CHAPTER III

RESEARCH METHOD

III.1. Research aspects

III.1.1 Field

In the field of human genetic, mainly molecular genetics. This research is also related with neurology, neuro-pediatrics and clinical genetics.

III.1.2 Location

Since this study is a joint program between the Medical Faculty of Diponegoro University (UNDIP) Semarang Indonesia and the Human Genetics Department of Radboud Universiteit Nijmegen (RUN) The Netherlands, this study took place both in Indonesia and in The Netherlands.

Routine chromosome analysis and DNA extraction performed in Molecular and Cytogenetic laboratory of Center for Biomedical Research, Faculty of Medicine Diponegoro University Semarang in 2007. DNA analysis of the Fragile-X syndrome, sub-telomeric deletions and deletions (STDs Analysis) with Multiplex Ligation Probe Amplification (MLPA), and sequencing performed in Radboud University Nijmegen.

III.1.3. Period

Within one yeardata collections from the previous study in Semarang and Bandung were performed. Molecular screening held in RUN MC The Netherlands one year after data collection.

III.1.4 Design

The study is designed as descriptive study, as an observational survey.

III.2 Population and Sample

III.2.1 Population

About 527 epilepsymentally retarded patients of several special schools in Semarang and Bandung Indonesia were included in this study, 32 of them selected.

III.2.2. Samples

Selection of 32 epilepsy mentally retarded patients from total 527 mentally retarded patients in Semarang and Bandung special schools from previous studies[1]. The parents/ guardian of the patients were also interviewed in order to obtain the family history. Moreover, samples of the parents from patients with a genetic abnormality were taken, in order to determine de novo or inherited occurrence of the mutation/variant.

III.2.2.1 Inclusion criteria

1. From the previous study, epilepsy mentally retarded pupils were included (clinically diagnosed MR with AAMR criteria), whose parents signed the informed consent form.
2. Physical examination and dysmorphology examinationfromclinicians and neurologist in Semarang and Bandung; also clinical geneticists of Human Genetic Departement Radboud Universiteit Nijmegen.

III.2.2.2 Exclusion criteria

1. Chromosomal abnormality screened from cytogenetic examination.

2. FMR-1 gene mutation.
3. Non-availability of clinical data, pictures, history and molecular screening information that already performed.
4. Epilepsy mental retardation patients/ parents that do not sign for informed concent.

III. 3. Variables

- Dependent : Clinical data of Mental retardation and epilepsy patients

Scale : Nominal

- Independent :Mutation screening result

Scale : Nominal

III. 4. Operational definitions

1. Clinical data : Phenotype or clinical features of all MR and epilepsy patients.

   Scale : Nominal
2. Mutation : A change of the DNA sequence within a gene or chromosome of an organism resulting in the creation of a new character or trait not found in the parental type.

   Scale : Nominal

III.5Data Collection

III.5.1 Subject selection

Subjects were selected based on epilepsy seizures data from previous study that were obtained from the Semarang and Bandung special schools database.

III.5.2 Clinical examination

Patients were examined for MR physically by thorough clinical examination using a protocol adapted from the RUNMC by clinical geneticist RUNMC. For epilepsy, neuro-developmental and detailed seizures history was taken from the patient's parents or guardians by the examiners (taken from the previous examiners). Also neurological physical examination performed for those with epilepsy by neurologist. Complete pictures and pedigree were taken from all examined patients.

III.5.3 Sample collection

DNA from the process of DNA extraction came from previous study were collected. Withdrawal of 5 mL heparinized blood was performed (for chromosome analysis) and 5-10 mL EDTA blood (for DNA analysis) from each individual. EDTA blood from the parents of positively screened patients were withdrawed in order to draw the conclusion.

III.5.4. Methods

III.5.4.1 General

Previous Study Test

Blood collection, cytogenetic analysis, DNA extraction, Fragile X screening, MLPA STD's screening mostly had done in the previous study [1]. DNA extraction was done using the method in Centre for Biomedical Research (CEBIOR) Semarang Indonesia [2, 3]. DNA was extracted from peripheral blood by means of "salting out" method [3]. Subsequently, completionof Fragile X and

MLPA STD’s screening for extra patients were done to move forward to the next step, which was sequencing to identify the presence of mutation.

Parents of the patients whom met criteria were asked to include in this research by signing the consents and participate on blood collection sampling. They were also informed that, their participation was highly important to conclude whether the mutation in their child was the underlying genetic ethiology.

III.5.4.2 Laboratory Methods

III.5.4.2.1 Preparation

1. DNA Concentration Measurements

DNA that already extracted in Semarang was then brought to the laboratory in The Netherlands. First centrifuge it 10000 rpm in 1 minute. After transferred the DNA to new tubes following the system in the DNA Diagnostic Department of RUN MC, then measuring the concentration and ratio using NanoDrop 1000 (ND-1000) spectrophotometer from ISOGEN. Overall, Semarang DNA ratio are range between 1.70 – 1.83, which were in a good range.

2. Working solution

Working solution in DNA diagnostic lab is 100ng/ul. Using simple chemistry equation for making dilutions to get the amount of DNA to put in with Tris EDTA (TE) buffer 0.1x or to MilliQ (MQ).

3. DNA Purification

In certain circumstances, for example like bad MLPA or sequencing results, although from the Nanodrop measurements the DNA ratio was good, purification was needed for the DNA as additional efforts to get better results.

III.5.4.2.2 Sequencing

Possible syndromic cases which are: *UBE3A, UPF3B, MED12, PTEN,* and*TCF4*genes sequenced. Continued with sequencing the patients list with*SCN1A, ARX, STXBP1,* and *LGI1* genes.

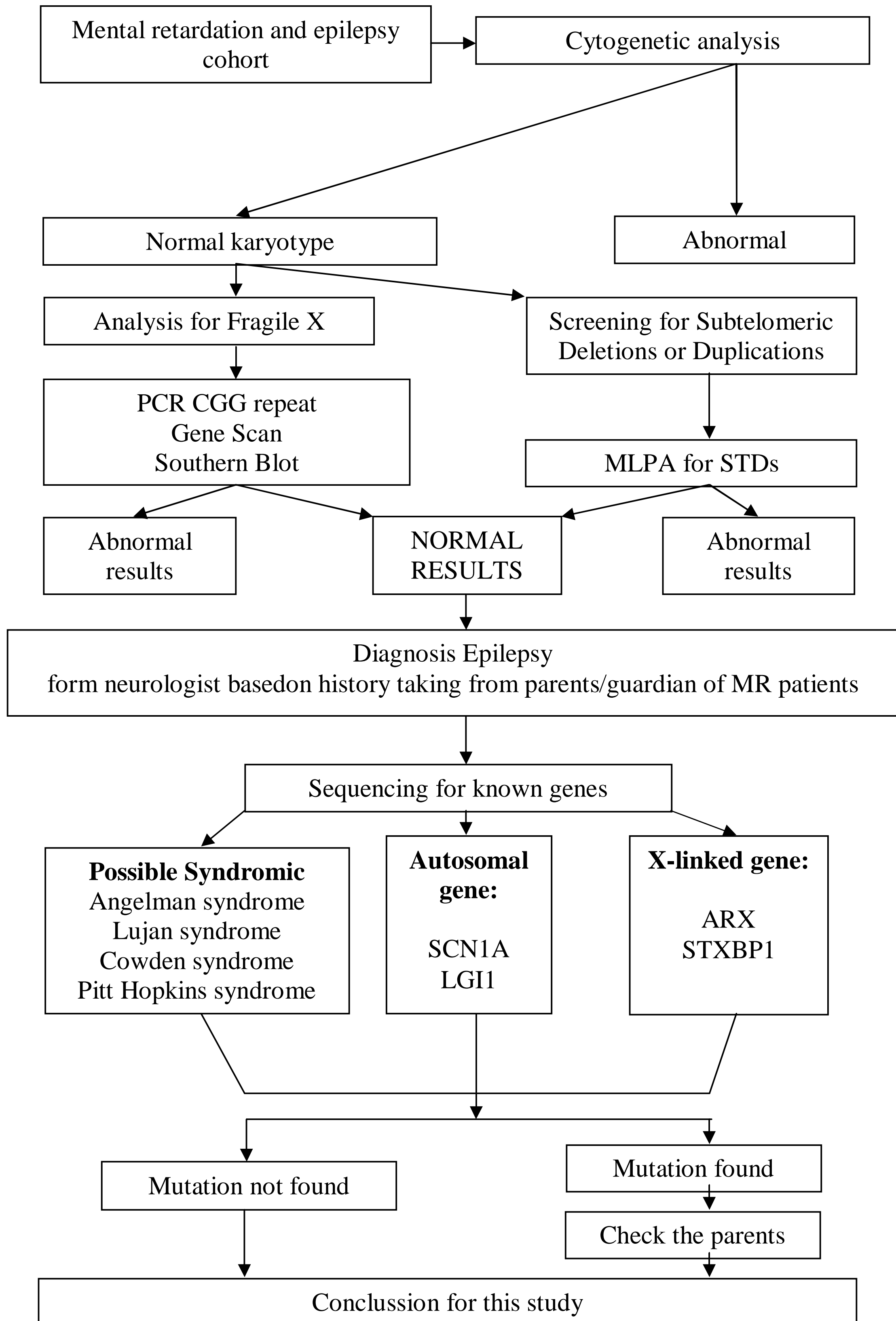
General process of sequencing were: preparation, design new primers for the genes, order and test the new primer using controls DNA, perform the test to the patients list samples, downloading results from the system, analyze them, then draw the conclusion after recheck it to the parents using sequencing method.

Screening then done for these genes:

1. Angelman syndrome*UBE3A*gene[4].
2. Lujan-Fryns Syndrome *MED12* and *UPF3B*genes[5].
3. Cowden syndrome *PTEN*gene[6].
4. Pitt–Hopkins syndrome *TCF4* gene [7].

Mutation screening for the *SCN1A*[8], *LGI1*[9], *ARX*[10], *STXBP1* [11] and *LGI1* [12]genes then performed.

III.5.4.2.3 Research Flow



III. 6. Data Analysis

Data analyzed using descriptive method and presented in tables and graphics.

III. 7. Research Ethics

Ethical Clearance approved by Ethic Commission from Medical Faculty Diponegoro University. Transfer of samples from laboratory CEBIOR to RUNMC with Material Transfer Agreement.

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