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Are GSTM1 Null and GSTT1 Null Risk Factor of Autism Spectrum Disorder? A Preliminary Study

Donna Hermawati *, Sue-Mian Then **, Tri Indah Winarni ***, Rahman Jamal **, Sultana MH Faradz ***

ABSTRACT

Background: *Low plasma total glutathione (tGSH) levels, elevated levels of oxidized glutathione (GSSG) and low ratios of tGSH to GSSG in autism were reported. Glutathione S-transferases (GST) are antioxidant enzymes that play important role in cellular detoxification and the excretion of environmental pollutants including heavy metals. Glutathione S-transferase mu (GSTM1) and Glutathione S-transferase theta (GSTT1) are known to be highly polymorphic. Homozygous deletions of these genes result in lack of enzyme activity and impaired the ability to excrete metals including mercury. Combined effects of mercury (Hg) accumulation coupled with decreased levels of antioxidants (low glutathione and antioxidant enzymes) contribute to the phenotypic presentation of autism spectrum disorder (ASD). Association of GSTM1 null genotype with autism has been reported. Therefore the preliminary study was performed to investigate the role of GSTM1 null and GSTT1 null as risk factor of ASD associated with phenotype expression.*

Method: *Fifty one ASD patients were recruited from special need & autism school and 45 controls from Semarang & Solo. Blood veins samples were collected and genomic DNA was extracted by salting-out method in CEBIOR Semarang. Genotyping for GSTM1 and GSTT1 gene was done in UMBI Malaysia. Multiplex PCR was performed and PCR products were separated on 1.2 % agarose gel, stained with ethidium bromide and visualized on UV transiluminator. GSTM1 & GSTT1 gene product is about 625 bp and 459 bp. Absence of GSTM1 and GSTT1 gene band was interpreted as GSTM1 null & GSTT1 null.*

Results: *The frequency of GSTM1 null and GSTT1 null in ASD higher compared with control group but the difference is not statistically significant (p=0.357, OR=0.504; 95% CI 0.117-2.168 and p=0.364, OR=0.674; 95% CI 0.287-1.580). There is also no statistically different in the distribution of GSTM1 null and GSTT1 null between mild to moderately autistic and severely autistic (p=0.983, OR=0.980; 95% CI 0.158-6.095 and p=0.439, OR=1.633; 95% CI 0.471-5.656).*

Conclusion: *GSTM1 null and GSTT1 null are not risk factor of ASD. 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.*

Keywords: *GSTM1 null, GSTT1 null, risk factor, autism spectrum disorder*

ABSTRAK

Apakah GSTM1 Null dan GSTT1 Null merupakan faktor risiko autism spectrum disorder? *Studi pendahuluan*

Latar belakang: *Pada autism ditemukan bahwa glutathion total plasma (tGSH) rendah, oxidized glutathione (GSSG) meningkat dan rasio tGSH terhadap GSSG rendah. Glutathione s-transferase (GST) merupakan enzim antioksidan yang berperan penting dalam proses detoksifikasi seluler dan ekskresi polutan lingkungan termasuk diantaranya logam berat. Polimorfisme gen GST mu (GSTM1) dan GST theta (GSTT1) cukup tinggi. Delesi homozigot gen GSTM1 dan GSTT1 yang menyebabkan berkurang sampai tidak adanya aktivitas enzim GST serta menurunnya level antioksidan berkontribusi terhadap risiko ASD. Oleh karena itu dilakukan penelitian pendahuluan untuk mengetahui hubungan antara gen GSTM1 null dan GSTT1 null sebagai faktor risiko ASD dengan ekspresi fenotip.*

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Metode: *Lima puluh satu pasien ASD dari SLB dan sekolah autis serta 45 kontrol dari Semarang dan Solo diambil darah vena, kemudian diekstraksi dengan metode* salting-out *di CEBIOR Semarang. Pemeriksaan genotip gen GSTM1 & GSTT1 dilakukan di UMBI Malaysia. Metode yang digunakan adalah PCR multipleks, setelah itu produk PCR dipisahkan pada 1,2% gel agarosa kemudian dicat dengan ethidium bromida dan hasilnya dilihat menggunakan transiluminator UV. Besar produk untuk GSTM1 & GSTT1 adalah 625 bp & 459 bp. Tidak adanya band untuk gen GSTM1 & GSTT1 diinterpretasikan sebagai GSTM1 null & GSTT1 null.*

Hasil: *Frekuensi gen GSTM1 null dan GSTT1 null pada ASD lebih tinggi dibandingkan kontrol tetapi tidak ditemukan perbedaan yang signifikan (p=0,357, OR=0,504; 95% CI 0,117-2,168 and p=0,364, OR=0,674; 95% CI 0,287-1,580). Distribusi gen GSTM1 null dan GSTT1 null pada autis ringan sedang dan autis berat juga tidak ditemukan perbedaan yang signifikan secara statistik (p=0,983, OR=0,980; 95% CI 0,158-6,095 and p=0,439, OR=1,633; 95% CI 0,471-5,656).*

Simpulan: *GSTM1 null dan GSTT1 null bukan merupakan faktor risiko ASD. Penelitian lebih lanjut sangat diperlukan dengan jumlah sampel yang lebih besar, analisis gen GST multipel dan pemeriksaan aktivitas GST untuk mendapatkan gen faktor risiko ASD dan faktor-faktor yang mempengaruhi ekspresi fenotip ASD.*

INTRODUCTION

Autism spectrum disorder (ASD) is neurodevelopmental disorder characterized by qualitative impairments in the development of social and communication skills, often accompanied by stereotyped and restricted patterns of interests and behavior, with onset of impairment before 3 years of age.[1] Epidemiological studies demonstrate that the prevalence of autism has increased in recent years[2] and occurs four times as frequently in males as females.[3] It is currently estimated that autism affects as many as 1 out of 150 children in the United States[4] and 14.8 per 10,000 in Asia Prevalence of ASD in Asia was 14.8 per 10,000 from 1980 to present.[5]

Investigators suggested that ASD may result from an interaction between genetic and environmental factors, with oxidative stress as a mechanism linking these risk factors.[6] There is evidence supporting the role of oxidative stress in autism[7] that affect brain development during gestation or possibly after gestation, contributing to expression of autism.[8] Some reviews support the hypothesis that oxidative stress contributed to the pathology of autism.[9,10]

Glutathione S-transferases are antioxidant enzymes that play important role in cellular detoxification and the excretion of environmental pollutants including many carcinogens and also in protection against oxidative stress[11,12], by its ability to conjugate *glutathione* (GSH) with compounds containing an electrophilic center.[11,13,14] 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* (θ), *kappa* (κ), and *zeta* (ξ).[13]

Glutathione S-transferase mu (GSTM1) and *glutathione S-transferase theta* (GSTT1) are known to be highly polymorphic. This genetic variation may change an individual's susceptibility to carcinogens and toxins.[11] Homozygous deletions of these genes, referred to as GSTM1 null and GSTT1 null, respectively, result in lack of enzyme activity.[15] Subjects with at least one functional allele for GSTM1 are designated as GSTM1 positive.[13]

Association of GSTM1 null genotype with autism has been reported.[16] Absence of the GSTM1 gene, resulting in impaired enzyme activity, could lead to failure of individuals with autism to detoxify xenobiotics. Mercury (Hg) coupled with decreased levels of antioxidants (low glutathione and antioxidant enzymes), leading to the increased production of free radicals. This can cause an increase in lipid peroxidation, protein oxidation, and DNA oxidation that lead to increased oxidative stress. All of that resulting in 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.[9]

Therefore the preliminary study was conducted to investigate the role of GSTM1 and GSTT1 polymorphism as risk factor of ASD associated with phenotype expression.

MATERIALS AND METHODS

Subjects

This is case control study with total 96 subjects comprised of 51 autism spectrum disorder subjects (48 males and 3 females; age range 4-18 years; mean 9.82 years) from special need and autism school and 45 unrelated healthy subjects 23 males and 22 females; age range 4-17 years; mean 12.25 years) from Public School in Semarang and Solo, Indonesia. Diagnosis of ASD was according to DSM IV (diagnosis and statistical manual of mental disorders, fourth edition) and phenotype expression of ASD was determined using childhood autism rating scale (CARS). This study was conducted with informed consent of all subjects' parent and approved by institutional review committee from the Medical Faculty Diponegoro University and Ethical Clearance of Dr. Kariadi Hospital Semarang, Indonesia. Research was done in Central Biomedical Research (CEBIOR) Medical Faculty Diponegoro University,

Indonesia and UKM Medical Molecular Biology Institute (UMBI), Malaysia.

Genotyping

Blood vein samples were collected from both groups of patients and controls in EDTA tubes. Genomic DNA was extracted from peripheral blood leucocytes by salting-out method. The purity and concentration of DNA were quantified using nanodrop spectro-photometer (ND 1000 spectrophotometer). Presence or absence of GSTM1 and GSTT1 genes in genomic DNA samples were detected by multiplex PCR technique. β-globin gene was coamplified as positive internal control.

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 dNTP and 0.75 mM $MgCl_2$. Concentration for each primer is 0.2 µM. Primers for GSTM1 gene (forward primer 5'-TCTGGGGAGGTTTGTTTTCA-3' and reverse primer 5' TCT CCAAAATGTCCACACGA-3'), GSTT1 gene (forward primer 5'-TTCCTTACTG GTCCTCACATCTC-3' and reverse primer 5'-TCACCGGATCATGGCCAGCA-3') and β-globin gene (forward primer 5'-GAGTCAAGGCTGAGAGATGCAGGA-3' and reverse primer 5'-CAATGTATCATGCCTCTTTGCACC-3').

The PCR for GSTM1 gene was performed with an initial denaturation 95^{O}C for 4 minutes, followed by 35 cycles with denaturation at 95^{O}C for 1 minute, annealing at 50^{O}C and extension at 72^{O}C for 2 minutes. Final extension was performed at 72^{O}C for 10 minutes and hold at 4^{O}C. PCR for GSTT1 gene, same with protocol above except the annealing temperature was changed at 61^{O}C.

PCR products were separated on 1.2% agarose gel, stained with ethidium bromide and visualized on an UV transiluminator. GSTM1 gene product is about 625 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.

Statistical analysis

Statistical analysis was carried out using SPSS software version 16.0 for Windows. Logistic regression test was used to calculate the differences in genotype prevalence and association between case and control groups. T-test and Kruskal-Wallis test were used to analyze association between CARS score and genotype. The odds ratio (OR) and its 95% confidence interval (CI) were used to illustrate the association. All tests were two sided and p value <0.05 was considered statistically significant.

RESULTS

The deletion polymorphisms for GSTM1 and GSTT1 were genotyped in 51 autism spectrum disorder cases and 45 controls. The frequency of ASD is 48 male and 3 female, while in control group 23 male and 22 female. Figure 1 and 2 shows the distribution of sex and age in ASD and control group. Frequency of male patients of ASD is higher than female, but in control group the number of male and female almost the same. The highest distribution of ASD patiens are in 4-10 years old age group. Meanwhile, in control group is 12-15 years old age group.

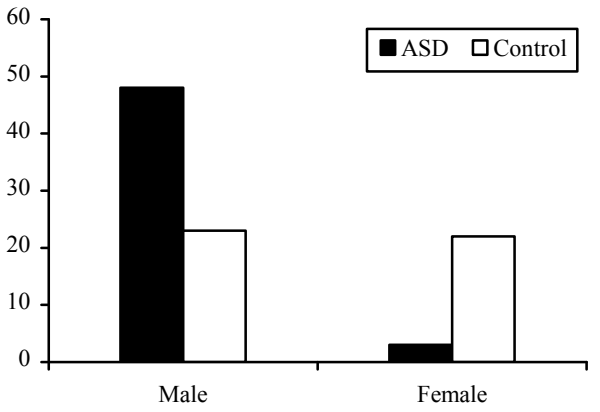

Figure 1. Distribution of sex in ASD and control

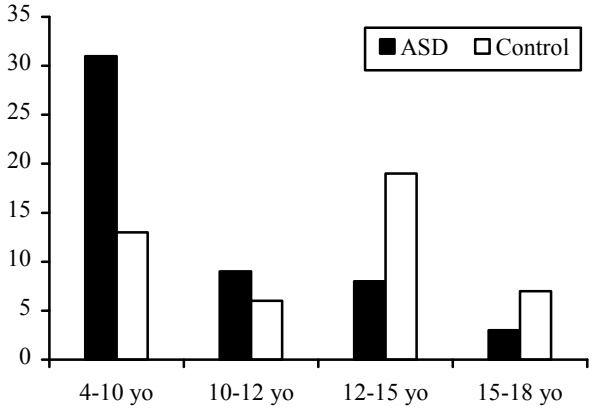

Figure 2. Distribution of age in ASD and control

Figure 3 shows the distribution of GSTM1 and GSTT1 gene in ASD and control. The frequency of both GSTM1 null and GSTT1 null gene in ASD is higher than control, meanwhile frequency of GSTM1 positive

and GSTT1 positive almost the same between ASD and control group. All female patients of ASD categorized in mild to moderate autistic using CARS. Therefore most of male ASD patients categorized in severe autistic (Figure 4).

Figure 5 and 6 shows PCR result for GSTM1 and GSTT1 gene. The presence of β globin gene (850 bp), GSTM1 gene band (637 bp) and GSTT1 gene band (459 bp) is interpreted as GSTM1 and GSTT1 positive. While absence of GSTM1 and GSTT1 band is interpreted as GSTM1 null and GSTT1 null.

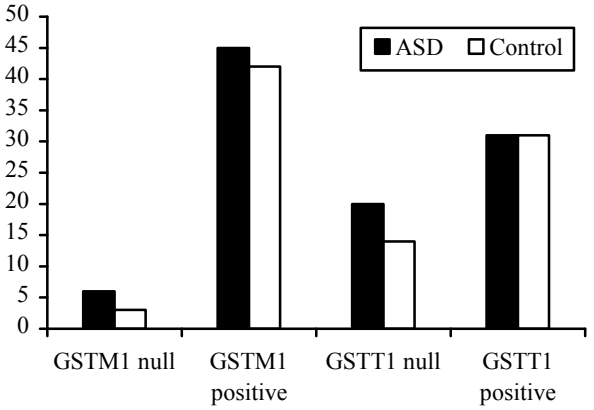

Figure 3. Distribution of GSTM1 and GSTT1 gene in ASD and control

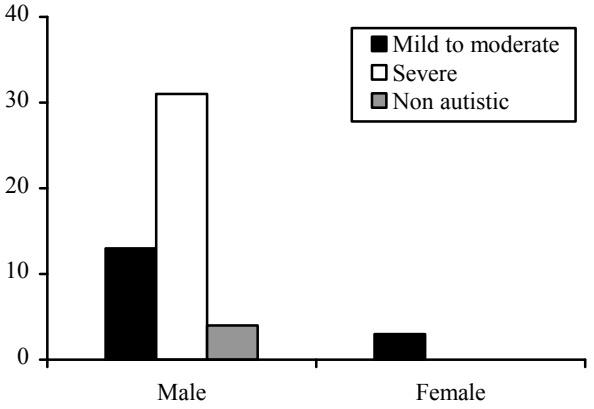

Figure 4. Distribution of sex according to phenotype expression

The frequency of GSTM1 null in ASD higher compared with control group but the difference is not statistically significant (p=0.357, OR=0.504; 95% CI 0.117-2.168). GSTT1 null genotype's frequency compared with control group is higher but also no significance different (p=0.364, OR=0.674; 95% CI 0.287-1.580). Additionally, combination of GSTM1 and GSTT1 was not reach statistically significance among cases and controls (p=0.299) [see Table 1].

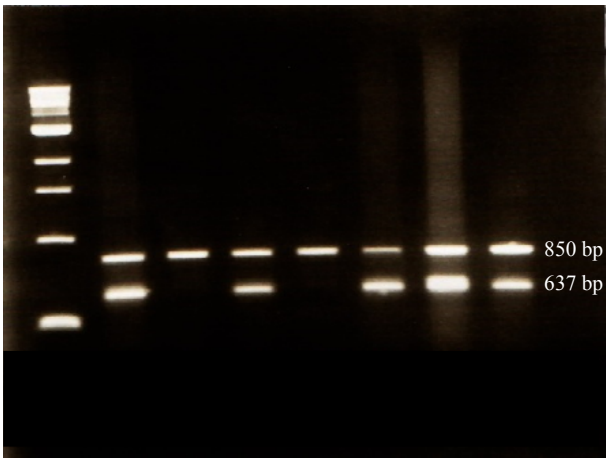

Figure 5. PCR result for GSTM1 gene

Lane 1	: marker (ladder 1 kb)
Lane 2,4,6,7	: GSTM1 positive (+/+ or +/-)
Lane 3 & 5	: GSTM1 null (-/-)
Lane 8	: positive control
Lane 9	: blank
β globin	: 850 bp; GSTM1 gene: 637 bp

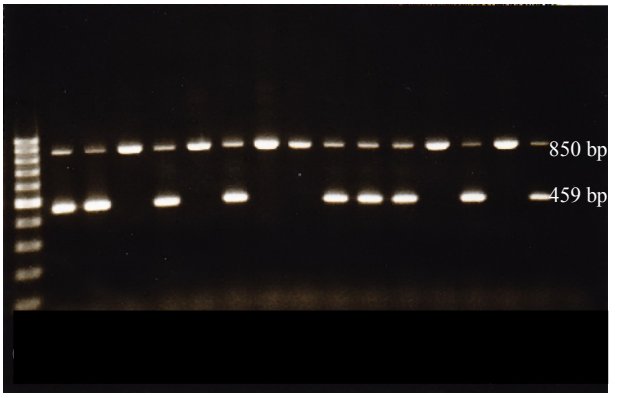

Figure 6. PCR result for GSTT1 gene

Lane 1	: marker (ladder 100 bp)
Lane 2,3,5,7,10,11,12,14	: GSTT1 positive (+/+ or +/-)
Lane 4,6,8,9,13,15	: GSTT1 null (-/-)
Lane 16	: positive control; lane 17: blank
β globin	: 850 bp; GSTT1 gene: 459 bp

Phenotype expression of ASD is represented by childhood autism rating scale (CARS), divided into two group as mild to moderately autistic and severely autistic. There is no statistically different in the distribution of GSTM1 null and GSTT1 null between mild to moderately autistic and severely autistic (p=0.983, OR=0.980; 95% CI 0.158-6.095 and p=0.439, OR=1.633; 95% CI 0.471-5.656), that also found in combination of GSTM1 and GSTT1 gene in which the p value is 0.667. Statistical analysis for GSTM1 null, GSTT1 null and combination of both gene compared with CARS score also showed no significance different (see Table 1).

Table 1. Odd ratios (OR) and p value of GSTM1, GSTT1 and combination both genes in association with group and phenotype expression

	GSTM1 null			GSTT1 null			GSTM1/GSTT1
	OR	95% CI	p	OR	95% CI	p	p
Group							
a. ASD	0.504	0.117 - 2.168	0.357[a]	0.674	0.287 - 1.580	0.364[a]	0.299[c]
b. Control							
CARS							
a. Mild to moderately autistic	0.980	0.158 - 6.095	0.983[a]	1.633	0.471 - 5.656	0.439[a]	0.667[c]
b. Severely autistic							
CARS score							
	GSTM1null	37.217 - 43.449	0.317[b]	GSTT1null	34.779 - 39.554	0.157[b]	0.476[d]
	Wild type	36.720 - 39.670		Wild type	37.604 - 40.815		

a. Logistic regression
b. T-test
c. Kendall tau
d. Kruskal-Wallis

DISCUSSION

This is preliminary study that was carried out to investigate the relationship between genetic polymorphisms of GSTT1, GSTM1 and phenotype expression of autism spectrum disorder (ASD).

Autism spectrum disorder is a complex and multifactorial disease. It is difficult to identify single elements that increase the risk. Autism may result from a combination of genetic susceptibility (reduced ability to remove metals including mercury or other neurotoxins from the system) and environmental exposure. [17-19] Several genes have been reported associated with autism, including genes involved in methionine transmethylation and transsulfuration pathways. Polymorphism of reduced folate carrier I (RFC1) gene, methylenetetrahydrofolate reductase (MTHFR), transcobalamin II (TCN2), catechol-O-methyl-transferase (COMT) and glutathione S-transferase mu 1 (GSTM1)[16] was significantly increased among autistic children compared to control.[20]

In the present study, we found that the frequency of the GSTM1 null genotype in control groups was 6.7%, quite different from previous study in Jakarta, Indonesian population (55.6%)[21] much lower than in Caucasian population (42%-60%) and other Asians population, in Japan 47.9%, Korea 52.2% and Singapore 56.2%.[22] The frequency of the GSTT1 null genotype was 31.1% in our control population, similar with Japan population (35.3%), lower than previous study in Jakarta, Indonesian population (41.4%)[21], in Koreans (51.5 %) and Singapore population (51.9%)[22], but higher than in Europeans (22%) and in African-Americans.[23]

Steven Buyske, *et al.* (2006) concluded an association of the homozygous GSTM1 deletion genotype with autism (p=0.028). Our study revealed that GSTM1 null and GSTT1 null are not risk factor of ASD. There is no significant association between GSTM1 and GSTT1 null gene with autism spectrum disorders (ASD), although the distribution of these genes among ASD higher than controls. The combination of GSTM1 null and GSTT1 null also do not contribute to ASD risk in this population.

The difference of result compared with others studies could be the fact that this study conducted in different race and population. Small total sample size is also the limitation of the present study, although it has fulfilled the sample size number requirement for statistical analysis. Another explanation for the differents in results is GST enzymes have a broad substrate overlap, therefore decreased in expression level of one GST may be compensated by increased expression of another. In spite of that ASD is multifactorial disease, in which there is complex environmental factors involved and multiple genes participated, results in heterogeneity. Therefore, this preliminary study needs further investigations with increased sample size, multiple genes and GST activity determination, to find out gene susceptibility of ASD and factors that contribute to the phenotype expression of ASD.

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