seconds, 66 °C for 60 seconds, 72 °C for 60 seconds, 25 cycles of 95 °C for 60 seconds, 64 °C for 60 seconds, 72 °C for 60 seconds and a final extension of 72 °C for 5 minutes.

The PCR products were separated on 2.5% agarose gel and visualized by ethidium bromide staining under a UV transilluminator.
<table>
<thead>
<tr>
<th>Primer name</th>
<th>Primer sequence</th>
<th>Amplified fragment length (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SRY forward</td>
<td>5’CAT GAA CGC ATT CAT CGT GTG GTC3’</td>
<td>254</td>
</tr>
<tr>
<td>SRY reverse</td>
<td>5’ CTG CGG GAA GCA AAC TGC AAT TCT T 3’</td>
<td></td>
</tr>
<tr>
<td>FRAXA-PSU forward</td>
<td>5’ CAGCGTTGATCACGTGACGTGGTTTCAGTG 3’</td>
<td>429 (30 repeat)</td>
</tr>
<tr>
<td>FRAXA-PSU reverse</td>
<td>5’ GATGGGGCCTGCCCTAGAGCCAAGTAC 3’</td>
<td></td>
</tr>
<tr>
<td>FRAXE-PSU forward</td>
<td>5’AAG CGG CGG CAG TGG CAC TGG GC 3’</td>
<td>384 (30 repeat)</td>
</tr>
<tr>
<td>FRAXE-PSU reverse</td>
<td>5’ CGCCCCCTGTGAGTGTCTAAGTGTGTGATG 3’</td>
<td></td>
</tr>
</tbody>
</table>
Figure 5 An example of Multiplex PCR results. Lane 1 shows the PCR product of a normal male. Lanes 2 and 4 show the PCR product of a normal female. Lane 3 is a 100 bp DNA ladder. Lane 5 shows the PCR product of a full mutation $FMR1$ male. Lane 6 shows the PCR product of a full mutation $FMR1$ female.