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Frequency of MTHFR GENE C677T polymorphism for non-syndromic autism spectrum disorder patients

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ABSTRACT

Background: The folate metabolism is a pathway that may involve in the non-syndromic Autism Spectrum Disorder (ASD). Methylenetetrahydrofolate reductase enzyme has a key role in folate metabolism. The C677T polymorphism of MTHFR gene could reduce the effectiveness of the enzyme.

Objectives: To evaluate the frequency of MTHFR geneC677T polymorphism for non-syndromic ASD patients.

Method: Thirty-four DNA samples were taken from each group. PCR mixture was consisted of 1µL DNA, 2.5µL PCR buffer, 0.5µL dNTP, 1.5µL MgCL2, 0.125µLTaqenzyme, 0.5µLofforwardandreverseprimerandaquabidesttoreach a volume of 25 µL. The PCR profiles were initiation 95ºC for 5 min, denaturation 94ºC for 1min, annealing 55ºCfor 45 seconds, and elongation 72ºC for30 seconds. The cycles were done in 35 times an dfinal elongation was at 72ºC for 5min. The PCR product was 198bp, and then digested by the Hinfl enzyme for 16hours at 37°C, and visualized using 2% agarose gel and then electrophoresed for 30 minutes at 100 volts.

Result: Non-syndromic ASD samples showed none had homozygote mutant type (677TT), 3 (8.8%) samples had heterozygote (677CT)and 31 (91.2%) samples had wild type (677CC). Meanwhile, normal control showed only 1 (2.9%)sample had homozygote mutant type(677TT), 9 (26.5%) samples had heterozygote (677CT)and 24 (70.6%) samples had wild type (677CC).

Conclusion: The frequency ofMTHFRgeneC677T polymorphism in patients with non-syndromic ASD and controls are not significantly different.

Keywords: ASD, MTHFR C677T, polymorphism

INTRODUCTION

Autism Spectrum Disorder (ASD) is group of neuro developmental disorders ranging from pervasive developmental disorders apart from attention deficit hyperactivity disorders and attention deficit disorders to autistic disorders.[1] Clinical, neuroimaging, neuropathology, and neurochemical examination show that ASD is a neuro-cortical system disorders that causedby disruption of information processing in the nervous system.

This may occur from the synaptic and dendritic process for connectivity to the brain structure, and seems to be influenced by genetic and environmental factors. [1]

Some risk factors may influence the gene-gene and gene-environment interactions.[2] These interactions can either be a toxic environmental factor or epigenetic factor, which may alter the gene functions, and in turn alters the neural network.

Specific examples of epigenetic factor may be derived from physical aspect (i.e. active biochemical substance) or psychological aspect (i.e. stress) that may influence the chemical composition of the brain, genes that became active/passive at the time of development, or changes in gene expression.[2]

Methylenetetrahydrofolate reductase (MTHFR) enzyme plays an important role in the chemical reaction for the synthesis of folic acid. This enzyme acts as a catalyst in the transformation of 5,10-methylenetetrahydrofolate to 5-methyltetrahydrofolate.(Ref) 5-methyltetrahydrofolate is a form of folate found in the main circulation and used in the remethylation of homocyste into methionine.[3] The folate-methionine reaction pathway plays a role in DNA synthesis, DNA methylation, and cellular oxidation-reduction balance.[4-6] Methionine is an amino acid that plays an important role in protein synthesis, because it serves as a start codon for protein synthesis. Impaired folate-methionine reaction pathway have been identified in individuals with ASD.[7] The reduction of cell proliferation ability to regenerate in the critical region of the brain was caused by the lost of nucleotide synthesis.[8,9] Currently, the screening for *MTHFR* gene is routinely performed among ASD because it has been well established that folate and methylation may influence the development of neural networks.[10]

MTHFR gene is located in the short arm of chromosome 1 at 1p36.3, and has 9 exons. Some common polymorphisms in *MTHFR* gene are A1298C (rs1801131) on exon 7 and C677T (rs1801133) on exon 4, both of which are known to reduce the activity of MTHFRenzyme.[11] C677T polymorphism resulted in the decrease of enzyme activity by 45% compared with the *wild type*. Heterozygocity for allele C677T (677CT) and allele A1298C (1298AC) showed biochemical characteristics more closely resembling the homozygous 677TT.[12]

SUBJECT AND METHOD

This was a cross sectional study that was carried out for three months from September 2012 to November 2012 at Center for Biomedical Research (CEBIOR) Faculty of Medicine Diponegoro University Semarang and at Health Research Unit (HRU) laboratory Faculty of Medicine Padjadjaran University Bandung. Samples was obtained DNA from previous ASD studyat CEBIOR laboratory Faculty of Medicine Diponegoro University Semarang and controls was obtained from DNA from Health Research Unit (HRU) laboratory Faculty of Medicine Padjadjaran University. Sex and age of the control group were matched to the non-syndromic ASDpatients. The sex ratio for male and female patients with non-syndromic ASD was1:6. Patient's age was ranging from6 to 17 years old.

Laboratory procedures for the *MTHFR* gene C677T polymorphism analysis were PCR (Polymerase Chain Reaction) - RFLP (Restriction Fragment Length Polymorphism).The materials used for the study were DNA extracts. Reagents used for PCR mixed were consisted of aquabidest, PCR buffer (P-2192; Sigma-Aldrich, St Louis, MO, USA), dNTPs (Promega, Madison, WI, USA), $MgCL_2$, TAG enzyme (D-6677; Sigma-Aldrich) and forward primer (5'-AGG-ACG-GTG-CGG-TGA-GAG-TG-3') and reverse primer (5'-TGA-AGG-AGA-AGG-TGT-CTG-CGG-GA-3'). *Hinf1*enzyme (Ferment as UAB, Vilnius, Lithuania) was used for RFLP and red buffer (R+) (Ferment as UAB). PCR products were then visualized using agarose gel 2%, TBE Buffer 0.5x, Ethidium Bromide 0.5%.[13] Making the PCR mixture for one reaction or one DNA sample with total volume of 25µl consisting of 1µL DNA, 2.5µL PCR buffer, 0.5µL dNTP, 1.5µL $MgCL_2$, 0.125µL Taq enzyme and 0.5µL forward and reverse primer with aquabidest to a total volume of 25µL. PCR amplification (Applied Biosystem) was performed using the following process: initiation at 95ºC for 5 minutes, denaturation at 94ºC for 1 minutes, annealing at 55ºC for 45 seconds and elongation at 72ºC for 30 seconds. The cycle was repeated 35 times. Final elongation at 72ºC for 5 minutes. The final PCR product is 198 base pair(bp). PCR product were then digested using restriction enzyme as follows: 30µl PCR product, 13.5µl aqua bidest, 1.5µl *Hinfl* enzyme and 5µl red buffer (R+). Incubated for 16 hours at 37°C. Next 25µl enzyme-digested PCR products will be planted in 2% agarose gel which were then electrophoresed at 100 volt for 30 minutes. *Hinf1* enzyme will cut the GAn↓TC sequences, so that the sample will have 2 fragments namely 175 and 23 bp identified as 677TT that is the homozygous polymorphism on both alleles. A 198 bp sample identified as 677CC was a *wild type* homozygous on both alleles, because *Hinf1* enzyme did not cut the PCR products. Samples consisting of 198, 175 and 23 bp fragments were identified as 677CT, or C/T heterozygous polymorphism.

Hypothesis was tested using Chi-square statistical test on SPSS 13.0, because this study was comparing two unpaired categorical variables using 2x2 table to compare the 677TT/CT and *wild type* 677CC polymorphism frequency characteristics, with 95% confidence interval and statistical significance level of $p<0.05$. The study had been approved by Ethical Committee Faculty of Medicine Diponegoro University/Dr. Kariadi HospitalSemarang and Human Research Ethic Committee Faculty of Medicine Padjadjaran University Bandung (appendix). Patient's data was obtained from the medical record from the previous study and was kept confidentially.

RESULTS

This research included 68 samples consisting of 34 non-syndromic ASD patients and 34 normal controls. The characteristics of the non-syndromic ASD patients were: 5 female (14.7%) and 29 male (85.3%). Samples from the normal control populations were consisted of 7 female samples (20.6%) and 27 male samples (79.4%) (Table 1).

Age distribution for the non-syndromic ASDpatient's samples ranged from 6 to 17 years with an average of 11.88 years, and from 7 to 17 years with an average of 12.09 years for the normal population samples. Sex ratio between male and female for the non-syndromic ASD patient group was1:6. Samples for the normal control group were selected based on the age and sex criteria of the non-syndromic ASD patients group.

Results for 34 samples of non-syndromic ASD patients showed no 677TT polymorphism, 3 677CT polymorphisms (8.8%) and 31 *wild type* 677CC (91.2%). Whereas the results for 34 normal population samples showed 1 677TT polymorphism (2.9%), 9 677CT

polymorphisms (26.5%) and 24 *wild type* 677CC (70.6%), as presented on Table 2.

Analysis for 677TT, 677CT and 677CC polymorphism showed p= 0.087 because p>0.05 it was concluded that there was no significant difference on the frequency of *MTHFR* gene C677T polymorphism between non-syndromic ASD patients and normal control group (Table 2).

The combined value of 677TT and 677CT polymorphisms also showed no significantly difference in the frequency of *MTHFR* gene C677T polymorphism between non-syndromic ASD patients and normal control (p= 0.062) (see Table 3).

Samples of non-syndromic ASD. M is the marker; Bl is the blank (internal control).Sample no 28 showed 175 bp and 198 bp bands. Most of the results in the non-syndromic ASD patients group were the *wild type* 677CC, as pictured on samples number 21, 22, 23, 24, 25, 26, 27, 29 and 30. It was a 198 bp band (Figure 1).

PCR results on 677TT polymorphism can be seen on sample number 12, with a 175 bp band. The 677CT polymorphism showed 175 bp and 198 bp bands, as pictured on sample number 7, 9 and 10. Whereas for 677CC or wild type, there are one 198 bp band, as pictured on sample number 8 and 11. Control on the K column was the PCR product with known size of 200 bp (internal control). The marker was a 100 bp band (Figure 2).

DISCUSSION

Distribution of 677TT polymorphism on the control group showed 1 sample (2.9%). The number of 677TT polymorphism on normal population differ based on ethnic and racial groups. Control group showed more 677TT and 677CT polymorphism compared to the non-syndromic ASD patients group, as pictured on Table 2. The different results obtained between the analysis of polymorphism and alleles may be caused by minimum sample size resulting in bias between the analysis of polymorphism and allele.

The minimum sample size in this study was calculated based on the number of homozygous 677TT on the normal control population, which was 11%, so the minimum sample size was 34 samples. Age and sex criteria of the normal control group were matched to the criteria for the non-syndromic ASD patients sample in Central Java. This study found more 677CT genotype than 677TT genotype, either on the non-syndromic ASD patients group or normal control group. There was also more C allele than T allele, either on the non-syndromic ASD or control group.

Sample size for the *MTHFR* gene C677T polymorphism study may affect the study results, where studies with large sample size (more than 168 samples) and minimum sample size (34 or 40 samples) showed different results. Sample size will also affect the frequency of 677TT polymorphism, where larger sample size is associated with higher frequency of 677TT polymorphism.

This can be observed from the study by Boris et al. (2004) who analyzed the *MTHFR* gene C677T and A1298C polymorphism on 168 Caucasian children with autism and found a significant C677T polymorphism on *MTHFR* gene, 23% 677TT allele on children with autism and 11% on control (P<0.0001). The study from Liu et al. also found a relationship between the reduction of MTHFR enzyme activity and the risk of autism. They found 42.9% 677TT allele on children with autism, compared with 32.3% 677TT allele on control group (P= 0.0004).This study involved 205 families in North America who has one or more children with autism. Both studies were done with large sample size, and the study by Boris et al. has analyzed two *MTHFR* gene polymorphisms, C677T and A1298C. The study by Gokcen C et al. showed no significant association between the reduction of MTHFR enzyme activity and *MTHFR* gene C677T polymorphism, but A1298C polymorphism showed significant association with the reduction of enzyme activity.[14]

In 1998, Abbate et al. studied 84 patients with heart disease and 106 normal samples in Italy, and found no association between *MTHFR* gene C677T polymorphism and heart diseases.[15] In 2001, another study by Brunelli et al. found that *MTHFR* gene C677T polymorphism showed no association with the Alzheimer.[16] Kostulas et al. studied 126 patients with ischemic cerebrovascular disease (ICVD) and 70 patients with internal carotid artery (ICA) stenosis, and found no significant association between *MTHFR* gene C677T polymorphism and ICVD or ICA stenosis, and also no causal relationship with the increase of homocystein levels in ICA stenosis.[17]The studies by Abbate et al., Brunelli et al. or Kostulas et al. only analyzed the *MTHFR* gene C677T polymorphism without another form of polymorphism, so there was a possibility of another polymorphism such as A1298C. These studies were also completed by the end of the 90s decade, while today we already found 34 polymorphisms with 9 frequently found polymorphisms for *MTHFR* gene. Gokcen et al. (2011) found different results where ADHD was not associated with *MTHFR* gene C677T polymorphism but was associated with *MTHFR*g ene A1298C polymorphism.

ASD can be caused by multiple factors, such as environment and genetic factors. Rio Garcia et al. found that *MTHFR* gene C677T and A1298C polymorphism status on a mother, and intake of folic acid and vitamin B12 in the early period of pregnancy is essential for the neurobiological development of a baby.[18]Considering that human brain is growing rapidly in the early period of pregnancy and the cortical and sub cortical structures was formed at weeks 5-25 of gestation, it is necessary to evaluate the folic acid level in the mother.

In this case we can see that the etiology of ASD, such as the environmental and genetic factors also include the environmental and genetic factors from the mother. Limitations of this study are the small sample size and the analysis was only performed on the non-syndromic ASD patients and not on their mothers. Non-syndromic ASD patient samples were obtained from CEBIOR laboratory FK UNDIP Semarang, whereas the normal control samples were obtained from UPK laboratory FK UNPAD Bandung, so there is a high possibility of bias. Non-syndromic ASD patient sample group did not come from the same race or ethnicity, so is the case for the normal control group. Although there are several studies about the relationship between *MTHFR* gene polymorphism variant and ASD, future studies should be done to find another genetic or environmental factor that may also contribute to

this disorders, considering the gene and gene or gene and environment interactions on the non-syndromic ASD.

Male normal control samples with homozygous 677TT may require counseling about the clinical effect of this homozygous 677TT. Individual with homozygous 677TT has higher risk for elevated plasma homocysteine levels, which may cause heart and blood vessels disorders. This was caused by an endothelial dysfunction. There are other risk factors that may accelerate and exacerbate this condition and need to be avoided such as smoking, high fat diets and overweight. They may also need to be informed about the possibility of cerebrovascular disorders, psychiatric disorders and colorectal cancer. It is highly recommended to increase folate intake from food and folate supplements, with minimum dose of 400µg/day, taken continuously and regularly.

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APPENDIX

Table 1. Sample characteristics

Characteristics	Non-syndromic ASD n(%)	Control n (%)
Sex		
Female	5 (14.7%)	7 (20.6%)
Male	29 (85.3%)	27 (79.4%)
Age		
Mean (Std Dev)	11,88 (±2.55)	12.09 (±2.40)

Table 2. Distribution of *MTHFR* gene C677T polymorphism

***MTHFR*gene polymorphism**	**Non-syndromic ASD**		**Control**		***p***
	n	**%**	**n**	**%**	
677 TT	0	0	1	2.9%	
677 CT	3	8.8%	9	26.5%	***p***= 0.087
677 CC	31	91.2%	24	70.6%	
Total	34	100%	34	100%	

Table 3. *MTHFR* gene C677T polymorphism analysis

***MTHFR*gene polymorphism**	**Non-syndromic ASD**		**Control**		***p***
	n	**%**	**n**	**%**	
677 TT/CT	3	8.8%	10	29.4%	
677 CC	31	91.2%	24	70.6%	***p***= 0.062
Total	34	100%	34	100%	

Table 4. *MTHFR* gene Allele C and T 677 analysis

Allele	**Non-syndromic ASD**	**Normal control**	
	%	**%**	***p***
Allele C	95.6%	83.8%	
Allele T	4.4%	16.2%	*p*=0.045
Total	100%	100%	

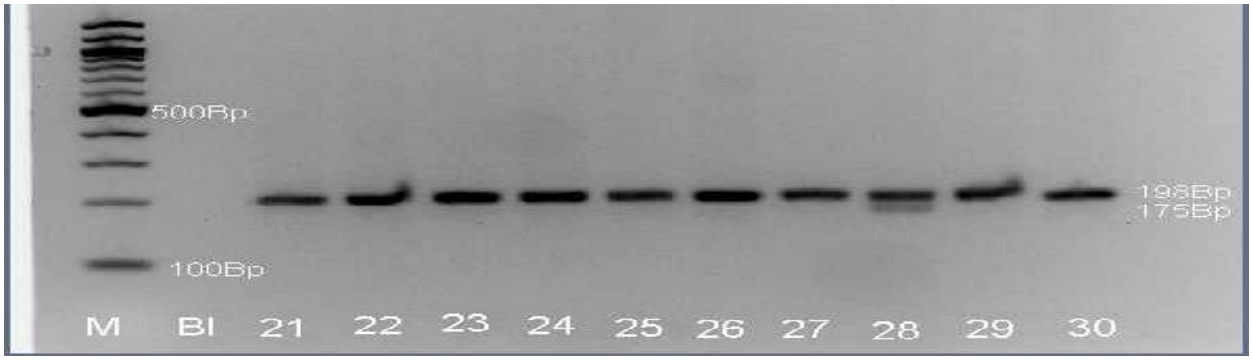
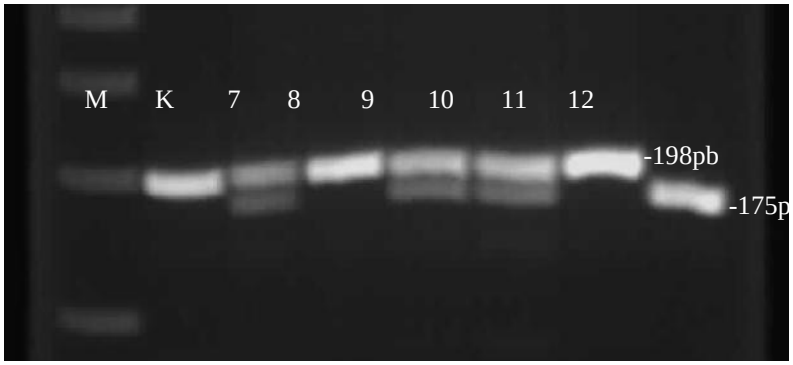


Figure 1.PCR-RFLP results for *MTHFR*C677T gene polymorphism.
Sample no 28 showed 175bp and 198bp bands. Most of the results in the non-syndromic ASDpatients group were the *wild type* 677CC, as pictured on samples number 21, 22, 23, 24, 25, 26, 27, 29 and 30. It was a 198bp band

300pb marker-



100pb marker-

Figure 2.PCR-RFLP results of normal samples.
PCR results on 677TT polymorphism can be seen on sample number 12, with a 175 bp band. The 677CT polymorphism showed 175 bp and 198 bp bands, as pictured on sample number 7, 9 and 10. Whereas for 677CC or wild type, there are one 198 bp band, as pictured on sample number 8 and 11. Control on the K column was the PCR product with known size of 200 bp (internal control). The marker was a 100 bp band