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Genetic Polymorphism of 5, 10-Methylenetetrahydrofolate Reductase C677T in Kashmiri Population

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Abstract: 5, 10-Methylenetetrahydrofolate Reductase (MTHFR) is one of the key enzyme in the metabolism of homocysteine, where it catalyses its remethylation. The autosomal recessive bp C677T mutation in the MTHFR gene leads to the substitution of valine for alanine. Individuals who are homozygous for this C677T mutation exhibit a decreased specific activity and increased thermolability of MTHFR. This leads to increased plasma levels of homocysteine, which is a known risk factor for atherosclerosis and related disease. This study was conducted to find out the distribution and frequency of C677T mutation in the general Kashmiri population by employing PCR-RFLP method for C677T mutation analysis. A group of 110 volunteers (75 males and 35 females) has been analyzed for the MTHFR polymorphism, which revealed the following distribution, 62% individuals were without mutation (C/C), 33% were heterozygous (C/T) and 5% homozygous (T/T).

Key words: 5, 10-Methylentetrahydrofolate reductase, homocysteine, C677T, atherosclerosis, PCR-RFLP

INTRODUCTION

Hyperhomocysteinaemia has been regarded as a new modifiable risk factor for atherosclerosis and vascular disease (Lievers *et al*., 2006). Moderate hyperhomocysteinemia has also been associated with premature cardiovascular disease in adults however, lacking the mental and skeletal symptoms of homocystinuric children (Refsum *et al*., 1998). The role of elevated total homocysteine levels as a risk for arteriosclerotic vascular disease has attracted growing interest since half of all deaths in humans are due to cardiovascular disease and its complications (Robinson *et al*., 1998). Homocysteine may be metabolized in one of two pathways: remethylation back into methionine or transulphuration to cystathionine, which is converted into cystein and ultimately, excreted in the urine. Elevation in plasma homocysteine is typically caused by either genetic defects in the enzymes involved in homocysteine metabolism or by nutritional deficiencies of vitamin cofactors. The normal blood level of homocysteine is controlled by enzymatic conversion of the homocysteine to methionine or cystathione. These reactions are dependent upon 3 key enzymes and the vitamin cofactors B_{12}, B_6 and folic acid. Cystathionine b synthase, 5, 10-Methylenetetrahydrofolate reductase (MTHFR) and Methionine synthase. A MTHFR variant with reduced activity was described by Kang *et al*. (1988). Later on Frosst *et al*. (1995) identified a mutation responsible for this MTHFR variant with transition of a nucleotide 677 (C677T) from cytosine to thymidine in the MTHFR gene. This mutation results in a substitution of alanine to valine at position 226 in the MTHFR. The encoded protein has a 50-60% reduced enzymatic activity at 37°C, hence the term thermolabile. Many studies have shown that (homozygous (T/T) mutant subjects had significantly elevated plasma total homocysteine concentrations, whereas the total homocysteine concentration in heterozygous (C/T) individuals and in subjects with no mutation (C/C) was indistinguishable (Friedman *et al*., 1999; Lorenzo *et al*., 2000). Other studies have shown that heterozygous (C/T) individuals had slightly elevated homocysteine levels in comparison to the homozygous wild (C/C) genotype (Mcquillan *et al*., 1999). The discovery of this mutation was of interest because elevated total homocysteine is associated with

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an increased vascular disease risk. Furthermore, some inverse associations between the TT mutations and gastric and proximal colon cancer have been established (Toffoli *et al*., 2003) as well as an increased risk for some severe neural tube defects such as spina bifida and anencephaly (Shaw *et al*., 1998).

MATERIALS AND METHODS

This study was conducted in the Department of Veterinary Biochemistry, Faculty of Veterinary Sciences and Animal Husbandry, Sheri Kashmir University of Agriculture Sciences and Technology of Kashmir-190006 during the period 2007-2008.

Sample collection: One hundred and ten randomly selected blood donors were analyzed for the MTHFR gene polymorphism. Venous blood was obtained by puncture of the cubital vein, collected in EDTA and immediately centrifuged at 2500 rpm for 10 min at 4°C.

DNA extraction: DNA was extracted with phenol-chloroform and precipitated with ethanol and was kept at 4°C until the genetic analysis was performed.

MTHFR polymorphism: The technique of polymerase chain reaction and restriction fragment linked polymorphism (PCR-RFLP) was used to detect the genotype and allele frequencies at mononucleotide site 667 of MTHFR gene. Each PCR mix contained 1x PCR buffer, 0.4 μmol L^{-1} of each primer, 2.0 mmol L^{-1} of $MgCl_2$, 0.4 μmol L^{-1} dNTPs, 100 ng of genomic DNA and 2.5 units of Taq DNA polymerase. The following primers were used: the forward primer 5'-TGA AGG AGA AGG TGTCTG CGG G A-3'; the reverse primer 5'-AGG ACG GTG CGG TGAGAG TG-3'. The PCR reaction mixture was pretreated at 95°C for 10 min followed by 35 cycles of 95°C for 1 min, 55°C for 1 min and 72°C for 1 min. The final extension was at 72°C for 10 min. An amplified product of 198 bp of C667T was run along with 100 bp of ladder strand on a 2% agarose gel for 1 h at 100 v. The PCR product was digested with Hinf1 enzyme at 60°C for 16 h.

Sample analysis: After digestion incubate the PCR product for 12 h or overnight, the reaction was stopped by placing at 62°C and the digested product was run on a 2% agarose gel for 2 h at 100 v and was stained with ethidium bromide.

Statistical analysis: From the observed genotypes among the individuals, allele frequencies were calculated with the help of BIOSYS-1 program (Swofford and Selander). The normal distribution of the data was analyzed using the Kolmogorov-Smirnov test. Allele frequencies were tested for the Hardy-Weinberg equilibrium. The expected genotype frequencies in the case of Hardy-Weinberg's equilibrium were determined.

RESULTS AND DISCUSSION

MTHFR genetic polymorphism among 110 individuals was analyzed and their allelic and genotypic frequencies was determined, 62% individuals were homozygous for the wild type (C/C), 33% were heterozygous (C/T) and 5% were homozygous for the mutation (T/T) (Table 1). The distribution of the MTHFR polymorphism was also analyzed separately in relation to gender. MTHFR genotype variants in males were: 45% individuals had the wild homozygous (C/C) genotype, 22% were heterozygous (C/T) and 4% had the mutant homozygous genotype (T/T). Among the 25 female individuals the distribution was as follows: 17% had the (C/C) genotype, 11% were heterozygous (CT) and 1% was T/T homozygous.

Study of 110 subjects of the Kashmiri population, as shown in Table 1, shows that the distribution of the C677T polymorphism of the MTHFR gene was as follows: 62% individuals were homozygous for the wild type (C/C), 33% were heterozygous (C/T) and 5% were homozygous for the mutation (T/T).

Interpretation for C677T

- Amplified product size --- 198 bp (Fig. 1)
- 175 and 23 bp after digestion-mutated (p6) (Fig. 2)
- 198 bp after digestion-normal wild type (p2, p3, p4, p5, p7) (Fig. 2)

Hyperhomocysteinemia is assumed to be an independent risk factor for cardiovascular disease and is thought to be responsible for about 10% of total risk (Boushey *et al*., 1995). Normal levels of fasting plasma homocysteine fall between 5 and 15 mmol L^{-1}. Moderate hyperhomocysteinemia refers to concentrations between 16 and 30 mmol L^{-1}, intermediate between

Table 1: Distribution of C677T allele of MTHFR gene in Kashmiri population

Gender	Male	Female	Total
No.	75	35	110
Genotype frequency (%)			
CC	45	17	62
CT	22	11	33
TT	4	1	5
Allele frequency (%)			
C	56	28	84
T	15	12	27

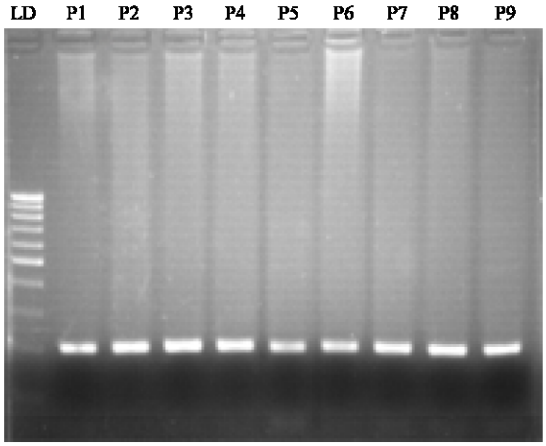

Fig. 1: An amplified product of 198 bp for C677T

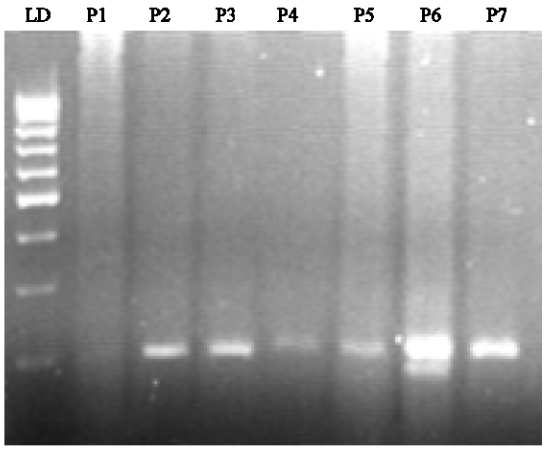

Fig. 2: For C677T:- 175 and 23 bp after digestion-mutated

31 and 100 mmol L^{-1} and severe hyperhomocysteinemia >100 mmol L^{-1}. Moderately elevated plasma homocysteine levels (>15 mmol L^{-1}) are considered cytotoxic and are found in 5-10% of the general population and up to 40% of patients with vascular disease (Ueland *et al*., 1992). Additional risk factors such as smoking, arterial hypertension, diabetes and hyperlipidemia interact with homocysteine and synergistically increase the overall risk. Hyperhomocysteinemia is associated with alterations in vascular morphology, loss of endothelial antithrombotic function and induction of a procoagulant environment (Denheijer *et al*., 1996; Wilcken, 1998). Furthermore, special importance of the role of MTHFR C677T mutation and hyper homocysteinemia represents a risk factor for development of arterial and venous thrombosis (Madonna *et al*., 2002). Besides homocysteine-mediated oxidative stress, numerous agents such as drugs, disease and life style factors also have an impact on homocysteine metabolism, when acting as direct or indirect antagonists of cofactors and enzyme activities (Girelli *et al*., 1998). The distribution of C677T allele is strikingly variable among different ethnic and racial groups (Giles *et al*., 1998).

CONCLUSION

The true role of mild elevation of homocysteine, which results from the homozygous C677T mutation of the MTHFR gene, in the genesis of cardiovascular disease, remains still somewhat controversial. The opinion is divided whereas this mild homocysteinaemia may be considered an important risk factor in pathogenesis of atherosclerotic disease or if it only represents a marker for increased risk. The contribution of low folate levels in homocysteinaemia in individuals who are homozygous for the T/T genotype is unquestionable; dietary folate supplementation may be recommended in these individuals as a strategy in normalizing the levels of homocysteine and preventing atherosclerotic and peripheral vascular disease. The distribution of MTHFR gene polymorphism studied in the Kashmiri population was as follows: approximately 62% were homozygous for the wild type (C/C), 33% were heterozygous (C/T) and 5% were homozygous for the mutation (T/T). These numbers may also be the foundation to a future preventive strategy in cardiovascular disease as well as guidance in treatment.

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