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Evaluation of Heterogeneity in the Association between Congenital Heart Defects and Variants of Folate Metabolism Genes: Conotruncal and Left-Sided Cardiac Defects

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Abstract

PURPOSE—Genetic variation in the folate metabolic pathway may influence the risk of congenital heart defects. This study was undertaken to assess the associations between the inherited and maternal genotypes for variants in folate-related genes and the risk of a composite heart phenotype that included two component phenotypes: conotruncal heart defects (CTDs) and left-sided cardiac lesions (LSLs).

METHODS—Nine folate-related gene variants were evaluated using data from 692 case-parent triads (CTD, n=419; LSL, n=273). Log-linear analyses were used to test for heterogeneity of the genotype-phenotype association across the two component phenotypes (i.e. CTD and LSLs) and, when there was no evidence of heterogeneity, to assess the associations of the maternal and inherited genotypes with the composite phenotype.

RESULTS—There was little evidence of heterogeneity of the genotype-phenotype association across the two component phenotypes or of an association between the genotypes and the composite phenotype. There was evidence of heterogeneity in the association of the maternal *MTR* A2756G genotype ($p = 0.01$) with CTDs and LSLs. However, further analyses suggested that the observed associations with the maternal *MTR* A2756G genotype might be confounded by parental imprinting effects.

CONCLUSIONS—Our analyses of these data provide little evidence that the folate-related gene variants evaluated in this study influence the risk of this composite congenital heart defect phenotype. However, larger and more comprehensive studies that evaluate parent-of-origin effects, as well as additional folate-related genes, are required to more fully explore the relation between folate and congenital heart defects.

Keywords

Phenotypic heterogeneity; congenital heart defects; folate; case-parent triads; maternal genetic effects

INTRODUCTION

Congenital heart defects (CHDs) are the most common birth defects, with a prevalence of approximately 8 per 1,000 births (Jenkins et al., 2007). Although CHDs have a major impact on pediatric morbidity and mortality (Christianson A 2006), there are no established strategies for reducing their public health impact and relatively little is known about their etiology (Jenkins et al., 2007). Familial aggregation studies suggest that there are risk factors that are specific to subgroups of CHDs (e.g. left sided lesions, conotruncal defects) as well as risk factors that have a more general influence on cardiac development. Specifically, while there is a tendency for affected relatives to be concordant for CHD subgroups (Hardin et al., 2009; Oyen et al., 2009), relatives of affected individuals are also at increased risk for discordant CHDs (i.e. CHDs that do not belong to the same subgroups) (Oyen et al., 2009). Consequently, there are no clearly established case definitions for epidemiological studies of CHDs.

Several studies suggest that the risk of CHDs (and other structural birth defects, such as neural tube defects and cleft lip) is influenced by maternal folate status (Botto et al., 2000; Botto and Correa 2003; Jenkins et al., 2007) as well as variants within genes that are involved in folate-homocysteine metabolism (Botto et al., 2000; Shaw et al., 2005; Hobbs et al., 2006; van Beynum et al., 2007; Goldmuntz et al., 2008; Lupo et al., 2010; Mitchell et al., 2010). However, it is unclear whether these factors affect the risk of all CHDs, or are specific to subgroups of CHDs. Given its critical role in cellular metabolism and association with other birth defects (Blom and Smulders 2011), it would not be surprising for folate to play a general role in embryonic development and thus be associated with a range of CHDs.

We have previously examined the association between several well-characterized variants in folate-related genes, separately, in case-parent triads ascertained through cases with either a conotruncal heart defect (CTDs) or a left-sided cardiac lesion (LSLs) (Goldmuntz et al., 2008; Mitchell et al., 2010). Based on these analyses, we identified several variants that were nominally associated with CTDs and/or LSLs, via either the maternal or the inherited (i.e. case) genotype, suggesting that variation in folate-related genes may be associated with a range of heart phenotypes. Given the lack of clear case definitions for epidemiological studies of CHDs, and the likelihood that folate plays a general role in embryonic development, we have extended our studies to include analyses of the combined data from the CTD and LSL triads. These data provided the opportunity to formally assess heterogeneity of the effects of the folate-related variants across two major CHD subgroups, and improve the power to detect associations with genetic variants that may influence the risk of both.

METHODS

Study Subjects

Details of the CTD and LSL case-parent triads used in this study have been provided previously (Goldmuntz et al., 2008; Mitchell et al., 2010). Briefly, cases were recruited between 1997 and 2007 from the Cardiac Center at The Children's Hospital of Philadelphia, in accordance with a protocol approved by the Institutional Review Board for the Protection of Human Subjects. Males and females, and individuals of all races and ethnicities were eligible to participate. The CTD cases included individuals with a diagnosis of: truncus arteriosus, d-transposition of the great arteries, tetralogy of Fallot, double outlet right ventricle, interrupted aortic arch, conoventricular or posterior malalignment type ventricular septal defects or an isolated aortic arch anomaly. The LSL cases included individuals with a diagnosis of: hypoplastic left heart syndrome, coarctation of the aorta with or without a bicuspid aortic valve, aortic valve stenosis, isolated mitral valve anomalies, or other left-

sided defect. All cardiac diagnoses were confirmed with the medical records including, when necessary, original imaging studies. Potential case subjects who had a chromosomal abnormality (e.g. 22q11 micro-deletion) or genetic syndrome were excluded from the study. Blood samples were collected from cases, before a blood transfusion, at the time of surgical or medical intervention. In addition, blood samples were collected from all available parents. When a parent was unavailable for a blood draw, buccal or saliva samples were collected by mail.

Genotyping Methods

DNA was extracted from blood, cell lines, saliva or buccals, using standard methods (Puregene DNA isolation kit, Gentra System, Minneapolis, MN). Genotyping methods for the nine variants genotyped in both the CTD and LSL triads: *MTHFR* C677T and A1298C, *MTR* A2756G, *MTRR* A66G, *NOS3* G894T, *BHMT* G742A, *SHMT* C1420T, *TCN2* C777G, and *MCP1* (-A2518G), have been previously described (Goldmuntz et al., 2008; Mitchell et al., 2010). Briefly, genotyping was performed in the High-Throughput Genotyping Core Laboratory of the Molecular Diagnosis and Genotyping Facility at the University of Pennsylvania, using the ABI 7900 HT Sequence Detection System (Applied Biosystems, Foster City, CA).

For each analyzed genetic variant, the proportion of samples for which a genotype could not be assigned, the proportion of samples that yielded discrepant results on repeated genotypes, and the proportion of triads that had genotype combinations that were incompatible with Mendelian inheritance were determined. For each sample, the number of genotyping failures (i.e., genotypes that could not be assigned or were discrepant across repeated genotypes) was determined.

Statistical Methods

For each variant, log-linear analyses were used to test for heterogeneity of the genotype-phenotype association across the two phenotypes (i.e. CTD and LSL). A log-linear model for gene-environment interactions (Umbach and Weinberg 2000) was adapted for this purpose:

$$\ln N_{cmfp} = \mu_i + \delta_i I_{\{P=1\}} + \alpha_m I_{\{M=m\}} + \beta_c I_{\{C=c\}} + \theta_m I_{\{M=m\}} I_{\{P=1\}} + \vartheta_c I_{\{C=c\}} I_{\{P=1\}} + \ln(2) I_{\{(M,F,C)=(1,1,1)\}} \quad (\text{Model 1})$$

In Model 1, M , F , C denote the number of copies of the variant allele carried by the mother, father and child, respectively and P is a dummy variable that denotes the component phenotype (CTD, $P = 0$; LSL, $P = 1$). In this model, N_{cmfp} represents the expected number of triads with child genotype c , maternal genotype m , paternal genotype f and case phenotype p . The μ_i are stratification parameters associated with parental mating types for triads with CTD ($P = 0$), and α_m and β_c index the maternal and child genotypes in such triads. The corresponding parameters for the LSL phenotype are: $\mu_i + \delta_i I_{\{P=1\}}$, $\theta_m I_{\{M=m\}} I_{\{P=1\}}$ and $\vartheta_c I_{\{C=c\}} I_{\{P=1\}}$ respectively. The $I_{\{expression\}}$ are indicator functions that are equal to 1 when the expression is true and are otherwise equal to 0, and $\ln(2) I_{\{(M,F,C)=(1,1,1)\}}$ is an offset term that accounts for the 1:2:1 ratio of genotypes among the offspring of double heterozygote matings.

To determine whether the maternal genotype-phenotype association differed for CTDs and LSLs, the likelihood ratio test was used to compare Model 1 and a reduced model that excluded the $\theta_m I_{\{M=m\}} I_{\{P=1\}}$ parameters. Similarly, to determine whether the inherited genotype-phenotype association differed for CTDs and LSLs, the likelihood ratio test was used to compare Model 1 and a reduced model that excluded the $\vartheta_c I_{\{C=c\}} I_{\{P=1\}}$ parameters.

When there was no evidence of heterogeneity for either the maternal or the inherited genotypes ($p \geq 0.05$), the associations between the composite phenotype (i.e. CTDs and LSL) and both the maternal and inherited genotypes for each variant were assessed using a log-linear model that excluded all of the parameters containing $I_{(P=I)}$:

$$\ln N_{cmf} = \mu_i + \alpha_m I_{\{M=m\}} + \beta_c I_{\{C=c\}} + \ln(2) I_{\{(M,F,C)=(1,1,1)\}} \quad (\text{Model 2})$$

Model 2 is the basic log-linear model for triad data (Weinberg et al., 1998; Wilcox et al., 1998). The association between each variant and the maternal and inherited genotypes was assessed using the likelihood ratio test to compare Model 2 with reduced models that included terms for only the inherited or only the maternal genotypes, respectively.

When there was evidence of heterogeneity for the association with the maternal genotype across the two component phenotypes, parental imprinting, which can confound associations with the maternal genotype, was assessed. Specifically, parental imprinting was assessed, separately in LSL and CTD triads, using: (1) the logistic based parent-of-origin test (PO-LRT), which is based on data from completely genotyped triads and provides a valid test for variants that are directly related to disease risk as well as those that are in linkage disequilibrium with such a variant; and (2) the log-linear likelihood ratio test (LL-LRT), which uses data from both complete and incomplete triads but is subject to bias when the evaluated variant is not directly related to disease risk (Weinberg 1999; van Den Oord and Vermunt 2000).

All log-linear analyses were performed using LEM (Vermunt JK 1997), a program that allows data from case-parent triads that have not been completely genotyped to be included in the analysis. Consistent with our previous analyses of these data, a general model of inheritance (i.e. no restrictions were placed on the relationships between alleles) was used for all of these analyses. All other analyses were performed using SAS v9.0 (SAS, Cary, NC). For simplicity, the most common genotype for each variant was used as the referent category. All analyses were conducted using data from a single case per triad and were restricted to include data from triads in which both parents were reported to be Caucasian. To our knowledge, the cases are all unrelated.

RESULTS

Results of the quality control assessments have been previously reported (Goldmuntz et al., 2008; Lupo et al., 2010; Mitchell et al., 2010). Briefly, genotype call rates ranged from 94% to 98%; the proportion of samples that provided discrepant results on repeat genotypes ranged from 0% to 0.8%; and the proportion of triads with genotype combinations that were incompatible with Mendelian inheritance ranged from 0.7% to 2.5%. All genotype data from families that included at least one genotype combination that was incompatible with Mendelian inheritance were omitted from all analyses ($n = 89$ triads). In addition, all genotype data from individual samples that failed or provided discrepant results on repeat genotyping for more than three variants were omitted from all analyses ($n = 83$ samples), resulting in the exclusion of an additional 45 triads. After making these exclusions, the study sample included 692 Caucasian, case-parent triads in which the case individual had either a CTD ($n = 419$) or LSL ($n = 273$), without a recognized genetic syndrome or chromosomal abnormality. The characteristics of the cases are described in Table 1 and the distribution of triads by the genotype combinations of the case, mother and father are provided for each variant in supplementary Table I.

There was no evidence of heterogeneity in the inherited genotype-phenotype association for any of the variants. Furthermore, there was no evidence of heterogeneity for the maternal genotype-phenotype association for any variant except *MTR* A2756G ($p = 0.01$) (Table 2). Consequently, the association between each variant, except *MTR* A2756G, and the composite genotype was assessed using data from all triads (i.e. CTD and LSL) (Table 3). The likelihood ratio tests, assessing the association between the maternal and inherited genotype for each variant with the composite phenotype, were all non-significant ($p \geq 0.05$) in the combined data.

There was evidence of heterogeneity in the association of the maternal *MTR* A2756G genotype with CTDs and LSLs. Consequently, parental imprinting effects were assessed for this variant, since such effects can confound analyses of maternal genetic effects. Using the PO-LRT, separately in CTD and LSL triads, there was no convincing evidence of imprinting in either group (Table 4). However, the imprinting parameter (I_m) was greater than 1.0 for both CTDs ($I_m = 1.85$, 95% CI 0.84–4.08) and LSLs ($I_m = 1.51$, 95% CI 0.63–3.61). In addition, the LL-LRT, which uses more of the available data (i.e. complete and incomplete triads) than the PO-LRT (complete data only), provided evidence for parental imprinting in the CTD triads ($p = 0.03$).

DISCUSSION

Previous studies suggest that the risk of congenital heart defects, or subsets of congenital heart defects, may be related to maternal folate status and variants of folate-related genes (Botto et al., 2000; Botto and Correa 2003; Shaw et al., 2005; Hobbs et al., 2006; Jenkins et al., 2007; van Beynum et al., 2007; Goldmuntz et al., 2008; Lupo et al., 2010; Mitchell et al., 2010). However, such associations have not been convincingly established. We have previously evaluated the association between specific subsets of congenital heart defects (i.e. CTDs and LSLs) and several folate-related variants (Goldmuntz et al., 2008; Mitchell et al., 2010). While these studies were based on relatively large samples, only one association (maternal *MTHFR* A1298C genotype and CTD risk in offspring) remained statistically significant after adjusting for multiple-testing (Goldmuntz et al., 2008; Mitchell et al., 2010).

Our previous analyses, which considered CTDs and LSLs separately, may have been under-powered (relative to analysis based on the combined CTD and LSL data), if folate-related gene variants influence the risk of congenital heart defects in general. To address this possibility, the present study evaluated heterogeneity of the association of nine folate-related genes variants with CTDs and LSLs, and, when there was no evidence of such heterogeneity (i.e. p -heterogeneity ≥ 0.05), the association of each variant with the composite CTD and LSL phenotype type was evaluated. With only one exception, there was no evidence of heterogeneity in the association of the folate-related genotypes and the risk of LSL and CTDs. However, analyses of the combined CTD and LSL triad data provided no evidence that the evaluated folate-variants were associated with the composite phenotype.

There was evidence of heterogeneity in the association of the maternal *MTR* A2756G genotype with CTDs and LSLs. This finding is consistent with the results from our previous analyses, which suggested that maternal *MTR* A2756G genotypes including a G allele were associated with an increased risk of CTDs (unadjusted p -value=0.04; $RR_{AG \text{ vs } AA}=1.40$, 95% CI 1.07–1.83; $RR_{GG \text{ vs } AA}=1.26$, 95% CI 0.69–2.29) and a decreased risk of LSLs (unadjusted p -value=0.01; $RR_{AG \text{ or } GG \text{ vs } AA}=0.66$, 95% CI 0.48–0.93) (Goldmuntz et al., 2008; Mitchell et al., 2010). As failure to account for parent of origin effects can bias estimates of maternal genetic effects (Hager et al., 2008), models accounting for the parental origin of the *MTR* 2756 G allele were evaluated. Although these models provided some evidence in favor of a parent of origin effect, *MTR* is not known to be imprinted or to fall

within an imprinted region of the genome. Hence, additional studies will be required to confirm and interpret these findings.

In summary, these analyses provide little evidence that the evaluated folate-related gene variants influence the risk of the composite (i.e. CTD + LSL) phenotype. Further, as CTDs and LSLs account for a substantial portion of all congenital heart defects, these findings suggest that the evaluated variants are unlikely to be major determinants of heart defect risk in general. However, given the limited number of variants evaluated in this study, it is possible that other variants within these genes or variants of other folate-related genes are related to the risk of congenital heart defects. Further, it is possible that folate-related gene variants are associated with defects other than CTDs and LSLs. In addition, these analyses provide some limited evidence that the relationship between folate-related gene variants and congenital heart defects may be more complex than anticipated (i.e. perhaps involving parent-of-origin effects). In conclusion, future studies of the relationship between folate-related gene variants and the risk of congenital heart defects should include a comprehensive assessment of folate-related gene variants, including evaluation of multiple genes, multiple variants per gene and evaluation of parent-of-origin effects in addition to both inherited and maternal genetic effects.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Table 1

Characteristics of the CTD and LSL cases, Children's Hospital of Philadelphia, 1997–2007.

Characteristic	N (%)	
	CTD (N= 419)	LSL (N=273)
Sex		
Male	240 (57.3)	172 (63.0)
Female	179 (42.7)	101 (37.0)
Diagnosis		
Tetralogy of Fallot	177 (42.2)	
D-transposition of the great artery	89 (21.2)	
Ventricular septal defect	74 (17.7)	
Double outlet right ventricle	39 (9.3)	
Isolated aortic arch anomaly	19 (4.5)	
Truncus arteriosus	11 (2.6)	
Interrupted aortic arch	10 (2.4)	
Hypoplastic left heart syndrome		124 (45.4)
Coarctation of the aorta		85 (31.1)
Aortic valve stenosis		59 (21.6)
Other		5 (1.8)

Table 2
association between folate-related genetic variants and CTDs and LSLs.

	LRT for heterogeneity (p-value)									
	MTHFR C677T (1801133) 487/165/48	MTR A2756G (1805087) 473/176/20	MTRR A66G (1801394) 461/183/25	MCPI A2518G (1024611) 477/172/19	BHMT G742A (3733890) 481/172/17	TCN2 C777G (1801198) 473/165/30	SHMT C1420T (1979277) 479/172/18	NOS3 G894T (1799983) 469/179/19		
1) ^a	1.57 (0.46)	1.55 (0.46)	0.73 (0.69)	0.24 (0.89)	1.96 (0.38)	0.08 (0.96)	0.66 (0.72)	0.80 (0.67)		
Birth Defects Res A Clin Mol Teratol.	0.40 (0.88)	10.16 (0.01)	1.77 (0.41)	0.19 (0.91)	0.50 (0.78)	0.40 (0.82)	1.69 (0.43)	2.10 (0.35)		

member.

Table 3

Log-Linear Modeling Results for the Combined CTD and LSL Case-Parents Triads.

MTHFR A1298C (1801131) ^a 481/168/20 ^b	MTHFR C677T (1801133) 487/165/18	MTRR A66G (1801394) 461/183/25	MCPI A2518G (1024611) 477/172/19	BHMT G742A (3733890) 481/172/17	TCN2 C777G (1801198) 473/165/30	SHMT C1420T (1979277) 479/172/18	NOS3 G894T (179983) 469/179/19
0.84 (0.68–1.04)	1.22 (0.98–1.52)	0.84 (0.64–1.09)	1.02 (0.82–1.27)	0.96 (0.77–1.19)	0.77 (0.62–0.97)	1.12 (0.9–1.4)	1.03 (0.83–1.29)
0.91 (0.63–1.20)	1.17 (0.82–1.66)	0.79 (0.56–1.09)	1.05 (0.68–1.61)	0.86 (0.58–1.28)	0.73 (0.52–1.02)	1.22 (0.83–1.79)	0.96 (0.65–1.41)
1.04 (0.82–1.36)	1.15 (0.90–1.47)	1.23 (0.90–1.69)	0.99 (0.78–1.27)	0.81 (0.64–1.04)	1.17 (0.91–1.51)	0.84 (0.66–1.08)	1.26 (0.98–1.63)
0.83 (0.55–1.20)	1.46 (1.01–2.12)	1.17 (0.84–1.64)	0.89 (0.55–1.44)	0.84 (0.55–1.28)	0.99 (0.71–1.37)	0.67 (0.43–1.05)	1.43 (0.94–2.20)
2.59 (0.27–26.4)	3.23 (0.20)	2.24 (0.33)	0.05 (0.97)	0.56 (0.76)	5.35 (0.07)	1.43 (0.49)	0.23 (0.89)
0.97 (0.61)	4.34 (0.11)	1.66 (0.44)	0.23 (0.89)	2.97 (0.23)	1.92 (0.38)	3.99 (0.14)	4.77 (0.09)

ids with genotype data for 1, 2, 3, 2 or 1 member.

ote the relative risk to cases with the heterozygous and rare homozygous genotypes, respectively, as compared to cases with the common homozygous genotype.

ote the relative risk to offspring of women with the heterozygous and rare homozygous genotypes, respectively, as compared to the common homozygous genotype.

Table 4
Summary of tests for parent of origin effects for *MTR* A2756G (rs1805087), in CTD and LSL triads.

	PO-LRT				LL-LRT			
	I_m^a (95% CI)	$S1^a$ (95% CI)	$S2^a$ (95% CI)	LRT (<i>p</i> -value)	I_m (95% CI)	$S1$ (95% CI)	$S2$ (95% CI)	LRT (<i>p</i> -value)
CTD	1.85 (0.84–4.08)	1.11 (0.64–1.92)	0.85 (0.29–2.47)	2.37 (0.12)	2.27 (1.06–4.87)	1.02 (0.61–1.71)	0.59 (0.20–1.70)	4.40 (0.03)
LSL	1.51 (0.63–3.61)	0.53 (0.30–0.94)	NA ^b	0.86 (0.35)	1.59 (0.69–3.66)	0.62 (0.36–1.07)	0.08 (0.01–0.74)	1.18 (0.28)

^a I_m denotes the imprinting parameter; $S1$ and $S2$ denote the relative risks for maternal heterozygous and rare homozygous genotypes, respectively.

^bThere were no mothers of LSL cases who were homozygous for the variant allele, consequently $S2$ could not be estimated.