Original Article

Polymorphisms in Maternal Selected Folate Metabolism-Related Genes in Neural Tube Defect-Affected Pregnancy

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Abstract

Background: Neural tube defects (NTDs) are abnormalities of the brain and spinal cord, which occur as a result of failure in neural tube closure during embryogenesis. Causes of NTDs are complex and multiple, with hereditary, lifestyle, and environmental factors appearing to play a role. In spite of their impact on public health, the role genetics play on NTDs in Ethiopia is lacking. In this study, the role of polymorphisms in MTHFR 677C > T (rs1801133), MTHFR 1298A > C (rs1801131), MTRR 66A > G (rs1801394), RFC1 80A > G (rs1051266), and TCN2 776C > G (rs1801198) on the risk of having NTD-affected pregnancy was investigated.

Materials and Methods: One hundred women with NTD-affected pregnancy and 100 women with normal pregnancy were included in the study. DNA was extracted from saliva and genotyping for five polymorphisms in four genes was analyzed by polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP). The departure of the genotype's distribution from Hardy–Weinberg equilibrium (HWE) was evaluated using the x^2 goodness-of-fit test. Frequencies of genotypes and alleles in case and control mothers were determined and differences between relative frequencies were evaluated by the x^2 or the Fisher's exact test.

Results: The statistically significant difference was absent in the genotype and allele frequencies for all the analyzed polymorphisms between cases and controls ($P > 0.05$).

Conclusion: MTHFR 677C > T, MTHFR 1298A > C, MTRR 66A > G, RFC1 80A > G, and TCN2 776C > G polymorphisms lack association with the risk of having a pregnancy affected by NTD. The role of other genes or environmental factors in NTD etiology needs to be investigated.

Keywords: MTHFR, MTRR, NTDs, RFC-1, TCN2

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Introduction

Genes involved in the metabolism of folate and homocysteine contribute to the problem of neonatal abnormalities.[1] It has been demonstrated that polymorphisms in the genes that produce the proteins or enzymes necessary for folate absorption and metabolism affect their expression or catalytic activity, which can significantly increase the chance of developing an abnormality. Despite substantial evidence demonstrating the importance of genetic variables in the etiology of neural tube defects (NTDs), there are currently no single candidate genes for NTDs that influence the management of NTDs in high-risk pregnancies.[2]

The methylenetetrahydrofolate reductase (MTHFR) enzyme, which is encoded by the MTHFR gene, catalyzes the conversion of 5,10-methylenetetrahydrofolate to 5-methyltetrahydrofolate, the donor of a methyl group for the conversion of homocysteine

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to methionine.[3] Due to its crucial roles in the one-carbon cycle, which include the metabolism of folate and methionine, the MTHFR enzyme is essential for maintaining cellular homeostasis.[4]

The two frequently investigated single nucleotide polymorphisms (SNPs) in the MTHFR gene are 677C > T and 1298A > C. The 677C > T SNP causes a missense mutation that changes a cytosine into thymine, resulting in an amino acid change from alanine to valine.[4] MTHFR polymorphisms result in reduced enzyme activity and have been linked to increased risks of several pathologies.[5] People with the TT genotype have around 30% of the MTHFR enzyme activity of the wild-type genotype (CC), while individuals with CT genotype have roughly 65% of enzyme activity.[6] Fetal NTDs were observed to be substantially associated with maternal MTHFR 677C > T polymorphism.[1] The 1298A > C SNP leads to the substitution of alanine for glutamate in the MTHFR enzyme and impairs enzyme activity to a lesser degree[7]; however, with 677C > T polymorphisms, it has a stronger impact on the reduction of MTHFR enzyme function. Previous studies have reported an association between 1298A > C and NTD.[8]

The MTRR gene codes methionine synthase reductase (MTRR) enzyme,[9] which is necessary for the regeneration of functional methionine synthase (MS) by reductive methylation in which the methyl donor is S-adenosylmethionine (SAM). The prevalent SNP in the MTRR gene is the substitution of G for A at position 66 which causes isoleucine to methionine substitution. This polymorphism negatively affects enzyme activity.[10] Previous research has suggested additive or synergistic action of MTHFR 677C > T and MTRR 66A > G polymorphisms on levels of plasma homocysteine[11] and an increased risk of NTD with low cobalamin status.[12]

The RFC-1 (SLC19A1) gene encodes a protein involved in folate metabolism, which serves as a bidirectional transporter, receiving folate cofactors while exporting numerous organic anions. In cells and tissues of mammals, the ubiquitously expressed reduced folate carrier (RFC) is the main system for transporting folate cofactors.[13] The 80A > G polymorphism, in the RFC-1 gene was reported to be a risk factor for NTDs.[14]

The TCN2 gene codes for transcobalamin, a protein required for transporting vitamin B12 into cells. The most extensively studied SNP in the TCN2 gene is 776C > G.[15] In the protein, this polymorphism causes the substitution of proline with arginine, which may change the secondary structure of the protein.[16] This SNP has been reported as strongly predictive of NTDs.[17]

Materials and Methods

Subjects

Pregnant women, 18+ years, with a diagnosis of fetal NTD on prenatal ultrasound, were considered as cases. Controls consisted of pregnant women of the same gestational age whose pregnancy was not affected by NTD. The study participants were chosen from three public hospitals in Addis Ababa, Ethiopia (Zewditu Memorial, Gandhi Memorial, and Tikur Anbessa Specialized Hospitals) between September 2019 and August 2020. The study included 100 cases and 100 controls.

Genetic analysis

Five polymorphisms in four selected folate metabolism-related genes were invetigated; MTHFR 677C > T (rs1801133), MTHFR 1298A > C (rs1801131), MTRR 66A > G (rs1801394), RFC1 80A > G (rs1051266), and TCN2 776C > G (rs1801198) were analyzed by polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP).

For DNA extraction, saliva was collected by the study participants themselves using Oragene® DNA self-collection kit (DNA Genotek inc, Ottawa, ON, Canada) after receiving instructions on how to collect saliva. The participants were advised not to eat, drink, or chew gum and requested to rinse their mouths with water before collection. Genomic DNA was extracted by ethanol precipitation using prepIT-L2P reagent according to the manufacturer's protocol (DNA Genotek Inc., Ottawa, ON, Canada). The concentration and purity of the DNA were measured by NanoDrop 2000 spectrophotometer (Thermo Scientific; Thermo Fisher Scientific, Waltham, MA). All of the extracted DNA yielded a 260/280 ratio of greater than 1.8.

PCR was performed in a mixture containing 6.25 µl of HotStarTaq Master Mix (Qiagen Inc., Chatsworth, CA, USA), 0.25 µl of each of forward and reverse primers obtained from previous studies (Inqaba Biotec East Africa Ltd, Nairobi, 00606, Kenya), 4.5 µl of RNase-free water, and 1.25 µl of template DNA. For all the tested polymorphisms, the PCR reactions included an initial step of activation of the HotStarTaq DNA Polymerase at 95°C for 15 min, and termination of the reaction by a final extension at 72°C for 10 min. The PCR conditions, primer sequences, and length of PCR amplification products are presented in Table 1. Electrophoresis of PCR amplified products and a DNA ladder of 100 bp were conducted on an agarose gel. The size of PCR products on the gel was estimated by comparing the bands of the sample lane against the DNA ladder. Samples that failed PCR amplification were repeated.

Restriction enzyme digestion of PCR amplified products

Restriction enzyme digestion of PCR amplified products was carried out in a mixture containing 2 µl of restriction enzyme digestion buffer, 1 µl of specific restriction enzyme (Inqaba Biotec East Africa Ltd, Nairobi, 00606, Kenya), and 7 µl of molecular grade water with 10 µl of PCR amplified product. The reaction was carried out at a digestion temperature of 37°C for 1 h and terminated by heat inactivation at 80°C for MTHFR 677C > T and at 65°C for MTHFR 1298A > C, MTRR 66A > G, RFC1 80A > G, and TCN2 776C > G for 20 min. The restriction enzymes used for each polymorphism and the products obtained after the restriction enzyme digestion are presented in Table 2. Electrophoresis of restriction enzyme

Table 1: PCR conditions, primer sequences, and PCR amplification products

SNP	Primer sequences	PCR conditions	Amplification products length
MTHFR 677CT	F: 5'-TGAAGGAGAAGGTGTCTGCGGGA-3'[18] R: 5'-TGAGAGTGGGGTGGAGGGAGCTT-3'	35 × (30 s, 94°C; 45 s, 58°C; 1 min, 72°C)	198 bp
MTHFR 1298AC	F: 5'-GCAAGTCCCCCAAGGAGG-3'[19] R: 5'-GGTCCCCACTTCCAGCATC-3'	35 × (30 s, 94°C; 45 s, 52°C; 1 min, 72°C)	137 bp
MTRR 66AG	F: 5'-CAGGCAAAGGCCATCGCAGAAGACAT-3'[20] R: 5'-CACTTCCCAACCAAAATTCTTCAAAG-3'	30 × (30 s, 94°C; 30 s, 55°C; 30 s, 72°C)	150 bp
RFC1 80AG	F: 5'-AGCGTCACCTTCGTCCC-3'[21] R: 5'-TCCCGCGTGAAGTTCTTG -3'	35 × (30 s, 94°C; 45 s, 52°C; 1 min, 72°C)	230 bp
TCN2 776CG	F: 5'-GGTCGGAGACAACGGATCACC-3'[22] R: 5'-CAAAGCAACCCTCGCCTTGA-3'	35 × (30 s, 94°C; 45 s, 52°C; 1 min, 72°C)	310 bp

F=Forward primer, R=Reverse primer

Table 2: Restriction enzymes and restriction enzyme digestion products

SNPs	Restriction enzymes	Restriction enzyme digestion products (bp)
MTHFR 677CT	HinfI	CC: 198 CT: 198, 175, 23 TT: 175, 23
MTHFR 1298AC	MboII	AA: 72, 37, 28 AC: 100, 72, 28 CC: 100, 28
MTRR 66AG	NdeI	AA: 150 AG: 150, 123, 27 GG: 123, 27
RFC1 80AG	HhaI	AA: 162, 68 AG: 162, 125, 68, 37 GG: 125, 68, 37
TCN2 776CG	ScrFI	CC: 164, 146 CG: 310, 164, 146 GG: 310

SNP=Single nucleotide polymorphism, bp=base pair

digestion products and a DNA ladder of 100 bp were carried out on agarose gel. The size of restriction enzyme digestion products on the gel was estimated by comparing the bands of the sample lane against the DNA ladder.

Statistical analysis

Statistical analysis was done using SPSS for windows program version 25.0 (Armonk, NY: IBM Corp.). Discrepancies in variables between cases and controls were evaluated by the x^2 test. The departure of the genotype's distribution from Hardy–Weinberg equilibrium (HWE) was measured using x^2 goodness-of-fit test. Genotypic and allelic frequencies of cases and control mothers were determined and differences between relative frequencies were analyzed by the x^2 or the Fisher's exact test where suitable. Statistical significance was set at $P < 0.05$.

Ethical approval

Ethical approval was obtained from the National Research Ethics Review Committee at the Ministry of Science and Higher Education-Ethiopia (Ref. No. MoSHE/RD/14.1/465/19, signed by Professor Afework Kassu) on July 17, 2019. The aim of the study was briefly described to the study participants and they were notified that their results will be managed with privacy. Data and samples had been collected after the study participants gave written informed consent.

Results

The study enrolled 200 pregnant women, of whom 100 were case mothers and 100 were control mothers. Among the 100 pregnancies affected by NTDs, there were 79, 17, and 4 spina bifida, anencephaly, and encephalocele cases, respectively.

Cases had a mean age of 26.8 ± 5.25 while that of controls was 26.5 ± 4 years. Forty-six percent of cases and 45% of controls were in the age group of 20–24 and 25–29 years, respectively.

Five percent of the cases reported they had a sibling with NTD out of which one had a previous child with NTD. None of the controls reported having an NTD sibling or a previous child with NTD. However, this result has no statistical significance ($P = 0.059$).

HWE matching

To test for the distribution of genotypes for HWE, the Chi-square goodness-of-fit test was used. For MTHFR 677C > T and MTHFR 1298A > C, the alleles were out of HWE in both the cases and controls. For MTRR 66A > G, RFC1 80A > G, and TCN2 776C > G, the alleles were according to HWE in both the cases and controls [Table 3].

Genotype and allele distribution between cases and controls

Genotypic and allelic distribution of the selected genes among cases and controls is presented in Table 4.

For the MTHFR rs1801133 polymorphism, the CT genotype frequency in cases was 12% while it was 7% in controls, however, this difference was not statistically significant ($P = 0.209$). Statistical significance was not seen for the TT genotype ($P = 0.434$) as well as the T allele ($P = 0.092$).

For MTHFR rs1801131 polymorphism, the AC genotype frequency was higher in case mothers than controls (27%

Table 3: Hardy–Weinberg equilibrium matching of case and control mothers

Gene polymorphisms	Genotypes	Cases		Controls	
		Observed	Expected	Observed	Expected
MTHFR 677C > T	CC	84	81	91	89.3
(rs1801133)	CT	12	18	7	10.4
	TT	4	1	2	0.3
X^2 (*P*)		11.11 (0.008)		10.777 (0.016)	
MTHFR 1298A > C	AA	46	35.4	49	32.5
	AC	27	48.2	16	49
(rs1801131)	CC	27	16.4	35	18.5
X^2 (*P*)		19.350 (<0.001)		45.318 (<0.001)	
MTRR 66A > G	AA	74	68.06	65	63.2
(rs1801394)	AG	21	28.88	31	32.6
	GG	5	3.06	4	4.2
X^2 (*P*)		1.801 (0.386)		0.241 (0.905)	
RFC1 80A > G	AA	49	46.24	48	49
(rs1051266)	AG	38	43.52	44	42
	GG	13	10.24	8	9
X^2 (*P*)		1.611 (0.447)		0.227 (0.893)	
TCN2 776C > G	CC	57	56.25	69	64.8
(rs1801198)	CG	40	37.5	29	31.4
	GG	3	6.25	2	3.8
X^2 (*P*)		0.018 (0.991)		0.585 (0.740)	

Table 4: Genotype and allele distribution of the selected genes of case and control mothers

Gene polymorphisms	Genotypes	Cases	Controls	X^2	OR (95% CI)	*P*
MTHFR 677C > T	CC	84	91			
(rs1801133)	CT	12	7	1.575	0.538 (0.202–1.432)	0.209
	TT	4	2	0.809	0.462 (0.082–2.585)	0.434
	C	180	189			
	T	20	11	2.832	0.524 (0.244–1.124)	0.092
MTHFR 1298A > C	AA	46	49			
(rs1801131)	AC	27	16	2.453	0.556 (0.266– 1.163)	0.117
	CC	27	35	0.358	1.217 (0.640–2.316)	0.55
	A	119	114			
	C	81	86	0.257	1.108 (0.745–1.649)	0.612
MTRR 66A > G	AA	74	65			
(rs1801394)	AG	21	31	2.501	1.681 (0.880–3.208)	0.114
	GG	5	4	0.018	0.911 (0.235–3.536)	1.00
	A	165	159			
	G	35	41	0.585	1.216 (0.737–2.006)	0.444
RFC1 80A > G	AA	49	48			
(rs1051266)	AG	38	44	0.310	1.182 (0.656–2.130)	0.578
	GG	13	8	0.898	0.628 (0.239–1.651)	0.343
	A	136	140			
	G	64	60	0.187	0.911 (0.596–1.392)	0.665
TCN2 776C > G	CC	57	69			
(rs1801198)	CG	40	29	2.891	0.599 (0.331–1.084)	0.089
	GG	3	2	0.422	0.551 (0.089–3.410)	0.660
	C	150	161			
	G	50	39	1.749	0.727 (0.452–1.168)	0.186

vs. 16%). However, this difference was not statistically significant (P = 0.117). In contrast, the CC genotype frequency was greater in controls than in the cases (35% vs. 27%). However, this difference was not statistically significant (P = 0.55). The distribution of the C allele between cases and controls also lacks statistical significance (P = 0.612).

For MTRR rs1801394 polymorphism, the AG genotype frequency was greater in controls than in cases (31% vs. 21%); however, this difference lacks statistical significance ($P = 0.114$). A statistically significant difference was not observed in the frequency of the GG genotype ($P = 1$) and the G allele ($P = 0.444$) between cases and controls.

For the RFC1 rs1051266 polymorphism, a relatively higher frequency is observed for the AG genotype (44% vs. 38%) but without statistical significance ($P = 0.578$). Neither the frequency of the GG genotype ($P = 0.343$) nor did the G allele frequency ($P = 0.665$) differ between the two groups.

For the TCN2 rs1801198 polymorphism, the frequency of the CG genotype was higher in cases than in controls but without statistical significance ($P = 0.089$). The GG genotype and the G allele frequencies were not different between the two groups ($P = 0.660$ and 0.186, respectively).

Discussion

In this study, the role of polymorphisms in MTHFR 677C > T, MTHFR 1298A > C, MTRR 66A > G, RFC-1 80A > G, and TCN2 776C > G genes on NTD risk in women with NTD-affected pregnancy was explored.

The association between maternal MTHFR 677 gene polymorphism and NTD susceptibility has been debated in many communities throughout the world. Our result indicated that there is no difference in the frequency of the CT ($P = 0.209$) and TT ($P = 0.434$) genotypes and the T allele ($P = 0.092$) between cases and controls implying that neither the genotypes nor the allele had an association with the risk of having NTDs affected pregnancy. Similar to our finding, lack of statistically significant difference in genotype or allele frequencies between the two groups was reported[14,23] and lack of association with NTD-affected pregnancy[17,24,25] was reported. Previous data suggest that it is the TT genotype in the NTD-affected embryo, rather than the maternal TT genotype, which is the primary predictor of NTD.[26]

For the MTHFR 1298A > C, no statistically significant difference was found in the frequency of the AC ($P = 0.117$) and CC ($P = 0.55$) genotypes and the C allele ($P = 0.612$) between cases and controls indicating a lack of association of the genotypes and the allele with risk of having NTD-affected offspring. Our finding agrees with previous reports.[23,25,27-29] Even the 1298A > C polymorphism was found to be protective.[17,30] However, a direct association between the MTHFR 1298C and NTD was reported.[8] These conflicting findings may be attributable to other factors that may influence the occurrence of NTDs. MTHFR 677C > T and 1298A > C polymorphisms may thus be a maternal risk factor for NTD-affected pregnancies in some populations but not others.

MTRR restores methylated vitamin B12 from the oxidized form and in so doing plays a vital role in sustaining the active state of the enzyme MS which catalyzes the methylation of homocysteine to methionine.[31] It has been indicated that MTRR 66A > G polymorphisms may potentially impair MS action resulting in increased homocysteine concentrations which is a recognized risk factor for NTDs. A meta-analysis indicated that MTRR 66A > G polymorphism may increase the maternal risk for NTDs.[32] It was reported that the G allele frequency was significantly higher in cases than in controls.[33] However, we found no difference in the distribution of the AG ($P = 0.114$) and GG ($P = 1.00$) genotypes as well as the G allele ($P = 0.444$) between cases and controls implying an absence of connection between MTRR 66A > G polymorphism and NTDs. Our result is similar to previous reports.[12,32] Even the MTRR 66A > G polymorphism was indicated to provide a protective effect in NTD cases.[18] In our study population, it is likely that other factors affected NTD risk.

Our findings indicated that there is no difference in the distribution of the AG genotype ($P = 0.578$) and the GG genotype ($P = 0.343$) as well as the G allele ($P = 0.665$) between cases and controls implying a lack of connection between RFC-1 80A > G polymorphism and NTDs, similar with previous studies.[34,35] A meta-analysis was unable to provide a positive relationship between RFC1 80A > G polymorphism and vulnerability to NTDs.[36] It appears that our study did not find any positive relationship between RFC1 80A > G polymorphism and the risk of having NTDs affected the fetus.

For the TCN2 776C > G (rs1801198), our finding indicated that the G allele ($P = 0.186$) and the CG ($P = 0.089$) and GG ($P = 0.660$) genotype frequencies did not differ between cases and controls; an indication for absence of a relationship between the polymorphism and NTD risk. Our finding is similar to previous reports.[16,24,37] A meta-analysis also indicated that the TCN2 776C > G polymorphism has no significant association with the risks of congenital abnormalities.[38]

Conclusion

The disagreements about the association of the different genotypes and alleles and NTDs among the various studies could be justified by the multiple causes of NTD. We investigated the association between five polymorphisms in four selected folate metabolism genes and the likelihood of having an NTD-affected pregnancy. A statistically significant difference was absent in the frequencies of all the tested genotypes and alleles between case and control mothers; hence, the MTHFR 677C > T, MTHFR 1298A > C, MTRR 66A > G, RFC1 80A > G, and TCN2 776C > G polymorphisms do not appear to have any role in NTDs in our study population, an observation that highlights the importance of other genes or environmental factors in NTD etiology. Additional studies are needed to get a deeper insight into the etiology of NTDs in Ethiopia.

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Conflicts of interest

There are no conflicts of interest.

References

1. Li J, Feng D, He S, Yang H, Su Z, Ye H. Association of MTHFR 677C>T gene polymorphism with neonatal defects: A meta-analysis of 81444 subjects. J Obstet Gynaecol 2022;42:1811-22.
2. Finnell RH, Caiaffa CD, Kim SE, Lei Y, Steele J, Cao X, *et al*. Gene environment interactions in the etiology of neural tube defects. Front Genet 2021;10;12:659612.
3. Zhang R, Huo C, Wang X, Dang B, Mu Y, Wang Y. Two common MTHFR gene polymorphisms (C677T and A1298C) and fetal congenital heart disease risk: An updated meta-analysis with trial sequential analysis. Cell Physiol Biochem 2018;45:2483-96.
4. Raghubeer S, Matsha TE. Methylenetetrahydrofolate (MTHFR), the one-carbon cycle, and cardiovascular risks. Nutrients 2021;13:4562.
5. Petrone I, Bernardo PS, Dos Santos EC, Abdelhay E. MTHFR C677T and A1298C polymorphisms in breast cancer, gliomas and gastric cancer: A review. Genes (Basel) 2021; 12:587.
6. Rozen R. Genetic predisposition to hyperhomocysteinemia: Deficiency of methylenetetrahydrofolate reductase (MTHFR). Thromb Haemost 1997;78:523-6.
7. Weisberg IS, Jacques PF, Selhub J, Bostom AG, Chen Z, Curtis Ellison R, *et al*. The 1298A ≥ C polymorphism in methylenetetrahydrofolate reductase (MTHFR): *In vitro* expression and association with homocysteine. Atherosclerosis 2001;156:409-15.
8. De Marco P, Calevo MG, Moroni A, Arata L, Merello E, Finnell RH, *et al*. Study of MTHFR and MS polymorphisms as risk factors for NTD in the Italian population. J Hum Genet 2002; 47:319-24.
9. Zhong G, Luo X, Li J, Liao Y, Gui G, Sheng J. MTRR rs1532268 polymorphism and gastric cancer risk: Evidence from a meta-analysis. J Int Med Res 2022;50:3000605221097486.
10. Gaughan DJ, Kluijtmans LA, Barbaux S, McMaster D, Young IS, Yarnell JW, *et al*. The methionine synthase reductase (MTRR) A66G polymorphism is a novel genetic determinant of plasma homocysteine concentrations. Atherosclerosis 2001;157:451-6.
11. Vaughn JD, Bailey LB, Shelnutt KP, von-Castel Dunwoody KM, Maneval DR, Davis SR, *et al*. Methionine synthase reductase 66A ≥ G polymorphism is associated with increased plasma homocysteine concentration when combined with the homozygous methylenetetrahydrofolate reductase 677C ≥ T variant. J Nutr 2004;134:2985-90.
12. Wilson A, Platt R, Wu Q, Leclerc D, Christensen B, Yang H, *et al*. A common variant in methionine synthase reductase combined with low cobalamin (vitamin B12) increases risk for spina bifida. Mol Genet Metab 1999;67:317-23.
13. Hou Z, Matherly LH. Biology of the major facilitative folate transporters SLC19A1 and SLC46A1. Curr Top Membr 2014;73:175-204.
14. Shang Y, Zhao H, Niu B, Li WI, Zhou R, Zhang T, *et al*. Correlation of polymorphism of MTHFRs and RFC-1 genes with neural tube defects in China. Birth Defects Res A Clin Mol Teratol 2008;82:3-7.
15. Stanisławska-Sachadyn A, Woodside JV, Sayers CM, Yarnell JW, Young IS, Evans AE, *et al*. The transcobalamin (TCN2) 776C > G polymorphism affects homocysteine concentrations among subjects with low vitamin B(12) status. Eur J Clin Nutr 2010;64:1338-43.
16. Afman LA, Lievers KJA, van der Put NMJ, Trijbels FJM, Blom HJ. Single nucleotide polymorphisms in the transcobalamin gene: Relationship with transcobalamin concentrations and risk for neural tube defects. Eur J Hum Genet 2002;10:433-8.
17. Godbole K, Gayathri P, Ghule S, Sasirekha BV, Kanitkar-Damle A, Memane N, *et al*. Maternal one-carbon metabolism, MTHFR and TCN2 genotypes and neural tube defects in India. Birth Defects Res A Clin Mol Teratol 2011;91:848-56.
18. Relton CL, Wilding CS, Pearce MS, Laffling AJ, Jonas PA, Lynch SA, *et al*. Gene-gene interaction in folate-related genes and risk of neural tube defects in a UK population. J Med Genet 2004;41:256-60.
19. Aly RM, Taalab MM, Ghazy HF. MTHFR A1298C and C677T gene polymorphisms and susceptibility to chronic myeloid leukemia in Egypt. Int J Clin Exp Pathol 2014;7:2571-8.
20. Brown CA, McKinney KQ, Kaufman JS, Gravel RA, Rozen R. A common polymorphism in methionine synthase reductase increases risk of premature coronary artery disease. J Cardiovasc Risk 2000;7:197-200.
21. Soghani B, Ebadifar A, Khorram Khorshid HR, Kamali K, Hamedi R, Aghakhani Moghadam F. The study of association between reduced folate carrier 1 (RFC1) polymorphism and non-syndromic cleft lip/palate in Iranian population. Bioimpacts 2017;7:263-8.
22. Meyer K, Fredriksen A, Ueland PM. High-level multiplex genotyping of polymorphisms involved in folate or homocysteine metabolism by matrix-assisted laser desorption/ionization mass spectrometry. Clin Chem 2004;50:391-402.
23. Félix TM, Leistner S, Giugliani R. Metabolic effects and the methylenetetrahydrofolate reductase (MTHFR) polymorphism associated with neural tube defects in southern Brazil. Birth Defects Res A Clin Mol Teratol 2004;70:459-63.
24. Candito M, Rivet R, Herbeth B, Boisson C, Rudigoz RC, Luton D, *et al*. Nutritional and genetic determinants of vitamin B and homocysteine metabolisms in neural tube defects: A multicenter case-control study. Am J Med Genet A 2008;146A:1128-33.
25. Boduroğlu K, Alanay Y, Alikaşifoğlu M, Aktaş D, Tunçbilek E. Analysis of MTHFR 1298A > C in addition to MTHFR 677C > T polymorphism as a risk factor for neural tube defects in the Turkish population. Turk J Pediatr 2005;47:327-33.
26. Shields DC, Kirke PN, Mills JL, Ramsbottom D, Molloy AM, Burke H, *et al*. The "thermolabile" variant of methylenetetrahydrofolate reductase and neural tube defects: An evaluation of genetic risk and the relative importance of the genotypes of the embryo and the mother. Am J Hum Genet 1999;64:1045-55.
27. Grandone E, Corrao AM, Colaizzo D, Vecchione G, Di Girgenti C, Paladini D, *et al*. Homocysteine metabolism in families from southern Italy with neural tube defects: Role of genetic and nutritional determinants. Prenat Diagn 2006;26:1-5.
28. Parle-McDermott A, Mills JL, Kirke PN, O'Leary VB, Swanson DA, Pangilinan F, *et al.* Analysis of the MTHFR 1298A → C and 677C → T polymorphisms as risk factors for neural tube defects. J Hum Genet 2003;48:190-3.
29. Volcik KA, Blanton SH, Tyerman GH, Jong ST, Rott EJ, Page TZ, *et al*. Methylenetetrahydrofolate reductase and spina bifida: Evaluation of level of defect and maternal genotypic risk in Hispanics. Am J Med Genet 2000;95:21-7.
30. Nasri K, Midani F, Kallel A, Jemaa NB, Aloui M, Boulares M, *et al*. Association of MTHFR C677T, MTHFR A1298C, and MTRR A66G polymorphisms with neural tube defects in tunisian parents. Pathobiology 2019;86:190-200.
31. O'Leary VB, Mills JL, Pangilinan F, Kirke PN, Cox C, Conley M, *et al*. Analysis of methionine synthase reductase polymorphisms for neural tube defects risk association. Mol Genet Metab 2005;85:220-7.
32. Ouyang S, Li Y, Liu Z, Chang H, Wu J. Association between MTR A2756G and MTRR A66G polymorphisms and maternal risk for neural tube defects: A meta-analysis. Gene 2013;515:308-12.
33. Cai CQ, Fang YL, Shu JB, Zhao LS, Zhang RP, Cao LR, *et al*. Association of neural tube defects with maternal alterations and genetic polymorphisms in one-carbon metabolic pathway. Ital J Pediatr 2019;45:37.
34. O'leary VB, Pangilinan F, Cox C, Parle-McDermott A, Conley M, Molloy AM, *et al*. Members of the Birth Defects Research Group. Reduced folate carrier polymorphisms and neural tube defect risk. Mol Genet Metab 2006;87:364-9.

35. Vieira AR, Murray JC, Trembath D, Orioli IM, Castilla EE, Cooper ME, *et al*. Studies of reduced folate carrier 1 (RFC1) A80G and 5,10-methylenetetrahydrofolate reductase (MTHFR) C677T polymorphisms with neural tube and orofacial cleft defects. Am J Med Genet A 2005;135:220-3.
36. Wang HG, Wang JL, Zhang J, Zhao LX, Zhai GX, Xiang YZ, *et al*. Reduced folate carrier A80G polymorphism and susceptibility to neural tube defects: A meta-analysis. Gene 2012;510:180-4.
37. Swanson DA, Pangilinan F, Mills JL, Kirke PN, Conley M, Weiler A, *et al*. Evaluation of transcobalamin II polymorphisms as neural tube defect risk factors in an Irish population. Birth Defects Res A Clin Mol Teratol 2005;73:239-44.
38. Oussalah A, Levy J, Filhine-Trésarrieu P, Namour F, Guéant JL. Association of TCN2 rs1801198 c. 776G > C polymorphism with markers of one-carbon metabolism and related diseases: A systematic review and meta-analysis of genetic association studies. Am J Clin Nutr 2017;106:1142-56.