Lower Erythrocyte GST Activity in Autism Spectrum Disorder (ASD) Patients Compared to Normal Controls

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ABSTRACT

Glutathione S-transferases (GST) are antioxidant enzymes that play an important role in the cellular detoxification and excretion of environmental pollutants including heavy metals. GST mu (GSTM1) and G theta (GSTT1) are known to be highly polymorphic and homozygous deletions of these genes result in the lack of enzyme activity and when combined with decreased levels of antioxidants, they have been associated with the Autism Spectrum Disorder (ASD). This preliminary study was performed to investigate the role of GSTM1 and GSTT1 polymorphisms as risk factors of ASD associated with GST activity and phenotype expression. Fifty one ASD patients and 45 controls were recruited for GSTM1 and GSTT1 genotyping while 6 ASD patients and 8 controls were assessed for GST activity. The results showed no significant differences in frequencies of GSTM1 null, GSTT1 null and combination both genotype between ASD patients and controls. However the mean erythrocyte GST activity in ASD is significantly decreased compared with controls (p = 0.043). The mean erythrocyte GST activity is lower in the severely autistic group compare to the mild to moderately autistic group, although it was not statistically significant. Further investigations are needed with a bigger sample size, analyzing multiple GST genes and GST activity determination to find out the gene susceptibility of ASD and factors that contribute to the phenotype expression of ASD.

INTRODUCTION

Autism spectrum disorder (ASD) is a neurodevelopmental disorder characterized by qualitative impairments in the development of social and communication skills which is often accompanied by stereotyped and restricted patterns of interests and behavior with the onset of impairment before the age of three. ASD includes the diagnostic categories of autistic disorder, pervasive developmental disorder not otherwise specified (PDD-NOS) and Asperger's syndrome (1). Childhood Autism Rating Scale (CARS) is often used for evaluating the child's body movements, adaptation to change, listening response, verbal communication, and relationship to people in children over 2 years of age. The child's behavior is rated on a scale based on deviation from the typical behavior of children of the same age (2).

Epidemiological studies demonstrate that the prevalence of autism has increased in recent years (3). It is currently estimated that autism affects as many as 1 out of 150 children (67 per 10,000) in the United States and occurs four times as frequently in males as females (4). Overall, the incidence of ASD in children age 0 to 4 years was 5.49 per 10 000 for Chinese children in Hong Kong (5) while the incidence of was estimated to be 4.3 to 5.5 per 10 000 in Australia (6) and as high as 27.2 per 10 000 children in Japan (7).

Investigators suggested that ASD may result from the interaction between genetic and environmental factors, with oxidative stress as a mechanism linking these risk factors (8). There is evidence supporting the role of oxidative stress in autism (9) that affect brain development during gestation or possibly after gestation, contributing to expression of autism (10). Studies have shown that in autism, the plasma total gluthatione (tGSH) levels were lower, while levels of oxidized GSH (GSSG) were elevated and the ratios of tGSH to GSSG were low (8, 11), which results in impaired ability to excrete metals including mercury for which GSH act as the major detoxification compound in the body (12).

Glutathione S-transferases (GST) are antioxidant enzymes that play important roles in the cellular detoxification, excretion of environmental pollutants as well as protection against oxidative stress by their ability to conjugate GSH with compounds containing an electrophilic

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center (13). The electrophilic functional group for conjugation reactions can be provided by a carbon, a nitrogen, or a sulfur atom (14-16). These enzymes detoxify a broad range of substances including carcinogens, environmental toxins, and drugs. At least seven distinct classes have been identified: alpha (α), mu (μ), pi (π), sigma (σ), theta (τ), kappa (κ), and zeta (ξ). This classification is according to the substrate specificity, chemical affinity, structure, amino acid sequence and kinetic behavior of the enzymes (14).

GST mu (GSTM1) and GST theta (GSTT1) are known to be highly polymorphic and have been investigated in relation to various types of diseases, including diabetes mellitus, hypertension, stroke, Parkinson's disease, rheumatoid arthritis and some types of cancer (16-20). Subjects with at least one functional allele are designated as GSTM1 or GSTT1 positive (14) but homozygous deletions of these genes, referred to as GSTM1 null (*GSTM1*$^{-/-}$) and GSTT1 null (*GSTT1*$^{-/-}$) respectively, result in reduced enzyme activity (21). Two Korean case-control studies found frequencies of 53% and 56% for the GSTM1 deletion genotype (22) while the Indian population showed a lower percentage of *GSTM1*$^{-/-}$ frequency (30.4-35.4%) when compared to other Asian and Caucasian populations which was reported in the range of 49-53.8% (23). Population-based study has reported that the frequency among Caucasians in the United States of America ranged from 48%-57% (22). The prevalence of the *GSTT1*$^{-/-}$ genotype has been shown to be 11% in European and North American Caucasians (24-27), while in Asia the prevalence is much higher, which is 49% in Shanghai, 47% in Taiwan and 44% in Japan (28-30).

The association of *GSTM1*$^{-/-}$ with autism has been reported in a previous study (31) which showed an increased genotype relative risk near 2 for the homozygous GSTM null genotype and autism. Meanwhile, a recent study with an autism animal model using GSTM1 knockout mice that were given valproic acid (VPA) treatment (which is known to cause autism-like behavioral deficits) showed higher rate of neuronal cell apoptosis compared to wild-type controls (32). VPA administration during pregnancy may result in fetal valproate syndrome which has features similar to autism and rodents exposed *in utero* to VPA showed both cellular damage and behavioral deficit that are analogous to the autism phenotype (33). The absence of the GSTM1 gene, resulting in impaired enzyme activity, is believed to lead to failure of individuals with autism to detoxify xenobiotics, including heavy metals such as mercury (Hg). Hg when coupled with decreased levels of antioxidants (low glutathione and antioxidant enzymes), leads to the increased production of free radicals which cause an increase in lipid peroxidation, protein oxidation, and DNA oxidation that finally leads to increased oxidative stress. Oxidative stress is associated with impaired neuronal development, increased inflammatory response, impaired energy production, cell death and decreased synaptic efficiency. These combined effects contribute to the phenotypic presentation of ASD (11). Therefore this preliminary study was conducted to investigate the role of GSTM1 and GSTT1 polymorphism as risk factor of ASD, the association with GST activity and phenotype expression in a cohort of Indonesian ASD patients.

MATERIALS AND METHODS

SUBJECTS

This was a case control study with a total of 96 subjects comprising of 51 Autism Spectrum Disorder subjects from the Special Need and Autism School and 45 unrelated healthy subjects from the public schools in Semarang and Solo, Indonesia. All the subjects for the study were aged between 2- 18 years old. Diagnosis of ASD was according to Diagnosis and Statistical Manual of Mental Disorders, fourth edition (DSM IV) and phenotype expression of ASD was determined using CARS. This study was approved by the institutional review committee from the Medical Faculty Diponegoro University and the Ethics Committee of Dr. Kariadi Hospital Semarang, Indonesia. The study was conducted with informed consent from all of the subjects' parents. The analysis was done at the Centre for Biomedical Research (CEBIOR) in the Medical Faculty Diponegoro University, Indonesia and the UKM Medical Molecular Biology Institute (UMBI), Universiti Kebangsaan Malaysia.

GENOTYPING

Venous blood samples were collected from patients and controls in EDTA tubes. Genomic DNA was extracted from peripheral blood leukocytes using the salting-out method (34). The genotype of GSTM1 and GSTT1 genes in the subjects were determined using a multiplex PCR technique with the co-amplification of β-globin gene as positive internal control. Primers pairs used in this study were: GSTM1 gene (forward primer 5'-TCTGGGGAGGTTTGTTTTCA-3' and reverse primer 5' TCT CCAAAATGTCCACACGA-3'), GSTT1 gene (forward primer 5'-TTCCTTACT GGTCCTCACATCTC-3' and reverse primer 5'-TCACCGGATCATGGCCAGCA-3), β-globin gene (forward primer 5'-GAGTCAAGGCTGAGAGATGCAGGA-3' and reverse primer 5'-CAATGTATCATGCCTCTT TGCACC-3'). The multiplex PCR was performed in a 25 μl reaction volume containing 30-50 ng of DNA, 12.5 μl HotStarTaq Master Mix (Qiagen) which contained 1.25 units of HotStarTaq DNA Polymerase, 1x PCR Buffer, 100 μM of each dNTP and 0.75 mM of $MgCl_2$. The final concentration for each primer is 0.2 μM.

The PCR conditions for GSTM1 gene was performed with an initial denaturation 95°C for 4 minutes, followed by 35 cycles with denaturation at 95°C for 1 minute, annealing at 50°C and extension at 72°C for 2 minutes with final extension at 72°C for 10 minutes. The PCR conditions for GSTT1 gene utilized the same with protocol above except

that the annealing temperature was changed to 61°C. A PCR product of 625 bp denotes the presence of GSTM1, a product of 459 bp denotes the presence of GSTT1 while a product of 850 bp denotes the internal control. The presence of GSTM1 and GSTT1 gene band was interpreted as GSTM1 and GSTT1 positive gene while the absence of GSTM1 and GSTT1 gene band was interpreted as *GSTM1*$^{-/-}$ and *GSTT1*$^{-/-}$.

GLUTATHIONE S-TRANSFERASE ACTIVITY

Blood samples were separated to 4 layers by lymphoprep. 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°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°C.

STATISTICAL ANALYSIS

Statistical analysis was carried out using SPSS (version 16.0). The data were reported as mean ± SD. 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 was analysed using Independent Samples T test and Mann-Whitney U test if the data is not normally distributed. Additionally, analysis between GST activity and CARS score was analysed by regression. The Odds Ratio (OR) and its 95% Confidence Interval (CI) were used to illustrate the association. All of the tests were two sided and p value < 0.05 was considered statistically significant.

RESULTS

Table 1 showed the frequency of the GSTM1 and GSTT1 genotypes in ASD and control group. The frequency of *GSTM1*$^{-/-}$ (11.8%) in ASD was higher compared to the control group (6.7%) but the difference was not statistically significant. The frequency of *GSTT1*$^{-/-}$ genotype (39.2%) was also higher among ASD patients compared to the control group (31.1%) but there was no statistically significant difference. In addition, the combination of *GSTM1*$^{-/-}$/ *GSTT1*$^{-/-}$ genotype was also not significant between cases and controls [refer Table 2]. The phenotype expression of ASD which were represented by CARS, were divided into two groups: mild to moderately autistic and severely autistic. We found no significant difference in the distribution of *GSTM1*$^{-/-}$, *GSTT1*$^{-/-}$as well as *GSTM1*$^{-/-}$/ *GSTT1*$^{-/-}$ genotype between the CARS score of mild to moderately autistic and severely autistic [refer Table 2].

There was no significant difference for the mean erythrocyte GST activity between *GSTM1*$^{-/-}$, *GSTT1*$^{-/-}$ as well as *GSTM1*$^{-/-}$/*GSTT1*$^{-/-}$ genotype respectively compared to the controls. Table 3 showed that the mean erythrocyte GST activity in ASD was significantly lower (0.374 ± 0.262 nmol/min/ml) compared with controls (1.486 ± 0.262 nmol/min/ml). Whereas in the severely autistic, the mean erythrocyte GST activity was lower (0.259 ± 0.037) compared with mild to moderately autistic (0.432 ± 0.317) , but this difference is not significant. The association of GST activity with CARS score was also not significant (p = 0.089).

DISCUSSION

Autism spectrum disorder is a complex and multifactorial disease, which leads to the difficulty of identifying significant risk factors. Autism may result from a

Table 1. Frequency of GSTM1 and GSTT1 genotype in ASD patients and control group

	GSTM1$^{-/-}$		Wild type		Total		GSTT1$^{-/-}$		Wild type		Total	
	N	%	N	%	N	%	N	%	N	%	N	%
Group												
a. ASD	6	11.8	45	88.2	51	100	20	39.2	31	60.8	51	100
b. Control	3	6.7	42	93.3	45	100	14	31.1	31	68.9	45	100
CARS												
a. Mild to moderately autistic	2	11.1	16	88.9	18	100	8	44.4	10	55.6	18	100
b. Severely autistic	4	12.9	27	87.1	31	100	10	32.3	21	67.7	31	100

Table 2. Odd ratios (OR) and p value of GSTM1$^{-/-}$, GSTT1$^{-/-}$ and GSTM1$^{-/-}$ / GSTT1$^{-/-}$ genotypes in association with groups and phenotype expression. All results were not statistically significant

	GSTM1$^{-/-}$			*GSTT1*$^{-/-}$			*GSTM1*$^{-/-}$/*GSTT1*$^{-/-}$
	OR	95% CI	p	OR	95% CI	p	p
Group							
a. ASD	0.536	0.126 - 2.280	0.495	0.700	0.301 - 1.630	0.407	0.299
b. Control							
CARS							
a. Mild to moderately autistic	0.844	0.139 - 5.138	1.000	1.680	0.508 - 5.558	0.394	0.667
b. Severely autistic							
CARS score			0.317			0.157	0.476
GST activity			0.770			0.180	0.377

Table 3. Mean erythrocyte GST activity in ASD patients and control group. * Denotes to P < 0.05 for ASD compared to wild-type controls

	GST activity		
	Mean (nmol/min/ml)	95% CI	p
Group			
a. ASD	0.374 ± 0.262	0.164 - 0.584	0.043*
b. Control	1.486 ± 1.269	0.607 - 2.365	
CARS			
a. Mild to moderately autistic	0.432 ± 0.317	0.121 - 0.743	0.509
b. Severely autistic	0.259 ± 0.037	0.207 - 0.311	
CARS score		24.990 - 47.410	0.089

combination of genetic susceptibility via the reduced ability to remove metals including mercury or other neurotoxins from the system, and environmental exposure (35-37). Several genes have been reported associated with autism including genes involved in methionine transmethylation and transsulfuration pathways (8). Polymorphisms of Reduced Folate Carrier I (RFC1) gene, Methylenetetrahydrofolate Reductase (MTHFR), Transcobalamin II (TCN2), Catechol-O-methyltransferase (COMT) and Glutathione S-transferase mu 1 (GSTM1) have all been shown to be significantly increased among autistic children compared to controls (31, 38).

In the present study, we found that the frequency of *GSTM1*$^{-/-}$ genotype in the control group was 6.7%, which was quite different from the previous study in the Indonesian population in Jakarta which reported a frequency of 55.6% (39). Geographical and ethnic variations in the distribution of *GSTM1*$^{-/-}$ and *GSTT1*$^{-/-}$ genotype frequencies has been shown different ethnic groups in India (40) and this may explain the difference in the frequency of *GSTM*$^{-/-}$ shown in this study. Indonesia is a huge archipelago of islands with many sub-ethnic groups from various regions and hence could explain the wide difference in the frequency. The frequency shown in our study is much lower than in the Caucasian population (42-60%) and other Asians population, including Japan (47.9%), South Korea (52.2%) and Singapore (56.2%) (41). Meanwhile, our study showed that the frequency of the *GSTT1*$^{-/-}$ genotype in the control group was 31.1%, which is similar with Japan (35.3%), but was lower than the previous study in Indonesia (41.4%) (39), South Korea (51.5%) and Singapore (51.9%) (41). However the *GSTT1*$^{-/-}$ frequency was higher compared to the Europeans (22%) and the African-Americans (21%) (42).

Steven Buyske et al. (2006) reported a significant association of the homozygous GSTM1 deletion genotype with autism (p = 0.028) in their study involving 54 complete case-parent trios and 172 controls, but our study did not show similar association (31). Their study showed a high frequency of the *GSTM1*$^{-/-}$ genotype, 69% compared to the 11.8% found in our study, which may explain for the lack of association of the GSTM1 null genotype with autism. Our study revealed that there is no significant association between *GSTM1*$^{-/-}$ and *GSTT1*$^{-/-}$ gene with ASD, although the distribution of these genes among our ASD patients was higher than controls . The *GSTM1*$^{-/-}$/*GSTT1*$^{-/-}$ genotype also did not contribute to ASD risk in our study population.

Our study also looked at the association between the erythrocyte GST activity among ASD patients and control groups as well the association between GST activity and phenotype expression of ASD. Our study showed lower GST activity in the *GSTM1*$^{-/-}$ and *GSTT1*$^{-/-}$ genotype groups compared to the control group which was in concordance to a recent study involving ischemic stroke patients, although the difference was not significant (43). According

to previous studies, there was no association between *GSTM1*$^{-/-}$ and reduced erythrocyte GST activity observed among Chinese patients, but combined *GSTP1*$^{-/-}$/ *GSTM1*$^{-/-}$ genotype resulted in significantly reduced GST activity (44). Another study in India showed that diabetes mellitus patients with double *GSTT1*$^{-/-}$ and *GSTM1*$^{-/-}$ genotypes have significantly low GST activity as compared to other genotypic groups (45). Studies among Caucasian patients and controls groups showed that those with double *GSTT1*$^{-/-}$/ *GSTM1*$^{-/-}$ genotypes had the lowest serum total GST activities (22, 25-27).

The difference in our results compared with others studies could be due to the fact that this study was conducted in a different race and population. Another explanation for the differences is that GST enzymes have a broad substrate overlap, therefore decreased in expression level of one GST may be compensated by increased expression of another isoform (46). Therefore, this preliminary study needs further investigations with increased sample size, full genotyping of the GST isoforms and GST activity determination, to explore further the genetic susceptibility of ASD and factors that contribute to the phenotype expression of ASD.

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