Research article

118 SNPs of folate-related genes and risks of spina bifida and conotruncal heart defects

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Abstract

Background: Folic acid taken in early pregnancy reduces risks for delivering offspring with several congenital anomalies. The mechanism by which folic acid reduces risk is unknown. Investigations into genetic variation that influences transport and metabolism of folate will help fill this data gap. We focused on 118 SNPs involved in folate transport and metabolism.

Methods: Using data from a California population-based registry, we investigated whether risks of spina bifida or conotruncal heart defects were influenced by 118 single nucleotide polymorphisms (SNPs) associated with the complex folate pathway. This case-control study included 259 infants with spina bifida and a random sample of 359 nonmalformed control infants born during 1983–86 or 1994–95. It also included 214 infants with conotruncal heart defects born during 1983–86. Infant genotyping was performed blinded to case or control status using a designed SNPlex assay. We examined single SNP effects for each of the 118 SNPs, as well as haplotypes, for each of the two outcomes.

Results: Few odds ratios (ORs) revealed sizable departures from 1.0. With respect to spina bifida, we observed ORs with 95% confidence intervals that did not include 1.0 for the following SNPs (heterozygous or homozygous) relative to the reference genotype: BHMRT (rs3733890) OR = 1.8 (1.1–3.1), CBS (rs2851391) OR = 2.0 (1.2–3.1); CBS (rs234713) OR = 2.9 (1.3–6.7); MTHFD1 (rs2236224) OR = 1.7 (1.1–2.7); MTHFD2 (hcv11462908) OR = 0.2 (0–0.9); MTHFD2 (rs702465) OR = 0.6 (0.4–0.9); MTHFD2 (rs7571842) OR = 0.6 (0.4–0.9); MTHFR (rs1801133) OR = 2.0 (1.2–3.1); MTRR (rs162036) OR = 3.0 (1.5–5.9); MTRR (rs10380) OR = 3.4 (1.6–7.1); MTRR (rs1801394) OR = 0.7 (0.5–0.9); MTRR (rs9332) OR = 2.7 (1.3–5.3); TYMS (rs2847149) OR = 2.2 (1.4–3.5); TYMS (rs1001761) OR = 2.4 (1.5–3.8); and TYMS (rs502396) OR = 2.1 (1.3–3.3). However, multiple SNPs observed for a given gene showed evidence of linkage disequilibrium indicating that the observed SNPs were not individually contributing to risk. We did not observe any ORs with confidence intervals that did not include 1.0 for any of the studied SNPs with conotruncal heart defects. Haplotype reconstruction showed statistical evidence of nonrandom associations with TYMS, MTHFR, BHMRT and MTR for spina bifida.

Conclusion: Our observations do not implicate a particular folate transport or metabolism gene to be strongly associated with risks for spina bifida or conotruncal defects.
Background

Periconceptional vitamin supplementation with folic acid substantially reduces risks of women having neural tube defect-affected pregnancies [1, 2] and has been implicated in reducing risks of several other congenital anomalies, including orofacial clefts and selected heart defects [3-11]. Mechanisms underlying these reduced risks have not been elucidated, although it has been speculated that supplementation with vitamins 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 developmentally protective 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 modest increased risk of neural tube defects (e.g., [12-17]), and possibly heart defects [18, 19]. Observed risks with the two principal 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 [17]. Thus, further investigation of other folate-related genes is necessary to reveal clues about mechanisms underlying the potential embryonic protective effects of folic acid supplementation.

We hypothesized that genetic susceptibility of fetal metabolism or transport of folate puts fetuses at risk for selected congenital anomalies. Using population-based data, we investigated 118 single nucleotide polymorphisms (SNPs) in 14 genes in the complex folate pathway as risk factors for spina bifida and conotruncal heart defects.

Methods

This population-based case-control study included infants with spina bifida or conotruncal heart defects diagnosed within 1 year after birth among infants and fetal deaths delivered to women residing in most California counties. Data were derived from the California Birth Defects Monitoring Program [20], a population-based active surveillance system for collecting information on infants and fetuses with congenital malformations. Diagnostic and demographic information was collected by program staff from multiple sources of medical records for all liveborn and stillborn fetuses (defined as >20 weeks gestation). Overall ascertainment for major malformations has been estimated as 97% complete [21]. Eligible were live born infants only because the source of DNA was from newborn screening cards.

Included were 259 infants with spina bifida and a random sample of 359 nonmalformed control infants born during 1983–86 and 1994–95 in selected counties in California. Also included for study were 214 infants with conotruncal heart defects, specifically d-transposition of the great arteries and tetralogy of Fallot. The random sample of 1983–86 controls for conotruncal heart defects included 220 of the overall 359. Newborn bloodspots were obtained from the State of California and their use in this study was consistent with the consent procedures at the time of sample collection. The protocol for this study was reviewed and approved by the State of California Health and Welfare Agency Committee for the Protection of Human Subjects.

Genomic DNA was extracted from dried blood spots on filter paper using the Puregene DNA Extraction Kit (Genta, Minneapolis, MN). Prior to genotyping, genomic DNA was amplified using a commercial multiple displacement amplification (MDA) kit, GenomePhi (GE Healthcare, Piscataway, NJ). The MDA method relies on isothermal amplification using the DNA polymerase of the bacteriophage phi29 and is a recently developed technique for high performance WGA. MDA has been demonstrated to be reliable for genotyping, with the most favorable call rates, best genomic coverage, and lowest amplification bias [22]. Studies indicate no discernable difference between WGA samples with GenomiPhi kit and the original DNA templates [23, 24]. The whole genome amplification (WGA) product was then quantified using RNase P method (AppliedBiosystems, Foster City, CA). 150 ng WGA product was then used for each SNPlex assay pool which contained about 48 SNPs.

Genotype analyses were performed using SNPlex assays (AppliedBiosystems, Foster City, CA). SNP markers were selected using the SNPBrowser™ program (version 3.0) provided by AppliedBiosystems Inc. This program allowed selection of SNP markers from the HapMap database. For each target gene, tagging SNPs were selected based on the pairwise r² > = 0.8. SNPs with minor allele frequencies lower than 10% in Caucasians were excluded. All validated non-synonymous SNPs were included. Successful rates for SNPlex assays were >96% for 75 SNPs, from 90% to 96% for 32 SNPs, from 70% to 90% for 7 SNPs. 15 SNPs suffered from more than 30% failure rates. In a subsequent effort to fill in the missing genotyping data and obtain higher call rate, we performed TaqMan SNP assays (Appliedbiosystems, Foster City, CA) for 22 of these SNPs on an ABI 7900 Genetic Analyzer.

All genotyping was performed blinded to subject’s case or control status. Case and control infants were genotyped for 129 SNPs. Failure to obtain unambiguous genotype data on >50% of the samples for 11 SNPs (CBS rs1801181 and rs12329790; MTHFR rs1537514 and rs7533315; MTR rs10925257, NOS3 rs1800780 and hcv11631000;
RFCI rs1051266, rs4819130, hcv16186310, and rs7278825) resulted in their elimination from further analyses. The remaining 118 SNPs are shown in Table 1. The percentage of control study subjects (percentages were similar for cases) for whom genotype could be assigned is also shown in Table 1.

Genotypes among controls were analyzed to verify that their distributions fit Hardy-Weinberg expectations. Genotypes for each SNP were statistically consistent with Hardy-Weinberg expectations. Odds ratios and 95% confidence intervals (CI) were used to estimate risks. These measures were calculated using SAS software (version 9.1). Information on maternal race/ethnicity was obtained for case and control infants from California birth certificates. Logistic regression was used to compute risk estimates adjusted for maternal race/ethnicity (white Hispanic; white nonHispanic, and other). Analyses estimated defect risks (spina bifida or conotruncal heart defects) for each SNP assuming a recessive model, i.e., homozygous variant genotype compared to homozygous reference genotype and heterozygous variant genotype compared to homozygous reference genotype. In addition to single SNP-at-a-time analyses, we explored haplotype block analyses. Haplotype analyses were performed using Haploview version 3.32. Identified blocks were assessed with odds ratios.

Results
Numbers of case and control infants stratified by race/ethnicity are shown in Table 2. These data show the expected greater frequency of Hispanics in the spina bifida case group.

We examined risks for each of the 118 SNPs and for each of the two birth defect outcome (Additional file 1). Few odds ratios (ORs) revealed sizable departures from 1.0. Given the large number of comparisons (n = 472) we expected more ORs to be substantially different from 1.0 by chance. With respect to spina bifida, we observed ORs with confidence intervals that did not include 1.0 for the reference genotype: BHMT (rs3733890) OR = 1.8 (1.1–3.1), CBS (rs2851391) OR = 2.0 (1.2–3.1), CBS (rs234713) OR = 2.9 (1.3–6.7), MTHFD1 (rs2236224) OR = 1.7 (1.1–2.7); MTHFD1 (hcv11462908) OR = 0.2 (0.0–0.9); MTHFD2 (rs702465) OR = 0.6 (0.4–0.9); MTHFD2 (rs7571842) OR = 0.6 (0.4–0.9); MTHFR (rs1801133) OR = 2.0 (1.2–3.1); MTRR (rs162036) OR = 3.0 (1.5–5.9); MTRR (rs10380) OR = 3.4 (1.6–7.1); MTRR (rs1801394) OR = 0.7 (0.5–0.9); MTRR (rs9332) OR = 2.7 (1.3–5.3); TYMS (rs2847149) OR = 2.2 (1.4–3.5); TYMS (rs1001761) OR = 2.4 (1.5–3.8); and TYMS (rs502396) OR = 2.1 (1.3–3.3). Each gene involving multiple SNP associations was investigated for linkage disequilibrium.

Modest to strong evidence for linkage disequilibrium was observed for SNPs in each gene, i.e., D’ ranged from 0.44 to 1.0 with all p values < 10^-4. With respect to conotruncal heart defects, we did not observe any OR with a confidence interval that did not include 1.0.

We did not observe evidence to indicate that risk patterns were confounded by race/ethnicity groupings, i.e., observed ORs were not substantially altered after adjusting for maternal race/ethnicity (not shown, available from authors upon request).

Haplotypes, reconstructed for each gene based on studied SNPs, were explored to assess risks for each case group. A total of 77 of the 118 studied SNPs formed 17 haplotype blocks. As shown in Table 3, blocks for TYMS, MTHFR, BHMT, and MTR showed some evidence of nonrandom effects for spina bifida. For each of these haplotypes we observed decreased risk associated with the lower frequency haplotype relative to the most frequent haplotype. Similar to SNP analyses, haplotype analyses for conotruncal heart defects did not reveal evidence of nonrandom effects, with the exception of one haplotype block for MTR (Table 4).

Haplotypes analyses were stratified by race/ethnic background (Hispanic white and nonHispanic white). We observed evidence of a nonrandom haplotype association with TYMS for spina bifida and conotruncal heart defects among nonHispanic whites. Lack of evidence for other haplotypes that were observed overall was likely the result of smaller sample sizes from stratification.

Discussion
In this California population we found only modest evidence that polymorphisms in 14 folate-related genes contributed to risk of spina bifida. SNPs contributing risks were in BHMT, CBS, MTHFD1, MTHFD2, MTHFR, MTRR, and TYMS. Haplotype association analyses further identified TYMS and MTHFR as potential contributors to spina bifida risk. In general, however, most of these folate-related genes showed little evidence for a gene-only effect on risk of spina bifida, and even less, on risks of conotruncal heart defects.

The 14 genes studied here have been implicated in the complex metabolic cycle involving folate (e.g., [25-27]). To our knowledge, this study contained the largest number of SNPs in folate-related genes interrogated as risk factors for human spina bifida or conotruncal heart defects. Previous studies have included some of the SNPs examined here. For example, Boyles and colleagues [28] studied 28 SNPs in 11 folate-related genes and found that only BHMT (rs3733890) was associated with increased
Table 1: Fourteen folate-related genes and 118 SNPs

| Gene | Change | Chromosome | Base Position | SNP_ID | Type/Comment | Percent Genotyped |
|------|--------|------------|---------------|--------|--------------|-------------------|
| BHMT | R (A/G) | 5          | 78457715      | rs3733890 | exon, nonsynonymous R239Q | 100               |
| BHMT | Y (C/T) | 5          | 78471967      | rs1915706 | Intergenic/Unknown | 96.4              |
| BHMT | (G/C)   | 5          | 78567093      | rs1316753 | Tag, BHMT    | 100               |
| BHMT | M (C/A) | 5          | 78453350      | rs617219  | intergenic   | 96.4              |
| BHMT | Y (C/T) | 5          | 78438303      | rs645112  | Intergenic/Unknown | 96.9             |
| BHMT | W (A/T) | 5          | 78462964      | rs585800  | untranslated region | 94.2             |
| BHMT | S (C/G) | 5          | 78559288      | rs3829809 | Tag, BHMT    | 100               |
| BHMT | Y (C/T) | 5          | 78452172      | rs567754  | intron      | 95.8              |
| BHMT2 | M (A/C) | 5          | 78405657      | rs626105  | intron      | 96.1              |
| BHMT2 | Y (C/T) | 5          | 78409187      | rs567754  | intron      | 95.5              |
| BHMT2 | M (A/C) | 5          | 78387392      | rs2253262 | exon, nonsynonymous | 96.4            |
| BHMT2 | W (A/T) | 5          | 78420828      | rs670220  | Validated   | 96.7              |
| BHMT2 | M (A/C) | 5          | 78404058      | rs592052  | intron      | 99.2              |
| CBS  | Y (T/C) | 21         | 43360473      | rs2851391 | intron      | 92.5              |
| CBS  | R (A/G) | 21         | 43359173      | rs2298759 | intron      | 72.4              |
| CBS  | Y (T/C) | 21         | 43361102      | rs234714  | intron      | 90                |
| CBS  | S (C/G) | 21         | 43346936      | rs1051319 | untranslated region | 91.9            |
| CBS  | Y (T/C) | 21         | 43376503      | rs234784  | Tag, CBS    | 99.7              |
| CBS  | N (A/G/C/T) | 21     | 43346760      | rs12613   | untranslated region | 92.5            |
| CBS  | S (C/G) | 21         | 43377074      | rs234785  | Tag, CBS    | 100               |
| CBS  | R (A/G) | 21         | 43360960      | rs234713  | intron      | 91.1              |
| CBS  | Y (C/T) | 21         | 43376312      | rs234783  | Tag, CBS    | 100               |
| DHFR | Y (C/T) | 5          | 79986337      | rs1650697 | Validated nsSNP | 92.6             |
| DHFR | W (A/T) | 5          | 79957572      | rs1210987 | Validated   | 94.2              |
| DHFR | Y (C/T) | 5          | 79987790      | rs380691  | Validated   | 95.5              |
| DHFR | M (A/C) | 5          | 79985331      | rs1478834 | Validated   | 96.4              |
| DHFR | Y (C/T) | 5          | 79966012      | rs164368  | Validated   | 92.8              |
| DHFR | M (A/C) | 5          | 79961366      | rs261372  | Validated   | 96.9              |
| DHFR | R (A/G) | 5          | 79980489      | rs13161245 | intron | 96.1            |
| DHFR | Y (C/T) | 5          | 79975899      | rs1643650 | intron      | 94.7              |
| DHFR | K (G/T) | 5          | 79981467      | rs836821  | Validated   | 97.5              |
| FOLR1 | Y (C/T) | 11         | 73373406      | rs1540087 | untranslated region | 95.8            |
| FOLR1 | W (T/A) | 11         | 73380857      | rs11235462 | Tag, FOLR1 | 100               |
| FOLR1 | R (A/G) | 11         | 73372879      | rs2071010 | untranslated region | 91.9            |
| FOLR2 | R (A/G) | 11         | 7340256       | rs2298444 | intron      | 92.2              |
| FOLR2 | R (A/G) | 11         | 73402049      | rs514933  | intron      | 100               |
| FOLR2 | W (A/T) | 11         | 73401368      | rs651646  | untranslated region | 100            |
| MTHFD1 | Y (C/T) | 14         | 63984935      | rs2236222 | intron      | 95.5              |
| MTHFD1 | Y (C/T) | 14         | 63978904      | rs2236222 | intron      | 97.8              |
| MTHFD1 | Y (C/T) | 14         | 63952133      | rs1950920 | exon, nonsynonymous | 90.5            |
| MTHFD1 | Y (C/T) | 14         | 63978598      | rs2236225 | exon, nonsynonymous G1958A (R653Q) | 100        |
| MTHFD1 | (T/A)  | 14         | 63990040      | hCV11462908 | Tag, MTHFD1 | 100               |
| MTHFD1 | R (A/G) | 14         | 63957808      | hCV1160794 | intron      | 95.3              |
| MTHFD1 | R (A/G) | 14         | 63988165      | rs11849530 | intron      | 95.8              |
| MTHFD1 | R (A/G) | 14         | 63990418      | rs1256146 | intron      | 95                |
| MTHFD1 | Y (C/T) | 14         | 63985918      | rs1037921 | exon, nonsynonymous | 96.4            |
| MTHFD1 | Y (C/T) | 14         | 63980547      | rs1256142 | intron      | 97.8              |
| MTHFD2 | Y (T/C) | 2          | 74304595      | rs1126426 | Intergenic, Tag | 100              |
| MTHFD2 | (T/A)  | 2          | 74280806      | rs702465  | Intergenic, Tag | 96.7              |
| MTHFD2 | R (A/G) | 2          | 74313429      | rs1667599 | Intergenic, Tag | 100              |
| MTHFD2 | W (A/T) | 2          | 74338849      | rs828858  | Intergenic, Tag | 96.1              |
| MTHFD2 | C (G)  | 2          | 74281605      | rs702466  | Intergenic, Tag | 99.7              |
| MTHFD2 | R (A/G) | 2          | 74372559      | rs7571842 | Intergenic, Tag | 100              |
| MTHFD2 | R (A/G) | 2          | 74348376      | rs828903  | Validated    | 94.4              |
| MTHFR | R (A/G) | 1          | 11801310      | rs3737964 | Validated    | 95.8              |
| MTHFR | R (A/G) | 1          | 11823734      | rs535107  | Intergenic, Tag | 93.3              |
| MTHFR | K (G/T) | 1          | 11798240      | rs1931226 | Validated    | 96.9              |
| Gene       | Position | RefSNP ID | Genotype | rsID       | Description                      | Validated | Genotype Percentage |
|------------|----------|-----------|----------|------------|----------------------------------|-----------|---------------------|
| MTHFR R(A/G) | 1        | 11780518  | rs4846048 | Validated  | 89.7                             |           |                     |
| MTHFR Y(C/T) | 1        | 11796598  | rs7525338 | Validated  | 97.5                             |           |                     |
| MTHFR R(A/G) | 1        | 11785193  | rs2274976 | exon, nonsynonymous          | 93       |                     |
| MTHFR Y(C/T) | 1        | 11792217  | rs4846052 | intron     | 96.9                             |           |                     |
| MTHFR Y(C/T) | 1        | 11790644  | rs1801133 | exon, nonsynonymous C677T     | 99.4     |                     |
| MTHFR R(A/G) | 1        | 11775209  | rs1889292 | Intergenic, Tag            | 100      |                     |
| MTHFR Y(C/T) | 1        | 11797323  | rs2066470 | exon, nonsynonymous          | 95.3     |                     |
| MTHFR R(A/G) | 1        | 11786566  | rs4846051 | exon, nonsynonymous          | 93       |                     |
| MTHFR R(A/G) | 1        | 11788723  | rs4846051 | intron     | 93.9                             |           |                     |
| MTHFR R(A/G) | 1        | 11785193  | rs2274976 | exon, nonsynonymous C677T     | 99.7     |                     |
| MTHFR Y(C/T) | 1        | 11785193  | rs2274976 | exon, nonsynonymous C677T     | 99.7     |                     |
| MTHFR R(A/G) | 1        | 11786566  | rs4846051 | exon, nonsynonymous          | 93.9     |                     |
| MTHFR Y(C/T) | 1        | 11785193  | rs2274976 | exon, nonsynonymous C677T     | 99.7     |                     |
| MTHFR R(A/G) | 1        | 11786566  | rs4846051 | exon, nonsynonymous          | 93.9     |                     |
| MTHFR Y(C/T) | 1        | 11785193  | rs2274976 | exon, nonsynonymous C677T     | 99.7     |                     |
| MTHFR R(A/G) | 1        | 11786566  | rs4846051 | exon, nonsynonymous          | 93.9     |                     |
| MTHFR Y(C/T) | 1        | 11785193  | rs2274976 | exon, nonsynonymous C677T     | 99.7     |                     |
| MTHFR R(A/G) | 1        | 11786566  | rs4846051 | exon, nonsynonymous          | 93.9     |                     |
| MTHFR Y(C/T) | 1        | 11785193  | rs2274976 | exon, nonsynonymous C677T     | 99.7     |                     |

Percent of 359 controls genotyped for each SNP.

Abbreviations: BHMT = betaine homocysteine methyltransferase; BHMT2 betaine homocysteine methyltransferase-2; CBS = cystathionine beta synthase; DHFR = dihydrofolate reductase; FOLR1 folate receptor 1; FOLR2 folate receptor 2; MTHFD1 = methylenetetrahydrofolate dehydrogenase 1; MTHFD2 = methylenetetrahydrofolate dehydrogenase 2; MTHFR = methylenetetrahydrofolate reductase; MTR = methionine synthase; MTRR = methionine synthase reductase; NOS3 = nitric oxide synthase; RFC1 = reduced folate carrier 1; TYMS = thymidylate synthase.
spina bifida risk. This BHMT association is consistent with our findings that showed an odds ratio of 1.8 (1.1–3.1).

Many studies have explored MTHFR 677 (rs1801133) polymorphism. A range of risks, including no-effect, has been reported for this SNP relative to spina bifida. Botto and Yang [15] in a meta-analysis demonstrated a pooled odds ratio of 1.8 for spina bifida among infants homozygous for 677T. A few studies have also explored this 677 SNP in MTHFR as a risk factor for selected congenital heart defects, with most investigations finding no or little association [18,19,29–31]. We did observe a 2-fold increased risk of spina bifida associated with this SNP for homozygous infants. Further, haplotype analyses showed some association for the MTHFR gene as well.

Methionine synthase (MTR) is a vitamin B12 dependent enzyme that is essential for the remethylation of homocysteine to methionine. The enzyme is required by cells for the essential accumulation of folate [32]. One particular SNP (A2756G; rs1805087) has been considerably investigated, with increased risks of NTDs reported in some studies [33–35], but not in others [36,37]. We did not find an increased risk for spina bifida or conotruncal heart defects associated with this SNP or any other SNP of MTR.

Cystathione beta synthase (CBS) is critical to the degradation of homocysteine to cysteine. Regulation of this pyridoxal phosphate-dependent enzyme catalyzes the hydroxyl group of serine with the thiolate of homocysteine [38]. The polymorphism in the CBS gene that has received the most study is a 68 bp insertion (844ins68), with predominantly no associations observed for NTDs [27]. This polymorphism was not investigated in the current study. We did observe, however, two CBS SNPs (rs2851391 and rs234713) that showed increased risks for spina bifida. Boyles et al [28], albeit using a different study design than ours, observed that these two SNPs were not differentially transmitted from parents of infants with spina bifida.

MTRR gene polymorphisms (particularly rs1801394) have been investigated as a risk factor for both spina bifida and congenital heart defects. Polymorphisms in MTRR could alter homocysteine levels because methionine synthase reductase participates in maintaining the vitamin B12-dependent conversion of homocysteine to methionine [32]. The most frequently studied MTRR polymorphism has been the 66A>G (rs1801394). This polymorphism in infants was associated with a 2.6-fold increased risk of spina bifida in an earlier study by us [33], it was associated with increased risk for spina bifida in another study only when vitamin B12 levels were low [39], or in combination with MTHFR CC genotype [35]. The polymorphism in mothers of infants with neural tube defects has been associated with increased risk in one study [40], but not in another study [41]. Recent work from the Netherlands has shown a lack of association between this polymorphism and risk for conotruncal heart defects [42] as well as no increased risks for a broader phenotypic group of heart defects [43]. In this study, the 66A>G polymorphism was not associated with increased risks for either spina bifida or conotruncal heart defects. We did observe, however, approximately 3-fold elevated risks for spina bifida associated with three other MTRR SNPs (rs162036, rs10380, and rs9332). The significance of these observations will have to be explored in future studies.

With respect to MTHFD1 and MTHFD2, two studies have demonstrated an association with one polymorphism (rs2236225) in MTHFD1 and NTD risk. One study showed a 1.5-fold increase in risk of an NTD-affected pregnancy in Irish women who were homozygous AA [44], a finding that confirmed an earlier increased risk that was identified in Irish women. Another study showed a similar risk for Italian women as well as a 1.9-fold risk for infants with the AA genotype to have spina bifida [45]. For this particular SNP, we observed a similar magnitude of risk (OR = 1.6) for infants with the homozygous genotype, but the estimate was relatively imprecise. We did observe a modestly elevated spina bifida risk for individuals who were homozygous for another MTHFD1 SNP (rs2236224) and modestly lowered risks for three others (hcv11462908, rs702465, and rs7571842). These observations will need to be replicated in future studies.

Polymorphisms in the DHFR gene have not been well-studied for their role in risks of birth defects. Three studies have investigated a 19-bp deletion with mixed results [46–48]. That particular polymorphism was not interrogated in the current study.
Table 3: Haplotype associations with risks of spina bifida

| Haplotype Block | Frequency | Odds Ratio (95% CI) |
|-----------------|-----------|--------------------|
| **TYMS**        |           |                    |
| CGC             | 0.500     | REF                |
| TAT             | 0.373     | 0.7 (0.6–0.9)      |
| TAC             | 0.115     | 0.5 (0.3–0.7)      |
| **MTRR**        |           |                    |
| ATTAGCAACAC     | 0.264     | REF                |
| ACTGGCAGTGT     | 0.213     | 1.4 (1.0–1.9)      |
| ACTAGCAACGC     | 0.201     | 0.8 (0.6–1.1)      |
| GCAGGGGGCGG     | 0.162     | 1.1 (0.7–1.5)      |
| ACAAGAGGCGC     | 0.055     | 1.1 (0.7–1.9)      |
| ACTAGCAGGCCG    | 0.034     | 0.6 (0.3–1.3)      |
| ACTAAGAGGCGC    | 0.027     | 1.2 (0.6–2.6)      |
| ACTGGCAGCGT     | 0.011     | 1.4 (0.5–4.1)      |
| **MTHFR**       |           |                    |
| GGG             | 0.656     | REF                |
| AGA             | 0.163     | 0.9 (0.6–1.2)      |
| AGG             | 0.121     | 0.9 (0.6–1.2)      |
| AAA             | 0.057     | 0.6 (0.3–1.0)      |
| **MTHFD1**      |           |                    |
| TCCCA           | 0.368     | REF                |
| CCCCA           | 0.231     | 0.7 (0.5–0.9)      |
| CTTGG            | 0.180     | 0.8 (0.6–1.1)      |
| CTCCG           | 0.099     | 0.6 (0.4–0.9)      |
| CTCCG           | 0.063     | 0.7 (0.5–1.2)      |
| CTCCA           | 0.037     | 1.0 (0.5–1.8)      |
| **CBS**         |           |                    |
| CG              | 0.889     | REF                |
| TC              | 0.055     | 1.2 (0.7–1.9)      |
| CC              | 0.053     | 0.6 (0.3–1.0)      |
| **RFC1**        |           |                    |
| CG              | 0.856     | REF                |
| GG              | 0.079     | 1.1 (0.7–1.7)      |
| GA              | 0.063     | 1.0 (0.6–1.6)      |
| **MTHFD2**      |           |                    |
| TG              | 0.486     | REF                |
| GA              | 0.463     | 0.9 (0.7–1.2)      |
| GG              | 0.046     | 0.6 (0.3–1.0)      |
| **FOLR2**       |           |                    |
| TA              | 0.549     | REF                |
| AG              | 0.356     | 1.0 (0.8–1.3)      |
| AA              | 0.093     | 1.0 (0.7–1.6)      |
| **BHMT2**       |           |                    |
| GGGTCA          | 0.466     | REF                |
| TAACTC          | 0.219     | 1.0 (0.7–1.3)      |
Our analyses did not show associations with SNPs in RFC1. Previous investigations of this gene have focused on a particular SNP, rs1051266, and have found mixed results [37,41,49-53]. This particular SNP was not analyzed here as a result of too many samples failing to be genotyped for this SNP using the SNPlex platform.

Recent studies have focused on the importance of TYMS in the folate metabolic pathway, including associations between TYMS polymorphisms and folate levels [54-56]. This folate-dependent enzyme catalyzes the reductive methylation of deoxyuridylate (dUMP) to thymidylate (dTMP), thereby playing a central role in DNA synthesis and repair by serving as the primary intracellular source of dTMP [54,57-59]. We previously [56] observed a 4-fold increased risk of spina bifida in non-Hispanic white infants who had a polymorphism for a 28 bp insertion in the promoter region. This observation, however, was not replicated in a population from the northern UK [55].

| Block 19 (MTR) | Frequency | Odds Ratios (95% CI) |
|---------------|-----------|---------------------|
| AATCTTTCTAGAGGCTTGG | 0.373 | REF |
| GTGCCCTCGAGAAGAGAT | 0.262 | 1.0 (0.7–1.3) |
| GTGCCCTAGGACTTTGG | 0.190 | 0.9 (0.7–1.3) |
| GTGCCCTGGAGAGAGAT | 0.045 | 1.4 (0.8–2.5) |
| GTGCCCTCGAGAGAGAT | 0.040 | 0.6 (0.3–1.2) |
| GTGCCCTCGAGAGAGAT | 0.032 | 0.3 (0.1–0.6) |

Block 19 included rs4659724, rs955516, rs4077829, rs12060570, rs1806505, rs6668344, rs3754255, rs10925252, rs3768139, rs3768142, rs1770449, rs7367859, rs1805087, rs2275565, rs1266164, rs2229276, rs10802569, rs4659743, rs3820571, rs1050993, and rs6676866.
current study. Three of the five TYMS SNPs (rs284179, rs1001761, and rs502396) investigated here showed elevated risks for spina bifida for both heterozygote and homozygote individuals. This finding and the corresponding haplotype finding (Table 3) will be important to explore in future studies.

The strengths of this study were: 1) it investigated the potential effects of a large number of folate pathway SNPs, as well as investigated haplotype associations; 2) it had population-based ascertainment of two case phenotypes and controls; and 3) it included cases and controls born before the US food supply was fortified with folic acid, thus we would expect a sizable proportion of cases to have been folate-responsive.

Conversely, our study was limited in its effect estimation owing to small sample sizes for some comparisons. For example, our study had 80% power to detect risks of 2.5 or more associated with genotypes that were observed in at least 4% of controls. Another potential limitation is the lack of information on maternal folate status. Our working hypothesis is that transient elevation in maternal serum folate from supplementation or dietary intake could prevent birth defects by overcoming metabolic inefficiencies or transport-related issues. Absence of information on low folate status would make it more difficult to find putative genotypes. It is also possible that the protective effect of folic acid relates to correction of a maternal metabolic defect, rather than the fetus. Our study was limited to infant genotype information. Thus, we were unable to investigate the potential effects of maternal genotype. As with any study that seeks to explore associations with a large number of genotypes, findings are subject to chance owing to multiple comparisons. As noted above, we conducted 472 analytic comparisons and thus expected more "statistically significant" findings to arise by chance alone. Further, our findings may have been influenced by uncontrolled confounding by population stratification undetectable in analyses stratified or adjusted by race/ethnicity [60,61]. Lastly, the selected SNPs represent only a fraction of the potential variation of the studied genes. Thus, full gene coverage was not achieved even though a large number of SNPs was studied.

Conclusion

Despite compelling evidence that folate intake by women in early pregnancy substantially reduces risks of selected birth defects, the underlying mechanisms have not been elucidated. Our study attempted to determine genetic mechanisms responsible for folic acid's preventive effects. Our observations do not implicate a particular folate transport or metabolism gene to be strongly associated with risks for spina bifida or conotruncal defects. Although we explored a sizable number of polymorphic areas in these genes, we clearly did not capture all the genetic variation. Thus, these genes may continue to be candidates for further inquiry. Alternatively, the preventive role of folate may be via other biological mechanisms such as methylation of nonfolate-related genes that participate in the closure of the neural tube or the development of the heart.

Competing interests

The authors declare that they have no competing interests.

Authors’ contributions

GMS conceived of the study and participated in the statistical analysis. WL conducted the molecular genetic studies. HZ conducted the molecular genetic studies and participated in the statistical analysis. WY conducted the statistical analysis. SLC participated in the statistical analysis. LFB designed and participated in the statistical analysis. EJL conceived of the study and participated in the statistical analysis. RHF conceived of the study and directed the laboratory molecular genetic studies. All authors read and approved the final manuscript.

Additional material

Additional file 1

Appendix. Risks of spina bifida and conotruncal heart defects among California infants associated with 118 SNPs in 14 genes involved in folate metabolism or transport relative to nonmalformed population-based controls.

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