MTHFR Gene Polymorphism In Patients Of Myocardial Infarction

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ABSTRACT :

Homocysteine is an emerging new risk factor for cardiovascular disease. A common polymorphism in the gene coding for the 5,10-methylenetetrahydrofolate reductase (MTHFR) is associated with a decreased activity of the enzyme due to thermolability. There is an accumulating evidence that adequate intake of folate may protect the risk of developing the athero-thrombotic disease and its complications. Hyperhomocysteinemia (HCA) either due to mutation of MTHFR gene or deficiency of vitamin B12 and folic acid, has been reported as a risk factor for coronary artery disease (CAD). the present study was aimed to determine plasma homocysteine (hcy) levels and to evaluate MTHFR C677T gene polymorphism as risk factors for CAD in younger Indians. the effect of vitamin B12 and folic acid supplements on the raised plasma hcy levels in patients with CAD was also assessed. In our results, 6 cases are effected with a mutation in MTHFR gene. among them 4 individuals may face the future threat of myocardial infarction remaining 2 individuals are mutated and risk factors are similar to that of myocardial infarction.

Keywords: myocardial infarction, MTHFR, cardiovascular disease, folic acid.

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INTRODUCTION:

A myocardial infarction(MI) is defined as obstruction of blood flow in a coronary artery resulting in cell death of myocardial tissues or myocytes. Obstruction of blood flow may cause by 'ATHEROSCLEROSIS'¹⁻⁵.

- MI may cause by homocysteine associated with coronary artery diseases.
- Homocysteine may develop the cardiovascular disease.

- Homocysteine causes damage to the lining of blood vessels increases clot formation & indicator of cardiovascular diseases.
- Classical symptoms of acute myocardial infarction include sudden chest pain, shortness of breath, nausea, vomiting, palpitation, sweating, and anxiety women may experience fewer typical symptoms than men may shortness of breath a feeling of indigestion.

A high level of blood serum homocysteine (homocysteinemia) is a powerful risk factor for cardiovascular disease. Hyperhomocysteinemia (HCA) either due to mutation of MTHFR gene or deficiency of vitamin B12 and folic acid, has been reported as a risk factor for coronary artery disease. the present study was aimed to determine plasma homocysteine (hcy) levels and to evaluate MTHFR C677T gene polymorphism as risk factors for CAD, and to study the role of hcy in conjunction with a few other risk factors for CAD in younger Indians. the effect of vitamin B12 and folic acid supplements on the raised plasma hcy levels in patients with CAD was also assessed⁶⁻⁸. CAD due to atherosclerosis is associated with increased mortality and morbidity. various risk factors have been found to be associated with the development of CAD. the role of diabetes mellitus, smoking, and hyperlipidemia as risk factors for CAD is well-established. hyperhomocysteinemia (HCA) Known to be associated with increased thrombotic tendency has been considered as a risk for coronary artery disease and atherosclerosis whereas others have found no association between acute myocardial infarction and HCA. mild to moderate HCA is known to be due to genetic factors like a mutation in methylene tetrahydrofolate reductase genes or due to environmental factors like deficiency of vitamin B12 or folic acid. Mutations in the MTHFR gene could be one of the factors leading to increased risk of developing schizophrenia. schizophrenic patients having the risk allele (T/T) show more deficiencies in executive function tasks¹²⁻¹⁵.

Mthfr Gene Polymorphism : C677T SNP (Ala 222val):

The MTHFR nucleotide at position 677 in the gene has two possibilities: C (Cysteine) or T (thymine). C at position 677 (leading to an alanine at amino acid 222) is the normal allele. the 677T allele (leading to a valine substitution at amino acid 222) encodes a thermolabile enzyme with reduced activity¹⁶⁻¹⁸. The degree of enzyme thermolability assessed as a residual activity after heat inactivation is much greater in 677TT individuals (18-22%) compared with 677CT(56%) and 677CC(66-67%). individuals of 677TT are predisposed to mild hyperhomocysteinemia (high blood homocysteine levels) because they have less active MTHFR available to produce 5-methyltetrahydrofolate (which is used to decrease homocysteine) low dietary intake of the vitamin folic acid can also cause mild hyperhomocysteinemia¹⁹⁻²⁴.

METHODS AND MATERIALS :

Simple collection :

The present study includes six patients with myocardial infarction and five healthy individuals as controls to analyse the mutation status in MTHFR gene at codon 677(c>T) that resulted in the

substitution of alanine by valine by PCR –RFLP analysis by using H1NF-I restriction enzyme which will yield 198bp for alanine variant and 175bp + 23bp for valine variant. on gel electrophoresis, the 23bp band cannot be visualized while 198bp and 175bp bands can be visualized .peripheral blood (2ml) samples were collected from the above patients and control groups with prior consent. samples were collected into sterile tubes containing EDTA. samples were stored at -20°C till DNA isolation.

DNA isolation:

- DNA was isolated by simple, rapid, non-enzymatic method (Lahiri and Nurnberger,1991).for all solutions use double distilled water, autoclaved. All solutions should be filtered and autoclaved except NaCl and SDS.

Procedure:

- To 2ml of blood add equal volume of TKMI buffer and add 2-3 drops of Triton 100(RBS lyses)
- Vortex well and spin at 2700 rpm for 10minutes. Discard the supernatant .to the pellet add an equal amount of TKMI buffer and spin at 2700 rpm for 10minutes.
- Discard supernatant and repeat the wash with TKMI buffer until white pellet is obtained.
- To the white pellet add 80µl of TKM2 buffer and 12.5µl of 10% SDS (WBC lysis)and vortex well and incubate in a water bath for 15 minutes at 55°C. After the pellet is dissolved add freshly prepared 35µl of NaCl (to remove contaminants).
- Vortex well and incubate for 10minutes at 55°C in a water bath.Centrifuge at 1200rpm for 5minutes.The supernatant is collected in a fresh vial and add double the amount of 100%ethanol.
- Vortex the tube until the DNA is precipitated.Centrifuge at 1200rpm for 3minutes.
- Discard the supernatant and add 200µl of 70%ethanol and vortex (by inverting the tube)thoroughly and spin for 2 minutes.Discard the supernatant and dry the pellet.
- After the DNA is dried add 100µl of TE buffer and dissolve at 65°C
- Store the DNA solution at -20°C.

DNA analysis :

Genomic DNA obtained from patients with MI was analyzed to determine the distribution of 677≥T mutation in MTHFR gene using PCR-RLFP.

The polymorphic locus was studied were genotyped by amplifying genomic DNA in 40cycles, three-step PCR appropriate annealing temperature and additives were optimized for the system.

Quantity assessment :

Prior to analysis, DNA sample should be quantitated and checked for purity and integrity.based on its structure DNA absorbs light in the ultraviolet range, specifically at a wavelength of 260nm.

A volume of 1 at OD 260nm is equal to 50ng/µl double-stranded DNA.

Therefore, to calculate the concentration of DNA the following formula can be used.

Concentration of DNA= 260abs x 50 x dilution factor(at the time of OD taken (OD at 260nm x 50 x 100).

PCR-RFLP(Restriction fragment length polymorphism –polymerase chain reaction):

PCR can be applied only when the nucleotide sequence of at least one short DNA segment on each side of the region of interest is known. the procedure of PCR technique involves using synthetic oligonucleotides (amplimers, primers) complementary to this known sequence of prime enzymatic amplification of the target DNA.

The method by which PCR works:

- Two small fragments of DNA -20bp long called oligonucleotide primers are synthesized
- These primers are complementary images to each end of the DNA sequence of interest.
- The reaction contains the source DNA, the primers, the four deoxynucleotide triphosphates- d ATP, d CTP, d GTP, d TTP, thermal stable DNA polymerase and its reaction buffer, the most critical component of which is Mg +2.

The reaction involves 3 stages carried out at different temperatures:

- Denaturation of the double-stranded DNA at 94°C for human genomic DNA .the thermal energy at 94°C is sufficient to overcome the hydrogen bonding between the base pairs of the two DNA strands.
- Annealing of primers to the resulting single-stranded templates carried out at =55°C, the optimal temperature depending upon the primer sequence .the primers bind specifically to complementary DNA sequence present on the denatured genomic DNA which serves as the template. The 3' end of both the primers face each other and they provide the free 3' –OH required for the covalent extension.
- DNA synthesis: primer extension is generally carried out at 72°C DNA polymerase replicates the DNA segment between the sites complementary to the oligonucleotide primers. The synthesis occurs at the rate 20 nucleotide /sec.
- Each group of three reactions is termed as PCR cycle and theoretically doubles the amount of the original target sequence.
- Amplification is exponential (2,4,8,16,32,...).the products of the first cycle of replication are then denatured, annealed to primers and replicated again..the PCR is a chain reaction because newly synthesized in subsequent cycles.these cycles are repeated 25-35 times resulting in the synthesis of millions of copies of the amplified DNA fragment .each round of amplification takes about 5-10mins .at the end of Apcr, a sample of the reaction mixture is usually analyzed by agarose gel electrophoresis.
- Initially, PCR was performed with DNA polymerase –I of E.coli as the replicate .because this enzyme is heat activated during the denaturation step, new enzyme had to be added at each cycle. Later a heat stable DNA polymerase was discovered in the thermophilic bacterium – thermos aquatics, which is called Taq polymerase (T-aquaticus polymerase). This microorganism which was found in the hot springs of the yellow store national park, thrive at 75°C temperatures and have enzymes with maximum activity at High Temperature.Taq resist inactivation even at 94°C.

PCR analysis:

The MTHFR gene was amplified by polymerase chain reaction in a 10µm Tris HCl, 50mm KCl, 0.2mm of each dNTPs, 200p mol of each primer, an optimal concentration of MgCl₂ & 0.5U of Taq DNA polymerase and 100ng of genomic DNA.

PCR protocol (Stoneking et al. 1970)

Agarose gel electrophoresis :

After PCR, the samples were subjected to 2% agarose gel electrophoresis at 100V for 20-30 minutes. Ethidium bromide stained gels were visualized under UV light and were documented. Band sizes were compared to molecular weight marker of 100-1000bp for confirmation. Agarose gel showing the 198bp (alanine) and 175 + 23bp (valine) at codon 677(C>T) mutation in MTHFR gene in myocardial infarction patients and control individuals.

lane 2-7: MI patients; lane 8-12: control group.

lane 1: 100bp DNA ladder; lane 2,3,5,7,10: 198bp+175bp bands (val/ala heterozygotes)

lane 4,8,11: 198bp (valine homozygotes); lanes 6,8,12: 175bp (alanine homozygotes)

Quality check of genomic DNA by agarose gel electrophoresis

RESULTS AND DISCUSSION:

A significant difference ($p \leq 0.001$) was found between mean fasting levels of plasma hcy in cases ($22.14 \pm 10.62 \mu\text{mol/liter}$) and controls ($17.38 \pm \mu\text{mol/l}$) with an odds ratio as 1.93 (95% CI, 1.27-2.94). Levels of cholesterol, LDL and triglycerides were significantly ($P \leq 0.001$) higher in cases compared with controls. Our study showed a significant correlation between hyperhomocysteinemia and coronary artery disease. Multivariate analysis by logistic regression of the various risk factors of a CAD when all other factors were controlled. Significant post-treatment decrease found in their CT or TT genotype of C677T MTHFR gene. Further studies to look at the plasma levels of folate and cobalamin and their association with hcy are required to be done. In an attempt to understand the association of 677C>T mutation with a myocardial infarction, genotyping of the polymorphic variants (alanine /valine) in MTHFR gene was carried out. MTHFR mutation (677C>T) in MTHFR gene was analyzed by PCR-RFLP in 6 MI patients and 5 healthy controls to determine the genotype. The genotype frequencies were identified as

for the II genotype, 40%(4 cases) for the heterozygous ID genotype respectively. the genotype and percentage frequencies are given in table 1.

Table-1: Type frequencies of 677C $\geq$ T mutation at codon 226 of MTHFR gene in cases and controls and their association of risk of MI patients and controls.

MTHFR 677 $\geq$ T genotype	Cases (n=6)	Controls(n=4)	X ² ; p value
CC	0	3	6.7:0.03
CT	4	1	
TT	2	0	

There is a significant difference between the distribution of CC (valine/alanine), CT (valine/alanine) and TT (alanine /alanine) genotypes in MI patients. table 2 shows the allele frequencies of C & T in MTHFR gene in a myocardial infarction.

Table-2: Allele frequencies of valine (c) and alanine (T) at codon 226 (677C $\geq$ T) in MTHFR gene in MI patients and controls.

	Cases(n=6)	Controls(n=4)
C allele	0.335	0.875
T allele	0.665	0.125

- In this regard d taken to analyze the mutation of 6 cases and 4 controls, individual blood samples are MTHFR gene and risk factor of myocardial infarctions. The DNA was isolated from blood samples and checked the DNA for purity by spectroscopy method.
- After that isolated DNA was subjected to DNA PCR method for amplification.
- These DNA samples were digested with restriction endonucleases of H1NF-1 to get the mutated fragments in DNA samples of tests.
- Finally, these samples will run in agarose gel electrophoresis for visualizing of digested or restricted fragments.

CONCLUSION :

In our results 6 cases are affected with a mutation in MTHFR gene among them 4 individuals may face the future threat of myocardial infraction remaining 2 individuals are mutated and risk factors are similar to that of myocardial infarction. the present study was carried out to evaluate plasma hcy levels and MTHFR C677T polymorphism as risk factors for CAD and to study the role of hcy in conjunction with a few other risk factors for CAD.

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