LETTER TO JMG

Changes in frequencies of heterozygous thermolabile 5,10-methylenetetrahydrofolate reductase gene in fetuses with neural tube defects

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Periconceptional folic acid (pteroylglutamic acid) supplementation or the fortification of staple foods with folic acid have been shown to reduce the risk for pregnancies with neural tube defects (NTDs).[1–4] However, mechanistic link(s) between this increased intake of folic acid and NTD prevention remains unclear. Homozygous or heterozygous variants of the thermolabile 5,10-methylenetetrahydrofolate reductase (*MTHFR*) gene have also been associated with a high risk for NTDs.[5–7] We recently reported the association of NTDs with *MTHFR* genotypes using fetal cells of NTD cases and their controls obtained between 1988 and 1998. The fetuses with either heterozygous or homozygous genotypes of *MTHFR* had a higher risk for NTDs than those with wild type.[7]

Further analysis of the data indicated a drastic change in the frequency of the heterozygous genotype of *MTHFR* among 77 NTD affected fetuses starting in 1994. However, no such change was found in 77 control fetuses (controls) without malformations, who were matched for race, maternal age, and time of amniocentesis. The mean maternal age was 28 years old and the subject population consisted of 84% whites and 16% African-Americans. The diagnosis of NTDs was made by ultrasound examination and amniocentesis and confirmed by neonatal examination or necropsy.[7] The amniotic fluid samples were obtained aseptically under ultrasound guidance at the University of Alabama Prenatal Genetics Clinic. The study was approved by the Institutional Review Board for Human Use of the University of Alabama at Birmingham. Amniotic fluid samples were stored at −70°C until analysis and those obtained between 1988 and 1998 were available for the analysis. Polymorphisms of the *MTHFR* gene were determined by polymerase chain reaction.[8] The fetuses homozygous and heterozygous for the alanine to valine mutation are designated Val/Val and Ala/Val, respectively, and those with wild type are designated Ala/Ala. The differences in genotype frequencies between the two periods were assessed by Fisher's exact test, and the risk ratios and 95% confidence intervals (CI) were calculated by contingency table analysis software (Statistical Solutions, Ann Arbor, MI, USA). A p value less than 0.05 was considered significant.

Table 1 shows the frequencies of *MTHFR* genotypes in cases and controls during the two periods of 1988-mid 1994 and mid 1994-1998. There was a significant difference among the cases in the frequencies of Ala/Val between the periods of 1988-mid 1994 (51%) and mid 1994-1998 (25%) (p=0.02). However, the frequencies of Val/Val among the cases increased from 41% to 63%, although the difference was not significant (p=0.07). No change in Ala/Ala genotypes in the cases was observed between the two periods (p=0.71). The frequencies of the three genotypes in the controls remained similar throughout the study period (p=0.75, 0.36, and 0.50). The risk ratio for NTDs in fetuses with Ala/Val was 9.8 (95% CI 2.8-34.0) between 1988 and mid 1994, whereas it was 1.9 (95% CI 0.63-5.7) between mid 1994 and 1998. The risk ratio in both periods combined was 4.1 (95% CI 1.9-9.4) as previously described.[7] The risk ratios in those with Val/Val for the two periods calculated independently were not significant, probably owing to a small sample size of the controls. However, the risk ratio in all fetuses with Val/Val combined for both periods was significant at 6.4 with 95% CI of 1.3-31.7, as previously reported.[7]

Weitkamp *et al*[9] reported a higher than expected frequency of Ala/Val in females with NTDs. We observed a similar trend in pre-1994 NTD cases, with more female than male fetuses having the Ala/Val genotype; however, the difference was not significant, probably because of a small sample size in our study. More importantly, there was a significant difference in the frequencies of Ala/Val between pre- and post-1994 in female cases. In the 1988-mid 1994 period, 11 of 36 (31%) female cases were Ala/Val, whereas only one of 35 (3%) was Ala/Val in the mid 1994-1998 period (p<0.003). There was also a significantly greater frequency of Ala/Ala in female

Table 1 Frequencies of *MTHFR* genotypes in NTD cases and controls during 1988-1998

	1988–mid 1994			Mid 1994-1998		
Genotypes	Ala/Ala (male/female)	Ala/Val (male/female)	Val/Val (male/female)	Ala/Ala (male/female)	Ala/Val (male/female)	Val/Val (male/female)
NTD cases	15/37 (41)* (10/5)§	19/37 (51)† (7/11)‡¶	3/37 (8) (1/2)	25/40 (63) (11/13)§	10/40 (25)† (5/1)¶	5/40 (13) (3/2)
Controls	31/35 (89) (16/15)	4/35 (11) (2/2)	0/35 (0) (0/0)	33/42 (79) (12/17)	7/42 (17) (3/3)	2/42 (5) (0/2)
Risk ratios (95% CI)	1.0	9.8 (2.8-34.0)	12.4 (0.58-263.6)	1.0	1.9 (0.63-5.7)	0.30 (0.05-1.7)

*Number of subjects/number in all three genotypes combined in each period (%).
†The difference in genotype frequencies between the two periods was significant (p=0.02).
‡Information on the gender of fetuses was not available for all subjects.
§The difference in male/female ratios between the two periods was significant (p<0.0025).
¶The difference in male/female ratios between the two periods was significant (p<0.003).

cases in the post-1994 group (37%) than in pre-1994 (14%) ($p<0.0025$).

We postulate that environmental changes including increased folic acid or dietary folate intake were responsible for the change over the period studied here, although we do not have any direct evidence to prove this. Since folates in the samples stored without reducing agents, such as ascorbic acid, are known to degrade over time, we did not measure folate concentrations in amniotic fluid. Furthermore, plasma samples of the subjects were unavailable for analysis. The majority of the mothers who participated in this study were from a well educated, middle class population receiving prenatal care from private obstetricians. These physicians were most likely aware of the recommendation of a sufficient amount of folate intake during the periconceptional period by the Centers for Disease Control and Prevention[10] and the American College of Obstetrics and Gynecology.[11] It is likely that the rate of taking periconceptional folic acid supplementation was much greater starting around 1993-1994 in our subjects than the other populations previously reported.[12] It is possible that this increased folic acid intake reverses abnormal folate metabolism, which could lead to NTD development. Based on our data, about 50% of fetuses with the Ala/Val genotype who had been possibly destined to have NTDs might not have developed NTDs, when a large dose of folic acid was taken by their mothers during the critical period of neural tube closure.

Similarly, the decreased frequency of Ala/Val in post-1994 female cases could be the result of increased folic acid intake. In the study reported by Weitkamp *et al*,[9] the gender difference in Ala/Val was attributed, in part, to the possibility that Ala/Val females with NTDs are less likely to abort than Ala/Val males with NTDs. Potential explanations of our results include: (1) improved maternal folate nutrition prevented NTD development in Ala/Val female fetuses; and (2) increased maternal folate intake led to an increased chance of abortion of Ala/Val female cases, as suggested by Weitkamp *et al*.[9] These hypotheses should be tested in the future. At present, there is no explanation for the greater frequency of the Ala/Ala genotype in female cases after 1994 (table 1). This shift may have been the result of the change in folate intake; however, its mechanistic basis requires further investigation.

Our findings may be analogous to those of Munoz-Moran *et al*,[13] who reported that the frequency of the Val/Val *MTHFR* genotype increased from 13% to 26% starting around 1982. They reported that this timing coincided with the recommendation of folic acid supplementation early in pregnancy by the Spanish National Health Service. However, Whitehead[14] argued against this report based on potential sampling biases. Is it possible that our finding is caused by sampling biases? We think that this is unlikely in our study. We selected all the NTD cases, diagnosed between 1988 and 1998, whose amniotic fluid samples were available for analysis, and controls were matched by race, maternal age, and timing of amniocentesis. Furthermore, the frequency of Val/Val and Ala/Ala *MTHFR* genotypes in cases and that of all three genotypes in controls did not significantly change during the entire period of 11 years. Considering the data by Spanish investigators, along with our findings, it is possible that the frequencies of *MTHFR* genotypes are sensitive to the changes in various environmental factors among certain populations. In addition to *MTHFR* genotypes, there is a report indicating the alteration in the frequencies of other polymorphisms over time among NTD cases. Shields *et al*[15] reported that the frequency of the *T* allelic variant *TIVS7-2* was highly associated with NTD cases which were diagnosed before, but not after, 1980, although the mechanism of the disappearance of this association is unknown.

Environmental changes, different geographical regions, racial/ethnic groups, and gender affect the frequency distribution of *MTHFR* genotypes.[9 16] Considering such possible factors affecting the frequency of *MTHFR* genotypes, caution should be used in the assessment of the risk ratios for NTDs by comparing the frequencies of *MTHFR* genotypes of the cases to those of the controls. Our finding suggests that it would be important to evaluate the frequency of *MTHFR* genotypes in fetuses or newborns with NTDs before and after folic acid fortification of foods, such as the programme started in the USA in 1998.

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