***MTHFR* rs1801133 polymorphism in Bangladeshi population – its prevalence and detection**

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Abstract. Methylenetetrahydrofolate reductase (*MTHFR*) has been reported as a key enzyme not only for intracellular folate homeostasis but also for metabolism. A particular variant (G677A) leads to an altered amino acid, which ultimately causes decreased enzyme activity and may modulate the risk of causing several chronic diseases. The purpose of this study was to detect the pervasiveness of this variant *MTHFR* rs1801133 G677A in the Bangladeshi population. We applied allele-specific polymerase chain reaction (AS-PCR) to determine the genotypes at the rs1801133 in the Bangladeshi population. We performed targeted sequencing of the AS-PCR product of randomly selected samples. Out of the 181 Bangladeshi individuals, 71.8% had homozygous 677GG genotype, while 28.2% comprised of heterozygous 677GA genotype. No individual with the homozygous 677AA genotype was found in this representative Bangladeshi population. The 677G alleles had higher frequency (0.856) compared to 677A alleles (0.144) at the rs1801133 locus. Though the more risky homozygous 677AA genotype at the rs1801133 locus is absent in the Bangladeshi population, further association studies can be performed to identify the role of *MTHFR* mutation in the susceptibility to different multifactorial diseases.

Keywords: Allele specific PCR, *MTHFR*, polymorphism, rs1801133

INTRODUCTION

Hyperhomocysteinemia, a type of genetic disorder, is manifested by elevated concentrations of homocysteine in plasma and act as a mediator in the development of several medical conditions such as cardiovascular and neurodegenerative diseases, renal dysfunction, diabetes, pregnancy-related complications, congenital defects, male infertility, osteoporosis, neuropsychiatric disorders and cancer (Yafei *et al.*, 2012). Homocysteine is formed when the amino acid methionine is naturally broken down (i.e., metabolized) to be excreted in the urine (Varga *et al.*, 2005). The produced homocysteine can be recycled within our body with the help of vitamins

B12, B6, folate and an enzyme methylenetetrahydrofolate reductase (*MTHFR*). Any abnormality of this element hampers the recycling process and therefore causes accumulation of homocysteine in the blood (Moll & Varga, 2015). Both of environmental and genetic factors influence the plasma levels of homocysteine. However, Dajani *et al.* (2013) has reported the *MTHFR* rs1801133 G677A mutation as a potential risk factor.

MTHFR is an enzyme-linked to folate pathway that transforms 5, 10-methylenetetrahydrofolate (5, 10-MTHF) to 5-methyltetrahydrofolate (5-MTHF). As a result,

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homocysteine is re-methylated to methionine. Conversely, methionine can be reversibly hydrolyzed into adenosine and homocysteine and catalyzes the synthesis of purine, DNA and RNA (Lima *et al.*, 2014). Since MTHFR affects several biochemical pathways, including distribution of folate, multiple enzymatic homocysteine metabolism and methylation reactions, a mutation in *MTHFR* can modify the risk of disease both in a positive and negative fashion (Esfahani *et al.*, 2003). Although a good number of polymorphisms have been identified in the *MTHFR* gene, the most common rs1801133 G677A missense mutation has been confirmed to decrease 65% of the enzymatic activity (Rozen, 1997). *MTHFR* rs1801133 G677A is a nonsynonymous polymorphism at the rs1801133 locus, located on chromosome 1p36.3, that substitutes valine for alanine at codon 222 of the MTHFR enzyme that leads to a mild-to-moderate hyperhomocysteinemia (Brezovska-Kavrakova *et al.*, 2013).

In current development, pharmacogenetics has also emerged as a novel tool for the individualization of treatment. Thus, various genetic polymorphisms and haplotypes in the *MTHFR* gene have been analyzed and applied as markers for optimizing treatment outcome that involved in drug transport, metabolism as well as the mechanism of action (De Mattia & Toffoli, 2009). Several published case-control investigations have demonstrated the probable influence of *MTHFR* rs1801133 G677A (*MTHFR* G677A) genetic variants on the efficacy and toxicity of several fluoropyrimidines and antifolate agents (Toffoli & De Mattia, 2008) such as methotrexate, raltitrexed and 5-fluorouracil that are used in the treatment of rheumatoid arthritis, bowel cancer and colon cancer respectively. In Bangladesh, almost 6.5% of males and 2.7% of females suffer from colorectal cancer (Hussain, & Sullivan, 2013) and the rate of rheumatoid arthritis prevalence was 0.7% per year in rural populations compared to 0.4% in urban communities where females were affected six times more than males (Rudan *et al.*, 2015).

The variations in the frequency of the polymorphisms were observed in different geographical gradient and ethnic groups. The pervasiveness of the *MTHFR* G677A polymorphism has already been studied in

Caucasian, Hispanic and Latino/Mexico-American populations as well as in many Asian countries like India, Pakistan, Sri Lanka, Japan and different ethnic groups of China (Yang *et al.*, 2013). In several studies, the homozygous variant allele *MTHFR* 677AA was found to be associated with severely impaired phenotype, with a frequency often reported to be high in Europe (0.241–0.643), and low in Africa (0–0.355) (Yang *et al.*, 2013). However, the homozygous 677GG and the heterozygote 677GA are quite common among various populations. In contrast, until recently no research was conducted on the frequency of *MTHFR* rs1801133 polymorphism in the Bangladeshi community. Realizing the need for the analysis of this variant, the study was designed to investigate the pervasiveness of *MTHFR* rs1801133 polymorphism in general and healthy Bangladeshi population.

MATERIALS AND METHODS

Blood sample collection and DNA isolation

A total of 181 blood samples were collected from random Bangladeshi individuals (105 females and 76 males) living in the Dhaka city. Subjects were unrelated, apparently healthy, with average age of 38.16 and 40.55 years for females and males, respectively. This study was approved by the ethics review committee of the Faculty of Biological Sciences, University of Dhaka. Blood samples were collected in K₂-EDTA coated collection tubes and DNA was extracted from the collected samples using the PureLink™ Genomic DNA Mini Kit (K1820-01, Thermo Fisher Scientific Corp.) following the manufacturer's protocol.

Allele-specific PCR

Allele-specific primers were designed for the rs1801133 SNP locus based on the principle described by Wangkumhang *et al.*, 2007 (Table 1). Primer-BLAST tool was used to check the specificities of the designed primers (Ye *et al.*, 2012). Temperature-gradient PCR was executed in a PCR machine to optimize the annealing condition for the primer pairs in PCR.

A sufficient amount (10–50 ng) of genomic DNA template was used for PCR reaction with

2.5 μ L of 10 $\times$ DreamTaq Green Buffer, 0.75 μ L of 10 mM dNTP mix (18427088; InvitrogenTM), 0.5 μ L of common forward primer (10 μ M), 0.5 μ L of allele-specific reverse primer (10 μ M), 0.2 μ L of DreamTaq Green DNA Polymerase (EP0712, Thermo Fisher Scientific), and PCR grade water. The final volume was 25 μ L. All the primers were obtained from Macrogen Inc (South Korea). An equal volume of PCR grade water was added as a negative control PCR in lieu of genomic DNA. A total of 34 cycles reaction condition was: an initial denaturation step at 95°C for 2 min, denaturation at 94°C for 30 sec, annealing at an appropriate temperature (Table 1) for 1 min, and elongation at 72°C for 20 sec, followed by a final hold at 4°C. An equal volume of the wild and variant allele-specific PCR products were mixed with loading buffer and resolved in 1.5% agarose gel electrophoresis with 0.5 $\times$ Tris-acetate-EDTA (TAE) buffer along with DNA markers. The agarose gel containing the separated PCR products was then stained with ethidium bromide in TAE buffer. Then, they were observed and photographed in a gel documentation system. The banding pattern image of gel photographs determined the genotype of *MTHFR* rs1801133 locus.

DNA sequencing

To assess the specificity of AS-PCR, a different primer (for sequencing) in combination with the common forward primer (Table 1) for the *MTHFR* rs1801133 locus was used to amplify the corresponding SNP encompassing region. A

sufficient amount (10–50 ng) of genomic DNA template was used for PCR reaction. The final volume was 25 μ L containing 2.5 μ L of 10 $\times$ DreamTaq Green Buffer 0.75 μ L of 10 mM dNTP mix 0.5 μ L of each primer (10 μ M), 0.2 μ L of DreamTaq Green DNA Polymerase and PCR grade water. The thermal cycler profile was as follows: initial denaturation at 95°C for 2 min, 34 cycles each with denaturation at 94°C for 30 sec, annealing at 58.5°C for 1 min, and elongation at 72°C for 30 sec followed by a single elongation step at 72°C for 5 min and a final hold at 4°C. 1.0% agarose gel was used to resolve the PCR amplified sequences with 0.5 $\times$ TAE buffer and DNA ladder (300003; GeneON). After staining with ethidium bromide in TAE buffer, the products were visualized in a gel documentation system. According to the manufacturer's protocol, FavorPrepTM GEL/PCR Purification Kit was used to purify the PCR products. The quality and quantity of the purified PCR products were analyzed in a NanoDropTM 2000 spectrophotometer. These purified PCR products were sent to Macrogen Inc. (South Korea) for sequencing. SnapGene Viewer was used to analyze the sequences.

Data Analysis

Microsoft Excel and GraphPad Prism[®] software were used to calculate and represent the allele frequencies and genotypes at *MTHFR* rs1801133 locus in the Bangladeshi population. The χ^2 analysis was performed to test the Hardy-Weinberg equilibrium.

Table 1. Primers used in this study.

PCR product	Primer	Sequence, 5'→3'	T _m (°C)	Product size (bp)
Wild type	rs1801133_Common Forward	ACTGTCATCCCTATTGGCAG	60.5	172
	rs1801133_W_Reverse	CTGCGTGATGATGAAATCCG		
Variant type	rs1801133_Common Forward	ACTGTCATCCCTATTGGCAG	58.5	118
	rs1801133_M_Reverse	CTGCGTGATGATGAAATCCA		
Sequencing	Forward	ACTGTCATCCCTATTGGCAG	58.5	252
	Reverse	GGGTGGAGGGGAGCTTATG		

RESULTS

In the current study, allele-specific polymerase chain reaction method was applied to determine allele frequencies and corresponding genotypes at the clinically significant rs1801133 locus (Figure 1). The confirmation of allele-specific genotypic data was achieved by targeted sequencing of DNA collected from randomly selected samples (Figure 2).

A total of 181 samples were collected randomly to perform this study. In this representative Bangladeshi population, the frequency of 677G and 677A alleles at the rs1801133 locus of *MTHFR* gene were found to be 0.856 and 0.144, respectively (Figure 3A). Among 181 subjects, 130 individuals were found with homozygous 677GG genotype, which covers 71.8% of the studied population (Figure 3B). Only 28.2% of the studied population comprised of heterozygous 677GA genotype, while there was no individual with the homozygous 677AA genotype. This result was significantly similar to the *MTHFR* rs1801133 genotype data of 86 Bangladeshi individuals collected from the 1000 Genomes Browser (Auton *et al.*, 2015) where 75.6% individuals were found to be homozygous 677GG genotype and 24.4% of the 86 studied individuals were heterozygous 677GA genotype, while there was no individual with the homozygous 677AA genotype.

Figure 1. AS-PCR of *MTHFR* rs1801133 variants. Sizes of the wild type 677G and variant 677A amplicons are 172 and 118 bps, respectively. In this representative figure, four individuals (1, 4, 6 and 7) have homozygous 677GG genotype and

four individuals harbor heterozygous 677GA genotype (2, 3, 5 and 8).

Figure 2. DNA sequence chromatograms of representative samples. A different pair of primers (Table 1) were used in PCR to amplify the region encompassing the *MTHFR* rs1801133 site for the sequencing purpose. In this representative figure, three individuals (1, 4 and 5) have homozygous 677GG genotype and two individuals carry heterozygous 677GA genotype (2 and 3).

Interestingly, data from both studies combined together with a total of 267 individuals, also showed a similar percentage pattern of genotype where 677G and 677A allele frequencies were

0.861 and 0.139, respectively (Figure 3A). The data of the combined population demonstrated that 73%, 27% and 0% of the individuals had homozygous 677GG, heterozygous 677GA and homozygous 677AA genotypes, respectively, which actually indicate the nearly concordant

result of the present study (Figure 3B). The genotype frequencies of this polymorphism were in compliance with the Hardy-Weinberg equilibrium ($p < 0.05$, $df=2$), reflecting that no selective mechanism for a specific genotype of the polymorphism existed.

Figure 3. Genetic architecture of the Bangladeshi population at the *MTHFR* rs1801133 locus. **A.** Frequencies of 677G and 677A alleles in our experiment and combined data with the 1000 Genomes Browser. **B.** Percentages of different genotypes at the *MTHFR* rs1801133 locus.

DISCUSSION

The present study examined the pervasiveness of the G677A variant in the *MTHFR* gene among randomly selected healthy subjects. Many epidemiological studies have indicated that *MTHFR* G677A is variable which depends on the location and ethnicity. A study on various ethnic groups in the United States of America reported a higher frequency of a minor allele in the Hispanic population while a lower frequency in the African Americans (Li *et al.*, 2005). The variation was also observed among Europeans ranging from 0.20 to 0.55 while the frequency was comparatively lower (0.04 to 0.38) in Asian populations (Rai *et al.*, 2012). The 677A allele frequency (0.144) in our target population actually supports the data, which was reported in other studies on Asian populations.

The genotype variation was also found even in different regions in a similar geographic location (Romero-Sánchez *et al.*, 2015). The world population shows a greater variation of 677AA

homozygosity, ranging from 0% to over 30% (González-Galofre *et al.*, 2010). Among the European populations, the highest prevalence of the mutant homozygous allele was observed in the Italian while the lowest persistence was found in the German population. Outside Europe, the variation in the prevalence of the homozygous polymorphism was 10 to 14% in America, Australia, Canada and Brazil (Liew & Gupta, 2015). The frequencies of *MTHFR* rs1801133 genotypes in countries of different continents are shown in Figure 4. The frequencies of homozygous 677AA genotype vary from 0% to 30% in the world populations (Romero-Sánchez *et al.*, 2015). From the 1000 Genome Browser study, it was found that two South Asian countries (SAS) such as Punjabi from Lahore in Pakistan (PJL) and Sri Lankan Tamil from the UK (STU) had no homozygous 677AA genotype in their population like Bangladeshi population (BEB). The homozygous 677AA genotype was also absent in some African populations, while the rest of the countries of different continents show a range of distributions (Figure 4).

Figure 4. The frequency of *MTHFR* rs1801133 homozygous 677AA genotype in world populations. Here, SAS=South Asian, EUR=European, EAS=East Asian, AMR=Admixed American, AFR=African.

In our present study, the wild type homozygous 677GG genotype (0.718) was most frequently observed, followed by heterozygous 677GA (0.282). The study did not find any homozygous 677AA genotype in the target population. The percentage of the homozygous mutant 677AA was reported approximately 0%, 1.9%, 6% and 3.9% in Tamil (Angeline *et al.*, 2004), Pakistani (Iqbal *et al.*, 2016), North Indian (Kohli *et al.*, 2007) and Lebanese (Sabbagh *et al.*, 2008) healthy population respectively. Considering the different ethnic groups, the Chinese Han population showed 23.2% overall frequencies of the homozygous 677AA genotype (Yang *et al.*, 2013).

Till now, the reasons behind these geographical and ethnic differences are not clear and there may be an involvement of genetic and environmental factors. The regional variation among populations may be indicative of selective advantages and disadvantages under particular conditions (Fodil-Cornu *et al.*, 2009). Several reports also suggest that in different parts of the world, environmental selective pressures condition the 677G and 677A allele frequencies (Fodil-Cornu *et al.*, 2009). It can be found from several investigations that UV radiation (Cordain & Hickey, 2006), evolution and migration (Underhill *et al.*, 2000), living environment (Botto & Yang, 2000) food habit especially folate intake

(Wang *et al.*, 2011), genetic drift (Rosenberg *et al.*, 2002), gene-gene (Diamond, 2005) and gene-environment interactions (Ke *et al.*, 2001) could be the reasons for various geographical gradient. Furthermore, accurate genotype patterns of this *MTHFR* rs1801133 locus may be helpful for further gene-disease correlations study, population genetics and health effect evaluation.

The reports of several case-control studies that focused on the association of genotype with diseases are complicated and not constant. The existence of *MTHFR* G677A mutation (heterozygous 677GA or homozygous 677AA form) may influence the incidence of acute myocardial infarction in the Tamil population and especially the Caucasians who are young/middle-aged (<50 years). In the same case, associations were not found in elderly male or female Caucasians, people of East Asia and South Asia and African-Americans (Xuan *et al.*, 2011). In the Chinese population, homozygous *MTHFR* 677AA and heterozygous *MTHFR* 677GA genotypes play a significant role in developing esophageal cancer. But the inverse relationship was found in Japanese and German people (Zhao *et al.*, 2011). The antagonistic association results obtained across independent studies may be partly explained by the distributional variation in genotype. This phenomenon reminds us that

association studies of *MTHFR* rs1801133 mutations with several diseases should warrant more attention in this region.

Here, a simple yet efficient AS-PCR-based method was reported to clarify the genotype of individuals at the *MTHFR* rs1801133 locus. The data of allele-specific genotype exhibited accordant results in their analogous DNA sequence chromatograms (Figure 2). The AS-PCR-based method is easy but precise. These are the prominent advantages of this method over the restriction enzyme-based or hybridization methods (Islam *et al.*, 2018). This simple, quick and cost-effective technique can be conducted in a fundamental laboratory setting and requires only a few hours to determine the *MTHFR* rs1801133 genotype.

Although the results of our study have potential importance, we are also concerned about the possible limitations of the sample size used and the gender effect. Tribal populations of our country should be considered in order to determine the real existence of the *MTHFR* rs1801133 genotype pattern.

CONCLUSION

Our findings represent the pervasiveness of the *MTHFR* rs1801133 polymorphism and the pattern of genotype in our community. This baseline data can be further helpful to design case-control studies and to explore whether there is any impact of genotype on several diseases. From public health standpoint, the data can be useful to help both government and health care professionals to generate the idea of preventive measures as well as to recommend an appropriate drug, dose and dietary habit on the basis of the patient's genotype.

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