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Role of MTHFR 677 C>T Polymorphism on Blood Homocysteine and Susceptibility to Hypertension

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ABSTRACT Genetic polymorphism of Methylenetetrahydrofolate reductase is found to associated with Stroke and other Cardiovascular diseases. This study analyzed Methylenetetrahydrofolate 677C>T polymorphism for association of blood homocysteine concentration and susceptibility to Hypertension in the Central Indian Population. This investigation has been conducted on 100 hypertensive cases and 223 healthy controls. 5ml blood sample has been taken from case and control and analyzed for blood homocysteine concentration by enzymatic assay and genotyping. The present study found statistically significant ($P<0.0001$) to establish hyperhomocystenimia is a risk factor for hypertension. The Methylenetetrahydrofolate 677C>T polymorphism is significantly ($P<0.0001$) associated with blood homocysteine level. The CT and TT genotype are showing association with hypertension susceptibility.

INTRODUCTION

In India 24 percent of the coronary heart disease and 57 percent of stroke patients have previous history of Hypertension. Cardiovascular disease (CVD) will be the largest cause of death and disability in India by 2020 and hypertension is emerging as a major health problem (Ramaswamy et al. 2016).The World Health Organization (WHO) World Health Statistics Report (2016) reveals that the worldwide prevalence of hypertension is 29.2 percent for males and 24.8 percent for females. Hypertension has become an important worldwide public health challenge because of its high prevalence and concomitant risks of coronary artery disease, congestive heart failure, stroke, end-stage renal disease, dementia, and blindness. People with hypertension possess two-fold higher risk of developing coronary artery disease, four times higher risk of congestive heart failure, and seven times higher risk of cerebrovascular disease compared to normotensive people (Ismail et al. 2016). Regional studies in India, documents that the prevalence of hypertension ranges from 20-40 percent among urban adults and 12-17 percent among rural adults and the number of hypertensive is projected to increase from 118 million in 2000 to 214 million in 2025 (Paul et al. 2017).

A recent study on hypertension prevalence in India demonstrated a significant increase in the prevalence of hypertension. The increase was significantly higher in urban than in rural populations and the prevalence of hypertension was higher in urban compared to rural areas. Prolonged uncontrolled or inadequate treatment of hypertension is a leading risk factor for the occurrence of heart attack, stroke, kidney failure and other cardiovascular diseases (Singh et al. 2017).

Homocysteine is largely known as potential biomarker for CVD because of it produces reactive oxygen species. Homocysteine (Hcy), an essential sulfur-containing amino acid is generated during metabolism of methionine (Met). In the regulation of homocysteine metabolism, homocysteine is remethylated into methionine and in this pathway Methylene-tetrahydrofolate reductase (MTHFR) plays most important role (Engbersen et al. 1995). The catalytic efficiency of an enzyme itself is depend on its three dimen-

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sional structure determined by amino acid sequence of protein which is determined by nucleotide sequence within the gene that transcribed and translated into specific enzyme. The C677T mutation of the MTHFR gene which leads to the synthesis of a thermo labile form of MTHFR that is responsible for 50 percent of the MTHFR activity (Kluijtmans et al. 1996).

METHODOLOGY

Sample Collection

(a) Patient Recruitment

Medically certified hypertensive patients were recruited from medicine department (OPD) of medical colleges of central India region, registered during the year 2010 and 2015. 100 hypertensive patients were recruited for present investigation. All the recruited patients were Central Indian origin.

(b) Healthy Controls

223 randomly selected healthy controls (HC) were enrolled in the study. The control group consisted of medical staff and healthy volunteers from central region of India. Hence, control group was drawn from same area with similar environmental and social factors with same mean age and sex ratio.

(c) Sample Collection Strategy

Approximately 5 ml. of blood sample was collected in 0.5 M EDTA tubes from each hypertensive patients as well as from healthy controls. These samples were stored frozen at -80^{0}C until DNA was extracted from them.

HomocysteineMeasurement

The Diazyme Homocysteine Enzymatic Assay (by DIAZYME laboratories, catalogue no. DZ1 12 A-K) is used for the measurement.

DNA Extraction

Genomic DNA was extracted from whole blood by the modification of salting out procedure described by Miller and coworkers (Miller et al. 1988).

Characterization of DNA

Integrity

The integrity of high molecular weight DNA is an important factor, which should be considered during extraction steps. Integrity was checked by electrophoresis on 0.8 percent Agarose prepared in 1X TBE buffer. The high molecular weight genomic DNA appeared as a single band near the well.

Concentration

DNA was quantified by measuring the optical density at 260nm. 5 µl of stock genomic DNA was taken and 995 µl of water was added (Dilution factor D.F. = 200), mixed well and OD was taken at 260 nm in a spectrophotometer (Systronic, India). DNA concentration was found160 µl/ml.

Purity of DNA

Purity of DNA was determined by taking OD of the sample at 280 nm for protein concentration and at 260 nm for DNA concentration. The ratio OD_{260} / OD_{280} was calculated. DNA sample for which the ratio was 1.7 or above was considered good.

The Detection of *MTHFR* C677T Polymorphism

The MTHFR C677T polymorphism was sought using a PCR-RFLP method (Saffroy et al. 2008). The transition of C→T at the position 677 produces restriction site for HinfI, by which the polymorphic MTHFR gene and wild type gene can be identified after PCR amplification, restriction digestion and gel electrophoresis. All the PCRs were carried out in a PTC 200 thermalcycler (MJ Research Inc. USA).

Primers

Primers were synthesized by Sigma Aldrich, India and amplification was carried out using MJ research thermalcycler those described by-

Primer sequence: - Sense: 5'-TGAAGGAGAAGGTGTCTGCGGGA-3'

Anti sense: 5'-AGGACGGTGCGGTGAGAGTG-3'

PCR Mix

25 µl of each PCR reaction mixture contained 2-5 µl template DNA (final concentration 100-200 ng/ µl), 2.5 µl of 10X *Taq* polymerase buffer (10 mMTrisHCl pH 8.8, 50 mMKCl, 1.5 mM MgCl2, 0.01 percent gelatin, 0.005 percent Tween-20, 0.005 percent NP-40; final concentration 1X; Genetix Biotech Asia Pvt. Ltd., India), 1 µl of 10 mMdNTPs (Bangalore Genei, Bangalore, India), 1 µl of 10 pm/µl of forward and reverse primers specific for MTHFR gene, 0.3 µl of 5U/µl of *Taq* DNA polymerase (final concentration 1.5U; Bangalore Genei, Bangalore, India) and sterile water to set up the volume of reaction mixture to 25 µl.

Thermal Profile

Thermal profile used for the amplification of desired segment of gene was as follows: Initial denaturation at 94^{0}C for 2 min and 35 cycles of denaturation at 94^{0}C for 1 min, annealing at 58^{0}C for 1 min and extension at 72^{0}C for 1 min, followed by final extension at 72^{0}C for 10 min.

Restriction Digestion by HinfI

The digestion mixture contained 1 µl of *HinfI*(10 units/µl) (Roche Diagnostics, Meylan, France), 5 µl of the digestion control PCR product and 10 µl of the patient's PCR product in a final volume of 25 µl. It was incubated at 37°C for 4 h.

Statistical Analysis

The researchers analyzed data by Fischer's exact test, unpaired t-test, and Odds ratio with 95 percent confidence interval and interpret results for P value.

RESULTS

The demographic parameters of study groups are presented in Table 1 which shows there are insignificant changes in these parameters and individuals in both the groups are same except the disease.

Association between Homocysteine Concentration and Susceptibility for Hypertension

The results of association between increased homocysteine concentration and susceptibility to hypertension are presented in Table 2. It was observed that the hyperhomocystenimia is an independent risk factor for hypertension. It is observed that homocysteine concentration in some persons of control group and normal homocysteine concentration in some persons of diseased group, so the results of this study indicated that hypertensive group contain higher percentage of hyperhomocystenimia individuals than control.

Table 1: Clinical features of hypertensive patients and healthy controls

Clinical features	*Hypertension patients*	*Healthy controls*
Total number	100	223
Sex (Male: Female)	68:32	138:82
Mean BMI ± SD	24.81 ± 5.39	22.75 ± 4.52
Age (Years)		
Mean ± SD	47.5 ± 5.54	49.79± 12.66
Age range	34-56	25-78
Mean Homocysteine (µmol/L) Value ± SD	15.89 ± 3.94	11.17 ± 2.40

Table 2: Comparison of total blood plasma homocysteine concentration (µmol/L) stroke, hypertension compare to control using t-test (unpaired) test

Study groups	*Mean Homocysteine (µmol/L) ± SD*	*t-test P value*
Control (n = 223)	11.17 ± 2.40	
Hypertensive (n = 100)	15.89 ± 3.94	$P^2<0.0001^{***}$

* = Statistically significant

Table 3 showing 63 persons out of 100 hypertensive patients had elevated level of tHyc (above 15µmol/L) whereas in control group only 51 persons out of 223 individuals (23%) had elevated level of homocysteine. The situation with normal level of homocysteine was opposite, it was found 37 percent and 77 percent for hypertension as compared with healthy control. The results of this study were statistically significant (P<0.0001) to establish hyperhomocystenimia as a risk factor for hypertension.

Detection of MTHFR 677c>t Polymorphism

The 677C>T polymorphism was investigated in 100 hypertensive patients and 223 healthy

Table 3: Association of normal and elevated concentrations of total blood plasma homocysteine (tHyc) (µmol/L) with susceptibility to hypertension compare to control using Fishers exact test

tHyc (µmol/L)		*n*	*%*	*P value*	*OR*	*95% CI*
Normal	Healthy control	172	77	$P^2<0.0001^{***}$	0.1754	0.094-0.325
< 15(µmol/L)	Hypertensive	37	37			
Elevated	Healthy control	51	23	$P^2<0.0001^{***}$	5.7	3.07- 10.58
> 15(µmol/L)	Hypertensive	63	63			

* = Statistically significant

controls. The 198 bp DNA fragment including the C to T transition at nucleotide position 677 of MTHFR gene was amplified by the PCR and afterwards analyzed by restriction digestion with the *HinfI* enzyme. DNA Fragment length analysis was done 10.2 percent polyacrylamide gel Electrophoresis. The wild genotype CC produce a single band of 198 bp whereas heterozygous mutant CT genotype produce three bands of 198bp, 175bp and 23 bp while homozygous mutant TT genotype produce two bands of 175 and 23 bp.

Association between MTHFR 677C>T Polymorphism and Blood Plasma Total Homocysteine Concentration

The balance between homocysteine formation and elimination is maintained by its remethylation into methionine and this step requires methylene-tetrahydrofolate (MTHF) as a substrate. The formation of MTHF from tetrahydrofolate is catalyzed by Methylene-tetrahydrofolate reductase (MTHFR). Hence wild type and mutated allele influences tHyc concentration according to heterozygous and homozygous state. The effect of MTHFR677C>T mutation on blood homocysteine concentration in case and control group is given in Table 4.

The results indicated a significant elevation of tHyc concentration with MTHFR677C>T mutation carrying individuals. The elevation of tHyc concentration was increasing with homozygosity of MTHFR677C>T allele. The mean homocysteine concentration values were normal in healthy control group, slightly elevated in Hypertensive group with CC (wild) carrying individuals, while heterozygous mutated CT and homozygous mutated TT carrying individuals were shown more sharp elevation in tHyc concentration. The R^2 values in Table 4 were 0.71 and 0.28 for hypertension and control group respectively indicated a significantly different mean value of tHyc for CC, CT and TT genotype within groups.

The comparison among wild CC and mutated CT and TT genotypes of MTHFR gene and tHyc concentration were done within case and control groups and the values of this comparison are given in Table 5. It is observed that wild 'CC' gene was able to regulate the tHyc concentration in blood whereas heterozygous mutated allele 'CT' observed with intermediate regulation ability while homozygous mutant 'TT' failed to regulate tHyc concentration in hypertensive patients. The mean tHyc values in CC, CT and TT genotype carrying individuals were 13.30 µmol/L, 19.23 µmol/L and 24.26 µmol/L for hypertension and 10.56 µmol/L, 12.57µmol/L and 18.66 µmol/L for healthy group indicated a strong association between genotype and tHyc concentration.

t-test has been done to analyze significant difference in mean value of tHyc between case and control groups carrying same genotype and results are given in Table 5.The researchers found different level of blood homocysteine between

Table 4: The mean tHyc concentration for CC, CT and TT genotype of MTHFR within hypertensive and healthy control group

Study group	*CC*	*CT*	*TT*	*ANOVA*
Hypertension tHyc (µmol/L) ± SD	13.30 ± 2.11	19.23 ± 2.05	24.26 ± 0.98	$P<0.0001^{***}$ $R^2 = 0.7193$
Healthy control tHyc (µmol/L) ± SD	10.56 ± 2.24	12.57 ± 1.6	18.66 ± 1.63	$P<0.0001^{***}$ $R^2 = 0.2892$

* Statistically significant

case and control carrying CC genotype, this observation was consistent with CT and TT genotype. The difference in mean values of tHyc (t-test) were 8.895 for hypertension as compared with HC group at the level of significant P<0.0001 in CC carrying individuals. The value of t = 13.89 for hypertension as compared with HC group at the level of significant P<0.0001 in CT carrying individuals indicated, same genotype carrying individuals had significantly different mean tHyc concentration due to belonging of cases or control.

Association between MTHFR 677 C>T Polymorphism and Susceptibility for Hypertension

The association was tested using 3 x 2 chi squire test. χ^2 values and its P values are presented in Table 6. Further this distribution confirmed comparing individual study group (Hypertensive patients and HC) for each genotype (CC, CT, TT) using 2x2 contingency table by Fishers exact P values, Odds Ratio (OR) and 95 percent confidence interval (CI)(Table 7).

Table 5: The mean value of tHyc between case and control groups carrying same genotype and analysis of difference of mean values by t-test.

MTHFR 677 C > T Genotype	*Hypertension (100)*		*Control (223)*		*t-test*	*95% CI*	*ANOVA*
	N	*tHyc (µmol/L) ± SD*	*N*	*tHyc (µmol/L) ± SD*			
CC	71	13.30 ± 2.11	184	10.56 ± 2.24	P<0.0001* t^2 = 8.895	-3.344 to -2.136	P<0.0001**
CT	24	19.23 ± 2.05	34	12.57 ± 1.6	P<0.0001** t^2=13.89	-7.621 to -5.699	P<0.0001**
T T	05	24.26 ± .98	5	18.66 ± 1.63	P=0.0002** t^2 =6.584	-7.561 to -3.639	P<0.0001**

*Statisticallysignificant

Table 6: Frequency distribution and association of genotypes, alleles carriage rate of MTHFR 677C>T polymorphism in hypertension and healthy controls in central India

MTHFR 677 C > T	*Hypertension (100)*		*Healthy controls (N=223)*	
	n	*%*	*n*	*%*
Genotypes				
CC	71	71	169	75.8
CT	24	24	49	21.9
TT	05	05	05	2.3
χ^2 (P Value)	2.034 (0.3616)			
Alleles				
Allele 'C'	166	83	387	86.8
Allele 'T'	34	17	59	13.2
χ^2 (P Value)	1.594 (0.2068)			
Carriage Rates				
Allele 'C'	95	95	218	97.75
Allele 'T'	29	29	54	24.21
χ^2 (P Value)	0.6421 (0.4229)			

N- Number of individuals carrying particular genotype, Allele in a study group.
percent - Genotype and allele frequencies and carriage rates in percentage
* - Statistically significant .
χ^2 (P Value) indicates χ^2 (P Value) when HC compared to hypertensive patients

The observed genotype frequencies, allele frequencies and carriage rates for MTHFR 677C>T polymorphisms are given in Table 6.The researchers did not found any association between MTHFR 677C>T genotype and disease susceptibility and overall distribution of MTHFR 677C>T genotype was similar between case and control. Overall genotypes (CC, CT and TT) difference of MTHFR gene were found statistically not significant for hypertensive groups (χ^2 = 2.034 P= 0.3616) as compared with HC group.

The researchers observed higher proportion of homozygous mutant 'TT' genotype in Hypertensive patients (5%vs 2.3%) than controls which was scientifically important, and higher odds ratio of 1.50 (95% CI, 0.44- 5.09) and 1.2 (95% CI, 0.34- 4.41) in stroke and hypertension patients respectively (Table 7) indicated its harmful effect and strong positive association of this mutant genotype with the disease. The heterozygous genotype 'CT' was slightly similar in hypertensive patients (24%vs 21.9%) and not found any association with disease.

To analyze allelic distribution of 'C' and 'T' allele among study groups the researchers applied χ^2 test and the values are presented in Table 7. The researchers observed that wild 'C' allele had a protective effect on disease and when this allele get mutated into 'T' its protective effect get lost and it makes an individual susceptible for hypertension. In this connection the researchers found more individuals carrying 'T' allele in hypertensive patients and an odds ratio for 'C' allele were 0.58 (95% CI, 0.38-0.90) for hypertension indicated significantly higher allele frequency among HC group whereas odds ratio for 'T' allele were 1.34 (95% CI, 0.84- 2.12) for hypertension group indicated approximately double frequency of 'T' allele among hypertensive patients suggesting role of 'T' allele for hypertension susceptibility (Table 7). The 'T' allele frequency was 17 and 13.21 in hypertensive patients and control group respectively. 'T' allele was present in significantly higher proportion in hypertension (P<0.02) as compared with HC group shows to a moderate association of 'T' allele with hypertension susceptibility.

Carriage rate distribution of both 'C' and 'T' alleles were not significantly distributed in hypertension χ^2= 2.357 P=0.1247 as compared with HC group (Table 7).With an odds ratio for carriage of 'C' allele 0.43 (95% CI, 0.12-1.54) hypertension whereas odds ratio for carriage of 'T' allele were 1.2 (95% CI, 0.75- 2.17) hypertension respectively.

DISCUSSION

Homocysteine is an inflammatory vasotoxin, at the damaged sites, homocysteine (Hcy) mediated enhanced lipid peroxidation and generation of free radicals results into inflammation (Dwivedi et al. 2011). An increased homocysteine in the blood is thus related with acute en-

Table 7: Association of individual MTHFR 677C >T genotypes, alleles and their carriage rates with susceptibility to disease in the groups of hypertension compare to control using Fisher's exact test

MTHFR 677	*Study group*	*n*	*%*	*P value*	*Ods ratio*	*95% CA*
C > T Genotypes						
CC	Healthy control	169	75.8	0.4089	0.7823	0.4606 to 1.329
	Hypertensive	71	71			
CT	Healthy control	49	21.9		1.121	0.6419 to 1.959
	Hypertensive	24	24	0.7737		
TT	Healthy control	5	2.2			
	Hypertensive	5	5	0.756	1.242	0.3492 to 4.419
Alleles						
Allele 'C'	Healthy control	387	86.8	0.0204*	0.5885	0.381 to 0.907
	Hypertensive	166	83			
Allele 'T'	Healthy control	59	13.21	0.0204*	1.343	0.84 to 2.12
	Hypertensive	34	17			
Carriage Rates						
Allele 'C'	Healthy control	218	97.75	0.2947	0.4358	0.12 to 1.54
	Hypertensive	95	95			
Allele 'T'	Healthy control	54	24.2	0.4089	1.278	0.75 to 2.17
	Hypertensive	29	29			

* Statistically significant

dothelial dysfunction, and oxidative stress is implicated in inflammatory process. Elevated Homocysteine in blood is well established factor for susceptibility to several cardiovascular diseases but it is not studied for hypertension in Indian population. In this study the researchers analyzed the effect of transition of cytosine to Thymine at 677 positions in MTHFR gene on blood homocysteine concentration and impact of the transition as well as elevated blood homocysteine concentration on susceptibility to hypertension. In the present study the researchers adopted model of case- control study. Medically certified hundred hypertensive patients were analyzed for this purpose. Genotyping was done and blood homocysteine concentration was measured. Obtained results were compared with age and sex and ethnicity matched healthy controls.

Studies with same objective but in different diseases have been done in different countries with almost all types of ethnic populations worldwide, but findings are entirely different from one another. A limited similar study has been done across the world in which the MTHFR C677T polymorphism is analyzed for hyperhomocystenimia as well as susceptibility to hypertension.

This study found elevated blood homocysteine as an independent risk factor for hypertension. Hypertensive patients were found with significantly elevated value ($P<0.0001$) of homocysteine as compared with healthy controls. In the present study elevated homocysteine level is found significantly higher in hypertensive patients and an independent risk factor for hypertension. A study in Chinese population found similar observation where the elevated homocysteine level in blood is significant risk marker for hypertension and MTHFR genotype play important role in this association (Xu et al. 2017). One of the two Chinese study found MTHFR gene expression is statistically different between hypertensive patients and (Fan et al. 2016) and the second study revealed that MTHFR C677T polymorphism is associated with hyperhomocystenimia only not with disease (Zhao et al. 2017). A study on European population showing association of blood homocysteine elevation with carotid artery disease but in this study hypertension and the Genetic polymorphism has been not observed (Catena et al. 2015). A study with the population of morocco showing a positive association between MTHFR TT genotype and susceptibility to hypertension (Sanaa et al. 2015).A study from south west Cameroon MTHFR C677T polymorphism is associated with the hypertension, homocysteine evaluation excluded in this study (Ghogomu et al. 2016).Whereas study on Algerian population showed that there is no association between MTHFR polymorphism and hypertension (Amrani et al. 2016).

India consists of ethnically, geographically, and genetically diverse populations consisting of 4,693 communities with several thousand endogamous groups, 325 functioning languages, and 25 scripts. Due to these diversities in the Indian population in habiting different regions and adapting vary different marriage cultures homozygous mutant allele is less frequent in India. Therefore in Indian scenario findings of studies from different regions are more variable. A study from Jammu region (Raina et al. 2016), North India region (Kumar et al. 2016) in India revealed that MTHFR genotype is significantly associated with diseases. In some other studies MTHFR genotype is not significantly associated especially with Stroke but found in positive correlation with hyperhomocystenimia (Mukherjee et al. 2002; Kumar et al. 2009; Mukhopadhyay et al. 2007).

In this study the researchers found distribution of the MTHFR genotype 71 percent, 24 percent and 5 percent for CC, CT and TT genotype respectively in hypertensive patients. The researchers' findings are consistent with other studies from north India (Kumar et al. 2009). Some studies from same population found 75 percent, 20 percent and 5 percent for CC, CT and TT genotype frequencies respectively (Nevin et al. 2008; Panigrahi et al. 2006).

The present study found a significant higher concentration of total homocysteine for CT and TT genotype. Significant correlation was found between CT genotype and hypertension and hyperhomocystenimia as an independent risk factor for hypertension. Among the different genotypes of MTHFR C677T alleles, CT alleles have shown a weak but significant interaction with hypertension but strong interaction with hyperhomocystenimia.

There were a few limitations in the present study. First- This study was conducted in a small region of North India. Therefore, a study with large sample size needed to confirm findings of this study. Second, the researchers selected only one single nucleotide polymorphism of the MTH-

FR gene whereas homocysteine metabolism is regulated by two other enzymes. Third, the researchers did not considered other parameters like lipid profile of patients with homocysteine. Despite these limitations, this study provides strong evidence for the independent association of MTHFR C677T gene polymorphism and elevated homocysteine level for risk of hypertension in the North Indian population.

CONCLUSION

Findings of the present case–control study suggest that polymorphism in C677T position of MTHFR gene and hyperhomocystenimia might be a risk factor for hypertension in North Indian population.

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