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Original Article

Study of C677T Methylene Tetrahydrofolate Reductase Gene Polymorphism as a Risk Factor for Neural Tube Defects

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Abstract

Introduction: Various genetic and environmental factors contribute to the development of neural tube defects (NTDs) which are a group of neurulation defects resulting from failure of closure of embryonic neural tube. Among genetic factors is polymorphism in methylene tetrahydrofolate reductase (MTHFR) gene, giving rise to a gene variant or mutant. However, in most studies directed at finding an association between MTHFR variants and NTD, there is no clear evidence of a cause-and-effect relationship. **Materials and Methods:** Forty diagnosed cases of NTDs and forty healthy individuals were investigated in a case–control study for presence of C677T MTHFR gene polymorphism. Serum folate and Vitamin B12 levels were estimated and MTHFR gene polymorphism was detected by polymerase chain reaction-restriction fragment length polymorphism. **Results:** It was found that 32 cases were homozygous with CC genotype and eight were heterozygous with CT genotype, whereas 35 controls had CC genotype and five had CT genotype. TT genotype was absent in both the groups. There was no statistically significant difference between both the groups. No evidence of association between MTHFR C677T polymorphism and NTDs was found. **Conclusion:** Although there was no evidence of association between MTHFR C677T polymorphism and NTDs, our study does not rule out the impact of MTHFR gene mutation on folate metabolism. The reason for absence of TT genotype and no association could be a small sample size. Larger, comprehensive, and well-designed multicentric but feasible studies involving proper subjects and appropriate and adequate controls from several hospitals may provide more meaningful data.

Keywords: *Allele, association, methylene tetrahydrofolate reductase, neural tube defect, polymorphism*

Introduction

Neural tube defects (NTDs) are group of severe congenital malformations that occur as a result of failure of closure of embryonic neural tube properly during early development. The identification of the causative factors is confounded by the complex interplay of the genetic, metabolic, and environmental components. Genetic abnormalities in folate-related enzymes could probably explain the role of folate in preventing NTDs. One of the critical genes to play significant role in folate metabolism is methylene tetrahydrofolate reductase (MTHFR). The hypothesis that an underlying genetic susceptibility interacts with folate-sensitive metabolic processes at the time of neural tube closure is very pertinent in context to the development of NTDs.[1] The risk that parents with known MTHFR mutations will have a baby with a NTD is extremely low and far less than 1% as the genetic variant alone may not be the causative factor in the development of NTDs.[2] An increased risk for NTDs has been predicted if the infant or the mother has homozygous MTHFR TT genotype. In many studies, it has been found that there is a significant association between MTHFR 677C>T and increased risk of NTDs.[3-5] Studies by De de Franchis *et al.* and Shields *et al.* supported the association between NTDs and MTHFR 677TT,[6,7] while Behunova *et al.*, Félix *et al.,* and Perez *et al*. did not find any such association.[8-10] To date, there are neither large-scale, well-designed epidemiological studies that explicitly prove that either of these MTHFR variants cause speculated health effects nor clear evidence establishing the clinical utility of genotyping for MTHFR in guiding drug

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therapy for any indication. Despite an exhaustive scientific literature on the effect of common MTHFR variants, there is apparently no convincing evidence linking MTHFR association to most of the health conditions.

With the above background, the present study was undertaken to investigate the levels of Vitamin B12 and folate in serum samples of patients with NTDs compared with healthy controls, to investigate genetic polymorphism of MTHFR in NTDs and to evaluate the correlation between Vitamin B12, folate levels, and pattern of gene polymorphism in NTDs in patients attending Post Graduate Institute of Medical Sciences (PGIMS) Hospital, Rohtak, Haryana.

Materials and Methods

Subjects

The present study was conducted in the Department of Biochemistry, in collaboration with the Department of Neurosurgery Microbiology, Biotechnology and Molecular Medicine, Pt. B. D. Sharma, PGIMS, Rohtak, and Department of Genetics, M. D. University, Rohtak, with strict accordance to the protocol approved by the ethical committee and after taking written consent from all the subjects. Forty diagnosed cases of NTDs and forty apparently healthy volunteers as controls were included in the study. In this study, 16 patients had meningomyelocele, eight had lipomeningocele, nine had dorsal dermal sinus, and seven had split cord malformations [Figure 1]. The mean and standard deviation (mean ± SD) of age of cases and controls were 12.32 ± 7.27 years and 28.42 ± 5.79 years, respectively. Among forty cases with NTDs, mothers of 16 cases had consumed folic acid in their periconceptional period, while the mothers of the remaining 24 patients did not consume folic acid at all.

Materials

All chemicals and reagents used were of analytical/molecular biology grade from Sigma, USA, unless mentioned otherwise and unless otherwise specified were used as per manufacturer's instructions. Ready-to-use ×20 TBE Buffer was obtained from Amresco, USA. SDS, Proteinase K, and ethidium bromide were obtained from Sigma, USA. Absolute ethanol was obtained from Merck (Germany). DNA molecular weight markers, 6X DNA loading dye, and dNTPs were obtained from MBI Fermentas/Thermofisher (USA). Primers for polymerase chain reaction (PCR) were got synthesized from Eurofins and Taq DNA polymerase and restriction Enzyme *Hinf I* were from Thermo Fisher, USA. Enzyme-linked immunosorbent assay (ELISA) kits for serum folate and Vitamin B12 were obtained from Weldon Biotech, USA.

Methylene tetrahydrofolate reductase gene

The MTHFR gene is located on the short (p) arm of chromosome 1 at position 36.3 from base pair 11,769,246 to base pair 11,788,568. The complete MTHFR gene comprising 4261 nucleotides as retrieved from the National Center for Biotechnology Information (NIH), USA; Acc. No. AH007464, is shown in Figure 2a. The location of the amplified fragment of 294 bp is shown on the complete MTHFR gene from position 1196 to 1489 and highlighted in gray [Figure 2b].

Methods

Extraction of genomic DNA from blood samples

DNA was extracted from EDTA anticoagulated 2-mL venous blood from all the patients and healthy individuals, participating in the study. DNA was extracted from buffy coat using phenol-chloroform method.[11] The concentration of DNA was measured using spectrophotometry and quality was checked through agarose gel electrophoresis by resolving the DNA on 0.8% agarose gel.

Polymerase chain reaction-restriction fragment length polymorphism

PCR was performed on a semi-quantitative end point thermal cycler (Applied Biosystems, USA). The C677T MTHFR polymorphism was genotyped as previously described.[12,13] Briefly, we used the forward primer 5'-CCT TGA ACA GGT GGA GGC CAG-3' and the reverse primer 5'-GCG GTG AGA GTG GGG TGG AG-3'. The reaction mixture was denatured at 95°C for 10 min, and the PCR reaction was performed for 35 cycles under the following conditions: denaturation at 95°C for 1 min, annealing at 65°C for 30 s, extension at 72°C for 1 min, and a final extension cycle of 72°C was for 7 min. The PCR products were digested with *HinfI* and analyzed on agarose gel (3%).

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Folate and Vitamin B12 estimation

Serum folate and Vitamin B12 levels were estimated by ELISA kit method, manufactured by Weldon Biotech. The experiment was performed on TECAN (Switzerland) plate reader equipment as per the manufacturer's protocol. Concentration of samples was calculated after entering the OD values of the samples plotted against the standard curve. Final results of serum folate/Vitamin B_{12} were calculated in ng/mL or pg/mL.

Statisitical analysis

The data were compiled and analyzed by IBM SPSS Statistics Software for Windows Version 19, Armonk New York, IBM Corp using appropriate statistical methods. Results were expressed as mean ± SD and the Chi-square test was applied for ordinal and nominal variables. Data were considered statistically significant if $P \leq 0.05$.

Results

A single fragment of 294 base pairs (bp) was identified as homozygous (CC); three fragments of 294, 168, and 126 bp

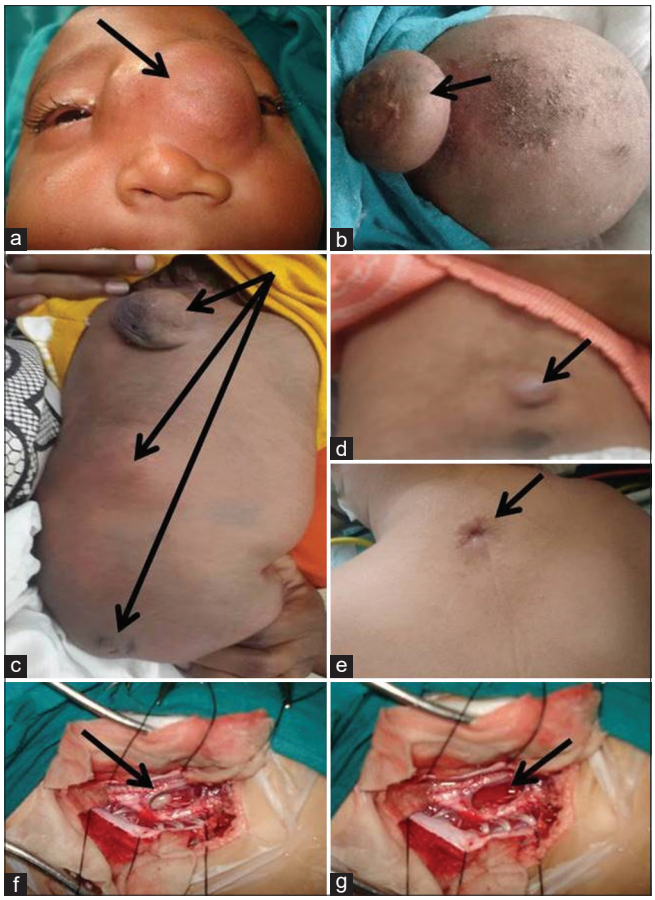

Figure 1: Spectrum of neural tube defects investigated in this study – arrows showing the specific neural tube defect lesions (a) Ethmoidal encephalocele (b) Occipital encephalocele (c) Cervical myelomeningocele, thoracic lipomeningocele with stain in sacral region (multiple neural tube defects). (d) Lumbosacral lipomeningocele (e) Dorsal dermal sinus (f and g) Intraoperative photographs of split cord malformation showing bony spur before and after surgical excision

were identified as heterozygous (CT); and two fragments of 168 and 126 bp were identified as homozygous (TT) genotype [Figure 3a and 3b].

The genotype distribution and the relative allele frequencies for the MTHFR gene C677T polymorphism in children with NTDs and healthy controls are shown in Tables 1 and 2, respectively and graphically represented in Figure 4a and b respectively. In our study, we found that 32 cases were homozygous with CC genotype and eight were heterozygous with CT genotype, whereas 35 controls had CC genotype and five had CT genotype. TT genotype was absent in both the groups. No statistically significant difference was found between both the groups. The Chi-square value was 0.83, $P > 0.05$ (0.36) [Table 1]. Serum folate levels were low in cases compared to controls (1.62 ± 0.34 ng/ml vs. 5.37 ± 1.67 ng/ml) as shown in Figure 5a. The mean ± SD of folate concentration in cases with homozygous CC genotype was 1.72 ± 0.30 ng/mL, whereas in heterozygous CT cases, folate concentration was 1.23 ± 0.21 ng/mL. The difference was statistically significant ($P < 0.001$).

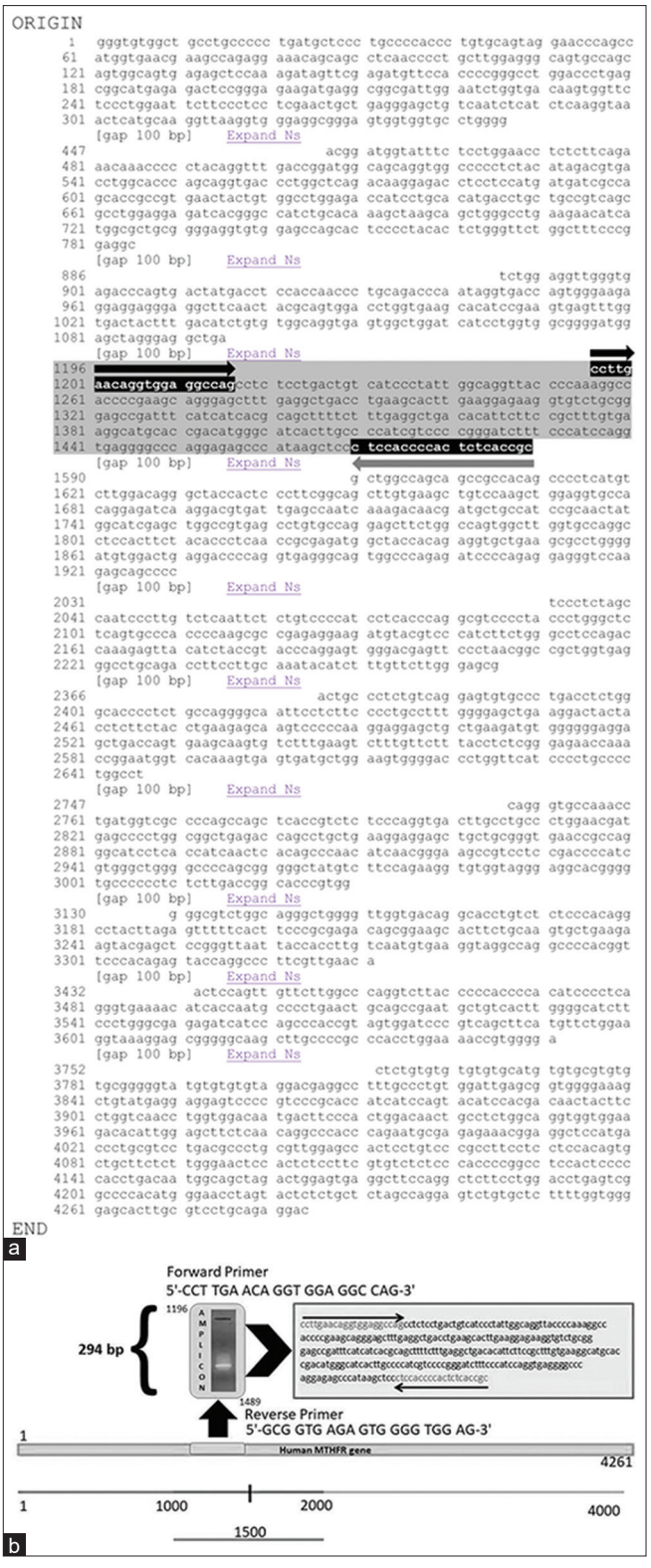

Figure 2: (a) Structure of human methylene tetrahydrofolate reductase gene (NCBI Acc. No. [AH007464]) with location of amplicon (b) Location of forward and reverse primers on amplified methylene tetrahydrofolate reductase gene fragment

Folate concentration was not measured genotype wise in controls because all healthy controls were supposed to give apparently similar results (in normal range 2–20

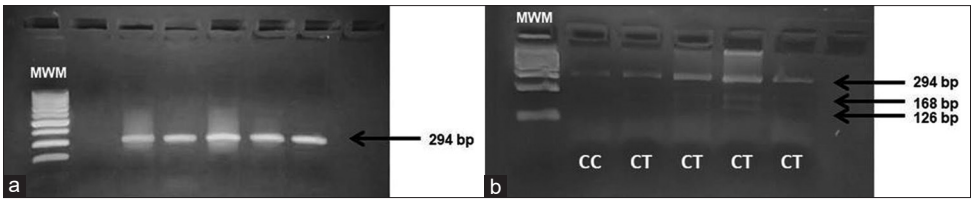

Figure 3: (a) Polymerase chain reaction analysis of methylene tetrahydrofolate reductase gene (a) Representative photograph showing amplification of a 294 bp amplicon from human methylene tetrahydrofolate reductase gene from human methylene tetrahydrofolate reductase gene (b) A representative photograph showing HinfI-restriction fragment length polymorphism analysis of methylene tetrahydrofolate reductase C677T amplicon restriction digests

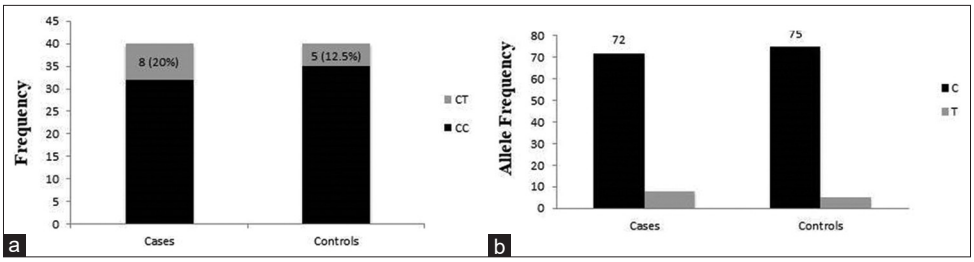

Figure 4: (a) Bar graph showing %age frequency of methylene tetrahydrofolate reductase genotype at nucleotide C677T (b) Bar graph showing allele frequency of the C677T variation

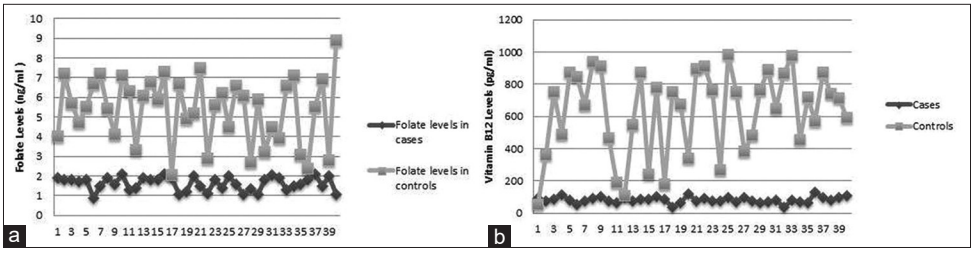

Figure 5: (a) Serum folate levels in case and control groups (b) Vitamin B12 levels in case and control groups

Table 1: Genotype frequencies of the C677T in methylenetetrahydrofolate reductase in cases and control groups

MTHFR genotype at nucleotide	Cases (%)	Controls (%)	χ^2	*P*
CC	32 (80)	35 (87.5)	0.83	0.36
CT	8 (20)	5 (12.5)		
Total	40	40		

Chi-square test was used to test the significance.
MTHFR – Methylenetetrahydrofolate reductase

Table 2: Allele frequency of the C677T variation in cases and control groups

MTHFR allele at nucleotide 677	Cases (%)	Controls (%)	χ^2	*P*
C	72 (90)	75 (93.75)	0.754	0.38
T	8 (10)	5 (6.25)		

P value was calculated using Chi-square test.
MTHFR – Methylenetetrahydrofolate reductase

ng/mL) as none of them were given additional folate supplementation externally. Serum Vitamin B12 levels were measured in both cases and controls. Vitamin B12 levels were low in the cases (82.77 ± 19.12 pg/mL) when compared to the controls (632 ± 261.06 pg/mL) and the difference was statistically significant ($P < 0.001$) as shown in Figure 5b. Serum folate levels showed a significant positive correlation with Vitamin B12 in cases ($r = 0.32$) ($P < 0.05$) [Figure 6]. Concentration of Vitamin B12 was also measured in cases genotype wise. The mean ± SD of Vitamin B12 concentration in cases with homozygous CC genotype was 84.62 ± 19.98 pg/mL, whereas in heterozygous CT cases, Vitamin B12 concentration was 75.38 ± 13.87 pg/mL. The difference was not statistically significant ($P > 0.05$) (0.22). Cobalamin deprivation may lead to functional folate deficiency which can be explained through methyl trap hypothesis which states that Vitamin B12 deficiency can cause lowered levels of methionine synthetase, which in turn results in a functional folate deficiency by trapping an increased proportion of folate as the 5-methyl derivative.[14] A positive correlation was found for folate and Vitamin B12 levels in both the genotype (CC, CT) groups, with the values of folate and Vitamin B12 being 1.72 ± 0.30 ng/mL and 1.23 ± 0.21 pg/mL and 84.62 ± 19.98 ng/mL and 75.38 ± 13.87 pg/mL, respectively. Hemoglobin, blood glucose, blood urea, serum fluoride, and serum calcium levels were comparable in both the groups.

Discussion

NTDs exert major burden on public health worldwide. In India, the incidence of NTDs ranges from 0.5 to 11/1000 live births with the highest incidence in North India.[15] This is due to its multiple patterns of complex inheritance involving nutritional, environmental, and genetic factors.[16,17]

Many studies have attempted to find out the association between MTHFR gene polymorphism and susceptibility to NTDs. Some of these studies are tabulated in Table 3. Whereas an association was found to be present between C677T and NTD risk factor in the studies by Relton *et al*., Kirke *et al.*, Mutchinick *et al*., Muñoz *et al*., Devi *et al*., and Cunha *et al*.,[18-23] no association was found in the studies conducted by Boduroğlu *et al.* (1999), Kondo *et al.*, Fisk *et al.*, Naushad and Devi, Behunova *et al.,* and Nauman *et al*.[24-28] Similarly, C677T MTHFR polymorphism was not found to be a risk factor in the studies conducted by Erdogan *et al.*, Barber *et al*., Mornet *et al.,* and Pardo *et al*.[29-32] Nasri *et al.,* in their study to find a possible association between MTHFR gene polymorphism and NTD in Tunisian parents found that, TT genotype and T allele in MTHFR C677T significantly decreased the incidence of NTDs in the mother group, but significantly increased this incidence in the father group.[33] A good deal of interaction occurs between Folate, Vitamin B12, Homocysteine and MTHFR [Figure 7]. Richter *et al.* investigated if the interaction of folate and homocysteine pathway genotypes leads to susceptibility to NTDs in a German population but did not find significant difference in allele and genotype frequencies for MTHFR gene polymorphism.[34] Nishio *et al*. while investigating the association between folate intake and serum folate levels in Japanese subjects found that Japanese people with the TT genotype had lower serum folate levels than those in people with the CT or CC genotypes, even after the folate intake was adjusted.[35] Deb *et al.* in a community-based case–control study from North India found that Muslim NTD mothers had higher TT genotype, showing an increased risk for NTDs and lower folic acid supplementation, whereas there was a marginalized increased risk for NTDs with vegetarian diet among Hindu counterparts.[36] A good deal of literature is available that demonstrates that individuals with the TT genotype have lower serum folate concentrations than those with the CC genotype. Nonetheless, we did not find any focused and well-designed study which could relate folate, Vitamin B12, hyperhomocysteinemia, and MTHFR gene mutation with regard to NTDs.

Table 3: Studies from 19 countries showing relationship between methylenetetrahydrofolate reductase gene polymorphisms and neural tube defects among subjects of different ethnicities

Study	Study type	Population type/ ethnicity	Subjects	Presence/absence of association or MTHFR polymorphism as a risk factor for NTD
Hayati *et al*., 2008	Case-control	Malay	22 cases, 20 controls	No association
Sadewa *et al*., 2002	Case-control	Indonesian Javanese	68 cases, 244 controls	No risk factor
Dutta *et al*., 2017	Case-control	North East Indian	40 cases, 80 controls	Possible risk factor
Boduroglu *et al*., 1999	Case-control	Turkish	91 cases, 93 controls	No association
Erdogan, 2010	Case-control	Turkish	33 children, 46 controls	No association, no risk factor
Behunova *et al*., 2010	Case-control	Slovak	93 cases, 290 controls	No association
Barber *et al*., 2000	Case-control	American Hispanic	149 cases, 195 controls	No risk factor
Mornet *et al*., 1997	Case-control	French	43 cases, 133 controls	No risk factor
Munoz *et al*., 2007	Case-control	Mexican	118 cases, 112 controls	Possible risk factor
Nasri *et al*., 2019	Case-control	Tunisian	119 cases, 127 controls	Risk factor in father group
Richter *et al*., 2001	Case-control	German	356 cases, 233 controls	No association
Nauman *et al*., 2018	Case-control	Pakistani	109 cases, 100 controls	Maternal TT homozygous genotype an independent risk factor
Kondo *et al*., 2014	Case-control	Japanese	115 mothers, 4517 controls	No association
Kirke *et al*., 2004	Case-control	Irish	397 cases, 848 controls	CT and TT genotype as possible risk factor
Naushad *et al*., 2010	Case-control	South India	100 cases, 160 controls	No association, T allele as possible risk factor
Cunha *et al*., 2002	Case-control	Brazil	46 cases, 75 controls	No association, no risk factor
Yu *et al*., 2014	Case-control	Chinese	271 cases, 192 controls	Some degree of association, risk factor
Deb *et al*., 2001	Case-control	North India	111 cases, 220 controls	TT genotype as risk factor depending on religion and nutritional habits
Rama Devi *et al*., 2004	Cross-sectional	South India	608 subjects	Strong association Maternal TT genotype as risk factor
Pardo *et al*., 2014	Cross-sectional	Chile	105 subjects	No risk factor
Green *et al*., 2013	Cross-sectional	Irish	331 subjects	No association
Mutchinick *et al*., 1999	Cross-sectional	Maxican	250 subjects	Marked association as well as risk factor
Nishio *et al*., 2008	Cross-sectional	Japanese	170 subjects	Marked association

MTHFR – Methylenetetrahydrofolate reductase; NTD – Neural tube defect

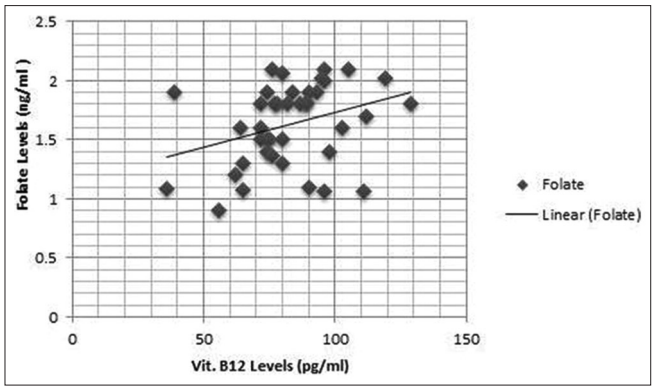

Figure 6: Correlation between folate and Vitamin B12 levels in cases

Our study is in perfect match with studies by Hayati *et al*., Sadewa *et al*., and Dutta *et al*.[37-39] where no MTHFR C677 TT genotype was found. Hayati *et al.* conducted a case–control investigation to ascertain the relationship of the MTHFR C677T polymorphism and incidence of NTDs in Malaysian Malay pediatric population.[37] The study comprised 22 subjects aged 5–12 years and 20 unrelated healthy individuals aged below 30 years. All subjects studied had lumbosacral NTDs. Among the 22 NTDs patients and 20 control subjects, the MTHFR C677TT genotype was found to be absent in both groups. The MTHFR 677CT heterozygous genotype was also absent in the NTDs patients, but was present in three of the control subjects Thus, in this study, no Malaysian Malay individual was found to have homozygous MTHFR C677TT genotype. Sadewa *et al*. studied the C677T mutation in the methylenetetrahydrofolate reductase gene among the Indonesian Javanese population.[38] Sixty-eight Indonesian Javanese were enrolled in this study along with 244 Japanese who constituted the control group. When data on the frequency of the mutation were compared, the frequencies of the three genotypes in Javanese and Japanese were C/C 0.84, C/T 0.16, and T/T 0.00 and C/C 0.39, C/T 0.48, and T/T 0.13, respectively. The authors of this study concluded that homozygosity for the C677T mutation in the MTHFR gene does not constitute a genetic risk factor for NTDs in the Indonesian Javanese population. Dutta *et al*. through a case–control study comprising forty anterior encephalocele (AE) cases and eighty controls investigated whether the interaction between MTHFD1 and MTHFR results into susceptibility to AE in population from Northeast India.[39] In this study, 677C>T was not found to be an independent risk factor of AE. Besides, similar to the other two studies cited above, the prevalence of the C677TT genotype among cases was found to be zero or close to zero. MTHFR C677T gene mutation is considered to be associated with increase in recurrent early pregnancy loss. The reason for this could be hyperhomocysteinemia in the absence of folate supplementation which is a risk factor for recurrent pregnancy loss. Fetuses homozygous for the T allele are often on survival disadvantage in the event of

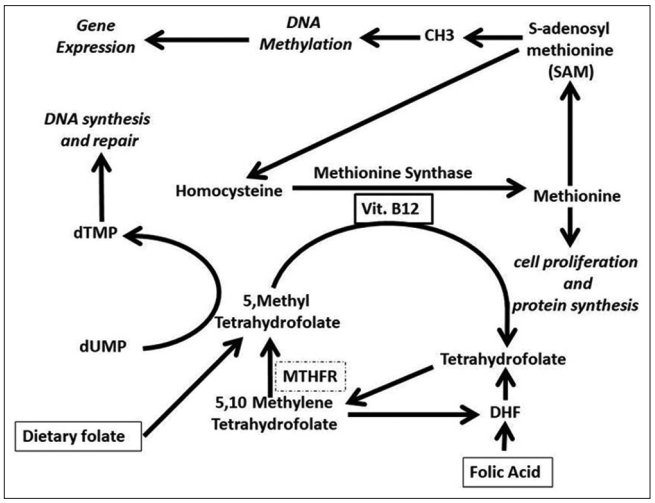

Figure 7: Pathway showing interaction between folate, Vitamin B12, homocysteine, and methylene tetrahydrofolate reductase

inadequate and insufficient folate consumption by pregnant mothers. Probably, the multifactorial etiology of NTDs and the complex interaction between genes and environment could present a plausible explanation of the controversy on the association of the C677T variant and NTDs worldwide.

We found no association between C677T MTHFR polymorphism and NTDs in our study. The presence or lack of association of 677C>T may be because of difference of mothers' folate consumption, the location of the NTDs,[40-42] difference in T allele frequency in population,[36] genetic homogeneity of the studied population,[4] and different gene–nutrient interaction.[43] Christensen *et al*. showed similar but statistically significant results which may be because their sample size was considerably large.[44] Results of Kirke's study where population-attributable fraction calculations revealed that the CT genotype was responsible for at least as many NTDs in the population as the TT genotype, are at times important for making approximations in cases where no TT genotype is present. Further, whereas MTHFR C677T polymorphisms are major factors influencing folate status, both the lower folate and increased homocysteine concentrations associated with CT and TT genotypes can be corrected by folic acid, even in relatively small doses. In cases with recurrent unexplained pregnancy loss, in spite of folate supplementation, levels of active folate in the serum, i.e., 5-methylene tetrahydrofolate as well as homocysteine, should be measured in next pregnancy. If homocysteine is raised, MTHFR is ineffective and folate supplementation would not help. Then, a genetic study for CT or TT genotype may be conducted. In addition, the role of these genotypes on paternal side should be explored to better understand the etiology of NTDs.

The reason for no TT genotype or no association in our study could be a small sample size. Nevertheless, CT genotype that is 20% in cases in our study can very much influence the development of NTDs as in Kirke's study

where population-attributable fraction calculations revealed that the CT genotype was responsible for at least as many NTDs in the population as the TT genotype. Whereas our study reconfirms results from three previous studies with one having comparable sample size that match our results, there are some studies which differ from us. One possible reason for the conflicting results could be difference in racial, geographical, and nutritional status in different parts of the world. Other reasons for these variations are the differences in the sample size, time, and way of sampling. Limitation of the present study was that, the sample size was very small with little power of analysis, mother's sample was not taken, and homocysteine levels were not measured. Besides, the sample was not representative of the entire population as all the samples came from only one tertiary care hospital and no reliable and precise estimate of live, still birth and pregnancy terminations was available.

Conclusion

We did not find evidence of the association between MTHFR C677T polymorphism and NTDs. Hence, we conclude that MTHFR variant may not be a risk factor for the selected population. However, our study does not rule out the impact of MTHFR gene mutation on folate metabolism. In our study, the frequency of prevalence of the homozygous 677CC genotype in cases and control was 80% and 87.5%, respectively, whereas that of heterozygous 677CT genotype was 20% in cases and 12.5% in controls. No TT genotype was found and no association between MTHFR 677C>T gene polymorphism and NTD was observed in our study. Larger and comprehensive multicentric but feasible studies involving proper subjects and appropriate and adequate controls from several hospitals may provide more meaningful data which can help in resolving some of the unresolved controversies of the relationship between MTHFR gene polymorphism and NTDs.

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Nil.

Conflicts of interest

There are no conflicts of interest.

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