MTHFR GENE POLYMORPHISM AMONG BHILS OF UDAIPUR, RAJASTHAN

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Methylenetetrahydrofolate reductase (MTHFR) ,an enzyme that in humans is encoded by the MTHFR gene, has a crucial role in regulating cellular methylation, through the conversion of 5,10-methyl-tetrahydrofolate(THF) to 5-methyl-THF, the methyle donor in the transformation of homocysteine to methionine. Reduced MTHFR activity due to the C677T change has been associated with different disorders, eg:, vascular disease, neural tube defects, Down's syndrome and preeclampsia and spontaneous abortions. Moreover, many studies have showed the association of this polymorphism with the risk of colon, ovarian and breast cancer. The aim of the study is to screen MTHFR C677T single nucleotide polymorphisms among Bhils of Udaipur, Rajasthan and to compare our results with Indian and world populations. In present study a total number of 78 samples (both blood and buccal) were taken from randomly selected individuals (both males and females, unrelated upto first cousins). DNA extraction from samples was done by using High Yeild Genomic DNA extraction kit (Real Biotech Corporation) and Gentra Puregene Buccal Kit. Further using other molecular techniques like PCR-RFLP using *Hinf I* and gel electrophoresis. In the present study MTHFR gene is found to be polymorphic among the Bhils of Rajasthan with maximum number of CC (92.31%) genotype followed by CT (6.41%) and least by TT (1.28%) genotype. The population is not in the Hardy Weinberg Equilibrium with respect to the MTHFR polymorphism ($P<0.05$).

INTRODUCTION

Methylenetetrahydrofolate Reductase is a critical enzyme that catalyzes the conversion of 5,10-methylenetetrahydrofolate into 5-methyletetrahydrofolate, a co-substrate which serves as a methyl donor in the reaction that converts homocysteine to methionine. Methionine is subsequently converted to S-adenosylmethionine, which serves as an essential methyl donor in reactions involving nucleic acids, proteins, and many other biological compounds. The gene encoding MTHFR is located at the position of p36.3 of chromosome no.1 and is 2.2 kb in length with a total of 11 exons. Molecular defects in the MTHFR gene reduce the enzyme's activity which results in increased plasma homocysteine , and supplementation of folate being an important co-factor in the conversion of homocysteine to methionine, may compensate for this effect . Hyperhomocysteinemia is a risk factor for many complex disorders like coronary artery disease (Frosst *et al.,* 1995, Sinha *et al.,* 2010), neural tube defects and recurrent pregnancy loss (Wilcken *et al.,* 1997). There are two commonly recognized polymorphic variants in the gene encoding for this enzyme: variant 665C→T (p.Ala222Val), historically more commonly referred to as C677T, and the c.1286A→C (p.Glu429Ala) variant; both are missense changes that are known to decrease enzyme activity. The *C677T* mutation exists in exon 4 at position 677, it is a point mutation that converts a cytosine (C) to a thymine (T), resulting in an amino acid substitution of alanine to valine (Frosst et al). The T variant codes for a thermolabile enzyme leading to an activity of 65% in the heterozygous state (CT) and ~30% in the homozygous state (TT), respectively. MTHFR gene mutation has been related to many diseases including colon cancer, leukemia, vascular disease, and depression, and schizophrenia, migraine with aura, glaucoma, Down syndrome, and neural tube defects. The frequency of the C677T allele is subject to considerable ethnic and geographic variation, with a high allele frequency being reported in California Hispanics and a low allele frequency in US Blacks (*Am J Epidemiol* Vol. 151, No. 9, 2000). There is also marked variation in the frequency of C677T homozygote variants between populations. The highest frequency (>20 percent) is found among US Hispanics, Colombians, and Amerindians in Brazil; conversely, in Black populations, less than 2 percent have the variant genotype. Among White populations in Europe, North America, and Australia, the frequency ranges from 8 percent to 20 percent, although interestingly in Europe there seems to be a drift in the occurrence of the homozygote variant from north to south. Because of the changing life styles and dietary habits, like in any developing country the number of patients suffering from complex disorders is increasing in India and there is a need of knowing the distribution pattern of various candidate genes for these disorders in general population. The Indian Genome Variation Consortium had included MTHFR C677T as one of its SNPs but it failed to demonstrate clear cut

population variation. Considering the implication of MTHFR gene in various complex disorders coupled with its high heterogeneity among different ethnic and regional population of the world, the present study was planned to investigate the heterogeneity of this gene in Bhil population of Udaipur, Rajasthan, India.

OBJECTIVES

To screen MTHFR C677T SNP among Bhils of Udaipur district in Rajasthan.

- To study genetic variations in terms of MTHFR gene polymorphisms among the Bhils of Udaipur.
- To understand the status of Bhils of Udaipur district of Rajasthan with special reference to MTHFR C677T single nucleotide polymorphisms in Indian and world context.

MATERIALS AND METHODS

Studied Area

A total of 78 random blood and buccal samples were collected from individuals irrespective of genders from Bhils of Udaipur, Rajasthan. Care was taken to avoid blood relatives up to the first cousin during the sampling. Ethical clearance was obtained from the Departmental Ethical Committee, Department of Anthropology University of Delhi. Consent forms were filled up.

Wet Lab

- Extraction of Genomic DNA from blood

Blood samples were collected into sterile EDTA vaccutainer and were stored at 4°C until processing. Genomic DNA was isolated from the peripheral blood as per the standard procedure that consists of erythrocyte lysis by Erythrocyte Lysis Buffer (ELB) till a white pellet free of heme was obtained. Leukocytes /lymphocytes lysis carried out by suspending white pellet in ELB and on mixing with 270µl of 20% SDS and 30µl of proteinase K (10mg/ml) were added and mixed. Samples were incubated at 370C, in water bath for overnight. After 16 h of incubation, DNA was precipitated by isopropyl alcohol. The total precipitated DNA appeared as a white thread like structure was

completely transferred to a sterile micro centrifuge tube containing 500µl of 80% alcohol and incubated at room temperature for 15 min. It was centrifuged at 12000 rpm for 5 min. The supernatant obtained was discarded and this step was repeated for three times to obtain purified form of DNA. The DNA was then air dried and dissolved in 500µl of Tris-EDTA buffer and then incubated at 65°C for 30 min. The dissolved fraction was refrigerated at 4˚C for one day and stored at -20^0 C until use.

- Genomic DNA extraction from buccal swabs:

To collect buccal cells, collection brushes were dispensed in 300 µl Cell Lysis Solution into a 1.5 ml microcentrifuge tube. For completing cell lysis then it was incubated at 65°C for at least 15 min. Then 1.5 µl Puregene Proteinase K (cat. no. 158918), was added and mixed by inverting 25 times, and incubated at 55°C for at least 1hour. Adding 100 µl Protein Precipitation Solution, and vortex was done vigorously for 20 s at high speed. After another Incubation, centrifugation for 3 min at 13,000–16,000 x g. The precipitated proteins should form a tight pellet. 300 µl isopropanol and 0.5 µl Glycogen Solution into a clean 1.5 ml microcentrifuge tube, and add the supernatant from the previous step by pouring carefully. 300 µl of 70% ethanol added. 100 µl DNA Hydration Solution added and vortexed for 5 s at medium speed to mix. Incubating at room temperature was done overnight with gentle shaking. Samples can then be centrifuged briefly and transferred to a storage tube.

- DNA quantificationby Agarose gel electrophoresis

The quantity of the DNA samples was checked in a 1% agarose gel. The solution was allowed to become lukewarm and 0.1mg/ml ethidium bromide was added. The gel was then poured on a gel-casting tray and allowed to solidify. The gel was placed in an electrophoresis tank with 1X TAE buffer. The DNA samples were mixed with 6X DNA loading dye and loaded on the gel. The gel was electrophoresed at 2 volts/cm and images were captured in a gel documentation system (Bio Rad Gel Doc).

- DNA purity – Spectrophotometric method

The quality and quantity of the DNA samples were assessed by spectrophotometric method by using Perkin Elmer Lambda 35 model. 50µl of TE buffer was pipetted into quartz cuvette and subjected for auto zero correction. 48µl of TE buffer and 2 µl of DNA sample were added in quartz cuvette, the absorbance was measured at 260 and 280nm. The absorbance at 260nm gives DNA concentration

and the ratio between 260/280 gives the purity of DNA. DNA sample with 260/280 absorbance ratio between 1.7-1.9 were considered for PCR procedure. However, DNA samples with absorbance ratio less than 1.7 were subjected for precipitation until the desired absorbance was obtained. Then after, DNA sample of expected purity was used for PCR procedure.

- Genotype Screening

For identification of MTHFR C677T gene mutation PCR-RFLP was performed in the present study.

- PCR-RFLP –Based Screening

PCR amplification of the MTHFR C677T gene was amplified by polymerase chain reaction using modified primers [primer sequence (F5-TCTTCATCCCTCGCCTTGAAC-3; R5-AGGACGGTGCGGTGAGAGTG-3) according to Frosst *et al*. (1995)]. PCR was carried out on 50µl volume, in an Eppendorf thermal cycler and subjected to standardized PCR. Initial denaturation at 950C for 5 min followed by 35 cycles of denaturation at 950C for 1 min; annealing at 610C (MTHFR C677T) and extension at 720C for one minute and final extension at 720C for seven minutes were performed. PCR Amplification was confirmed by 2% agarose gel electrophoresis. 100bp DNA molecular weight marker was used to confirm the amplicon size. Electrophoresis was carried out at 80V for one hour and the gel was visualized in the gel documentation system. Finally, the amplified products were digested with 10U Hinf 1 restriction enzyme as shown in and digested products were separated on 2% agarose gel stained with Ethedium Bromide and visualized in the Gel Doc System.

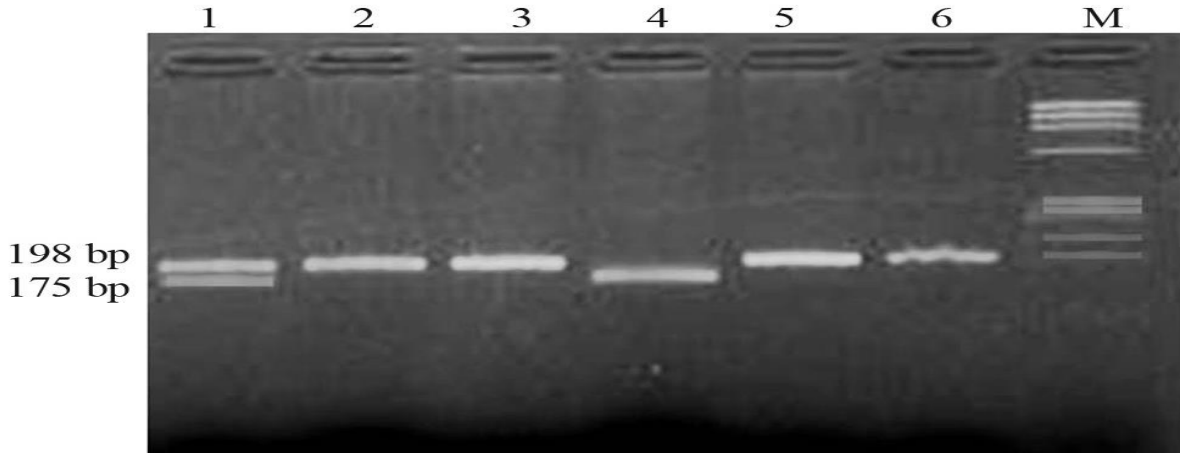


Figure : Detection of MTHFR polymorphism. Lanes 2, 3, 5 and 6 showing wild type (CC) genotype, lane 1 showing mutant heterozygous (CT) genotype and lane 4 showing mutant homozygous (TT) genotype, M showing molecular weight Marker**.**

- Statistics

Genotype and allele frequencies were calculated by gene counting method using POPGENE software. Conformity to Hardy–Weinberg equilibrium (HWE) of the population was performed by goodness of fit Chisquare test. Statistical significance was checked at 5% level of probability.

RESULTS

After applying the genotypic data of MTHFR gene polymorphic to statistical analysis, we get the following results:

- ❖ Homozygous C genotype is present in the population with the frequency of 92.31%
- ❖ Heterozygous CT genotype accounts for 6.41% frequency in the population.
- ❖ Homozygous T genotype is present at 1.28% in the population of the sample size of 78 individuals.

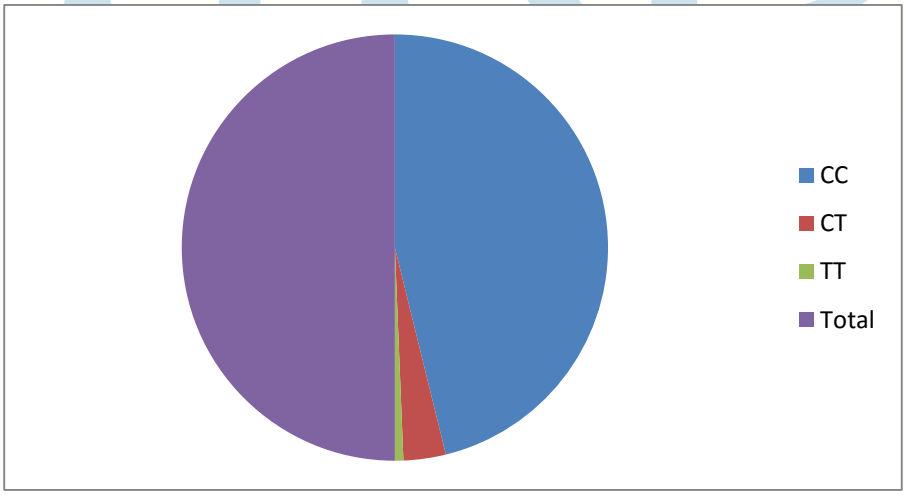


Figure: Graphical representation of allele frequency of MTHFR C677T polymorphism among Bhils of Udaipur, Rajasthan.

On testing for Hardy- Weinberg ,the chi-square value for Bhils of Dhinkli Panchayet, Udaipur we found that the population is not in the Hardy Weinberg Equilibrium with respect to the MTHFR polymorphism ($P<0.05$). Here alleles are 2 (two categories), so degree of freedom is one less than

that: 2-1=1. The table value for 1 d.f. is 3.841. The calculated value 1.14 is smaller than the table value (3.841), therefore the differences between expected and observed values are NOT significant. So the hypothesis is that the observed and expected numbers are statistically non-significant.

ALLELE/LOCUS	**FREQUENCY**
ALLELE C	0.96
ALLELE T	0.05

Table: Allele frquencies of MTHFR C677T polymorphism Bhils of Dhinkli Panchayet, Udaipur.

The allele frequency of the Bhils for the "C" allele is 0.96, whereas for the "T" allele it is 0.05.

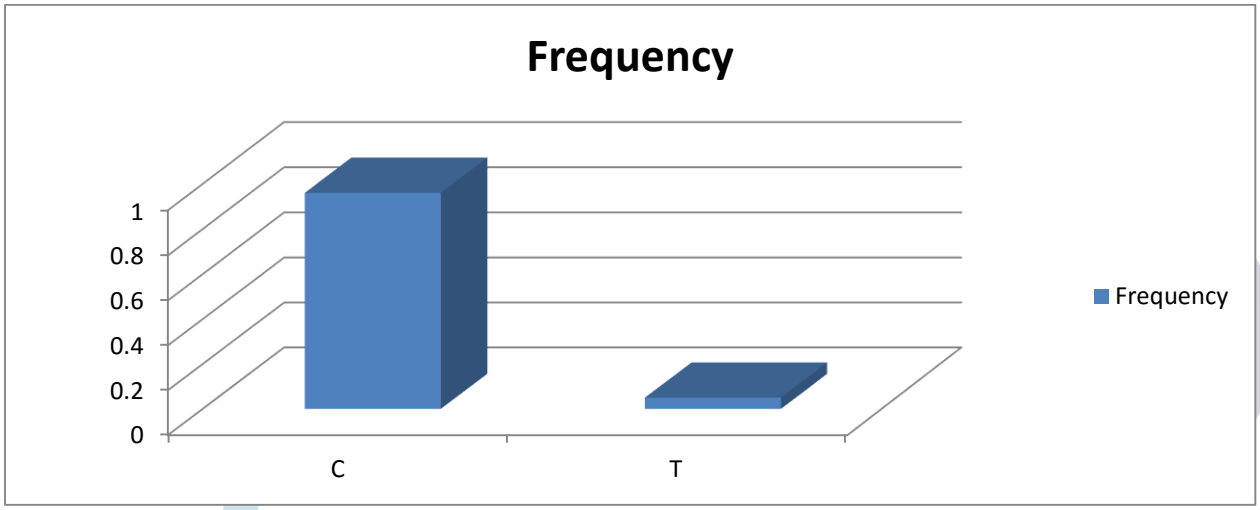
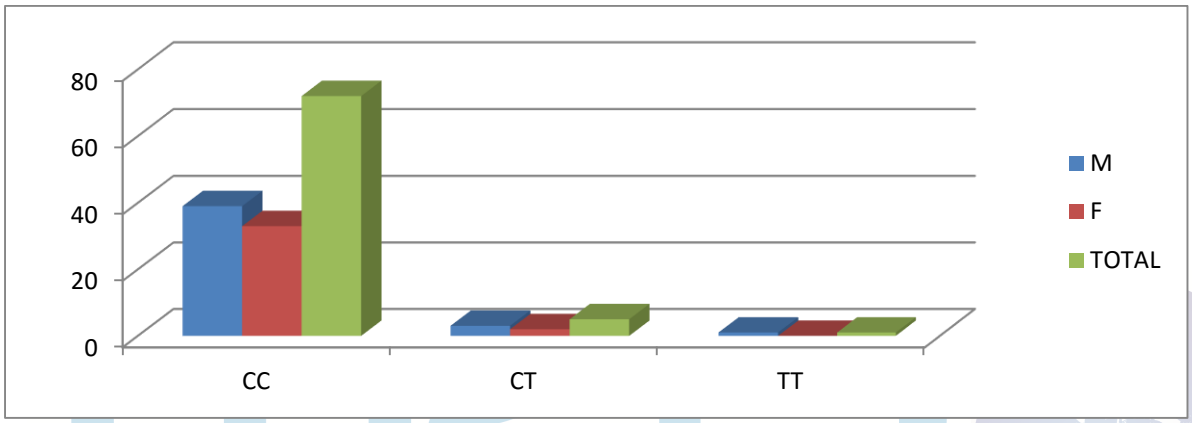


Figure : Graphical representation of allele frequency of MTHFR C677T polymorphism among Bhils of Udaipur, Rajasthan.

Sl No.	Sex	Source Of DNA	Number of individuals			Percentage		
			CC	CT	TT	CC (%)	CT (%)	TT (%)
1	M	Blood and Buccal	39	3	1	54.17	60	100
2	F	Blood and Buccal	33	2	0	45.83	40	0
Total			72	5	1	100	100	100

Table : Distribution of the Genotypes and Allele Frequency of MTHFR C677T Polymorphism among the Bhils of Dhinkli Panchayet, Udaipur



Distribution of the Genotypes and Allele Frequency of MTHFR C677T Polymorphism among the Bhils of Dhinkli Panchayet, Udaipur.

Among 78 subjects total number of homozygous C male is 39 and homozygous female is 33. On the other hand, heterozygous CT male is 3 and female is 2 in numbers. Homozygous T male is found to be 1 in number whereas no female homozygous T subject was found. Hence the homozygous C male among 72 individuals of Bhil population is 54.17% and female has 45.83% homozygosity of C. Homozygosity for T allele in male is of 100% and females have no homozygosity for T allele. Heterozygous CT in male individuals was found in 60% and females have 40% of heterozygous CT. MTHFR gene was found to be polymorphic at C677T site in the population, studied.

DISCUSSION

MTHFR 677T allele frequency in the present study (1.28%) was lower than the average frequency (14%) reported by Indian Genome Variation Consortium. The incidence of 677T allele was found to be invariably higher among castes compared to tribes. The incidence was also found to be comparatively higher among the north Indian populations, followed by those of north-east, west,

south and east India. The presence of TT genotype among all non-tribal populations except Meitei with Mongoloid background, and also a high frequency of 677T allele among the caste populations particularly from north India compared to tribal populations, is indicative of the gene flow into caste populations of north India from Eurasian populations where the 677T allele frequency is very high. Globally, 677T allele is found to be highest in Europe with a frequency ranging from 24.1 to 64.3%, followed by North America (6–64.3%), East Asia (2–55%), South America (2–48.7%), Asia (2.5–45%), Africa (0–35.5%), Siberia (8–31.5%) and Oceania (2.9–28.6%). In African populations the allele is often reported absent. Considering the higher frequencies of 677T observed in Europe and North America, it can be presumed that the allele originated in Europe in the late stage of human evolution. The migration, settlements and colonisation might have helped in spreading this allele to other regions. North America which has more of European migrants has higher 677T allele frequency than South America, mainly inhabited by indigenous populations. Higher frequency of this allele among the Caucasoid groups compared to Mongoloid and Negroid groups also supports the above hypothesis. However, we cannot rule out the possible multi centric origin of this mutant allele as it is observed to be relatively high among some South East Asian populations and African populations. Lighter skin colour and more exposure to ultraviolet rays is a disadvantage for 677T allele carriers because of dermal photolysis of folate and thus a south to north cline of decrease in the allele frequency is apparent in European populations. On the other hand, in the present study and as reported elsewhere, a reverse trend was observed, i.e., lower frequency of 677T among dark skin coloured tribes of south, west and east India, and higher frequency of the allele among the light skin coloured Castes of north and north-east India. Moreover, since this allele is an important clinical marker associated with many complex disorders, the dietary habits might have had a significant influence in the propagation of 677T in the world populations. The T allele frequency of MTHFR C677T is found to vary across world populations. The frequency of the T allele of the MTHFR C677T polymorphism reaches frequencies as high as 64.3% among European populations while registering low frequencies in African populations. Among Indian populations, Caucasian populations of North India (3% among Ahirs to23.75% among Sindhi) and Mongoloid populations of North East India (0% among Koms to 23.1% among Lothas) are found to have relatively higher frequency of T allele as compared to Central (0% among Munda, 2.22% among Oraon) and South Indian (4.29% among Nayakpod to 11.7% among Thoti), Dravidian populations and including our studied population (1.28%). In comparison with the Mongoloid populations of Southeast Asia (16.7% among Japanese to as high as 55.2% among Chinese) the T allele frequency of our studied population was found to be low. In general, Mongoloid populations of India show very high frequencies of the T allele as compared to other non-Mongoloid populations of India. However, they

show a relatively lower frequency of T allele when compared with the Mongoloid population of Southeast Asia. Furthermore, T allele frequency among these Mongoloid populations was found lower than that of European population. Considering the higher frequencies of T allele observed in European populations, people have hypothesized that the T allele originated in Europe in the late stage of human evolution and later spread to various parts of the world. T allele is found to be implicated in various complex disorders as MTHFR is involved in a metabolic pathway that, if blocked, results in hyperhomocysteinemia, which could lead to many pathological conditions. However, some studies report a positive association of MTHFR T allele with various diseases, while some report no association. Undoubtedly, the T allele is increasing in populations, which goes against selective disadvantage that it should have, when associated with various diseases.

Moreover, these complex diseases often set in later life (ie, after reproductive age) and, by then, the allele is already passed on to the next generation, which could be partly responsible for the increased frequency of the T allele. Further, recent studies have also reported a heterozygous advantage for this allele, which would be another reason for the increase of this allele in various populations. In addition to the relatively low mutant allele frequency in our study population, it is likely that the more sedentary lifestyles they are beginning to adopt will further put them at little or no risk of complex diseases as proposed by Murry et al.15 Mayor-Olea et al20 reviewed reported data on individuals born in the southern Spain in four groups according to birth date (1900 to 1925; 1926 to 1950; 1951 to 1975; 1976 to 2000); they found an increase in the frequency of T allele and TT genotype in those born in the last quarter of the century. In our study population, the T allele frequency was found to be slightly lower in the younger age group (50 years) than in the older age group (50 years); however, the difference was not statistically significant. This is suggestive of the selective disadvantage of this allele in recent years. Thus, data generated in our study offers more information on possible causes of complex diseases among the Bhils. Health planners can use this information to develop health programming.

In recent years a large number of studies on MTHFR gene, particularly on C677T SNP has been conducted because of clinical importance of the gene. The enzymatic function of the gene is reduced up to 70% by 677T mutation which leads to hyperhomocysteinemia. Similarly, the MTHFR 1298C mutation also reduces the enzyme activity but to a lesser extent. However, this detrimental effect of mutations can be compensated by the supplementation of folic acid. In other words, apart from mating patterns, migratory histories and ethnic backgrounds, environment, i.e., folic acid in the present case plays an important role in the propagation of these two mutant alleles in human populations. The role of MTHFR in the patho-physiology of various complex disorders is still debatable despite the fact that hyperhomocysteinemia is associated with these disorders. The

homozygote 677TT genotype was reported to have a four-fold higher frequency among neonates in comparison to aborted foetuses. In another study, both T allele and TT genotype were reported to be significantly higher in the age group 0–24 years compared to 25 years and above. A similar study showed that these allele and genotype gradually increased in each quarter and were found to be highest in subjects born in the last quarter of the twentieth century. These studies indicate a selective advantage for T allele in human populations or a lesser disadvantage of carrying it, which could possibly be because of the improved nutritional status, especially with folic acid and vitamin B12 supplementation in course of time.

In conclusion, the present study demonstrates that the MTHFR gene is polymorphic among the Bhils of Rajasthan. Significant departures from HWE in the population indicated that selective pressures were acting on these alleles. Since MTHFR C677T is important clinical markers influenced by food habits, further studies are desirable taking into account the dietary habits of populations for an in depth understanding of these SNPs in India. Nonetheless, this study has provided baseline data for future studies from multi-ethnic India, where each population group has its own strict mating pattern, dietary habit and history of origin. The present study provides baseline data on haplotypes of C677T SNPs in people of India and also suggests that sub-lethal alleles of MTHFR gene may attain balanced polymorphism in a population depending on the surrounding cultural and environmental variables. Therefore, unlike the present population genetics study, the disease association studies need to be comprehended taking all the bio-social determinants of the population into consideration.

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