Reduced Folate Carrier Gene is a Risk Factor for Neural Tube Defects in a Chinese Population

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BACKGROUND: There is a considerable body of data demonstrating that periconceptional supplementation of folic acid can prevent a significant proportion of neural tube defects (NTDs). At present, the mechanism by which folic acid exerts its beneficial effect remains unknown. Folate transporter genes, including the reduced folate carrier gene (RFC1), have been proposed as NTD risk factors.

METHODS: The study population included 104 nuclear families with NTDs and 100 nonmalformed control families. We investigated the possible association between a common RFC1 polymorphism (A80G) and NTD risk among offspring, as well as potential gene-environment interactions between the infant RFC1 genotype and maternal periconceptional use of folic acid through a population-based case-control study.

RESULTS: We observed that the infants of the GG genotype were associated with a 2.56-fold increased risk of NTDs when compared to the AA genotype (odds ratio [OR], 2.56; 95% confidence interval [CI], 1.04 – 6.36) in our study population. Among mothers who did not utilize folic acid supplements, the risk for having a child with an NTD was 3.30 (95% CI, 1.15–9.65) for offspring with the GG genotype, compared to the reference (AA) genotype. Children who had the GG genotype and whose mothers did not take folic acid had an elevated risk for NTDs (OR, 8.80; 95% CI, 2.83–28.69), compared to offspring with the AA and GA genotypes whose mothers utilized folic acid supplements.

CONCLUSIONS: Our findings suggest that the RFC1 G allele is likely to be an important genetic factor in determining folate transport and subsequently may be a risk factor for NTDs in this Chinese population. Birth Defects Research (Part A) 73:430 – 433, 2005. © 2005 Wiley-Liss, Inc.

Key words: reduced folate carrier gene (RFC1); neural tube defects; folic acid; folate transport; birth defects

INTRODUCTION

Folic acid supplementation can effectively reduce the occurrence and recurrence of neural tube defects (NTDs) (MRC Vitamin Study Research Group, 1991; Czeizel and Dudas, 1992; Berry et al., 1999), yet the mechanism of this effect remains unknown. Previous work attempting to better understand folate’s protective effects has shown that mothers of NTD-affected babies have elevated plasma homocysteine (HCY) levels, suggesting that a disturbed folate-dependent HCY metabolism may be contributing to the increased risk (Steegers-Theunissen et al., 1991, 1994). There have been numerous studies focusing on genes encoding the enzymes involved in folate and HCY metabolism. In addition, variation in genes involved in cellular folate transportation, such as the reduced folate carrier gene (RFC1), represent excellent candidates for explaining susceptibility to folate-regulated NTDs (Shaw et al., 2002). Previous work in California provided evidence of a gene-nutrient interaction between the infant RFC1 GG genotype and maternal periconceptional intake of vitamins containing folic acid; this affected the risk for spina bifida (Shaw et al., 1995, 2002). Based on this work and the hypothesis that infants with the RFC1 G80/G80 genotype would be at increased risk for NTDs secondary to an impaired ability to transport folates to the cytoplasm of a critical cellular population, we sought to examine the role of the RFC1 GG
genotype and NTD risk in a Chinese population with a notably high rate of NTDs.

SUBJECTS AND METHODS

Study Subjects

The study area included 4 counties of Shanxi province and 5 counties of Hebei province in northern China. This study used data collected from the computerized perinatal health care (PHC) surveillance system established in 1993. Specific details about the PHC surveillance system have been previously published (Liu et al., 1999). For the present study, 206 NTD cases were identified from 47,695 births in the PHC surveillance system in these 9 study counties, between 1993 and 2002. Blood samples were obtained from 104 case families with NTD-affected children (50.5%). Additionally, 100 families with nonmalformed control children were selected through random sampling in order to represent the population from which the case group had been derived (from 47,695 births of the PHC surveillance system). Among the 104 triads of NTD-affected children, biological specimens (dried blood spot) were obtained from all infants, 93 fathers, and 99 mothers. For this study, NTDs were defined as anencephaly (43.3%), spina bifida (45.2%), and encephalocele (11.5%). The average age of the NTD cases at the time of the biological sampling was 2.55 years, with the range extending from birth to 8.84 years. The collection of biological samples from the control triads included 99 children, 97 fathers, and 100 mothers. The average age of control infants at the time of sampling was 5.88 years, ranging from 2.08 to 9.42 years. The study protocol was reviewed and approved by Institutional Review Board of Peking University Health Science Center, and written informed consent was obtained from all subjects.

Definition of the Use of Folic Acid

Trained pediatric clinicians completed the interviews with study subjects between January 2001 and December 2003. The information about folic acid supplementation was obtained directly from data that were self-recorded in the PHC handbook prior to the onset of pregnancy (Liu et al., 1999) and was supplemented with a questionnaire administered by the clinicians. Women who had taken a pill containing 0.4 mg of folic acid daily, from a time point 3 months before conception and continuing until completion of the first trimester of pregnancy were defined as regular users of folic acid. Women who never took folic acid were defined as nonusers.

RFC1 A80G Genotyping

The common polymorphism found in the RFC1 gene is located at nucleotide position 80, and changes the amino acid from glutamine (CAG) to arginine (CGG). To study this single nucleotide polymorphism (SNP), genomic DNA was extracted from whole blood using the Wizard Genomic DNA Purification Kit (Promega, Madison, WI). A 230-bp fragment in exon 2 of the RFC1 gene was amplified with the forward primer 5’-agt gtc acc ttc gtc ccc tc-3’ and reverse primer 5’-ctc cgg cct gaa gtt ctt-3’ (Chango et al., 2000). The PCR mixture included: 1× PCR buffer, 1.5 mM MgCl2, 0.2 mM each dNTP, 0.005–0.075 mM forward primer and reverse primer, 1 U Taq DNA polymerase, and 50 ng of genomic DNA. The PCR conditions were as follows: 1 cycle of 95°C for 5 min, followed by 35 cycles of 95°C for 15 sec, 60°C for 30 sec, 72°C for 20 sec, and 1 cycle of 72°C for 5 min. The A to G transition at position 80 creates an Hha I (CCGG/G) restriction site. Therefore, it was possible to genotype the samples using RFLP analysis with Hha 1 (Promega, Madison, WI). Genotyping was performed blinded to the subjects’ case or control status and maternal folic acid usage.

Statistical Analysis

Odds ratios (ORs) and their 95% confidence intervals (95% CIs) as estimates of relative risk for NTDs associated with RFC1 genotypes were calculated by logistic regression analysis. NTD risk was estimated for both GG and GA genotypes versus AA genotype among infants. NTD risk for either the GG or GA genotypes versus the AA genotype was estimated for infants and mothers who were either users or nonusers of folic acid.

The transmission/disequilibrium test (TDT) (Spielman et al, 1993), which evaluates the frequency with which heterozygous parents transmit different alleles to their affected offspring using the McNemar’s chi-square statistic, was performed using data from nuclear families to assess any possible association between RFC1 genotype and NTDs. Only heterozygous parents were included in the analysis. All analyses were performed using a two-tailed test with the aid of the SPSS software package version 10.0 for Windows (SPSS, Chicago, IL). Probability values were accepted as significantly different at \( p < 0.05 \).

RESULTS

A total of 592 DNA samples, which included samples from 104 NTD case infants or children, 93 case fathers, 99 case mothers, 99 control children, 97 control fathers, and 100 control mothers, were analyzed for the RFC1 A80G polymorphism. The number of cases and controls and their genotypes are shown in Table 1. The frequency of the AA genotype among NTD cases (13.5%) was lower than that observed in the control children (22.2%), whereas the percentage of GG genotypes (42.3%) was significantly higher among case children than controls (27.3%) \( p < 0.05 \). The children with the RFC1 GG genotype had a 2.6-fold higher risk for NTDs than the

<table>
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<tr>
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<th>Cases n (%)</th>
<th>Control n (%)</th>
<th>Odds ratio 95% CI</th>
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<tbody>
<tr>
<td>Infant genotype</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AA</td>
<td>14 (13.46)</td>
<td>22 (22.22)</td>
<td>Ref</td>
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<tr>
<td>GA</td>
<td>46 (44.23)</td>
<td>50 (50.51)</td>
<td>1.45</td>
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<tr>
<td>GG</td>
<td>44 (42.31)</td>
<td>27 (27.27)</td>
<td>2.56</td>
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<tr>
<td>Maternal genotype</td>
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<tr>
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<td>8 (8.08)</td>
<td>22 (22.00)</td>
<td>Ref</td>
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<tr>
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<td>60 (60.61)</td>
<td>47 (47.00)</td>
<td>3.51</td>
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<tr>
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<td>31 (31.31)</td>
<td>31 (31.00)</td>
<td>2.75</td>
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<td>Paternal genotype</td>
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<tr>
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<td>50 (53.76)</td>
<td>46 (50.52)</td>
<td>1.38</td>
</tr>
<tr>
<td>GG</td>
<td>28 (30.11)</td>
<td>32 (32.99)</td>
<td>0.99</td>
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infants with the AA genotype (OR, 2.56; 95% CI, 1.04–6.36). Mothers who were heterozygous (GA) at this locus had a 3.5-fold higher risk of having NTD-affected babies compared to mothers who were homozygous AA (OR, 3.51; 95% CI, 1.34–9.89).

The NTD risk for infants with the GG genotype was 3.30 (95% CI, 1.15–9.65) compared to the AA genotype when compared to the nonsupplemented offspring with the AA genotype. Our current results are in line with those of Shaw et al. (2002), who reported an increased risk for spina bifida among California newborns with the GG genotype whose mothers had not used vitamins in the periconceptional period. Although the spina bifida risk was not significantly altered, the study did reveal a modest gene-nutrient interaction between infant homozgyosity for the RFC1 GG genotype and maternal periconceptional intake of vitamins containing folic acid on the risk of spina bifida (Shaw et al., 2002). More recently, Morin et al. (2003) reported in an Italian population that the combination of the RFC1 GG genotype and low red blood cell (RBC) folate levels were associated with a highly significant 4.6-fold increase in NTD risk (OR, 4.6; 95% CI, 1.47–14.37).

In addition to the estimation of the OR for the GA and GG genotypes, we performed a TDT using family data to assess gene-disease associations. This approach can avoid biases induced by inappropriate controls and population stratification (Spielman et al., 1993; Martin et al., 1997; Lazzeroni and Lange, 1998). The TDT conducted in our study revealed a significant association between NTD risk and the RFC1 80G allele. This observation was consistent with results of the case-control study.

In this study, we observed a significant interaction with respect to NTD risk between offspring with the GA genotype and lack of maternal folic acid use, compared with the AA and GA genotypes and maternal folic acid nonusers.
interactions between the based study that was also designed to identify potential RFC1 of an control study was that it investigated the potential effects China (Pei et al., 2004). A major strength of this case-consistent with a previous report in a population in northern China (Pei et al., 2004). A major strength of this case-control study was that it investigated the potential effects of an RFC1 polymorphism on NTD risk in a population-based study that was also designed to identify potential interactions between the RFC1 polymorphism and maternal folic acid supplement intake. The study also involved the population-based ascertainment of both cases and controls. Cases with NTDs were actively ascertained through the Chinese Birth Defects Surveillance System, and control children were selected through random sampling to represent the population from which the case group had been derived. It is unlikely that any significant recall bias exists because the information concerning folic acid supplementation was self-recorded in the PHC handbook by the women themselves during the period of supplementation prior to the onset of pregnancy (Liu et al., 1999).

In interpreting these findings, one should take into account the limitations and strengths of the study. Due to ethical and privacy limitations, participation rates for this study were not high for NTD cases (104/206; 50.5%), but this is unlikely to have created a selection bias because the RFC1 genotype distribution of the control group was consistent with a previous report in a population in northern China (Pei et al., 2004). A major strength of this case-control study was that it investigated the potential effects of an RFC1 polymorphism on NTD risk in a population-based study that was also designed to identify potential interactions between the RFC1 polymorphism and maternal folic acid supplement intake. The study also involved the population-based ascertainment of both cases and controls. Cases with NTDs were actively ascertained through the Chinese Birth Defects Surveillance System, and control children were selected through random sampling to represent the population from which the case group had been derived. It is unlikely that any significant recall bias exists because the information concerning folic acid supplementation was self-recorded in the PHC handbook by the women themselves during the period of supplementation prior to the onset of pregnancy (Liu et al., 1999).

Investigation of candidate genes encoding specific folate-related pathways, such as those involved in metabolism, absorption, and transport, are important to describe fundamental aspects of embryonic development, especially when considering folate-responsive birth defects (Botto et al., 2000, 2001). Although the majority of association studies have focused on folate metabolic enzymes and NTD risk, it is possible that the genes involved in folate binding and transport may turn out to be important risk factors for NTDs and other folate-responsive birth defects (Finnell et al., 1998).

(OR, 8.80; 95% CI, 2.83–28.69). Additionally, our findings suggested that the G allele was likely to be a genetically susceptible marker for NTD risk (OR, 1.5; 95% CI, 1.03–2.09). In general, these findings support the conclusion that the RFC1 genotype (GG) is associated with an increased NTD risk in a Chinese population.

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REFERENCES


