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Thymidylate Synthase (TYMS) Polymorphisms and Risk of Conotruncal Heart Defects

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Abstract

In this study, we investigated whether the two *TYMS* functional variants (28bp VNTR and 1494del6) (275 cases and 653 controls) and six selected SNPs (265 case infants, 535 control infants; 169 case mothers and 276 control mothers) were associated with risks of conotruncal heart defects. Further, we evaluated interaction effects between these gene variants and maternal folate intake for risk of CTD. Cases with diagnosis of single gene disorders or chromosomal aneusomies were excluded. Controls were randomly selected from area hospitals in proportion to their contribution to the total population of live-born infants. DNA samples were collected using buccal brushes or drawn from the repository of newborn screening blood specimens when available. Genetic variants were treated as categorical variables (homozygous referent, heterozygote, homozygous variant). Odds ratios and 95% confidence intervals (CI) were computed to estimate risks among all subjects, Hispanic and non-Hispanic whites, respectively using logistic regression. Gene-folate interactions were assessed for these variants by adding an interaction term to the logistic model. A dichotomized composite variable, "combined folate intake", was created by combining maternal peri-conceptional use of folic acid-containing vitamin supplements with daily dietary intake of folate. In general, the results do not show strong gene-only effects on risk of CTD. We did, however, observe a 3.6-fold increase in CTD risk (95%CI:1.1 – 11.9) among infants who were homozygotes for the 6bp deletion in the 3'-untranslated region (UTR) (1694del6) and whose mothers had low folate intake during the peri-conceptional period.

Keywords

thymidylate synthase; *TYMS*; congenital heart defects; conotruncal defects; folate

INTRODUCTION

Congenital heart defects are the most common type of birth defects, affecting approximately 81.4 of every 10,000 live births [Reller et al., 2008], and account for a significant proportion of infant mortality [Lee and Kang 2001; Miller et al., 2011; Yang et al., 1997]. Of all nonsyndromic congenital heart defects, ~25% are conotruncal heart defects (CTD), caused by abnormal development of cardiac outflow tract during embryogenesis [Johnson 2010]. The most common conotruncal heart defects include tetralogy of Fallot (TOF), d-transposition of the great arteries (d-TGA), truncus arteriosus communis, and double-outlet right ventricle. Women who use vitamin supplements containing folic acid in early pregnancy are at approximately a 30% reduced risk to deliver offspring with conotruncal defects [Bailey and Berry, 2005; Botto et al., 1996; Botto et al., 2000; Botto et al., 2004; Shaw et al., 1995]. The exact mechanism(s) by which folic acid may reduce risks of CTDs is unclear. Potential folate-related processes that may contribute to normal outflow tract development include homocysteine metabolism, cellular methylation, and nucleotide biosynthesis.

Thymidylate synthase (TYMS) is a folate-dependent enzyme that 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 [Kawate et al., 2002; Liu et al., 2002; Trinh et al., 2002; Ulrich et al., 2002]. This process also requires 5,10-methylenetetrahydrofolate. The human *TYMS* gene locus is at chromosome 18p11.32. The minor alleles of two common *TYMS* polymorphisms adversely affect *TYMS* gene expression and enzyme activity: (1) rs45445694: a VNTR (variable numbers of tandem repeats) polymorphism consisting of varying numbers of a 28-bp tandem repeat in the promoter enhancer region of the 5'-untranslated region (UTR); and (2) rs16430: a 6-bp deletion in the 3'-UTR (1494del6) [Takeishi et al., 1989; Trinh et al., 2002; Ulrich et al., 2002]. Being a promoter *cis*-acting enhancer element, 2-repeat allele of the 28-bp VNTR was thought to have lower expression than the triple repeat [Ulrich et al., 2002]. The 6bp deletion allele (–) is associated with decreased mRNA stability *in vitro* and lower gene expression *in vivo* [Mandola et al., 2004]. These *TYMS* polymorphisms have been previously associated with increased risk of neural tube defects in a California population-based case-control study [Volcik et al., 2003]. A recent study reported an association between maternal *TYMS* 1494del6 genotype and risk of conotruncal and related heart defects (CTRDs) [Lupo et al., 2011]. An earlier study by our group examined potential infant genotype effects of five tagSNPs markers using the SNPlex™ genotyping assays (Life Technologies/AppliedBiosystems) in *TYMS* did not find a strong association [Shaw et al., 2009]. Three of the five SNPs were examined in the current study for potential maternal and infant effects.

In this study, we investigated whether these two *TYMS* functional variants (rs45445694 and rs16430) were associated with risks of CTD. We also investigated whether 6 selected SNPs in the *TYMS* gene among the mothers and infants were associated with the risk of CTD. Further, we evaluated interaction effects between these gene variants and maternal folate intake for risk of CTD.

METHODS

Patients

This case-control study included deliveries that had estimated due dates from July 1999 to June 2004. Included were live-born, stillborn (fetal deaths at ≥ 20 wk gestation), and prenatally diagnosed, electively terminated cases that occurred to mothers residing in Los Angeles, San Francisco and Santa Clara counties in California. Cases were ascertained by

the California Birth Defects Monitoring Program using stringent multiple source and population-based ascertainment approaches as previously described [Croen et al., 1991; Schulman et al., 1993]. Case information was abstracted from multiple hospital reports and medical records reviewed by a clinical geneticist (EJL). Infants diagnosed with single gene disorders or chromosomal aneusomies (based on information gathered from chart reviews) were ineligible [Lammer et al., 2009]. Cases included the conotruncal heart defects d-TGA and TOF. Infants with d-TGA or TOF associated with an atrioventricular canal defect or with double outlet right ventricle were excluded. For each case, anatomic and physiologic features were confirmed by reviewing echocardiography, cardiac catheterization, surgery, or autopsy reports. Non-malformed, live-born controls were selected randomly from birth hospitals, to represent the population from which the cases were derived. Specifically, controls were randomly selected from area hospitals in proportion to their contribution to the total population of live-born infants (i.e., the number of eligible control infants from each hospital was in proportion to that hospital's contribution to the most recent birth cohort for which vital statistics data were available).

Maternal Interview

Mothers were eligible for interview if they were the biologic mother and carried the pregnancy of the study subject, they were not incarcerated, and their primary language was English or Spanish. Maternal interviews were conducted using a standardized, computer-based questionnaire, primarily by telephone, in English or Spanish, and no earlier than 6 weeks after the infant's estimated date of delivery. A variety of exposures were assessed, focusing on the periconceptional time period, which was defined as 2 months before through 2 months after conception. The interview also included a modified version of the National Cancer Institute's Health Habits and History Questionnaire, a well-known, semi-quantitative food frequency questionnaire with demonstrated reliability and validity [Block et al., 1986; Block et al., 1990]. The food frequency questionnaire was modified to include ethnic foods appropriate to a diverse study population and its use previously validated [Mayer-Davis et al., 1999].

Study Population for Analysis

420 conotruncal defect cases (186 d-TGA and 234 TOF) and 907 controls were eligible for the study. Eleven percent of eligible case mothers and 12% of control mothers were not locatable, and the remainder of non-participants declined interview. In total, 76% of eligible case mothers (142 dTGA, 176 TOF) and 77% of control mothers (700) were interviewed. Median time between estimated date of delivery and interview completion was 11 months (Interquartile range: 8 months) for cases and 8 months (Interquartile range: 8 months) for controls. For the analyses described here, we limited the study population to women who were interviewed and their liveborn infants.

DNA Samples and Genotyping Approach

DNA was available from newborn screening dried blood spots obtained from linkage efforts made by the California Birth Defects Monitoring Program. DNA was also available from buccal brushes that were collected by mail after the maternal interview. If both the dried blood spot and buccal brush were available for an infant, DNA from the buccal brushes was used in genotyping. All DNA samples for mothers were from buccal brushes.

Genomic DNA was extracted from dried blood spots using the Puregene DNA Extraction Kit (Qiagen, Germantown, MD) or the MasterPure DNA Purification Kit (Epicentre Biotechnologies, Madison, WI). Cell lysates were obtained from buccal brushes using an NaOH protocol [Richards et al., 1993]. Non-synonymous SNPs and tagSNPs with minor allele frequencies (MAF) ≥ 0.05 and an available TaqMan genotyping assay

(<http://www.appliedbiosystems.com>) were selected. For SNP genotyping, 10ng of isolated genomic DNA from bloodspots was amplified using GenomiPhi™ multiple displacement amplification according to the manufacturer's instructions (Amersham Biosciences, Sunnyvale, CA). DNA from buccal brush samples was used for SNP genotyping assays without whole genome amplification. SNPs were assayed using TaqMan (Life Technologies, Carlsbad, CA) and genotypes were read and discriminated on the ABI PRISM® 7900HT Sequence Detection System (Life Technologies, Carlsbad, CA). As quality assurance, genotyping assays were duplicated for a 10% subset of blood spots DNA samples and all buccal DNA samples. All genotyping was performed blinded to case and control status.

For the analysis of the 1494del6 polymorphism, a fragment spanning the 6 bp insertion or deletion was amplified by PCR using forward primer: 5'-CAAATCTGAGGGAGCTGAGT-3' and reverse primer: 5'-CAGATAAGTGGCAGTACAGA-3'. The PCR reactions contained 1X AbGene buffer (Thermo Fisher, Waltham, MA), 1.5 mM MgCl₂, 200 μM deoxyribonucleotide triphosphates (dNTPs), 100 nM each primer, 1 unit of AbGene ThermoStart Taq DNA polymerase (Thermo Fisher, Waltham, MA) and 10ng genomic DNA. Step-wise cycling conditions were as follows. One cycle of 95°C for 10 min then 40 cycles of 95°C for 30 s, 58°C for 45 s, and 72°C for 45 s, with a final extension at 72°C for 5 min. The amplified fragments were digested with *Dra* I (Fermentas Life Sciences, Glen Burnie, MD) and the products separated on a 3% NuSieve 3:1 Agarose gel (Lonza, Rockland, ME). The expected fragment sizes are 70 bp and 88 bp for the wild-type allele containing the 6 bp insertion (+), and 152 bp for the mutant allele containing the 6 bp deletion (-).

For the analysis of the 28-bp VNTR polymorphism, a fragment containing the repeats was amplified using following primers: Forward primer: 5'-GCCGCGGGAAAAGGCGCG-3'; Reverse primer: 5'-GGACGGAGGCAGGCGAAGTG-3'. The PCR reactions contained 1X AbGene buffer (Thermo Fisher, Waltham, MA), 1.5 mM MgCl₂, 200 μM deoxyribonucleotide triphosphates (dNTPs), 100 nM each primer, 1 unit of AbGene ThermoStart Taq DNA polymerase (Thermo Fisher, Waltham, MA) and 10ng of genomic DNA. Step-wise cycling conditions were as follows. One cycle of 95°C for 10 min; 3 cycles of 98°C for 15 s, 64°C for 30 s, and 72°C for 30 s; and 34 cycles of 95°C for 30 s, 64°C for 30 s, and 72°C for 30 s, with a final extension at 72°C for 5 min. The amplified fragments were separated on a 4% NuSieve 3:1 Agarose gel (Lonza, Rockland, ME). The fragments containing four, three and two repeats were 144bp, 116 bp and 88 bp, respectively.

Ethics Review

This study was approved by the California State Committee for the Protection of Human Subjects as well as Institutional Review Boards at Stanford University, Children's Hospital Oakland, and UT Austin.

Statistical Methods

First we analyzed the two functional variants (rs45445694 and rs16430) and six SNPs among 318 case infants and 700 control infants whose mothers provided interview information. Genotypes of rs45445694 and rs16430 were available for 275 case infants (86%) and 653 control infants (93%). DNA samples that failed to produce genotype calls on both variants were excluded. These two variants were not tested in mothers' samples due to the limited DNA quantity and quality that was available from buccal brushes. SNP genotyping results were available for 265 case infants (83%), with 138 from bloodspots and 127 from buccal samples, and 535 control infants (76%), with 366 from bloodspots and 169 from buccal samples, after exclusion of subjects with >3 missing genotyping calls. In

addition, the SNP genotyping results were available for 169 case mothers (53%) and 276 control mothers (39%); these results were also included in the final analyses. No substantive differences in race/ethnicity, education, and sex were noted between study subjects for whom genotyping was or was not possible.

For each SNP, the Haploview Program (<http://www.broadinstitute.org/scientific-community/science/programs/medical-and-population-genetics/haploview/haploview>) [Barrett et al., 2005] was used to calculate minor allele frequencies and to evaluate deviations from Hardy-Weinberg equilibrium (HWE) among control mothers and control infants separately. These analyses were conducted for subjects of all race/ethnicity, Hispanics and non-Hispanics. When a deviation from HWE (chi-square p-value = 0.01) was observed in a particular subgroup (e.g. Hispanic mothers), data for that subgroup was excluded from further analyses for that SNP. Specifically, SNP rs2847149 (HWE p=0.0031) was excluded for analysis for Hispanic mothers.

All genetic variants were treated as categorical variables (homozygous referent, heterozygote, homozygous variant). Odds ratios and 95% confidence intervals (CI) were used to estimate risks among all subjects, Hispanic and non-Hispanic whites, respectively. These measures were calculated using SAS software (version 9.2). Gene-folate interactions were assessed for the two functional variants as well as the 6 SNPs. A dichotomized composite variable, “combined folate intake”, was created by combining maternal peri-conceptional use of folic acid-containing vitamin supplements with daily dietary intake of folate. Information on “maternal multi-vitamin use” (yes/no) during the peri-conceptional period (from two months before conception to two months after conception) was obtained from the interview questionnaire. Dietary folate intake data were obtained from the food frequency questionnaire. For this variable, two categories were constructed corresponding to 25th centile and >25th centile, as determined from dietary folate intake levels among control mothers from each group (292.09 mcg among the 276 controls mothers whose genotype data were included in analysis and 283.81 mcg among the 673 control mothers whose infants’ genotype data were included for analysis). Combined folate intake was defined as low for women in the lowest quartile (≤ 25th centile) of folate intake who did not take supplemental folic acid in the peri-conceptional period. “Not low” folate intake was defined as dietary folate intake above the lowest quartile (>25th centile) regardless of maternal vitamin use. Interactions between each variant and the combined folate intake variable were tested among all subjects, Hispanics, and non-Hispanic whites, respectively. An interaction with a p-value of less than 0.05 was considered statistically meaningful.

RESULTS

Compared to controls, case mothers were more frequently non-Hispanic white, aged >34, represented in the category of greater than high school education, and observed to deliver male infants (Table I).

Infant *TYMS* 28-bp VNTR and 1494del6 and CTD risk

Table II summarizes infant genotypes for the 1494del6, the 28-bp VNTR, and the two variants combined. Since the allele with 6-bp deletion is associated with decreased RNA stability, homozygotes with no deletion (+/+) were considered the reference group for the 1494del6 polymorphism. The 2 28-bp repeat allele is associated with lower gene expression level, therefore subjects homozygous for the 3 28-bp VNTR repeats (genotype 3/3) plus the heterozygotes with 3 and 4 repeats (genotype 3/4) were considered as referent for the 28bp VNTR polymorphism. The 1496del6 did not appear to influence risk of CTD among Hispanic infants, but was associated with increased risk among heterozygous non-Hispanic white infants OR=1.8, 95%CI: 1.0–3.4). However, homozygotes for the 6bp deletion

showed lesser, rather than greater, risk compared to heterozygotes. The 28bp VNTR variant did not appear to influence CTD risk. The combined influence of the two variants appeared to confer a reduced risk of CTD among Hispanic infants but not among non-Hispanic white infants.

Maternal and infant *TYMS* SNPs and CTD risk

Table IIIa–c summarizes maternal and infant genotype results of each SNP among all subjects, Hispanics and non-Hispanic Whites, respectively. Hispanic infants who were homozygous for the minor alleles of rs2847153 or rs2847326 showed a >2-fold increase in CTD risk [(OR=2.3 (1.0–5.3) and OR=2.4 (1.2–4.8), respectively)]. All other OR estimates were consistent with random variation.

Gene-folate interactions and CTD risk

We explored possible interactions between the two functional variants and combined folate intake for risk of CTD. No interaction was observed between the 28bp VNTR and combined folate intake. Among all subjects, CTD risk is somewhat lower for those with higher folate intake (OR=0.7, 95%CI: 0.4–1.1). While risk for conotruncal defects was not independently associated with the 1486del6 variant, we found a more than 3-fold increased risk, albeit imprecise, associated with the combination of low folate intake and homozygous 6bp deletion (OR=3.6, 95%CI: 1.1–11.9). In contrast, genotypes of the 6bp deletion showed similar risks for conotruncal defects among mothers with normal maternal folate intake. The sample sizes were insufficient to explore this possible interaction further stratified by race-ethnicity (Tables II and IV).

Similarly, we explored interactions between maternal and infant genotypes of each SNP and the combined maternal folate intake. Results for the comparisons with interaction terms that had at least one associated p-value for interaction that was <0.05 are shown in Table V (a complete set of results is included in a Supplementary eTable file – see Supporting Information online). We expect the highest ORs would be seen among women who had low folate intake and who carry one or two minor alleles of a given SNP. For SNP rs699517, a 4-fold increase risk of CTD was observed among those who were homozygous for minor alleles and whose mothers had low combined folate intake (OR=4.0, 95%CI: 1.1–14.8). No other risk estimate, however, fit the expected pattern. SNP rs1001761 showed an interaction with low maternal folate intake among all infants, but the 95% confidence interval of the OR includes 1 (OR=2.0, 95%CI: 0.6–6.9).

DISCUSSION

In this study, the two functional variants in the *TYMS* gene, 1494del6 and VNTR, were evaluated among infants to determine whether they were associated with the risk of CTD alone (gene-only) or in combination with maternal folate intake (gene-folate interactions). We also evaluated maternal and infant genotype for six SNPs in *TYMS*. In general, the results do not show strong gene-only effects on risk of CTD. We did, however, observe a 3.6-fold increase in CTD risk among infants who were homozygotes for the 1494del6 and whose mothers had low folate intake during the peri-conceptional period.

The two functional variants in the *TYMS* gene have been associated with a variety of health conditions including coronary artery disease [Vijaya Lakshmi et al., 2011], birth defects [Blanton et al., 2011; Volcik et al., 2003] and cancer survival [Canalle et al., 2011; Pietrzyk et al., 2011; Shi et al., 2005]. These two variants, both located in regulatory regions of the gene, are thought to affect gene expression, enzyme levels, and plasma folate and homocysteine levels [Trinh et al., 2002; Ulrich et al., 2002].

Being a promoter, *cis*-acting enhancer element, 2-repeat allele of the 28-bp VNTR was thought to have lower expression than the triple repeat [Ulrich et al., 2002]. The 2/2 genotype (lower expression) appeared to be beneficial for coronary artery diseases [Vijaya Lakshmi et al., 2011] and drug responses of certain cancer treatment [Pietrzyk et al., 2011; Uchida et al., 2004; Ulrich et al., 2002] via interactions with folate intake. Recent studies also suggested post-transcriptional mechanisms of the functional role of this variant [Ghosh et al., 2011; Kawakami et al., 2001; Kawakami and Watanabe, 2003].

A previous California population-based case-control study from our group demonstrated an increase in the risk of spina bifida among infants with the 2/2 genotypes [Volcik et al., 2003]. Our results from the current study, however, didn't show an association between CTD risk and the 2/2 genotype alone. It is noteworthy that a G>C substitution has been identified within the repeats abolishing the putative E-box binding site (CACTTG) for upstream stimulatory factors (USF-1/USF-2) [de Bock et al., 2011; Lincz et al., 2007; Mandola et al., 2003]. Because we did not analyze the G>C substitution within the 28-bp VNTR in the current study, we were unable to include this variant when categorizing the "high" and "low" expression genotypes.

The 1494del6 variant in the 3'UTR is thought to affect RNA stability and translation. One study suggested that the 6bp deletion allele (–) is associated with decreased mRNA stability *in vitro* and lower gene expression *in vivo* [Mandola et al., 2004]. Another study showed that individuals with the del/del (–/–) genotype had higher red blood cell folate levels and lower plasma homocysteine levels compared to the other genotypes (+/+ or +/-) [Kealey et al., 2005]. The –/– genotype appeared to be associated with reduced risks of certain cancers [Shi et al., 2005; Skibola et al., 2004; Zhang et al., 2004]. The –/– genotype was observed to be associated with a reduced risk of spina bifida in a previous California case-control study. The +/- genotype conferred a 3.6-fold increase in risk of spina bifida compared to the –/– genotype [Volcik et al., 2003], which appeared to be consistent with the relationship between genotype and folate/homocysteine level [Kealey et al., 2005]. Conversely, our current study showed a modest increased CTD risk among non-Hispanic White infants who were heterozygotes for the 1494del6 variant (+/-) compared to infants with the wild type (+/+) genotype. The risks increases among homozygotes (–/–), however, were statistically imprecise.

It is not clear why the –/– genotype appears to be beneficial compared to the +/- genotype in some circumstances even though the +/- genotype results in higher gene expression and mRNA stability. The TYMS protein is auto-regulatory - the protein binds to its messenger RNA (mRNA) directly and inhibits mRNA translation. Thus, even though higher level of gene expression and mRNA stability would result in a direct increase in protein production, the increased binding of protein and its own mRNA can also lead to greater suppression under certain cellular conditions. Another possible explanation may be attributable to the linkage disequilibrium between the allele with deletion (–) of 1494del6 and the triple repeat allele (3) of the 28-bp VNTR in the 5'UTR [Mandola et al., 2004; Stoehlmacher et al., 2008]. In one study, the majority of subjects with the VNTR genotypes with low mRNA expression also possessed the 1494del6 +/- genotype, which correlates with high mRNA expression [Stoehlmacher et al., 2008]. Although we did not observe as strong a linkage disequilibrium as did Stoehlmacher and colleagues, when we analyzed the combined effect of these two variants, we observed a "protective" effect among Hispanic infants who carried the low expression allele of 1494del6 (–) and high expression genotypes of 28-bp VNTR (3 or 4) compared to those who carried the high expression alleles.

Our study identified an interaction between *TYMS* 1494del6 and maternal folate intake. That is, given low maternal folate intake in combination with, the low expression genotype

($-/-$) a much higher CTD risk is observed. The strong effect of folate intake may also be explained by the auto-regulatory mechanism. It is known that translation of *TYMS* mRNA is controlled by its own protein end product in an autoregulatory manner, while the co-factor for *TYMS*, 5,10-methylene-tetrahydrofolate (methylene-THF), completely relieves the inhibition of mRNA translation by the *TYMS* protein [Chu et al., 1991; Tai et al., 2004]. When the folate level is low, there is not enough methylene-THF to relieve the inhibition of mRNA translation, therefore the auto-regulatory mechanism may exacerbate the already low level of mRNA expression and translation caused by the $-/-$ genotype.

Our previous study has interrogated five SNPs in *TYMS* gene and found no significant genotype effect on risk of CTD [Shaw et al., 2009]. Although moderate increases in CTD risk were observed in two SNPs (rs2847163 in intron 2; rs2847326 in 3' near gene region) in a subgroup (Hispanic infants), the current study was consistent with the observed lack of effect of infant genotype for three previously studied SNPs (rs502396 in intron 1, rs1001761 in intron 2 and rs2847149 in intron 3); and suggested no maternal genotype effect. The interaction between infant rs699517 (in 3'UTR) genotype and low maternal folate intake which resulted in a 4-fold increase in CTD risk (Table V) is consistent with the similar observation of the immediately adjacent (428bp apart) functional 1494del6 variant (Table IV).

The strengths of our study include its population-based ascertainment of cases and controls, its relatively short period for maternal recall between peri-conceptional event of interest and interview, and its relatively high participation by study subjects. A potential limitation is that data used in the gene-nutrient interaction analysis relied upon a food frequency questionnaire to assess nutrient intake. Limitations of this type of instrument have been described [Block, 1982; Willett, 1998; Willett et al., 1987]. However, recent studies have demonstrated the successful utility of this type of instrument for estimating most nutrients, for example, glycemic load and choline [Cho et al., 2006; Flood et al., 2006]. Another limitation is that we only interrogated a limited number of SNPs. For example, the G>C substitution (MAF=0.06) within the repeats of the VNTR, plays functional roles, therefore warrants future investigation. In addition, the power to detect gene-nutrient interaction effects was low. We used a p-value cut-off of 0.05, which is relatively stringent for an interaction.

Our study did not observe gene-only effects but did observe an interaction effect of *TYMS* functional variants and maternal folate intake on CTD risk. Although these findings are consistent with the biological mechanisms, they were based on relatively small sample sizes and may represent false-positive discoveries. Therefore replication of this study is warranted in other populations.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Table 1
Demographic distribution of conotruncal heart defects cases and non-malformed controls, California 1999–2004.

Variable	Interviewed with maternal genotyping data available		Interviewed with infant genotyping available	
	Cases N=169 (%) [*]	Controls N=276 (%) [*]	Cases N=275 (%) [*]	Controls N=653 (%) [*]
Maternal Race/Ethnicity				
Hispanic	87 (51.5)	163 (59.1)	144 (52.4)	396 (60.6)
White	58 (34.3)	73 (26.4)	78 (28.4)	132 (20.2)
Black	5 (3.0)	17 (6.2)	14 (5.1)	51 (7.8)
Other	19 (11.2)	23 (8.3)	39 (14.2)	71 (10.9)
Maternal age at delivery (years)				
13–24	41 (24.3)	81 (29.3)	72 (26.2)	210 (32.2)
25–29	39 (23.1)	65 (23.6)	52 (18.9)	154 (23.6)
30–34	47 (27.8)	75 (27.2)	84 (30.5)	172 (26.3)
35–55	41 (24.3)	55 (19.9)	66 (24.0)	114 (17.5)
Maternal education				
Less than high school	45 (26.6)	75 (27.2)	69 (25.1)	187 (28.6)
High school	27 (16.0)	70 (25.4)	51 (18.5)	156 (23.9)
Greater than high school	95 (56.2)	130 (47.1)	150 (54.5)	299 (45.8)
Infant Sex				
Male	98 (58.0)	144 (52.2)	156 (56.7)	346 (53.0)
Female	71 (42.0)	132 (47.8)	119 (43.3)	307 (47.0)
Maternal vitamin use				
No	59 (34.9)	98 (35.5)	110 (40.0)	260 (39.8)
Yes	110 (65.1)	177 (64.1)	164 (59.6)	391 (59.9)
Maternal dietary folate intake[§]				
25 percentile	45 (26.6)	65 (23.6)	71 (25.8)	151 (23.1)
>25 percentile	121 (71.6)	195 (70.7)	195 (70.9)	455 (69.7)
Combined maternal folate intake				
No vitamin use & dietary folate intake ≤25%	18 (10.7)	25 (9.1)	38 (13.8)	57 (8.7)
Any vitamin use & dietary folate intake >25%	149 (88.2)	243 (88.0)	234 (85.1)	575 (88.1)

Variable	Interviewed with maternal genotyping data available		Interviewed with infant genotyping available	
	Cases N=169 (%) *	Controls N=276 (%) *	Cases N=275 (%) *	Controls N=653 (%) *
Maternal BMI (kg/m ²)	24.5 (5.4)	25.0 (4.8)	24.7 (5.4)	24.7 (5.6)
Maternal Dietary Folate (mcg)	413.7 (181.9)	434.6 (187.9)	413.9 (186.9)	432.5 (188.6)
Maternal Energy Intake (kcal)	2600.0 (971.7)	2659.0 (947.7)	2579.7 (937.9)	2681.4 (961.2)

SD: Standard Deviation

* Percentages may not equal to 100 due to missing data or rounding.

[§]Two percentile categories were constructed corresponding to percentile categories 25, and >25. These categories were determined from dietary folate intake levels among control mothers from each group, with 291.47 mcg for mom controls, 283.81 mcg for infant controls.

Table II
TYMS functional variants: Crude ORs among infants of all interviewed subjects and stratified by White/Hispanic race-ethnicity.

TYMS	All Infants			Hispanic Infants			Non-Hispanic White Infants		
	Case N=275	Control N=653	OR (95%CI)	Case N=144	Control N=396	OR (95%CI)	Case N=78	Control N=132	OR (95%CI)
1494del6 (rs16430)*									
	102	263	REF	69	174	REF	26	62	REF
+/+									
	112	272	1.1 (0.8–1.5)	51	169	0.8 (0.5–1.2)	38	50	1.8 (1.0–3.4)
+/-									
	45	110	1.1 (0.7–1.6)	16	46	0.9 (0.5–1.7)	10	20	1.2 (0.5–2.9)
-/-									
28bp VNTR (rs45445694)**									
3/3 or 3/4	105	247	REF	55	141	REF	24	41	REF
2/3 or 2/4	124	263	1.1 (0.8–1.5)	63	165	1.0 (0.6–1.5)	36	54	1.1 (0.6–2.2)
2/2	43	116	0.9 (0.6–1.3)	25	70	0.9 (0.5–1.6)	16	34	0.8 (0.4–1.8)
1494del6 & 28bp VNTR combined									
+/+ & (3/3 or 3/4)	28	52	REF	21	33	REF	5	11	REF
+/- & (3/3 or 3/4)	38	124	0.6 (0.3–1.0)	19	78	0.4 (0.2–0.8)	12	21	1.3 (0.4–4.5)
-/- & (3/3 or 3/4)	30	68	0.8 (0.4–1.5)	11	27	0.6 (0.3–1.6)	5	9	1.2 (0.3–5.6)
+/+ & (2/3 or 2/4)	43	116	0.7 (0.4–1.2)	28	80	0.6 (0.3–1.1)	11	24	1.0 (0.3–3.6)
+/+ & (2/3 or 2/4)	65	112	1.1 (0.6–1.9)	29	68	0.7 (0.3–1.3)	21	22	2.1 (0.6–7.1)
-/- & (2/3 or 2/4)	14	31	0.8 (0.4–1.8)	5	14	0.6 (0.2–1.8)	4	8	1.1 (0.2–5.4)
+/+ & 2/2	30	83	0.7 (0.4–1.2)	19	51	0.6 (0.3–1.3)	10	25	0.9 (0.2–3.2)
+/+ & 2/2	8	27	0.6 (0.2–1.4)	3	17	0.3 (0.1–1.1)	4	6	1.5 (0.3–7.6)
-/- & 2/2	0	5	N/A	0	1	N/A	0	3	N/A

* 1494del6: +: allele with no deletion; -: allele with deletion
 ** 28bp VNTR: the 2-repeat allele is considered as the “variant” allele; the 3-repeat and 4-repeat allele are considered as the “wildtype” alleles

Table III

TYMS tagSNPs: Crude ORs among all subjects and stratified by White/Hispanic race-ethnicity.

a. All Subjects						
SNP_ID	Genotypes	Maternal genotyping data		Infant genotyping data		
		Cases (N=169)	Controls (N= 276)	OR(95%CI)	Cases (N= 265)	Controls (N= 535)
rs502396	Wildtype	54	82	REF	79	142
	Heterozygote	73	105	1.1 (0.7–1.7)	99	226
	Mutant	36	79	0.7 (0.4–1.2)	71	151
rs2847153	Wildtype	103	161	REF	158	328
	Heterozygote	50	95	0.8 (0.5–1.3)	79	167
	Mutant	11	17	1.0 (0.5–2.2)	21	34
rs1001761	Wildtype	47	87	REF	86	159
	Heterozygote	75	100	1.4 (0.9–2.2)	105	213
	Mutant	40	73	1.0 (0.6–1.7)	71	147
rs2847149	Wildtype	46	87	REF	82	166
	Heterozygote	71	93	1.4 (0.9–2.3)	95	210
	Mutant	41	76	1.0 (0.6–1.7)	76	148
rs699517	Wildtype	65	118	REF	107	215
	Heterozygote	74	103	1.3 (0.9–2.0)	109	219
	Mutant	26	45	1.0 (0.6–1.9)	46	96
rs2847326	Wildtype	94	142	REF	149	299
	Heterozygote	53	97	0.8 (0.5–1.3)	80	181
	Mutant	17	27	1.0 (0.5–1.8)	26	42

b. Hispanics							
SNP_ID	Genotypes	Maternal genotyping data			Infant genotyping data		
		Cases (N=87)	Controls (N=163)	OR(95%CI)	Cases (N=146)	Controls (N=310)	OR(95%CI)
rs502396	Wildtype	34	61	REF	52	103	REF
	Heterozygote	33	65	0.9 (0.5–1.6)	51	136	0.7 (0.5–1.2)
	Mutant	18	31	1.0 (0.5–2.1)	34	62	1.1 (0.6–1.9)
		.	.		.	.	
rs2847153	Wildtype	59	101	REF	87	198	REF
	Heterozygote	22	55	0.7 (0.4–1.2)	42	96	1.0 (0.6–1.5)
	Mutant	4	5	1.4 (0.4–5.3)	12	12	2.3 (1.0–5.3)
		.	.		.	.	
rs1001761	Wildtype	32	64	REF	61	111	REF
	Heterozygote	32	62	1.0 (0.6–1.9)	50	128	0.7 (0.5–1.1)
	Mutant	18	29	1.2 (0.6–2.6)	34	59	1.0 (0.6–1.8)
		.	.		.	.	
rs2847149	Wildtype	excluded	excluded	excluded	61	116	REF
	Heterozygote	excluded	excluded	excluded	47	127	0.7 (0.4–1.1)
	Mutant	n/a	n/a	n/a	35	57	1.2 (0.7–2.0)
		.	.		.	.	
rs699517	Wildtype	41	79	REF	73	138	REF
	Heterozygote	34	60	1.1 (0.6–1.9)	51	128	0.8 (0.5–1.2)
	Mutant	11	19	1.1 (0.5–2.6)	20	40	0.9 (0.5–1.7)
		.	.		.	.	
rs2847326	Wildtype	38	73	REF	68	150	REF
	Heterozygote	34	65	1.0 (0.6–1.8)	51	132	0.9 (0.6–1.3)
	Mutant	13	19	1.3 (0.6–2.9)	21	19	2.4 (1.2–4.8)

c. Non-Hispanic Whites					
SNP_ID	Genotypes	Maternal genotyping data		Infant genotyping data	
		Cases (N=58)	Controls (N=73)	Cases (N=71)	Controls (N=106)
rs502396	Wildtype	16	14	REF	25
					REF

c. Non-Hispanic Whites		Maternal genotyping data			Infant genotyping data		
SNP_ID	Genotypes	Cases (N=58)	Controls (N=73)	OR(95%CI)	Cases (N=71)	Controls (N=106)	OR(95%CI)
	Heterozygote	28	32	0.8 (0.3–1.8)	27	42	0.8 (0.4–1.6)
	Mutant	13	23	0.5 (0.2–1.3)	17	35	0.6 (0.3–1.3)
		.	.		.	.	
rs2847153	Wildtype	35	44	REF	48	65	REF
	Heterozygote	19	25	1.0 (0.5–2.0)	19	33	0.8 (0.4–1.5)
	Mutant	3	3	1.3 (0.2–6.6)	2	6	0.5 (0.1–2.3)
		.	.		.	.	
rs1001761	Wildtype	13	18	REF	21	31	REF
	Heterozygote	32	31	1.4 (0.6–3.4)	34	41	1.2 (0.6–2.5)
	Mutant	12	18	0.9 (0.3–2.6)	14	33	0.6 (0.3–1.4)
		.	.		.	.	
rs2847149	Wildtype	13	18	REF	18	32	REF
	Heterozygote	30	30	1.4 (0.6–3.3)	28	41	1.2 (0.6–2.6)
	Mutant	10	19	0.7 (0.3–2.1)	18	33	1.0 (0.4–2.2)
		.	.		.	.	
rs699517	Wildtype	22	30	REF	27	49	REF
	Heterozygote	27	33	1.1 (0.5–2.4)	34	39	1.6 (0.8–3.1)
	Mutant	7	7	1.4 (0.4–4.5)	9	17	1.0 (0.4–2.4)
		.	.		.	.	
rs2847326	Wildtype	37	45	REF	48	62	REF
	Heterozygote	16	22	0.9 (0.4–1.9)	16	31	0.7 (0.3–1.4)
	Mutant	4	3	1.6 (0.3–7.7)	4	12	0.4 (0.1–1.4)

Gene-folate interactions and risk for conotruncal heart defects: infant *TYMS* functional variant 1494del6 and maternal folate intake§

Table IV

Variables		Case (N=266)	Control (N=606)	OR (95% CI)
maternal folate intake§	Low	38	57	REF
	Not Low	228	549	0.7 (0.4–1.1)
1494del6 (rs16430) *	+/+	99	243	REF
	+/-	107	252	1.0 (0.8–1.4)
	-/-	44	103	1.0 (0.7–1.6)
1494del6 (rs16430)	Maternal Folate Intake§			
	Low	12	20	REF
	Low	11	30	0.6 (0.2 – 1.6)
	Low	13	6	3.6 (1.1 – 11.9)
	Not Low	87	223	REF
	Not Low	96	222	1.1 (0.8 – 1.6)
	Not Low	31	97	0.8 (0.5 – 1.3)

* This data is the same as listed in table 2, excluding subjects without maternal intake data

Table V

Gene-folate interactions and risk for conotruncal heart defects (P value<0.05 for interaction): *TYMS* tagSNPs and combined folate intake[§] **

SNP ID	Race/ ethnicity	Mother/infant genotype	Maternal Folate Intake [§]	Genotype [*]	Case	Controls	Odds ratio ^{§§} (95% CI)
rs1001761	ALL	infant	Low	WW	7	11	REF
	ALL	infant	Low	MW	9	25	0.6 (0.2 – 1.9)
	ALL	infant	Low	MM	14	11	2.0 (0.6 – 6.9)
	ALL	infant	Not Low	WW	76	138	REF
	ALL	infant	Not Low	MW	93	172	1.0 (0.7 – 1.4)
	ALL	infant	Not Low	MM	55	125	0.8 (0.5 – 1.2)
rs699517	ALL	infant	Low	WW	10	20	REF
	ALL	infant	Low	MW	11	24	0.9 (0.3 – 2.6)
	ALL	infant	Low	MM	10	5	4.0 (1.1 – 14.8)
	ALL	infant	Not Low	WW	93	179	REF
	ALL	infant	Not Low	MW	95	179	1.0 (0.7 – 1.5)
	ALL	infant	Not Low	MM	35	86	0.8 (0.5 – 1.2)

[§] combined folate intake variable

For dietary variable, two percentile categories were constructed corresponding to percentile categories 25 percentile, and >25 percentile, and were determined from dietary folate intake levels among control mothers with 289.39 mcg. A dichotomized composite variable, “combined folate intake”, was created by combining maternal vitamin use with daily dietary intake of folate. Combined folate intake was defined as low for women in the lowest quartile (25 percentile) of folate intake who did not take supplemental folic acid in the periconceptional period. “Not low” folate intake was defined as dietary folate intake above the lowest quartile (>25 percentile) and/or any maternal vitamin use during the periconceptional period.

^{§§}OR adjusted for total energy intake

^{*}W: major allele; M: minor allele.

^{**}Only interactions with p values less than 0.05 are presented.