CHAPTER 3

MATERIALS AND METHODS

3.1 Research field

This research is in the field of Molecular and Clinical Genetics.

3.2 Location

The research was done in Central Biomedical Research (CEBIOR) Medical Faculty Diponegoro University and UKM Medical Molecular Biology Institute (UMBI).

3.3 Research Period

The research was done in January 2010 - September 2010, as part of on going research entitled “Identifikasi Gen Culprit Autis pada Anak-anak dengan Autistic” starting from 2009 by dr. Tri Indah Winarni and group.

3.4. Method

This is observational and analitical study with case control approach.

3.5 Population and Samples

3.5.1 Population

The population of this research are Autism Spectrum Disorders (ASD) children in School for Children with Special Need (SLB and Autism School) in Semarang and Solo. While, the population of control group are children who live in Semarang and Solo.

3.5.2 Sample

Samples from previous study in Cebior was included. Sample was determined by purposive sampling and the minimal quantity of sample is 40 for ASD and 40 for normal control calculated using formula below :

$$m' = \frac{[\, c_{\alpha/2} \sqrt{(r+1)\,PQ} - c_{1-\beta} \sqrt{r\,P_1Q_1 + P_2\,Q_2}\,]^2}{r\,(P_2 - P_1)^2}$$

$$m' = \frac{[1.96 \sqrt{(1+1)\,0.515x0.485} - (-0.842) \sqrt{(1x0.65x0.35)+(0.32x0.68)}]^2}{1\,(0.32 - 0.65)^2}$$

$$m' = 33.8$$

$$m = \frac{m'}{4} \left[\, 1 + \sqrt{1 + \frac{2\,(r+1)}{m'\,r\,|P_2 - P_1|}}\,\right]^2$$

$$m = \frac{33.8}{4} \left[\, 1 + \sqrt{1 + \frac{2\,(1+1)}{33.8x1x0.33}}\,\right]^2$$

$m = 39.8$
$m = n_1 = 40$
$n_2 = r \times m = 1x\ 40 = 40$
$n_1 + n_2 = 80$

$m = n_1$ = size of sample from population 1
$n_2 = rm$ = size of sample from population 2
P_1 = proportion of exposure in population 1 = 0.65
P_2 = proportion of exposure in population 2 = 0.32
α = " significance" = 0.05
β = chance of not detecting a difference = 0.2
$1 - \beta$ = power = 0.8
$P = (P_1 + rP_2) / (r + 1)$
$r = n_2 / n_1$ = ratio of cases to controls = 1/1
$Q = 1- P$
$1 - \alpha = 0.95 \rightarrow c_{\alpha/2} = 1.960$
$1 - \beta = 0.80 \rightarrow c_{1-\beta} = - 0.842$

3.6 AUTISM SPECTRUM DISORDERS (ASD) SAMPLES

Inclusion Criteria

The inclusion criteria of Autism Spectrum Disorder (ASD) is according to DSM IV, age 4 - 18 years old and parents agree to join the reseach by signing written informed consent.

Exclusion Criteria

The exclusion criteria are ADHD, Childhood Psychosis, mental retardation with known etiology, parent or guardian do not agree to join the research and failed to drawn the blood due to any reason.

3.7 NORMAL CONTROL SAMPLES

Samples was determined by simple random sampling from public school in Semarang and Solo with inclusion and exclusion criteria below:

Inclusion Criteria

Inclusion criteria of normal control sample are they whose age 4 – 18 years old, live in Semarang and Solo, have normal physical examination and developmental milestone.

Exclusion Criteria

The exclusion criteria are parents do not agree to join the research and failed to drawn the blood due to any reason.

3.8 Data collection and Research Protocol

3.8.1 Collecting data

Anamnesis to fill medical record about family history, antenatal, perinatal and postnatal care, behavior, medical and developmental history. Diagnosing Autism Spectrum Disorders (ASD) with DSM IV. Phenotype expression of autistic disorder was measured by CARS (Childhood Autism Rating Scale). Dysmorphology examination and cytogenetic analysis was done on previous study for excluding chromosomal abnormality and mental retardation with known etiology.

3.8.2 Collecting samples

Ten milliliter (10 ml) Blood peripheral ethylenediaminetetraacetic acid (EDTA) samples of Autism Spectrum Disorder children in Semarang and Solo was collected after diagnosed Autism using DSM IV. In which, 5

ml blood peripheral EDTA samples was used for DNA extraction and another 5 ml was used for Glutathione s-transferase activity measurement.

3.8.3 DNA extraction

DNA extraction was done by salting out method according to CEBIOR manual.

Salting out method

Add 5 ml whole blood (EDTA) with 5 ml Ammonium Chloride (NH_4Cl) lysis buffer, shake gently, incubate for 10-30 min in room temperature, and centrifuge at 3000-3500 rpm for 10 min at 4º C. Remove supernatant (blood waste), add NH_4Cl lysis buffer, resuspend the pellet, and centrifuge for 10 min at 4°C (3500 rpm).Repeat step 2 until we get white pellet.

Add white pellet with 2 ml strong Tris EDTA (TE) buffer lysis and resuspend the pellet, add 50 µl proteinase K (10 mg/ml) and 100 µl sodium dodecyl sulphate (SDS) 10% , shake gently, and incubate overnight at 50° C in a water bath.Add 6 M Natrium Chloride (NaCl) (1/3 volume supernatant) and centrifuge 10 min at 4°C (4000 rpm).

Transfer the supernatant into a new tube, add ethanol absolute (2x volume). shake gently until the DNA precipitated, like ‘cotton wool’. Wash the DNA in 70% ethanol and wait until dry in room temperature 30 min. Dissolve the DNA in 300-500 µl normal TE-buffer. The purity and concentration of DNA were quantified using Nanodrop Spectrophotometer (ND 1000 spectrophotometer).

3.8.4 Genotyping GSTM1 and GSTT1 gene by Multiplex Polymerase Chain Reaction (PCR) and gel electrophoresis.

PCR protocol

The multiplex PCR was performed in 25 µl reaction volume containing 30-50 ng of DNA,12,5 µl HotStarTaq Master Mix (Qiagen) in which containing of 1.25 units HotStarTaq DNA Polymerase, 1x PCR Buffer, 100 µM of each Deoxyribonucleotide triphosphate (dNTP) and 0.75 mM Magnesium Chloride ($MgCl_2$). Concentration for each primer is 0.2 µM. Primers for GSTM1 gene were designed using computer program Primer3 (forward primer 5'-TCTGGGGAGGTTTGTTTTCA-3' and reverse primer 5' TCTCCAAAATGTCCACACGA-3') and primers for GSTT1 gene according to Mohammad Ebrahimi et al, 2004 (forward primer 5'-TTCCTTACTG GTCCTCACATCTC-3' and reverse primer 5'-TCACCGGATCATGGCCAGCA-3')[1] and β-globin gene primers were follow UMBI's manual for internal control gene (forward primer 5'-GAGTCAAGGCTGAGAGATGCAGGA-3' and reverse primer 5'-CAATGTATCATGCCTCTTTGCACC-3').

The PCR for GSTM1 gene was performed with an initial denaturation 95^0 C for 4 minutes, followed by 35 cycles with denaturation at 95^0 C for 1 minute,annealing at 50 ^{0} C and extension at 72^0 C for 2 minutes. Final extension was performed at 72 ^{0} C for 10 minutes and hold at 4 ^{0} C. PCR for GSTT1 gene was performed with an initial denaturation 95^0 C for 4 minutes, followed by 35 cycles with denaturation at 95^0 C

for 1 minute, annealing at 61 ^{0} C and extension at 72^{0} C for 2 minutes. Final extension was performed at 72 ^{0} C for 10 minutes and hold at 4 ^{0} C.

Gel electrophoresis

PCR products was separated on 1,2 % agarose gel and stained with ethidium bromide and visualized on an UV transiluminator. GSTM1 gene product is about 637 bp,GSTT1 product is 459 bp and 850 bp for β-globin gene as internal control. In the presence of GSTM1 and GSTT1 gene band was interpreted as GSTM1 and GSTT1 positive gene. Absence of GSTM1 and GSTT1 gene band was interpreted as GSTM1 null and GSTT1 null.

3.8.5 Determining activity of Glutathione s-transferase using Enzyme-Linked Immunosorbant Assays (ELISA) in Erythrocyte

Five milliliter (5 ml) whole blood was separated into 4 layers by separation medium (Lymphoprep). Erythrocyte or red blood cells (RBC) at the bottom,followed by lymphoprep ,mononuclear cells (MNC) and serum on the top. Remove serum, MNC and lymphoprep layer. Briefly, the red blood cells (RBC) were lysed in equal volumes of ice-cold double distilled water and then centrifuged at 10,000 g for 15 minutes at 4 ^{0}C. The supernatant was collected for the glutathione-s transferase (GST) assay.

GST activity was determined using Glutathione S-Transferase Assay Kit (Cayman, USA) by measuring the conjugation of 1-chloro-2,4-dinitrobenzene (CDNB) with reduced GSH. The GST assay was performed according to assay protocol provided. Briefly, 170 µl of assay

buffer and 20 µl of GSH were added to the non-enzymatic wells. In the positive control wells, 150 µl of assay buffer, 20 µl of glutathione and 20 µl of diluted GST (control) were added together. For the sample wells, 150 µl of assay buffer, 20 µl of glutathione and 20 µl of sample were added. The reactions were initiated by adding 10 µl of CDNB to all of the wells. The absorbance was read once every minute at 340 nm using a plate reader to obtain values at 8 time points. GST activity in nmol/min/ml was calculated with the CDNB extinction coefficient at 340 nm as 0.00503 µM-1. One unit of enzyme will conjugate 1.0 nmol of CDNB with reduced glutathione per minute at 25 ^{0}C.

3.9 Research variable

Independent variable : GSTM1 gene and GSTT1 gene

Dependent variable : Glutathione s-transferase enzyme activity and Phenotype Expression of ASD

3.10 Definition and Measurement of the variable

1. GSTM1 gene :

   Gene located in chromosome 1p13.3, consist of 8 exon, measured by PCR and gel electrophoresis. The presence of 637 bp PCR product is interpreted as wild type (GSTM1 positive) and absence of PCR product interpreted as homozygous deletion (GSTM1 null).

2. GSTT1 gene :

   Gene located in chromosome 22q11.2, consist of 5 exon, measured by PCR and gel electrophoresis. The presence of 459 bp PCR product is interpreted as wild type (GSTT1 positive) and absence of PCR product interpreted as homozygous deletion (GSTT1 null).

3. Glutathione s-transferase enzyme activity :

   The activity of Glutathione s-transferase enzyme in nmol/min/ml measured by ELISA using GST assay kit.

4. Phenotype expression of ASD :

   Severity level of ASD according to Childhood Autism Rating Scale (CARS), divided into mild to moderate and severe stage.

3.11 Data analysis

Statistical analysis was carried out using computer. Chi-square test was used to calculate the differences in genotype prevalence and association between case and control groups. Association between GST activity and phenotype expression of ASD, case-control group and genotype were analysed using Independent samples T test and Mann-Whitney U test if the data not normally distributed. Additionally, ANOVA test was used to analyzed the association between GST activity and CARS score. The Odds Ratio (OR) and its 95 % Confidence Interval (CI) were used to illustrate the association. All tests are two sided with a p value < 0.05 is considered statistically significant.

3.12 Ethical Implication

This study is part of umbrella research previous study and was carried out under permission of the local ethical committee Medicine Faculty Diponegoro University, Semarang. Samples with Material Transfer Agreement (MTA) document was brought for laboratory examination in UMBI, Malaysia. The ethical clearance document is attached in the appendix.

3.13 RESEARCH FLOW