Genetic Variation of Infant Reduced Folate Carrier (A80G) and Risk of Orofacial and Conotruncal Heart Defects

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How folate reduces the risks of congenital anomalies is unknown. The authors focused on a gene involved in folate transport—reduced folate carrier-1 gene (RFC1). Using data from a California case-control study (1987–1989 births), the authors investigated whether the risks of orofacial clefts or conotruncal heart defects were influenced by a polymorphism of infant RFC1 or by an interaction between the RFC1 gene and maternal periconceptional use of vitamins containing folic acid. A total of 305 liveborn infants with cleft lip with or without cleft palate, 123 with cleft palate, 163 with conotruncal heart defects, and 364 nonmalformed controls were genotyped. Odds ratios of 1.6 (95% confidence interval: 0.9, 2.8) for the G80/G80 genotype and of 2.3 (95% confidence interval: 1.3, 3.9) for the G80/A80 genotype were observed relative to the A80/A80 genotype for conotruncal defects. Among mothers who did not use vitamins, the risk of conotruncal defects was 2.1 (95% confidence interval: 0.7, 5.9) for infants with genotype G80/G80 compared with those with the A80/A80 genotype. Among mothers who did use vitamins, the risk was 1.3 (95% confidence interval: 0.7, 2.7). Substantially elevated risks for either cleft group were not observed irrespective of genotype and use/nonuse of vitamins. Thus, this study found modest evidence for a gene-nutrient interaction between infant RFC1 genotype and periconceptional intake of vitamins on the risk of conotruncal defects.

abnormalities; case-control studies; cleft lip; cleft palate; folic acid; heart defects, congenital

Abbreviations: CLP, cleft lip with or without cleft palate; CP, cleft palate.

Periconceptional vitamin supplementation with folic acid substantially reduces the risk of women’s having neural tube defect-affected pregnancies (1, 2), and it has been implicated in the reduced risk of several other congenital anomalies, including orofacial clefts and selected congenital heart defects (3–9). A mechanism underlying this reduced risk has not been elucidated, although it has been speculated that supplementation with multivitamins containing folic acid restores some normal developmental function that is genetically compromised in selected infants.

Investigating genetic variation that influences cellular absorption, transport, and metabolism of folate may offer insight into this unknown mechanism. Indeed, numerous investigations of genes that are specifically involved with folate metabolism have yielded at least one gene, 5,10-methylenetetrahydrofolate reductase (MTHFR), that has been associated with a modestly increased risk of neural tube defects (10–15) and possibly heart defects (16, 17). Observed risks with MTHFR variants, however, do not appear to account for a large proportion of the etiologic fraction of any of these defects, under the assumption that MTHFR variants have a causal role (15). Thus, further investigation of folate-related genes is necessary to reveal clues about mechanisms underlying the potential embryonic protective effects of folic acid supplementation.

Here we explore a single nucleotide polymorphism of the infants’ reduced folate carrier-I (RFC1) gene. This single nucleotide polymorphism is an A-to-G (A80G) change at nucleotide position 80, replacing a histidine (CAC) with an arginine (CGC) in the protein (18). It is unknown whether this change alters the transport function of RFC1. However, higher plasma folate levels were observed in A80/A80 indi-
viduals when compared with G80/G80 individuals (18). In addition, we observed elevated risks of spina bifida among infants who were homozygous G80/G80 and whose mothers did not use vitamin supplements (19). Thus, we explored whether an interaction existed between this single nucleotide polymorphism of the infant’s RFC1 gene and maternal use of vitamin supplements containing folic acid on risk of orofacial clefts or conotruncal heart defects. We hypothesized that infants homozygous for the RFC1 G80/G80 genotype would be at increased risk of these defects, because of an impaired ability to transport folates to the cytoplasm of a critical cellular population. We further hypothesized that elevations in maternal serum folate levels resulting from periconceptional supplementation of folic acid might improve the activity of the poorly functioning variant form of the transport protein. Recent observations suggested that a similar phenomenon might occur with a variant of the other major classes of folate transport molecules, the folate-binding protein (20). Therefore, our hypothesis predicted that risk would be higher among infants homozygous for RFC1 G80/G80 whose mothers did not use periconceptional vitamin supplements containing folic acid, compared with infants homozygous for RFC1 A80/A80 whose mothers used folic acid supplements in the periconceptional period.

MATERIALS AND METHODS

Details of the population-based case-control data used in these analyses have been described (3, 4, 21). This study included conotruncal heart defects and orofacial clefts diagnosed within 1 year after birth among infants and fetal deaths delivered to women residing in most California counties. Eligible were all deliveries of infants or fetal deaths (≥20 weeks’ gestation) that occurred between January 1987 and December 1988 (n = 344,214) for conotruncal defects and between January 1987 and December 1989 (n = 552,601) for orofacial cleft cases. Case eligibility was determined by medical geneticists using detailed diagnostic information from medical records.

Eligible as conotruncal heart cases were all infants and fetuses with anomalies resulting from aorticopulmonary septation (includes tetralogy of Fallot, d-transposition of the great arteries, truncus arteriosus communis, double outlet right ventricle, pulmonary valve atresia with ventricular septal defect, subaortic ventricular septal defect type I, and aorticopulmonary window). Each case was confirmed by review of echocardiography, cardiac catheterization, surgery, or autopsy records. Eligible as orofacial cleft cases were those infants or fetuses with cleft palate (CP) or with cleft lip with or without cleft palate (CLP) confirmed by surgery or autopsy. CP and CLP cases were further classified by the nature of accompanying congenital anomalies. Cases with no other major anomaly or with anomalies considered minor were classified as isolated. Cases with at least one accompanying major anomaly were classified as multiple. Only isolated CP and CLP cases were considered in these analyses.

Infants diagnosed with single-gene disorders, trisomies, or Turner’s syndrome (45,X) were excluded.

As controls, 972 infants were randomly selected from all infants born alive in the same geographic area and time period (1987–1989) as cases. Control infants had no major congenital anomalies identified before the first birthday. Interviews were completed with 207 (87 percent of eligible) conotruncal case mothers, 489 (85 percent) orofacial (isolated) cleft case mothers, and 734 (76 percent) control mothers. Interviews were completed within an average of 3.7 years from the date of delivery for the cases and 3.8 years for the controls.

Telephone interviews with case mothers and control mothers elicited information on medical and reproductive histories and on activities associated with various lifestyles. The interviewer assisted each woman in establishing a 4-month periconceptional period, from 1 month before to 3 months after conception, that was referred to throughout the interview to elicit information. Women were asked whether they used vitamin and mineral supplements and which supplement (type or brand) they used in each month during this period. We divided women into two categories relative to their use of vitamins containing folic acid: 1) “Use” was defined as starting vitamin use anytime during the 1 month before conception through the end of the second month after conception, and 2) “nonuse” was defined as starting vitamin use in the third month after conception (postdating the relevant embryologic timing of the studied phenotypes) or absence of use during pregnancy.

Our analyses were restricted to 1) cases and controls whose mothers were interviewed and 2) liveborn case and control infants, because the source of DNA was residual newborn-screening blood specimens (filter paper). For the 207 infants with conotruncal defects whose mothers were interviewed, a blood specimen was obtained and genotyped for 163. For the 489 infants with orofacial clefts, a blood specimen was obtained and genotyped for 428 (305 with CLP and 123 with CP). To reduce the number of genotyping analyses needed among the control infants, we randomly sampled and genotyped 364 (239 for control infants born in 1987–1988) infants among the 652 control infants for whom DNA was available.

To genotype infants for the RFC1 A80G single nucleotide polymorphism, genomic DNA was extracted from dried blood spots using the Puregene DNA Extraction Kit (Gentra Systems, Minneapolis, Minnesota). A 140-base pair fragment in exon 2 of the RFC1 gene was amplified with forward primer 5′-AGC GGT GGA Aaa GCA GGT-3′ and reverse primer 5′-GGA GGT AGG GGG TGA TGA AG-3′. The polymerase chain reaction conditions were as follows: a single cycle of 95°C for 5 minutes, followed by 36 cycles of 95°C for 15 seconds, 61°C for 30 seconds, 72°C for 15 seconds, and one cycle of 72°C for 5 minutes. The magnesium concentration was maintained at 1.5 mmol per liter for all reactions. The A-to-G transition at position 80 creates an I (GCG/C) restriction site, allowing samples to be genotyped by restriction fragment length polymorphism analysis (New England Biolabs, Beverly, Massachusetts). All genotyping was performed blinded to information about subjects’ case or control status and other information such as maternal vitamin use.
Cases and controls were genotyped G80/G80, G80/A80, or A80/A80 on the basis of results of the restriction fragment length polymorphism analysis-based determinations of the nucleotide base at position 80 of the RFC1 gene. Analyses estimated the risks of isolated CLP, isolated CP, or conotruncal defects among infants with either the G80/G80 or G80/A80 genotype compared with infants with the A80/A80 genotype, independent of maternal vitamin use as well as in conjunction with information on maternal vitamin use. Risks were estimated by odds ratios and their 95 percent confidence intervals. Logistic regression was used to compute risk estimates adjusted for maternal race/ethnicity (White Hispanic, White non-Hispanic, and other).

**RESULTS**

The numbers of case infants and control infants classified by their RFC1 genotypes are shown in tables 1–3. The percentages of G80/G80 genotypes among White Hispanic infants and White non-Hispanic infants were as follows: 27.0 percent and 29.1 percent for conotruncal cases, 34.4 percent and 30.5 percent for CLP cases, 36.6 percent and 39.1 percent for CP cases, and 33.3 percent and 31.8 percent for control infants (data not shown).

We observed 1.6- and 2.3-fold elevated risks of conotruncal defects among infants with the G80/G80 and G80/A80 genotypes, respectively, compared with infants with the A80/A80 (referent) genotype (table 1). Elevated risks of CLP (table 2) or CP (table 3), however, were not observed for the G80/G80 and G80/A80 genotypes.

We also assessed whether a potential association between the RFC1 genotypes and maternal folic acid intakes existed for each defect type. For this assessment, we hypothesized that, among infants with the G80/G80 genotype, defect risk would be substantially higher (compared with the A80/A80 genotype) for those infants whose mothers did not use vitamins early in pregnancy (“nonusers”), compared with mothers who did use vitamin supplements. The results shown in tables 1–3 provide support for this hypothesis, but only for conotruncal defects. That is, compared with the infants with the A80/A80 genotype, the odds ratios for conotruncal defects among infants with the G80/G80 genotype were 1.3 (95 percent confidence interval: 0.7, 2.7) for those infants whose mothers were “users” of vitamins and 2.1 (95 percent confidence interval: 0.7, 5.9) for those infants whose mothers were “nonusers” of vitamins containing folic acid. Among infants heterozygous for RFC1 genotype, G80/A80, effect estimates for conotruncal defects were of a somewhat greater magnitude than observed for the G80/G80 homozygotes (table 1). We did not observe statistical evidence for heterogeneity of effect by race/ethnic groupings, although risks were highest among the “other” race/ethnic group (not shown). We did not observe evidence to indicate that the risk patterns were confounded by race/ethnic groupings either; that is, risk estimates were not substantially altered after adjusting for maternal race/ethnicity. Further, observed risk patterns were similar for the finer phenotypic categories, d-transposition and tetralogy of Fallot (data available from the primary author).
We also investigated the effects of the RFC1 genotype in combination with maternal intake of cereal, because cereal intake represents a significant source of dietary folate. These analyses were restricted to women who did not use vitamin supplements periconceptionally. For many of the comparisons, however, numbers were small, yielding imprecise risk estimates. In general, we did not observe higher risks for the three studied defects associated with either the GG or GA

<p>| TABLE 2. Infant reduced folate carrier-1 (RFC1) A80G genotype, maternal use of multivitamins containing folic acid, and risk (odds ratio) of isolated cleft lip with or without cleft palate, California, 1987–1989 |
|-------------------------------------------------|------------------|------------------|-----------------|-----------------|
| Infant’s A80/A80 genotype                       | Cases            | Controls†         | Odds ratio      | 95% confidence interval |</p>
<table>
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<th>%</th>
<th>No.</th>
<th>%</th>
<th></th>
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<td>40</td>
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<td>33.9</td>
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* Control infants reflect the same birth years as do cases, 1987–1989.  
† “No vitamin use” is defined as the mother’s not using or starting her use of a vitamin supplement containing folic acid after the second month of pregnancy. “Use” is defined as the mother’s beginning use in the preconceptional period or postconceptional period prior to the beginning of the third month of pregnancy.

<p>| TABLE 3. Infant reduced folate carrier-1 (RFC1) A80G genotype, maternal use of multivitamins containing folic acid, and risk (odds ratio) of isolated cleft palate, California, 1987–1989 |
|-------------------------------------------------|------------------|------------------|-----------------|-----------------|
| Infant’s A80/A80 genotype                       | Cases            | Odds ratio†       | 95% confidence interval |</p>
<table>
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<th>No.</th>
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* Refer to table 2 for the numbers of control infants used in the calculation of odds ratios.  
† “No vitamin use” is defined as the mother’s not using or starting her use of a vitamin supplement containing folic acid after the second month of pregnancy. “Use” is defined as the mother’s beginning use in the preconceptional period or postconceptional period prior to the beginning of the third month of pregnancy.
genotype among women who did not consume cereal, compared with the AA genotype infants whose mothers did consume cereal.

DISCUSSION

Our results indicate an increased risk of conotruncal defects but not isolated cleft defects among infants homozygous or heterozygous for RFC1 A80G polymorphism. We observed some evidence suggestive of an interaction between the infant G80/G80 genotype and maternal supplemental vitamin use on risk in this California population-based sample of births, but, again, only for conotruncal defects. Observing elevated risks for only one of the defect phenotypes studied is perplexing, particularly given that these data reveal a stronger association between maternal vitamin use and reduced risks for isolated CLP and CP than for conotruncal defects (tables 1–3). Such differences in results may reflect a true association with one potentially folate-responsive defect but not with another. Alternatively, such differences may reflect a chance finding between RFC1 and conotruncal defects. Indeed, the elevated risks for conotruncal heart defects associated with the G allele could be interpreted as too few conotruncal case infants with the A80/A80 (referent) genotype (table 1). Thus, one could infer that the A80/A80 genotype is associated with a reduced risk of conotruncal defects. Given that this appears to be the first epidemiologic study to investigate the contribution of RFC1 to the risk of conotruncal and orofacial cleft defects, we cannot substantiate one possibility (true association vs. chance) over the other. We also do not have a tenable explanation for why heterozygotes were observed to have larger risks than homozygotes for conotruncal defects.

A paucity of human epidemiologic evidence exists for this folate transport gene. In fact, we are aware of only one report that provides genotype frequencies for RFC1 in ethnic subgroups (22). Reported frequencies were similar to those observed in the current study. One rationale to investigate RFC1 was based on our earlier work that identified elevated risks of spina bifida among infants who were homozygous G80/G80 and whose mothers did not use vitamin supplements (19). The biologically plausible rationale for exploring genetic variation of RFC1 is based on the knowledge that RFC1 regulates the delivery of 5-methyltetrahydrofolate from the cell’s endocytotic vesicle into the cytoplasm (18, 23–26) and is one of the few identified mechanisms responsible for internalizing and transporting folate molecules (18). 5-Methyltetrahydrofolate is required for the remethylation of homocysteine. Inheriting one, or even two, variant alleles of RFC1 might not always result in elevated anomaly risks, because such variants would be expected to retain some level of function. Thus, increasing maternal serum folate from either supplements or diet could “correct” reduced kinetics of transport that result from a variant form of a folate membrane transport protein. If a putative genetic defect were severe enough to eliminate RFC1-mediated folate transport through these systems, it is likely that it would be embryolethal. This has been substantiated recently by investigations using knock-out mouse models for the folate receptor proteins (20, 27), as well as for RFC1 (28).

The strengths of this study were as follows: 1) It investigated the potential effects of an RFC1 polymorphism on anomaly risks, as well as a potential interaction between RFC1 polymorphism and maternal vitamin supplement intake on risks of three congenital anomalies; and 2) it had population-based ascertainment of both cases and infant controls. Conversely, our study was limited in its effect estimation because of small sample sizes for some comparisons, in particular our inability to adequately investigate whether a genotype-vitamin association was more or less evident in certain race/ethnic groups within the study population. Another potential limitation is the lack of information on other potential environmental factors that could interact with variant forms of RFC1, for example, dietary folate intake or maternal plasma folate concentration. Maternal plasma folate may be particularly important because the RFC1 variant alone may not be sufficient to alter circulating folate pathway precursors that may adversely affect homocysteine levels, but it may depend on accompanying low folate status for the development of elevated homocysteine levels much like that described for the C677T polymorphism of MTHFR (29). It is also possible that the protective effect of vitamins relates to correction of a maternal metabolic defect, rather than that of the fetus. Our study was limited to infant genotype information. Thus, we were unable to investigate the potential effects of the maternal RFC1 genotype.

The mechanism underlying the observed association between maternal multivitamin/folic acid supplementation and reduced occurrences of congenital anomalies remains an important question. Our study investigated the hypothesis that the adverse effects of a polymorphism of infant RFC1 may be “corrected” by maternal supplemental vitamin use. Investigations of candidate genes encoding specific folate-related pathways, such as folate binding and transport, are important to describe fundamental aspects of embryonic development, and they may lead ultimately to a better understanding of how to prevent malformations.

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REFERENCES