Research Article

Genetic Polymorphisms in Methionine Synthase and Methionine Synthase Reductase, their Metabolic Effects, and Risk of Neural Tube Defects in Algerian Population

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ABSTRACT

Impairment of folate and homocysteine metabolism has been observed in mothers of fetuses with neural tube defects (NTDs) and infants affected; they are considered as risk factors. The novelty of this study is to investigate, in Algerian population, the contribution of two polymorphic variations in genes involved in the folate dependent homocysteine metabolism in the aetiology of neural tube defects. Methionine synthase (MTR) A2756G and methionine synthase reductase (MTRR) A66G variants have been identified using PCR-RFLP analysis on a group of 48 NTD infants, 38 mothers of NTD cases and 67 normal controls. The effects of mutant variants on total plasma homocysteine (tHcy), serum folate and RBC (red blood cell) folate concentrations have also been evaluated in studied mothers of NTD cases. The calculated odds ratios (OR) show that the polymorphism A2756G of the MTR gene was not associated with NTD risk for both mothers and NTD cases. It seems not to influence homocysteine or serum folate concentrations but it does for RBC folate. The mean RBC folate level was significantly less in mothers of NTD cases having AG genotype (148.25 ± 47.55 ng/ml) with respect to mothers harbouring AA genotype (403.78 ± 213.18 ng/ml) ($p < 0.05$). Thus, MTRA2756G mutation could disrupt folate metabolism in mothers of our population. MTRR A66G mutationhas no significant influence on NTD risk or on metabolites levels.

Keywords: methionine synthase, methionine synthase reductase, Folate, Homocysteine, Neural tube defects.

INTRODUCTION

Neural tube defects (NTDs) are a common group of central nervous system anomalies that appear to result from a combination of genetic and environmental factors.[1] Maternal supplementation with folic acid during pregnancy reduces NTDs frequency[2,3] leading to folate metabolism to be the focus of intense researches aiming to clarify the mechanisms underlying this observation. In addition, several studies have indicated that mothers of NTD-affected infants exhibit elevated plasma homocysteine (Hcy) levels, suggesting a disturbed folate-dependent homocysteine metabolism as one of the hypothesized mechanisms.[4,5]

Folate and homocysteine metabolic cycles are closely related and involve over 25 proteins, many of which have been investigated for association with an increased NTD risk.[6]

Homocysteine is metabolized to methionine by a remethylation pathway. The latter is catalyzed by the cobalamin-dependent methionine synthase (MTR) requiring folate as a methyl donor.[7] Indeed, the active complex MTR bound to cobalamin, called cbl(I)MTR, can bind the methyl group of 5 methyl THF to form cbl(III)MTR that transfers the methyl group to homocysteine. Cbl(I)MTR can be oxidized into the inactive cbl(II)MTR and the enzyme becomes inactivated.[6]

The enzyme methionine synthase reductase (MTRR) catalyzes the regeneration of the methyl cobalamin cofactor from the oxidized cbl(II)MTR, and thus maintains methionine synthase in an active state for the remethylation of homocysteine to methionine.[6,7]

Consequently, mutations in MTR or MTRR genes could influence the methylation cycle, homocysteine and folate levels or predispose to NTD susceptibility.

MTR A2756G mutation (A to G transition) located at position 919 of the protein results in the substitution of aspartic acid into glycine.[8] It is located in a domain of the protein involved in cofactor binding.

It could have an effect on their secondary structure and therefore leads to functional consequences.[9] Mutant G allele frequency was approximately equal to 0.15 - 0.20.

MTRRA66G mutation (A to G transition) results in the substitution of isoleucine by methionine.

It is located in the putative flavin mononucleotide-binding domain of the MTRR enzyme, which interacts with MTR[10], and thus, disrupts the binding of MTRR to the MTR-cobalamin-complex, decreasing the rate of homocysteine remethylation.[11] The mutant G allele has a frequency of approximately 0.50.

In the current study, we examined a group of patients with NTDs, mothers of NTD infants and a group of controls for MTR A2756G and MTRR A66G polymorphisms to assess their impact on NTD risk.

The effect of the two polymorphisms on homocysteine and nutrients (RBCand serum folate) levels has been investigated in mothers at risk.

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This topic is poorly investigated in Algerian population. These polymorphisms are for the first time considered in relation with NTD risk through this paper.

MATERIALS AND METHODS

Subjects

The study population included 48 infants with NTDs, 38 mothers of NTD infants aged between 21 and 41 years and a control group including 67 mothers aged between 19 and 44 years. There were recruited from the nursery and maternity services of Constantine University Hospital and Ouargla Hospital, both located in the eastern part of Algeria.

None of the mothers who had an affected NTD infant were pregnant at the time of blood sampling or under treatment of any kind, nor use vitamin supplements. The control group is composed of women who had at least one normal pregnancy, and never had children affected by NTD. They have been selected to fill the same age, socio-economic level and conditions mentioned above as those of mothers of NTD patients. Local ethics committee approved the protocol and informed consent was obtained from the participant relatives.

Metabolites Analysis

Metabolites concentrations were determined for mothers of NTD cases, since the NTD patients had a lower mean age and were often taking medicines, possibly affecting the folate/homocysteine metabolism. Fasting blood samples were collected for:

Homocysteine Analysis

blood samples were collected on ethylene diamine tetra acetic acid (EDTA) tubes, transported on ice and centrifuged at 4000 rpm for 8 min, the plasma obtained was frozen at – 20 °C.

RBC (Red Blood Cell) Folate Analysis

fresh whole blood treated by ascorbic acid was used for RBC folate determination. The concentration is first determined in whole blood then precisely in RBC cell.

Serum folate Analysis

blood samples were collected on dried tubes and the serum obtained after centrifugation at 4000 rpm during 10 minutes was stored 6 to 8 weeks at -20°C.

Total plasma homocysteine (tHcy), RBC folate and serum folate levels were determined by a competitive immunoassay using the IMMULITE 2000 system.

Genetic Analyses

DNA was extracted from blood required in vacutainer K3EDTA tubes using NaCl method. Polymorphisms in genes were examined using PCR-RFLP analysis.

For MTR A2756G polymorphism (rs1805087), amplification was performed using forward 5'CATGGAAGAATATGAAGATATTAGAC3' and reverse5'GAACTAGAAGACAGAAATTCTCTA3' primer sequences. Cycling conditions were : initial denaturation at 95°C for 2 min followed by 35 cycles of 94°C for 40 s, 50°C for 40 s, and 72°C for 1 min with a final extension for 10 min at 72°C. PCR was performed in a total volume of 50 µl containing genomic DNA, 50 mM $MgCl_2$, 2.5mMof each deoxynucleotide triphosphate, 7 pmol of each primer, 1.2 U Taq DNApolymeraseand 5µl of 10X reaction buffer. PCR products were digested by HaeIII for 4 hours at 37°C. The products were visualized by ethidium bromide staining in a 3% agarose gel electrophoresis. The mutation creates a restriction site causing a cleavage of the amplified 189 bp fragment on two fragments 159 and 30 Pb.

For MTRR A66G Polymorphism (rs1801394) a sequence of 150 pb was amplified using both the forward primer: 5'CAGGCAAAGGCCATCGCAGAAGA<u>C</u>AT-3' and reverse primer 5' CACTTCCCAACCAAAATTCTTCAAAG 3'which are previously described by Jacques (2003).[12] Forward primer contains a mismatch (underlined base), which creates a restriction site for AflIII when the methionine-containing allele is present.

Cycling conditions of PCR were : initial denaturation at 95°C for 5 min followed by 45 cycles of 94°C for 20 s, 58°C for 40 s, and 72°C for 20 s with a final extension for 3 min at 72°C.

PCR amplification was performed in 22.5 µl reaction volumes, containing genomic DNA, 2.5 mM of each deoxynucleotide triphosphate, 20 µM of each primer, 5 UTaq DNA polymerase and 2.5 µl of 10X reaction buffer with $MgCl_2$.

Digestion is performed by AflIII one hour at 37°C, the normal allele A fragment remains undigested (150Pb) and mutant allele G gives two fragments 123 and 27 Pb.

Statistical Analyses

The risk of NTD associated with genotype was estimated by odds ratio (OR), with 95 % confidence intervals. The level of significance was set at $P < 0.05$. The test for significant differences in metabolites levels stratified according to genotypes was performed using analysis of variance (ANOVA) in the case of equal variance and using the Mann Witney test in the case of unequal variance. Statistics were conducted using Epi info logiciel (version 6.0).

RESULTS

Distribution of Genotypic and Allelic Frequencies of the Polymorphisms in Groups of NTD Cases, Mothers of NTD Cases and Control Group

The observed prevalences of gene mutations in controls versus mothers and NTD cases are given in **Table 1**. All distributions were in agreement with Hardy-Weinberg equilibrium.

The frequencies of the G allele of the MTR A2756G polymorphism are equal to 0.1, 0.1 and 0.13 among NTD

cases, mothers of NTD cases and controls, respectively. For the G allele of MTRR A66G polymorphism, frequencies are of 0.55, 0.47, and 0.45 among patients, mothers, and controls, respectively. There is no significant difference of mutant allele's frequencies between infants with NTDs and controls and between mothers of NTD cases and controls for the two polymorphisms. Concerning genotypes, the mutant homozygous MTR 2756GG genotype is absent in both mothers of NTD cases and controls. It is present only in one NTD case (2.08 %). Heterozygous genotype frequencies are of 16.66 % for infants with NTDs, 21.05 % for mothers of NTDs and 25.37 % for controls.

Statistically, there was no significant difference of homozygous or heterozygous genotype frequencies of this polymorphism between cases and controls or between mothers and controls (p > 0.05).

For A66G polymorphism, there was a slightly higher percentage of case mothers with the GG genotype, compared with controls, the frequency was equal to 21.05 % for mothers versus 13.4 % for controls.

The estimated odds ratio was 1.30(0.30-5.71) and the difference was not statistically significant. The others mutant genotype frequencies for this polymorphism in mothers or cases were not significantly different from those of control subjects (p > 0.05).

Thus, no mutant variant for the two polymorphisms represented a significant risk factor for mothers or NTD cases in our population.

Effect of Polymorphisms on Metabolites Levels

Metabolites levels were stratified according to genotypes in mothers of NTD infants. For the polymorphism A2756G, mothers do not have GG genotype why comparison was only realized between mothers having AG genotypes and those having AA genotypes **(Table 2).**

For the MTR A2756G polymorphism, it was observed that the mean RBC folate level was significantly less in mothers possessing AG genotype with respect to mothers having AA genotype (p value = 0.02).

MTR 2756AG genotype resulted also in a decreased serum folate levels but with a lack of significance (p > 0.05), values were of 4.5 ± 2.74 for AG vs 10.10 ± 6.29 for AA genotype.

The mean homocysteine concentration was slightly higher in mothers possessing MTR2756AG genotype compared with mothers having AA genotype, but the difference was not statistically significant, suggesting that there is no association between this polymorphism and increased total homocysteine levels.

About MTRR A66G polymorphism, we did not observe any significant influence of genotypes on metabolites concentrations.

Table 1: Genotypic distribution and allelic frequencies of the polymorphisms in NTD cases, mothers of NTD cases and controls

	Genotypic Frequencies %(n)			Allelic Frequencies	
MTR A2756G	**AA**	**AG**	**GG**	**A**	**G**
Infants with NTD	81.25 (39)	16.66 (8)	2.08 (1)	0.89	0.1
Mothers of NTD cases	78.94 (30)	21.05 (8)	0	0.89	0.1
Controls	74.63 (50)	25.37 (17)	0	0.87	0.13
OR Infants x Controls					
Genotype GG		----		0.8 (0.35-1.83)	
Genotype AG	0.72 (0.22-2.37)				
OR Mothers x Controls					
Genotype GG		----		0.81 (0.34-1.95)	
Genotype AG	0.75 (0.21-2.62)				
MTRR A66G	**AA**	**AG**	**GG**	**A**	**G**
Infants with NTD	41.66 (20)	52.08 (25)	6.25 (3)	0.67	0.55
Mothers of NTD cases	26.31 (10)	52.63 (20)	21.05 (8)	0.53	0.47
Controls	22.4 (15)	64.2 (43)	13.4 (9)	0.54	0.45
OR Infants x Controls					
Genotype GG		0.29 (0.05-1.53)		0.98 (0.66-1.46)	
Genotype AG		0.43 (0.16-1.12)			
OR Mothers x Controls					
Genotype GG		1.30 (0.30-5.71)		1.08 (0.61-1.91)	
Genotype AG		0.74 (0.25-2.21)			

OR, Odds Ratio; NTD, Neural Tube Defect

Table 2: Impact of Polymorphims on Serum Folate, RBC Folate and Homocysteine Levels in Mothers of NTD Cases

	Mothers of NTD cases		
	RBC folate (ng/ml)	Serum Folate (ng/ml)	Homocysteine (µmol/l)
MTR A2756G			
AA	403.78 ± 213.18	10.10 ± 6.29	12.83 ± 10.18
AG	148.25 ± 47.55	4.5 ± 2.74	14.28 ± 10.89
P	0.02*	NS	NS
MTRR A66G			
AA	405.48 ± 169.05	11.2 ± 7.08	16.53 ± 15.76
AG/GG	345.46 ± 225.22	9.06 ± 6.17	11.10 ± 6.73
P	NS	NS	NS

*P < 0.05, NS: not significant

DISCUSSION

In this study, we analysed data on mutant variants of two genes involved in homocysteine remethylation; methionine synthase A2756G polymorphism and methionine synthase reductase A66G polymorphism which emerged as a possible genetic risk factor for NTDs.

In our population, the MTR A2756G polymorphism was not shown to be associated with NTD risk, both in mothers and infants consistently with many other studies. Indeed, van der Put (1997) and Morrison (1998) failed to demonstrate a significant association between A2756G polymorphism and NTD risk in Netherlands and UK populations, respectively.[9,13] Later, several studies[14-22] on European, American and Asiatic populations suggested that mothers or children with A2756G polymorphism have no risk for NTDs development.

In addition, a recent meta-analysis undertaken by Ouyang (2013) on a total of 11 studies has revealed no significant association between maternal MTR A2756G polymorphism and NTD susceptibility.[23]

Authors have also conducted a meta-analysis including numerous studies (13 articles) evaluating the association between this polymorphism and infants NTD risk. The work has shown no significant association with a risk for NTDs in Caucasian children.[24]

Only few studies found a relationship between this polymorphism and the risk of NTDs. The presence of the G allele has been shown to be associated with an increased risk for mothers to have a child with NTD.[25,26] Similarly, the genotypes AG/GG appear to increase the risk of children with spina bifida.[26,27]

Therefore, although methionine synthase play a crucial role in the metabolism of folate and homocysteine and despite the AG transition at bp 2756 occurs near the crucial vitamin B-binding site and might influence the enzyme secondary structure, it generally appears not to be a risk factor for NTDs development. However, this polymorphism may be a risk factorthrough its influence on the rate of folate and homocysteine.

According to the literature, the A2756G polymorphism may decrease the rate of the conversion of 5-methyl-THF to THF then results in physiological folate deficiency. Indeed, in their recent study, Li (2015) found that the MTR 2756AG + GG genotypes reduced the serum folate level and increased the folate deficiency risk.[28]

In our population, the MTR 2756AG genotype does not seem to influence serum folate levels, certainly, the level was lower in AG mothers compared to AA mothers but there is no significant difference. However, a significant decrease in RBC folate level associated to the mutation (AG genotype) was observed in mothers of NTD infants. RBC folate responsiveness is an important aspect of these birth defects. It is generally considered to be a more useful indicator of folate status than serum folate, since it reflects levels of intracellular folate and folate turnover during the past 120 days.[29] We conclude that, in Algerian population, MTRA2756G polymorphism contributes to NTD risk through the disruption of folate metabolism resulting in depletion of the long-term storage pools for folate in RBC cell.

Obtained results about the plasma concentration of homocysteine with respect to genotypes are consistent with several works that failed to demonstrate any association between the A2756G mutation and this metabolite.[9,12,30-35]

This finding can be explained by the fact that the mutation to have a significant effect on the levels of homocysteine, it has to be associated to another one. Laraqui (2006) found that combined effect of the two mutations MTR A2756G and MTRR A66G increase the risk for having hyperhomocysteineamia.[36]

For MTRR A66G polymorphism, we found that there is no influence on NTD risk among infants. This result is consistent with the paper published by O'Leary (2005).[18] Later, in their meta-analysis carried out on children with

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NTDs of different populations, Ouyang (2013) found no significant correlation between this polymorphism and NTD risk.[24] Concerning mothers, we could not observe a significant association between this polymorphism and NTD risk, which is in a good agreement with others studies.[18,37]

Nonetheless, in our population, we noted a slightly higher proportion of case mothers who possessed the 66G allele in the homozygous state compared with controls, even if statistically the difference is not significant.

Several papers support the presence of a relationship between A66G polymorphism and an increased risk of having an affected infant.[17,26,38] Indeed, the large and recent meta-analysis undertaken by Yadav (2015) (based on 57 articles) found that the maternal A66G polymorphism was a risk factor for producing offspring with NTDs.[39]

Accordingly, we can say that Algerian mothers with MTRR homozygote 66GG genotype may be at an increased risk for NTD susceptibility. Future studies with a larger sample size are needed to validate this statement.

Stratification according to genotypes has shown no association of AG and GG genotypes with any of the nutrients and homocysteine parameters. Our results are consistent with those found in the literature, the MTRR A66G polymorphism itself may not affect the plasma folate level[28,40-43] or the incidence of folate deficiency.[28] Similarly, in most studies, the effect of this polymorphism on plasma homocysteine could not be observed.[12,40,41,44-46]

Olteanu (2002) found that the mutation does not lead to a major change in protein conformation. However, in the presence of a mutation a higher ratio of 3 to 4 fold of MTRR to MTR was required to reach maximal enzyme activity.[11] Thus, MTRR deficiency will adversely affect normal function of MTR which may result in a disturbance of homocysteine remethylation. Despite this, we assume the mutation does not affect metabolites levels because of its low independent effect.

CONCLUSION

We expected to know through this work if A2756G and A66G polymorphisms are involved in the development of NTDs in Algerian population.

Although, investigation of MTR A2756G polymorphism as a NTD risk appears to be negative, association of this polymorphism with a decrease of RBC folate level strengthen the contribution of A2756G mutation in NTDs occurrence.

Data on the association between MTRR A66G polymorphism, plasma homocysteine and serum/RBC folate concentrations as well as the relationship between this polymorphism and NTD risk are inconclusive.

As an interesting perspective for this study, the enlargement of the sample size in order to better show the influence of MTR and MTRR mutations on metabolite levels by studying their interactive effect.

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