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Investigation of Folate Pathway Gene Polymorphisms and the Incidence of Neural Tube Defects in a Texas Hispanic Population

Robert Barber,*,† Stuart Shalat,‡ Kate Hendricks,§ Brigitte Joggerst,‡ Russell Larsen,§ Lucina Suarez,§ and Richard Finnell*,†,[1]

**Center for Environmental and Rural Health, Texas A&M University, College Station, Texas; †Center for Human Molecular Genetics, University of Nebraska Medical Center, Omaha, Nebraska 68198-5455; ‡Environmental and Occupational Health Sciences Institute, UMDNJ, Rutgers, Piscataway, New Jersey; and §Texas Neural Tube Defect Project, Texas Department of Health, Austin, Texas*

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Neural tube defects (NTDs) are multifactorial in their etiology, having both genetic and environmental factors contributing to their development. Recent evidence demonstrates that periconceptional supplementation of the maternal diet with a multivitamin containing folic acid significantly reduces the occurrence and recurrence risk for having a pregnancy complicated by NTDs. Unfortunately, the mechanism underlying the beneficial effects of folic acid remains unknown. NTD surveillance data from the Texas–Mexico border show that the high NTD rate (28/10,000 live births) noted during the 1990–1991 Cameron county NTD cluster was superimposed on a background Cameron county NTD rate (16/10,000 live births) which is considerably higher than that generally noted in the United States (8–10/10,000 live births). These data suggest that genetic factors as well as transient environmental factors may contribute to the etiology of the NTDs. Furthermore, clinical and experimental evidence imply that allelic forms of genes involved with folate metabolism and/or transport may explain some of the observed variation in the NTD rates found across different populations. Two folate pathway genes were selected for evaluation in this study. The loci investigated included two known alleles of the 5,10-methylenetetrahydrofolate reductase (MTHFR) gene, as well as the promoter region of the folate receptor-α (FR-α) gene. Odds ratios (ORs) for the C677T polymorphism in the MTHFR gene were 1.8 (CI 0.47–6.8) for heterozygosity and 1.8 (CI 0.35–9.4) for homozygosity for the mutant 677T allele, relative to wildtype homozygotes. The odds ratio for the heterozygosity for the A1298C polymorphism in the same gene was 1.1 (CI 0.09–14). No individuals homozygous for the 1298C allele were observed. The OR for heterozygosity of FR-α gene polymorphisms detected at nucleotide 762 and at nucleotides 610/631 was 1.4 and 0.7, respectively. Neither of the FR-α polymorphisms was observed in the homozygous condition. No statistically significant associations were observed for any of the polymorphisms examined, as the 95% confidence intervals for all of the ORs included one. However, the frequency of the MTHFR 677T allele in the largely Hispanic control group from Texas was significantly different from other populations ($P < 0.005$), and among the highest reported for any control populations examined.

It is well established that periconceptional folate supplementation reduces the risk of a neural tube defect (NTD) in the fetus (1–12). The fact that increased folate intake lowers this risk indicates that folic acid supplementation is a significant modulator of NTD risk. Although folate levels of women having an NTD-affected pregnancy have often been observed to be low, they are still within the clinically normal range (13). However, periconceptional folate supplementation eliminates at most 50–70% of NTD risk (2–11), and this does not occur equally in all populations (14,15).

It has been suggested that altered folate metabolism or transport within either the mother or child

[1] To whom reprint requests should be addressed at Center for Human Molecular Genetics, 985455 Nebraska Medical Center, Omaha, NE 68198-5455. Fax: (402) 559-4001. E-mail: rfinnell@unmc.edu.

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could be responsible for NTDs (16–18). It has also been suggested that genetically determined deficiencies of enzymes involved in folate and homocysteine metabolism could explain the borderline low folate levels and increased homocysteine levels observed among the mothers of NTD infants. Elevated homocysteine levels could impair the closure of the neural tube and lead to a NTD, perhaps by working as an *N*-methyl-D-aspartate (NMDA) receptor antagonist (19). Multivitamins containing folic acid supplementation may provide sufficiently high levels of folate to compensate for malfunctioning enzymes, thereby rescuing the normal phenotype.

Several enzymes involved in folate metabolism have been investigated as candidate genes for increased NTD susceptibility. These include methionine synthase (MS), cystathionine β-synthase (CBS), and methylenetetrahydrofolate reductase (MTHFR). While mutations in the genes coding MS and CBS have not been shown to be associated with NTDs (20–22), a mutation in MS has been shown to be associated with increased plasma homocysteine levels that may indirectly contribute to NTD risk (20).

Most attempts to resolve the genetic factors influencing NTD etiology have focused on the MTHFR gene, which encodes an enzyme that converts homocysteine to methionine while transforming the primary circulating form of folate (5-methyltetrahydrofolate) into the metabolically active form (tetrahydrofolate). In this way, it serves to help regulate the partitioning of folate metabolites within the cell (23). It has been discovered that a C > T transition at nucleotide 677 (677C > T) of this gene leads to the substitution of valine at residue 222 (A222V) in the processed protein (24). This substitution renders the enzyme thermolabile, and less active than the wild-type form (24–26). Reduced activity is apparent in homozygous and, to a lesser extent, heterozygous individuals. Although homozygous MTHFR 677T individuals have been shown to possess decreased serum folate levels and increased homocysteine levels (26,27), red blood cell folate was found to be increased in one study (25) and decreased in another (28). Homozygosity, and to a lesser extent heterozygosity, for the MTHFR 677T allele in children has been linked to an increased risk of NTDs in Dutch and Irish populations (25,29). However, numerous other studies have failed to observe an association (14,15,30–34).

Although a second polymorphism in the MTHFR gene (1298A > C) was not shown to be associated with an elevated NTD risk (26), a combination of heterozygosity for both polymorphisms (677C > T and 1298A > C) has been observed to slightly increase homocysteine levels, as well as the risk for NTDs (26). The two polymorphisms have never been observed to occur on the same allele (26), and consequently, homozygosity for both polymorphisms, if it occurs, must be exceedingly rare.

A second candidate gene, folate receptor-α (FR-α), which is responsible for the receptor-mediated transport of folate into selected cell types, was also investigated. FR-α is one isoform within a family of folate receptors that is primarily expressed in the fetal part of the placenta, as well as in the maternal kidney and choroid plexus. The FR-α regulates folate transport from maternal blood into fetal circulation (35). A mutation in this gene could therefore result in a diminished fetal folate uptake. However, recent studies suggest that mutations in the FR-α coding region are not associated with an increased risk of NTDs (18,36). This finding is a result of several years of investigation that revealed surprisingly little variation at the nucleotide level within the coding region of the FR-α gene. Only two nucleotide changes within the FR-α coding region have been detected after examination of nearly 2000 individuals. These observations were made in five separate studies, conducted in two laboratories, utilizing single-strand conformational polymorphism analysis (SSCP), dideoxyDNA fingerprinting (ddF), or DNA sequence analysis. Of the two nucleotide changes discovered, only one occurred in a NTD-affected individual. This change was a synonymous polymorphism that arose *de novo* within the stop codon. The second alteration involved an amino acid substitution, but was detected in an individual who was phenotypically normal with respect to neural tube closure. We focused on the presumed promoter region of this gene, believing that differences in the extent of gene expression may regulate susceptibility to NTDs.

The purpose of this study was to identify the prevalence of selected polymorphisms in two folate pathway genes and determine if they were associated with an increased risk for neural tube defects in a largely Hispanic population residing on the Texas–Mexican border.

MATERIALS AND METHODS

A 1990–1991 cluster of NTD births in Brownsville, Texas was the impetus for the CDC, the Texas

Department of Health, and Texas A&M University to initiate a case–control study in several Texas counties. The population-based case–control study was designed to identify maternal and paternal risk factors including environmental contaminants, occupational exposures, infectious agents, and genetic factors that might be contributing to a high prevalence of neural tube defects which continues to be a concern in this population. In this article, we present the results of the genetic analysis of a subpopulation of these studies.

Study Population

Cases and controls were drawn from pregnancies in the 14 Texas counties that directly border Mexico, that delivered or were terminated between June 1, 1995 and September 30, 1998. Cases were identified through the following data sources: hospitals, birthing centers, ultrasound centers, abortion centers, prenatal clinics, genetics clinics, and birth attendants (midwives and nonhospital physicians). Cases were defined as terminations (spontaneous or elective abortions) and live or stillbirths with a diagnosis of an NTD. An NTD was defined as spina bifida (ICD 741), anencephaly (ICD 740), or encephalocele (ICD 742.0). Controls were healthy live births that occurred in the same counties during that time period. A more detailed description of the study population has been previously reported (37).

With this protocol, 189 case-women and 289 control-women were identified and 185 and 288, respectively, were ascertained. Informed consent and blood samples could be obtained for 101 case families and for 139 control families. The Texas Department of Health maintains consent forms. The study protocol was reviewed and approved by the Texas A&M University internal review board (IRB) and the Texas Department of Health IRB. An additional IRB approval was obtained from the University of Medicine and Dentistry New Jersey concerning the statistical analysis of the genetic data.

Genetic Analysis

Blood was dried on Gutherie cards and stored at −20°C until genomic DNA was extracted by the Puregene method (Gentra, Minneapolis, MN) and resuspended in 20 μl of TE (pH 7.5). Approximately 0.5–1.0 μl of DNA was used as template in each PCR amplification.

To detect polymorphisms associated with the FR-α gene, the P1 promoter region was amplified with the following primers: FR-αP1.UP: 5′GAGTTGGGGATGGAAGGAGAGC3′ FR-αP1.DN: 5′AGGCAGGGAGTGGGAATG3′. Amplifications were run in 25-μl reactions on a Robocycler Gradient thermocycler (Stratagene, La Jolla, CA). The following thermal profile was used: one cycle at 95°C for 5 min, followed by 40 cycles at 95°C for 1 min, 61°C for 1 min, 72°C for 2 min, followed by a final, 5-min extension at 72°C. Sigmataq DNA polymerase (Sigma, St Louis, MO) was used with $MgCl_2$ at a final concentration 1.5 mM.

Dideoxy fingerprinting of the FR-α P1 PCR product was performed using three primers and the ThermoSequenase cycle sequencing kit (Amersham Life Sciences, Arlington Heights, IL), following the manufacturer's protocol, adapted from four bases to one base per sample. The primer sequences, annealing temperatures, and ddNTP used in each reaction were: S1: 5′TGTGGCTGGTGATGGGTGAC3′, 62°C, ddATP; S2: 5′GGATCTCATTTTCCTCATCTGC3′ 63°C, ddGTP; S3: 5′GATGAGGAAAATGAGATCCG3′ 59°C, ddCTP. Electrophoresis was carried out on a native 8% polyacrylamide gel (Genomyx, Foster City, CA) at 30W and 4°C for 2.5 h. Gels were then dried and exposed to Biomax MR film (Kodak, Rochester, NY) at room temperature for 2 days.

To detect polymorphisms within the MTHFR gene, the regions surrounding nucleotides 677 and 1298 were amplified with the following primers: MTHFR677.UP: 5′TTGAGGCTGACCTGAAGCAC3′, MTHFR677.DN: 5′ATGTCGGTGCATGCCTTCAC3′, MTHFR1298.UP: 5′ GGACTACTACCTCTTCTACCT 3′, and MTHFR1298.DN: 5′ ATGAACCAGGGTCCCCACTC 3′ (38). Amplifications were run in 25 μl reactions under the same conditions used for amplification of the FR-α P1 region.

Dideoxy fingerprinting was performed with sequencing primers located between the PCR primers and downstream of the respective polymorphic nucleotides: MTHFR677.ddf: 5′CATGCCTTCACAAAGCGG3′ for the C677T polymorphism and MTHFREX7.ddf: 5′TCCCCACTCCAGCATCAC3′ for the A1298C polymorphism. Dideoxy termination reactions were carried out as described for FR-α. The use of ddATP and ddGTP in conjunction with a reverse-oriented sequencing primer enabled the unambiguous detection of the C677 and T677 as well as the A1298 and C1298 alleles in either the homozygous or heterozygous state.

Statistical Analysis

Data analysis was performed with SAS software program. To assess relative risk associated with ge-

TABLE 1
Combinations of Sampled Family Members

Family members included	Case	Control	Total
Child only	4	48	52
Mother and child	16	43	59
Mother, father, child	4	5	9
Mother only	52	40	92
Father only	0	1	1
Mother and father	24	3	27
Total	101	139	240

notype, odds ratios (ORs), 95% confidence intervals, and exact probabilities with Fisher's Exact test were performed. A χ^2-Goodness-of-Fit test was used to compare the background prevalence of different genotypes. To test for data consistency, the data were checked for impossible combinations of genotypes for mothers and children (a mother homozygous for one genotype could not be a parent to a child with homozygosity for the other genotype). No such impossible combinations were detected.

RESULTS

A total of 101 case and 139 control families were sampled, including 149 members of case families (24 children, 96 mothers, and 29 fathers) and 195 members of control families (96 children, 91 mothers, and 8 fathers). The combinations of different family members are shown in Table 1. The participation rate was higher for case mothers (95%) than for control mothers (61%). 94.3% of all cases and 92% of all controls sampled between 1993 and 1998 were born to a mother of Hispanic origin.

Folate Receptor-α P1 Promoter Region

Genetic analysis revealed two variant alleles, comprising three different polymorphisms (631T > C, 610A > G, 762G > A) in the promoter region of the FR-α gene, two of which were always observed in tandem (631T > C/610A > G). We failed to detect a statistically significant association or trend either as a risk, or a protective factor, for children or mothers with any of the different allelic forms of this gene (Table 2). This pattern did not change after stratification for different familial combinations. Stratification for counties was not attempted due to the small number of subjects observed with variant alleles. We noted, however, that of 13 subjects displaying the tandem mutation (610A > G and 631T > C), 9 originated from the NW border region (2 cases and 7 controls). This is a statistically significant difference from the pooled proportion of the 14 counties ($P < 0.005$). The frequencies of polymorphic individuals were as follows: NW border 19.6%, Mid border counties 1.6%, and Lower Rio Grande counties 1.3%. The difference among the last two was not significant ($P > 0.05$).

TABLE 2
Odds Ratios and 95% CIs for Children and Mothers from Different Familial Sampling Combinations for Folate Receptor-α Mutations

	Wildtype*		762G > A			613T > C + 610A > G		
	OR	*n*	OR (95% CI)	Exact *p*	*n*	OR (95% CI)	Exact *p*	*n*
Children								
Only children†	1	3/41	3.4 (0.29–41)	0.36	1/4	N/A		0/3
Mother available‡	1	18/43	1.2 (0.1–14)	1	1/2	0.8 (0.08–8.2)	1	1/3
All children§	1	21/84	1.3 (0.25–7)	0.66	2/6	0.67 (0.08–5.8)	1	1/6
Mothers								
Only mothers¶	1	64/37	1.3 (0.4–4.5)	0.77	9/4	0.87 (0.14–5.4)	1	3/2
Child available‡	1	19/43	0.57 (0.06–5.4)	1	1/4	N/A		0/1
All mothers§	1	83/80	1.2 (0.45–3.2)	0.8	10/8	0.96 (0.19–4.9)	1	3/3

Note. Exact *p*, probability for Fisher's Exact test for small samples; *n*, number of case subjects/number of control subjects.
* Reference group.
† Information available only for genotype of children.
‡ Information available for genotype of mothers and children.
§ All subjects, regardless of information about genotype of family member.
¶ Information available only for genotype mothers.

TABLE 3
ORs and 95% CIs for Children and Mothers from Different Familial Sampling Combinations for MTHFR 677 Polymorphism

	CC*		CT			TT		
	OR	*n*	OR (95% CI)	Exact *p*	*n*	OR (95% CI)	Exact *p*	*n*
Children								
Only children†	1	1/12	0.8 (0.07–10.0)	1	2/29	2.4 (0.12–46)	1	1/5
Mother available‡	1	2/7	1.7 (0.31–9.2)	0.71	15/31	1.2 (0.15–9.0)	1	3/9
All children§	1	3/19	1.8 (0.47–6.8)	0.55	17/60	1.8 (0.35–9.4)	0.68	4/14
Mothers								
Only mothers¶	1	15/9	0.9 (0.35–2.3)	1	42/28	1.8 (0.44–7.3)	0.5	12/4
Child available‡	1	8/7	0.3 (0.07–0.94)	0.049	8/27	0.15 (0.02–0.89)	0.05	2/12
All mothers§	1	23/16	0.6 (0.30–1.33)	0.26	50/55	0.6 (0.23–1.59)	0.34	14/16

Note. Exact *p*, probability for two-tailed Fisher's Exact for small samples; *n*, number of case subjects/number of control subjects.
* Reference group.
† Information available only for genotype of children.
‡ Information available for genotype of mothers and children.
§ All subjects, regardless of information about genotype of family member.
¶ Information available only for genotype mothers.

Methyleneterahydrofolate Reductase

MTHFR 677C > T polymorphism. Of 24 case children, 3 were homozygous CC (677CC) compared to 19 of 93 controls. There were 17 case heterozygotes (60/93 controls), while 4 cases were homozygous for the T allele (14/93 controls). With homozygosity for CC considered to be the reference group, ORs for heterozygosity (CT) and homozygosity (TT) were 1.8 (95% CI 0.5–6.8) and 1.8 (95% CI 0.4–9.4), respectively (Table 3). Thus, the presence of the 677TT genotype, although elevated in cases, was not significantly associated with NTD risk ($P > 0.05$).

To assess sampling bias, we computed ORs for children sampled within different family combinations. Either there was no information about other family members available, or there were children from families where additional information about mothers could be obtained. Although confidence intervals were quite large due to the small number of case children, the trends were similar for each stratification. Among children, there was a trend towards a slightly higher NTD risk associated with the 677T allele. This trend was not statistically significant. Interestingly, the same 677T allele in mothers (homozygous and heterozygous) was observed to possess a slight, nonsignificant protective effect (Table 3). We also analyzed the data following stratification by county of residence. We found similar trends for ORs for mothers and children, but none reached statistical significance (data not shown).

MTHFR 1298A > C polymorphism. No association or trend was observed for the 1298A > C polymorphism, either as a risk, or as a protective factor ($P < 0.05$). The mutant 1298C allele was observed in the homozygous condition in only four control children (Table 4).

Combined heterozygosity for MTHFR 677C > T and 1298A > C polymorphisms. No individual was observed to be homozygous for both the 677T and the 1298C polymorphisms. Combined heterozygosity (heterozygosity for both loci) was detected in 55 subjects (22 cases and 33 controls). Of those, 16 were case children and 16 control children. The OR for combined heterozygosity for children is 0.8 (95% CI 0.3–2.5), which is not statistically significant ($P > 0.05$).

Comparison of MTHFR 677C ~ T polymorphism in control populations. The frequencies of the MTHFR genotypes in our control population were compared with those of control populations from previous publications (Table 5). We used a χ^2 analysis to compare the frequencies of the MTHFR genotypes in our control population with control populations from other publications (Table 5). We observed that the frequencies for the MTHFR 677C and 677T alleles were statistically significantly different between studies ($P < 0.005$). In the χ^2 values for comparison of Texas data (from the present study) with data from California (15) and Ireland (39), Hispanic controls were more likely to be ho-

TABLE 4
ORs and 95% CIs for Children and Mothers from Different Familial Sampling Combinations for MTHFR 1298 Polymorphisms

	AA*		AC			CC	
	OR	*n*	OR (95% CI)	Exact *p*	*n*	OR	*n*
Children							
Only children†	1	2/25	1.14 (0.09–14)	1.0	1/11	N/A	0/3
Mother available‡	1	13/31	0.51 (0.13–2.1)	0.52	3/14	N/A	0/1
All children§	1	15/56	0.6 (0.18–2.0)	0.58	4/25	N/A	0/4
Mothers							
Only mothers¶	1	44/24	1.2 (0.49–2.95)	0.82	22/10	N/A	0/0
Child available‡	1	11/38	1.38 (0.36–5.3)	0.73	4/10	N/A	0/0
All mothers§	1	55/62	1.5 (0.7–2.9)	0.30	26/20	N/A	0/0

Note. Exact *p*, probability for Fisher's Exact test for small samples; *n*, number of case subjects/number of control subjects.
* Reference group.
† Information available only for genotype of children.
‡ Information available for genotype of mothers and children.
§ All subjects, regardless of familial combinations.
¶ Information available only for genotype of mothers.

mozygous for either allelic form than were controls in the present study. MTHFR 677C and 677T alleles differed significantly between studies. The highest frequency of the 677T allele was found in our population (48.9%), compared to 41.7% in the Hispanic control population (15) and to the 30.6% in the Irish control populations (39).

DISCUSSION

No significant increase in NTD risk was observed among individuals possessing the MTHFR 677T allele. This finding is consistent with several previously published studies (14,30–34), but in disagreement with others (25,29). Nonetheless, a trend for slightly increased NTD risk was observed for children who possessed the 677T allele in either the homozygous or heterozygous state. Although our data suggested that homozygosity and heterozygosity for the MTHFR 677T allele in mothers was associated with a lower risk of NTDs among their offspring, it seems likely that this finding was more of a statistical aberration rather than a biological phenomenon. Even in studies in which the MTHFR 677T allele has been shown to increase NTD risk, only about 20% of NTD cases can be attributed to this gene variant (40). When compared to the 50–70% of NTDs that can be prevented with periconceptional folate supplementation, it appears that the preventive effect of folate must involve additional biological mechanisms (40). In our study, we saw no association between combined heterozygosity for MTHFR (677C > T and 1298A > C) and an increased risk for NTDs, as reported by van der Put *et al.* (26). However, we did corroborate their finding that no person (case or control) was observed with homozygosity for both polymorphisms.

TABLE 5
Comparisons of MTHFR Genotype Frequencies in Different Studies

Genotypes	Our data	Shaw (Hispanic)	Shields (Irish)
CC	35/186 = 18.8%	63/169 = 37%	114/242 = 47.1%
CT	120/186 = 64.5%	71/169 = 42%	108/242 = 44.6%
TT	31/186 = 16.7%	35/169 = 21%	20/242 = 8.3%
χ^2_(E-O)_/E	0	40.37	63.9
P value (*df*)	1	<0.005 (2)	<0.005 (2)

The high prevalence of the MTHFR 677T allele in our control population (48.9%) was of interest. Generally, European populations are reported to have a frequency of this allele that ranges from 24 to 40%, significantly higher than that found in Asian populations, whose frequencies range from 4.1 to 37.5%, and higher than African populations, which range from 0 to 9.4% (41,42). Studies regarding the distribution of this polymorphism among different ethnic groups found the highest prevalence of the MTHFR 677T allele (54.5%) in a Spanish population (41).

Papapetrou *et al.* (14) have suggested that homozygosity for MTHFR 677T is only a risk factor for NTDs in some ethnic groups and not in others. They note that in populations where the T allele is found to be a risk factor for NTDs, specifically the Dutch and Irish populations, the background frequency of this allele is low (Dutch, 26%; Irish, 28%). In populations with high background frequencies of the 677T allele, such as the British (36%) or Mexican–American (49%), an association between MTHFR genotype and NTDs could not be established.

The conflicting data that have been reported for MTHFR genotype and NTD risk could be partially explained by the complexity of NTD etiology. Under the multifactorial threshold model, MTHFR would represent one locus among many that contribute to NTD liability. Within this model, high background frequencies of the MTHFR 677T allele could function to increase the overall susceptibility of a population to NTD risk, while not closely segregating with the disease. In this hypothetical instance, alleles at additional loci that are associated with NTD risk would occur at low frequencies and represent the critical alleles that could, along with key environmental factors, "push" an individual over the disease threshold and determine the final NTD prevalence in a population. In this example, it would be these additional, rare alleles that would be observed to segregate with the disease phenotype. In other populations where the MTHFR 677T allele occurred infrequently, the MTHFR genotype might be the allele that would be observed to segregate with the disease phenotype. If this hypothetical situation were accurate, specific alleles that influenced NTD sensitivity would be expected to show a statistically significant association with the disease phenotype in populations where the background frequency of the allele was low, but only be weakly associated in populations where the allele was more common.

Increasingly, studies of possible causes of NTDs report that interactions among different factors (gene–gene, gene–nutrition, gene–environment) may confer a higher risk for NTDs than any of these factors alone. Our data suggest that if there is an NTD risk associated with the MTHFR 677T allele, it is small, and insufficient to explain the vast majority of NTDs in Hispanics. It is also possible that different NTD types may have different etiologies and different known risk factors may explain the different etiologic fractions of these birth defects. Future studies are needed which include genetic, nutritional, and environmental data to better understand the complex interactions leading up to the expression of neural tube defects. Novel hypotheses of NTD etiology, including the role of homocysteine and NMDA receptor antagonists (19), are currently being investigated and might provide new genetic targets to explore in this Texas–Mexican border population.

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