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Penetrance of MTHFR, MTRR and SHMT Gene Polymorphism Modulate Folate Metabolism in Maternal Blood and Increases "Risk Factor" in Neural Tube Defects in Eastern India

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Abstract

Background: Neural Tube Defects (NTDs) are a multifactorial disorder that arises during first month after conception due to complex interactions between genetic and environmental factors. Role of folate metabolism plays a significant role in determining genetic predisposition of NTDs.

Materials and Methods: Present study was conducted to evaluate the allele frequency of folate regulatory candidate genes methylenetetrahydrofolate reductase (MTHFR), methionine synthase reductase (MTRR) and serine hydroxymethyltransferase (SHMT) as "risk factors" in NTD cases in Indian population.

Results: Genomic DNA was isolated from NTD cases, NTD mothers and respective controls. PCR-RFLP analysis was performed using specific set of primers to determine the frequency of genotypes and their alleles after using restriction enzymes- Hinf, MboII, Nde I & EarI. The DNA fragments were separated on agarose gel and visualized by Gel documentation system. MTHFR 667CT genotype reveals variable frequency between homozygous (CC genotype, wild type) 64.00% and heterozygous (CT) condition (32.00%) in NTDs cases. MTHFR 1298AC genotype showed a frequency 35.78% in heterozygous (AC) and 5.54% in homozygous (CC) conditions. Statistical analysis was performed by calculating CC/TT genotype O.R (0.113) and C.I. at 95% (0.0054-2.367) of and of AA/AC genotype O.R. (3.24) at 95% C.I (0.690-15.205) that showed significant ($p<0.05$) differences between NTD mothers and their respective controls in MTHFR gene. Data was further analyzed by adding "T/C" alleles in MTHFR gene to increase statistical power which further showed significant ($p<0.001$) differences between NTD cases with respect to controls. MTRR 66A→G gene showed significant ($p<0.05$) difference between NTDs cases and NTD mothers after combining the genotypes (AA vs. AG+GG). SHMT 1420CT gene showed lack of significant differences between homozygous and heterozygous conditions in NTD cases and NTD mother with their respective control groups.

Conclusion: Present study suggests that the variations in the genotype frequency are due to the penetrance of defective allele into maternal gene pool, affecting DNA synthesis during organogenesis leading to the onset of NTDs.

Keywords: Neural tube defects; MTHFR; MTRR; SHMT; Genotype; Alleles and gene pool

Introduction

In human, neural tube defects (NTDs) are one of the most severe congenital anomalies of the central nervous system where neural tube fails to close during fourth week of intra uterine life. The severity of NTDs is classified as – first anomalies that develop into either anencephaly or exencephaly and second category includes either myelomeningocele or meningomyelocele. In NTDs, the genetic predisposition is polygenic and influenced by genetic and epigenetic factors. The etiopathology of NTDs is regulated by couple of genes regulate folate metabolism associated with developing "Risk" in infants suffering from NTDs.

Methylenetetrahydrofolate reductase (MTHFR) gene is mapped on chromosome 1 (1p36.3) with 2.2 kbs long DNA sequence having 11 exons which transcribe to 77 kd protein. MTHFR catalyzes the conversion of 5, 10-methylenetetrahydrofolate into 5-methyltetrahydrofolate, is a major circulating form of folate in maternal blood. Most common site of mutation of MTHFR gene is at nucleotide 677 position in homozygous condition as reported in Caucasian populations (~10%). The alanine convert into valine at codon 225 and the enzymatic activity decreases up to 60% to 70% with an increase of homocysteine (Hcy) levels in blood. Hence, MTHFR gene polymorphism C→T677/A→C1298 increase "risk factor" for the

development of NTDs [1-5]. MTHFR gene implicated to make complex biochemical pathways for the synthesis of S-adenosyl-methionine i.e. remethylation of Hcy to methionine which is required for DNA methylation. Deficiencies in thymidylate have been shown to increase genetic instability due to misincorporation of uridylate into DNA [6,7]. Another essential enzyme which regulate folate metabolism is methionine synthase reductase (MTRR) that influence enzyme activity during homocysteine remethylation pathway act as predisposing factors leading to "high risk" for NTDs. The common polymorphism of MTRR is A66G substitution leading to a change of isoleucine to methionine and mutation in the alleles leads to reduce the enzymatic activity and decrease methylation of homocysteine to methionine. Similarly, serine hydroxymethyl transferase (SHMT), another key factor to regulate folate metabolism by catalyzing the reversible conversion of serine and tetrahydrofolate (THF) to glycine and 5, 10-methylene THF with thymidylate biosynthesis [8-11]. It has been observed that mutated SHMT alleles are responsible to enhance Hcy levels and changed the distribution of different folate derivatives leading to increase "risk factor" for NTDs. However, it is not clear whether mutated form of cSHMT or mSHMT are responsible to enhance the "risk" for developing NTDs. Because of high frequency of NTDs in the eastern part of the country, the present study was designed with an aim to evaluate the frequency of candidate genes-MTHFR, MTRR and SHMT polymorphism in NTD cases and NTD mothers in comparison with respective controls. Such studies become relevant to determine the "risk factors" due to penetrance of defective alleles in to maternal gene - pool responsible for the development of NTDs in eastern part India.

Materials and Methods

Subjects

Blood samples (0.5 ml) were collected in sterile EDTA vials from the OPD of department of Obstetrics and Gynecology/Plastic Surgery/ Pediatric Medicine and Surgery at AIIMS, Patna and Institute of Medical Sciences, BHU, Varanasi and stored at -20°C till further study. The total population for present study (n=323) that includes clinically diagnosed cases of NTDs (n=87), their (NTDs) mothers (n=87), age matched case control (n=80) and case control mothers (n=76). The samples were received after informed written consent from the participant's attendant/guardians and the study was cleared by IEC (Institute Ethical Committee). Fathers were also invited to participate in the study; however, due to lack of cooperation that group (paternal) could not be included in the present study.

Isolation of Genomic DNA and PCR - RFLP analysis

Genomic DNA was isolated from Quigen Kit (USA) protocol for further analysis and PCR RFLP analysis of MTHFR 677C→T was carried out by using specific forward (f) 5'TGAAGGAGAAGGTGTCTGGGGGA3' and reverse (r) 5'TGAGAG TGGGGTGCAGGGAGCTT3' with conditions 38 × (95°C 1', 61°C 1', 72°C 1') and Hinf-I and for MTHFR 1298 A→C f 5'CTTTGGGGAGCTGAAGGACTA3' and r 5'CAGGGGATGAACCAGGGTCC3' with PCR conditions of 38 × (94°C 1', 58°C'1', 72°C 1'), Mbo-II was used for RFLP analysis. For MTRR66 A→G gene analysis included (f) 5'GCAAAGGCCATCGCAGGAGACAT3' and (r) 5'CACTTCCCAACCAAAATTCTTGAAAG3' with PCR conditions 38 × (94°C 1', 59°C'1', 72°C 1') and Nde I was used for RFLP analysis. SHMT gene analysis was performed by using (f) 5'AGAGTTCAAGGAGAGACTGGCAG3' and (r) 5'TTTGCCCTACACCACCATCT3' with PCR conditions as 38 × (95°C 1', 56°C' 1', 72°C 1'), Ear-I was used for RFLP analysis. The total volume of 50 µl containing 50-100 ng of genomic DNA, 20 pmole of each primer, 200 µM of each dNTPs mix with Taq buffer (10mM Tris HCl pH 8.3, 50mM KCl), 3.0 mM MgCl2 and 3 unit of Taq polymerase (New England Biolab). The PCR product was separated and visualized on 1.5% agarose gel. RFLP analysis of MTHFR C677T gene after cleavage with Hinf-I restriction enzyme formed 198 bp and 175 bp bands showing three types of genotypes (CC, TT, CT). Similarly, restriction enzyme Mbo-II used for RFLP analyses of A1298C allele showed three fragments of 84bp, 56bp & 30bp of different genotypes. Nde I and Ear I enzyme were used for the study of SNP analysis of MTRR and SHMT gene that shows fragments of 106 bp, 66 bp, 44 bp and 433 bp, 343 bp and 90 bp respectively. These fragments after digestion were separated on 3% agarose gel stained with Et.Br and characterized on Gel Documentation system (Bio red system, USA).

Statistical analysis

Two tailed fisher exact probability test was used to find out the significant differences ($p<0.05$) between cases of NTDs, mothers of NTDs and their respective controls. The odd ratio (O.R) was calculated at 95% confidence interval (C.I.) in between three different genotypes and their individual allele's frequency was calculated by Hardy Weinberg Equilibrium to find relative "risk factor" between cases and their mothers.

Results

Table 1 shows the detail frequency of MTHFR C667T and A1298C genotypes and their alleles, odd ratio and confidence interval (C.I. at 95%) between NTDs cases and controls, NTDs mothers and controls mothers.

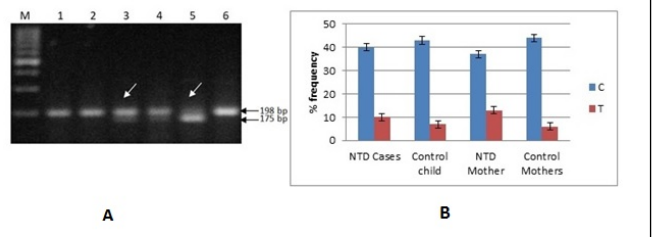

Figure 1: (A) PCR RFLP analysis of MTHFR C677T showing the appearance of bands 198 bp and 175 bp after use of restriction enzyme Himf I as shown in lane 3 and 5 in NTDs with controls (lane-6). **(B)** Bar diagram showing the frequency (%) of C & T alleles of MTHFR C667T genotype in NTD cases, mothers and their respective controls. In the NTD mothers frequency of "T" (red bar) higher than NTD cases, while the frequency of "C" alleles are seeming to be similar in NTD case, NTD mother and their respective controls.

MTHFR genotype and allele C677T frequency						
Groups (n=25)	**Genotype and their (%) frequency**			**Allele frequency (%)**		**p-value**
	CC	**CT**	**TT**	**C**	**T**	
NTD Cases	17 (64.00)	6(32.00)	2 (4.00)	40 (80)	10 (20)	0.2113
Control child	19 (73.96)	5 (24.08)	1 (1.96)	43 (86)	07 (14)	0.3969
NTD Mother	15 (54.76)	7 (34.48)	3 (6.76)	37 (74)	13 (26)	0.1733
Control Mothers	19 (77.44)	6 (21.12)	0 (1.44)	44 (88)	06(12)	0.4954
MTHFR genotype and allele A1298C frequency						
Groups (n=15)	**Genotype & their (%) frequency**			**Allele frequency (%)**		**p-value**
	AA	**AC**	**CC**	**A**	**C**	
NTD Cases	9 (58.78)	5(35.78)	1 (5.44)	23 (76.67)	7 (23.33)	0.7913
Control child	5 (40.11)	9 (46.44)	1 (13.44)	19 (63.33)	11 (36.67)	0.2583
NTD Mother	5 (47.93)	8 (42.60)	0 (09.47)	18 (69.23)	8 (30.77)	0.1091
Control Mothers	8 (58.78)	7 (35.78)	0 (05.44)	23 (76.67)	7(23.33)	0.2385
MTHFR C677T allele						
NTD Cases/Control	**CC/TT Homozygous condition**			**CC/CT Heterozygous condition**		
	O.R	**95% C.I.**	**p- value**	**O.R**	**95% C.I.**	**p- value**
	0.4474	0.037-5.385	0.5263	0.7456	0.192-2.891	0.6712
NTD Mother/Control	**CC/TT**			**CC/CT**		
	0.1136	0.005-2.367	0.1604	0.6767	0.187-2.442	0.5509
MTHFR A1298C allele						
NTD Cases/Control	**AA/CC**			**AA/AC**		
	O.R	**95% C.I**	**p- Value**	**Odd Ratio**	**95% C.I**	**p- Value**
	1.8	0.091-35.42	0.699	3.24	0.690-15.20	0.0881
NTD Mother/Control	**AA/CC**			**AA/AC**		
	O.R.	**95% C.I**	**p- Value**	**Odd Ratio**	**95% C.I**	**p- Value**
	0.6471	0.011-37.66	0.8337	0.5469	0.120-2.473	0.4331

Table 1: MTHFR gene polymorphism showing the frequency of genotypes, alleles, Odd Ratio (O.R) and Confidence Interval (C.I) between homozygous (CC) and heterozygous (CT) conditions and. Their p- values between NTD cases, NTD mothers and their respective controls.

The data was further analyzed after combining the genotype between homozygous (CC) and heterozygous (CT) conditions to increase the "power" and to evaluate the level of significance as documented in Table 2.

MTHFR C677T allele showed variable frequency of three genotypes i.e. CC (wild type), CT and TT (rare types) between homozygous and heterozygous conditions after digestion of PCR product using Hinf-I leading to formation of 198bp and 175bp fragments as shown in (Figure 1A).

In NTDs cases, the highest frequency (64.00%) was observed in the CC genotype (wild type), while TT genotype (rare type) existed at a frequency of 4.00% in homozygous condition and CT genotypes with a frequency of 32.00% in heterozygous condition.

Interestingly, MTHFR C677T the "T" allele frequency increased more than two folds (26%) in NTD mothers as compared to control mothers (12%) (Figure 1B).

Similarly, MTHFR A1298C the frequency of "C" allele was higher in NTD mothers as compared to controls suggesting an involvement in increasing "risk" for development of NTDs during organogenesis (Table 1). MTHFR A1298C allele after digestion of PCR product with restriction enzyme Mbo-II showed fragments of 84 bp, 56 bp and 30

bp (Figure 2A) indicating three different genotypes – AA (wild type), AC (heterozygous) and CC (rare type).

Highest frequency were observed in AA genotype (58.78%) followed by AC (35.78%) and CC (5.44%) in NTD cases. Further O.R. and C.I. at 95% was calculated between NTD cases, NTD mothers and their respective controls. For the genotypes of MTHFR C677T/A1298C the values of O.R at 95%, C.I. 1.8 (0.0915-35.42) in CC/TT (homozygous condition) in NTDs cases vs. controls showed lack of significant difference ($p<0.05$).

S.No.	Types/Groups	Combination of genotype (C677T)	p-values
1	NTDs Cases	CC vs. CT CC vs. TT CC vs. CT+TT	0.0250 0.0001** 0.0500*
2	NTDs Mother	CC vs. CT CC vs. TT CC vs. CT+TT	0.1585 0.1533 0.3350
3	Case Control	CC vs. CT CC vs. TT CC vs. CT+TT	0.0006** 0.0001** 0.001**
4	Mother Control	CC vs. CT CC vs. TT CC vs. CT+TT	0.0001** 0.0001** 0.0002**
S.No.	**Types**	**Combination of genotype (A1298C)**	**p-Values**
1	NTDs Cases	AA vs. AC AA vs. CC AA vs. AC+CC	0.21070 0.0023* 0.3062
2	NTDs Mother	AA vs. AC AA vs. CC AA vs. AC+CC	0.7732 0.0221* 0.8687
3	Case Control	AA vs. AC AA vs. CC AA vs. AC+CC	0.7442 0.0995 0.2870
4	Mother Control	AA vs. AC AA vs. CC AA vs. AC+CC	0.2148 0.0021* 0.3118
**Statistically analysis showing highly significant difference ($p<0.001$).* Significant difference ($p<0.05$).			

Table 2: Statistical analysis showing significant relationship after combining of MTHFR genotypes between homozygous and heterozygous condition in NTD Cases, NTD mother and their controls.

The individual allele's frequency was also calculated to determine "risk factor" by Hardy Weinberg Equilibrium which again showed lack of significant difference between cases and controls. To increase the "statistical power", data was further analyzed after combining the genotypes between homozygous and heterozygous conditions i.e. CC vs. TT, CC vs. CT and CC vs. CT+TT, which showed highly significant difference ($p<0.001$) in NTDs cases and controls (Table 2). The genotype frequency was slightly increases between NTDs mothers with respective controls, however showed lack of significant differences in the MTHFR A1298C genotype (Table 2).

The MTRR gene polymorphism was determined as described in material and methods, the detailed data is documented in (Tables 3 and 4). The highest frequency of genotype AG (49.92%) was observed in NTD cases in heterozygous condition.

The calculated value of O.R. (0.024) and C.I. at 95% (0.0011-0.5379) in AA/GG homozygous condition seems to increase between NTD cases and NTD mother with O.R (0.12) at 95% C.I=0.0108-1.448, showed lack of significant differences ($p<0.05$), but when the power of "G" allele between NTD cases and NTD mother in AA vs. AG+GG conditions was increased, the statistical value showed highly significant differences ($p<0.001$) with respect to controls as shown in (Table 4).

MTRR genotype & allele frequency						
Groups	**Genotype & their (%) frequency**			**Allele Frequency (%)**		**p-value**
	AA	**AG**	**GG**	**A**	**G**	
NTD Cases (n=25)	4(23.04)	16(49.92)	5 (27.04)	24 (48)	26 (52)	0.1585
Control child (n=19)	4 (36.63)	15 (47.78)	0 (15.58)	23 (60.53)	15 (39.47)	0.0045**
NTD Mother (n=20)	2 (25)	16 (50)	2 (25)	20 (50)	20 (50)	0.0073**
Control Mothers (n=16)	3 (28.22)	11 (49.8)	2 (21.97)	17 (53.13)	15(46.88)	0.1281
MTRR genotype O.R and C.I						
NTD Cases Vs. Control	**AA/GG**			**AA/AG**		
	O.R	**95% C.I**	**p- Value**	**O.R**	**95% C.I**	**p- Value**
	0.0248	0.001-0.537	0.139	0.9375	0.193-4.437	0.9352
NTD Mother Vs. Control	**AA/GG**			**AA/AG**		
	O.R	**95% C.I**	**p- Value**	**O.R**	**95% C.I**	**p- Value**
	0.125	0.0108-1.448	0.7646	0.4583	0.065-3.211	0.4322
*Highly significant difference (p<0.001)						

Table 3: MTRR gene polymorphism showing the frequency of genotypes, alleles, Odd Ratio and Confidence Interval and p- values between homozygous/heterozygous condition in NTDs Cases, NTDs mothers and their respective controls.

S.No.	**Types**	**Combination of genotype**	**p-Values**
1	NTDs Cases	AA vs. AG AA vs. GG AA vs. AG+GG	0.0507 0.7490 0.0002**
2	NTDs Mother	AA vs. AG AA vs. GG AA vs. AG+GG	0.1069 1.000 0.0018**
3	Case Control	AA vs. AG AA vs. GG AA vs. AG+GG	0.5226 0.1449 0.1087
4	Mother Control	AA vs. AG AA vs. GG AA vs. AG+GG	0.2181 0.6887 0.2000
**Highly significant difference (p<0.001).			

Table 4: Statistical analysis showing significant relationship after combining of MTRR genotypes between homozygous and heterozygous condition in NTD Cases, NTD mother and their controls.

Similarly, SHMT gene polymorphism showed variable frequencies of genotypes (CC, CT and TT) after digestion of PCR product with restriction enzyme Ear I as depicted in (Table 5).

The highest frequency was observed in CT heterozygous condition (49.00%) in NTD cases followed by homozygous CC wild type condition (29.34%) and TT rare condition (21.01%). The calculated value of O.R. (0.23) and C.I. at 95% (0.00-8.6156) showed lack of significant differences between NTD cases and their respective control.

SHMT Genotype and Allele Frequency						
Groups	**Genotype and their (%) frequency**			**Allele Frequency (%)**		**p-value**
	CC	**CT**	**TT**	**C**	**T**	
NTD Cases (n=12)	2 (29.34)	9(49.53)	1 (21.01)	13 (54.17)	11 (45.83)	0.077
Control Child(n=10)	3 (42.25)	7 (45.50)	0 (12.25)	13 (65)	7 (37)	0.0886
NTD Mother(n=10)	3 (42.25)	7 (45.50)	0 (12.25)	13 (65)	7 (37)	0.0886
Control Mothers(n=3)	2 (69.44)	1 (27.78)	0 (2.78)	5 (83.33)	1(16.67)	0.729
SHMT GENOTYPE O. R and C. I						
NTD Cases Vs. Control	**CC/TT**			**CC/CT**		
	O.R	**95% C.I**	**p- Value**	**O.R**	**95% C.I**	**p- Value**
	0.2381	0.006-8.615	0.4332	0.5185	0.0672-4.002	0.5288
NTD Mother Vs. Control	**CC/TT**			**CC/CT**		
	O.R	**95% C.I**	**p- Value**	**O.R**	**95% C.I**	**p- Value**
	1.4	0.020-97.435	0.8765	0.2143	0.0136-3.369	0.2732
Lack of significant difference (p<0.05) were observed						

Table 5: SHMT gene polymorphism showing the frequency of genotypes, alleles, values of Odd Ratio and Confidence Interval between homozygous and heterozygous conditions and their p values between NTD cases, NTD mothers and their respective controls.

S.No.	**Types**	**Combination of genotype**	**p-values**
1	NTDs Cases	CC vs. CT	0.3219
		CC vs. TT	0.6454
		CC vs. CT+TT	0.0512
2	NTDs Mother	CC vs. CT	0.9038
		CC vs. TT	0.142
		CC vs. CT+TT	0.5043
3	Case Control	CC vs. CT	0.8865
		CC vs. TT	0.142
		CC vs. CT+TT	0.4993
4	Mother Control	CC vs. CT	0.3514
		CC vs. TT	0.1207
		CC vs. CT+TT	0.3378
Lack of significant difference (p<0.05) were observed.			

Table 6: Statistical analysis showing significant relationship after combining of SHMT genotypes between homozygous and heterozygous conditions in NTD Cases, NTD mother and their controls.

However, individual allele "T" frequency was combined between homozygous and heterozygous conditions i.e. CC vs. CT+TT to increase the statistical power of "T" allele again showed the lack of significant difference differences (p<0.05) between NTD cases, their mothers and respective controls as documented in (Table 6).

Discussion

Globally, NTDs are the most severe congenital malformations of central nervous system that develop during early embryogenesis due to genetic mutations. Epidemiologically, study reveals that folic acid deficiency in to maternal blood leads to increase in incidence of NTDs. Besides genetic factor, other factors that include environmental factors, maternal age, sex, and racial, rural/urban population, socioeconomic and geographical conditions also play a significant role in NTDs [12-15].

Anencephaly and myelomeningocele (MMC) are the most common forms of NTDs, where a large number of genetic variants (copy number variation) in population have been identified between different racial/ethnic groups. Earlier our group has identified that in the eastern part of India, the disruption of cystathionin β synthase (CβS) enzyme activity due mutation i.e. insertion of 68bp and replacement of nucleotides sequences (TCCATGGGG) were associated with the development of lumbosacral myelomeningocele [16].

Importantly, in this region, the maternal population is highly prone for developing disease like NTDs due to poor socioeconomic status leading to folate deficient diet. Interestingly, the present study identifies the variation in frequency of genotypes in three different candidate genes MTHFR, MTRR and SHMT regulating folate metabolism in different clinical lesion on the basis of severity of NTDs. The genetic susceptibility is associated with variation of gene mutation in heterozygous condion increasing "risk" in the population due to abnormal DNA methylation.

Similarly, our studies suggested that MTHFR gene polymorphism increase "risk factor" in heterozygous condition for several diseases like mental retardation, recurrent miscarriages and cancers [17-21]. In present study, NTD mothers showing the significant differences in the genotype of MTHFR A1298C with respect to control mother suggesting increasing "risk" of developing NTDs.

Several relevant enzymes including MTHFR, methionine synthase (MTR), CβS has been involved in abnormal homocystine accumulation due to mutation in the gene [22-24]. MTRR deficiency leads to abnormal metabolism of homocystine or methionine which has been associated with A66G changes the 22nd amino acid from isoleucine to methionine. Such mutation results in lowering the amount of cofactor cobalamin which is required to maintain intracellular folate pool [25].

Our study demonstrated that in case of MTRR A66G gene after combining the genotypes to increase the power of "G" allele which showed highly significant differences in NTD mothers with respect to controls, suggesting that the "mutated" allele travelled from the maternal gene pool contributing to NTD cases. Interestingly, our data also suggests that MTHFR or MTRR mutated alleles either single locus or multiple loci are synergistically associated with increased "risk" of NTDs in this region.

The polymorphism of SHMT C1420T lead to increase Hcy levels and uneven distribution of folate derivatives that might be responsible for increase in the "risk" for NTDs, however, the mutation of 4-bp of the mSHMT gene fail to altered Hcy or folate levels [26]. The present study showed lack of significant difference in NTD cases and their mothers with respective controls suggesting no direct evidence involving the function of folate of defective SHMT in the onset of disease.

However, the present study showing higher frequency of "C" allele in NDTs mother when compare to NTD cases (Figures 2A and 2B) suggesting that it might play a role in increasing the incidence of NTDs due to penetrance of defective allele in to maternal gene pool. Previously, functional aspect of SHMT SNP (rs 1979277 c allele) has also been associated to the inhibition of nuclear import and consequently affecting thymidylate pool [27].

Present study after combining with all three folate regulatory metabolism genes i.e. MTHFR, MTRR and SHMT demonstrated variable frequency of alleles (rare type/wild type) in homozygous/heterozygous condition. Such mutations might be responsible for abnormal cellular differentiation during organogenesis. The variability in frequencies of C677T allele due to heterogeneous group of population or different ethnic or racial background, suggested that about half of the general population carries at least one mutated allele in homozygous condition (TT) ranges from 1 to 20% [2,5]. However, present study shows lack of similar findings either due to small sample size or unknown environmental factors. Authors hypothesized that infants with homozygous (TT) or heterozygous (CT) state of C677T genotype would be at "higher risk" in the cases of mental retardation where, the linkage analysis from mother to the offspring is complicated and onset of disease might be due segregation of alleles [17].

These mutations are responsible for reduced enzymatic activity and increased homocysteine levels resulting in disturbance of folate equilibrium with increases "risk" as an independent factor for neuronal disorders [28]. Earlier studies clearly reveal that pre-conceptional folic acid supplementation may reduce risk factor up to 75%, however, environmental factors might have direct impact on the variation of severity and frequency of NTDs (0.2-3.5 per 1000 births) [2,3]. MMC is the most severe form of spina bifida and carriers have difficulty to survive because of dysplastic spinal cord with lack of neuronal functions. Our earlier studies showed that glutamate carboxypeptidase II (H475Y) and reduced folate carrier (SLC19A1) gene polymorphism modulates the enzymatic activity in heterozygous condition and increase "risk" for the development of MMC, where both meanings and the spinal cord protrude through a gap in the vertebral column and the lesion is not covered by the skin [29].

The severity of disease in the proband might be due to the penetrance of defective allele frequency in maternal gene pool leading to disequilibrium either in homozygous or heterozygous conditions [30-32]. Furthermore, earlier we identified chromosomal abnormalities in mothers develop NTDs during pregnancies [33].

The etiology of NTDs remains complex involving genetic and epigenetic factors leading to gene - protein interaction during early organogenesis [13-15]. The three genes studied in the present study shows penetrance either in single gene locus or multiple loci increasing risk for the development of NTDs. The frequency of MTHFR CT varies in different populations, i.e. 8.4% population was found to be homozygous for the mutation in Dutch population as opposed to non-Dutch (Australian/American) population at 11% [34].

Additionally, previous studies investigating the association of the genes investigated in the current study with patient/maternal risk for NTDs have generated inconsistent data as regards the penetrance frequency as well as their role as a risk factor [35], which may be contributed to the complex nature of the genetic and epigenetic factors involved as well as environmental and social backgrounds [36], and changing frequency of alleles in different geographical locations, which necessitates further studies to generate population specific data.

Further, the genetic heterogeneity of stem cells is also responsible for commencement of the severity of the disease and understanding the role of stem cells during the development of NTDs [37]. There is still limited progress in delineating the molecular basis underlying NTDs that can pave way for prevention, diagnostics and therapeutics.

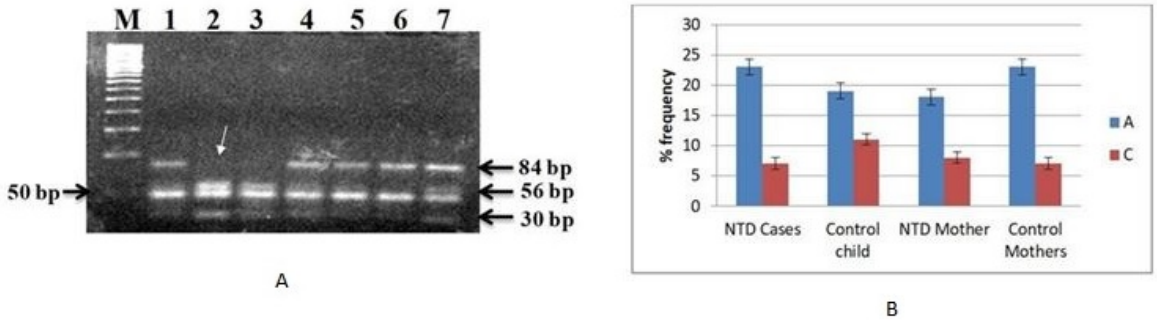

Figure 2: (A) PCR RFLP analysis of MTHFR A1298C showing the appearance of bands 84 bp, 56 bp and 30 bp after use of restriction enzyme Mbo-II as shown in lane 2-3 with respect to controls (Lane -7) in NTD cases. **(B)** Bar diagram showing the frequency (%) of A & C alleles of MTHFR A1298C genotype in NTD cases, mothers and their respective controls. In the NTD mothers frequency of "C" allele (red bar) higher than NTD cases as well control mother, while the frequency of "A" alleles are again showing almost similar frequency in NTD case, NTD mother and their respective controls.

Conclusions

The present study suggest following reasons associated with the genetic susceptibility of NTDs in the families belonging to Eastern part of India:

1) MTHFR polymorphism in heterozygous (CT) condition increases "risk" for the development of NTDs as an independent factor or carrying additional dose of mutant "T" allele in maternal gene-pool leading to decrease in folate levels in blood.

2) SHMT gene shows lack of significant contribution in the development of NTDs in this population either due to unknown epigenetic modifications such as histone modifications or DNA methylation, or environmental factors.

3) Similarly, MTRR gene showing significant values ($p<0.001$) in the case of NTD mothers after increasing the value of 'G' allele suggests a failure to maintain equilibrium between mutated/non-mutated allele, and penetrance of defective allele into the maternal gene pool either in homozygous (rare type) or in heterozygous condition are responsible for dysregulation of folate metabolism resulting in the deficiency of folate in maternal blood, increasing the risk of developing NTDs during early organogenesis. The socio-economic condition of the family or poor nutritional status i.e. folate deficient diet is equally important for developing complications during early embryogenesis. Hence, supplementation of folate rich diet to the mothers may reduce the incidences of NTDs in this region. However, further studies are required to understand the underlying mechanisms of allelic expression of the genes by increasing the sample size.

Declarations

The ethical approval and consent to participate

Present study has been approved by Institute Ethical committee. Before the collection samples parental/guardian consent have been taken in a prescribed consent form in both in English and Hindi languages.

Consent to publish

The participants of the present study have provided approval for participation as well as use of the data for further research work.

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Competing interests

The authors declare no conflict of interest in the present study.

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Authors' Contributions

AKS and MT were initially involved for experimental design, analysis and interpretation of the data. RK and AC were responsible for laboratory procedures and analysis of the cases. VK, VS., MA and PP were responsible for clinically diagnosing the cases. All the authors have approved and finalized the manuscript.

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Conflicts of Interest

There are no conflicts.

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