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Variants of Folate Metabolism Genes and Risk of Left-Sided Cardiac Defects

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Abstract

Background—Congenital heart defects (CHD) are the most common, serious group of birth defects. Although relatively little is known about the causes of these conditions and there are no established prevention strategies, evidence suggests that the risk of CHD may be related to maternal folate status as well as genetic variants in folate-related genes. Efforts to establish the relationships between these factors and CHD risk have, however, been hampered by a number of factors, including small study sample sizes and phenotypic heterogeneity.

Methods—The present study examined the relationship between nine genetic variants in eight folate-related genes and a relatively homogeneous group of left-sided cardiac defects in a cohort of 386 case-parent triads. Log-linear analyses were used to assess both maternal and inherited genetic effects.

Results—Analyses of the study data provided marginal evidence that the maternal *MTR* A2756G (unadjusted p=0.01) and the inherited *BHMT* G742A genotypes (unadjusted p=0.06) influence the risk of this subset of CHD. However, neither association achieved significance when the false-discovery rate was controlled at 0.05.

Conclusions—These results, which are based on the largest study sample and most comprehensive assessment of the relationship between left-sided cardiac defects and folate-related genes reported to date, provide little evidence that this subset of CHD is folate-related. However, even larger studies and more comprehensive evaluations of the folate pathway genes are required to fully explore the relationship between folate and left-sided cardiac defects.

Keywords

birth defect; folate; folic acid; gene; heart

Introduction

Congenital heart defects (CHD) are the most common, serious birth defects of genetic or partially genetic origin (Christianson and others, 2006). Despite medical and surgical improvements, CHD continue to be associated with significant lifelong morbidity and early mortality (Boneva and others, 2001). In addition, relatively little is known about the causes

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of these conditions, and there are no established strategies for reducing their public health impact (Jenkins and others, 2007).

As a group, CHD include a range of malformations that are anatomically, epidemiologically, developmentally and clinically heterogeneous. Nonetheless, there is evidence that subgroups of CHD share common risk factors and are, therefore, more similar to each other than to other forms of CHD (Botto and others, 2007). One such subgroup includes malformations of the left sided structures of the heart, or left-sided lesions (LSL). Component members of this subgroup of CHD (i.e. LSL) include hypoplastic left heart syndrome (HLHS), coarctation of the aorta (COA) and aortic valve stenosis (AS). Family studies, which demonstrate that the affected relatives of individuals with LSLs are more likely to have a LSL than other type of CHD, indicate that the various LSL share common genetic underpinnings (Botto and others, 2007; Corone and others, 1983; Cripe and others, 2004; Ferencz and others, 1989; Fraser and Hunter, 1975; Hinton and others, 2007; Lewin and others, 2004; McBride and others, 2005).

The established causes of CHD are individually quite rare and for most affected individuals a specific causative agent or gene cannot be identified (Jenkins and others, 2007; Pierpont and others, 2007). However, there is evidence that, similar to neural tube defects, the risk of CHD, including LSL, may be influenced by maternal folate status [reviewed in (Botto and others, 2000; 2003; Jenkins and others, 2007)] as well as variation within genes that are involved in folate-transport and metabolism (Botto and others, 2000; Goldmuntz and others, 2008; Hobbs and others, 2006; Pei and others, 2006; Shaw and others, 2005; Shaw and others, 2003; van Beynum and others, 2007; Verkleij-Hagoort and others, 2008). However, associations with maternal folate status and folate-related genes have not been established for CHD in general, or for specific subsets of CHD (Pierpont and others, 2007). Hence, the present study was undertaken to assess the associations between a relatively homogeneous subset of CHD (i.e. LSL) and variants within folate-metabolism genes that have previously been identified as potential risk factors for CHD and other common birth defects.

Methods

Study Subjects

The present study is based on data from 368 case-parent triads, recruited between 1997 and 2007 from the Cardiac Center at The Children's Hospital of Philadelphia and in accordance with a protocol approved by the Institutional Review Board for the Protection of Human Subjects. Study subjects included those with the left-sided cardiac lesions: hypoplastic left heart syndrome (HLHS), coarctation of the aorta with or without a bicuspid aortic valve (CA), aortic valve stenosis (AS), isolated mitral valve anomalies, and a small number with other left sided defects. Patients with so-called "HLHS variants" such as malaligned atrioventricular canal defects or double outlet right ventricle with mitral valve atresia were excluded.

Males and females, and individuals of any racial/ethnic group were eligible to participate in the study. Blood samples were collected from study subjects, prior to a blood transfusion, at the time of surgical or medical intervention, and blood, buccal or saliva samples were collected from each available parent. Cases with a recognized genetic syndrome or chromosome anomaly, including those with Turner syndrome, were excluded from the current analyses. Medical records including, when necessary, original imaging studies, were reviewed to confirm the cardiac diagnosis and identify additional medical issues.

Genotyping Methods

Case and parental DNA was extracted from whole blood, buccal swabs or lymphoblastoid cell lines using standard methods (Puregene DNA isolation kit by Gentra System Inc., Minneapolis MN). Duplicate samples were included both within and across plates such that 5% of the samples were genotyped three times. The study cohort was genotyped for nine single nucleotide polymorphisms (SNPs) from eight genes: *MTHFR* C677T and A1298C, *MTR* A2756G, *MTRR* A66G, *NOS3* G894T, *BHMT* G742A, *SHMT* C1420T, *TCN2* C777G and *MCP1* (-A2518G). The genes selected for analysis are involved in folate-homocysteine metabolism. Specific variants were selected for genotyping based on previous studies demonstrating an association between the variant and risk of CHD or another structural birth defect and/or evidence that the variant influences protein function.

SNP genotyping was conducted in the High-Throughput Genotyping Core Laboratory at the Molecular Diagnosis and Genotyping Facility at the University of Pennsylvania, using the ABI 7900 HT Sequence Detection System (Applied Biosystems, Inc., Foster City, CA). Taqman 5' nuclease polymerase chain reaction (PCR) primers and probes for the variant of interest were ordered (Assays-on-Demand) or custom designed (Assay-By-Design) by Applied Biosystems (Foster City, CA) or Epoch Biosciences (Nanogen and currently Sigma Aldrich; St. Louis, MO). To validate each assay, the primer and probe sets were tested on a panel of DNA samples composed of CEPH family members (Family 1331, XC01331) obtained from Coriell Human Variation Collection (Coriell Institute, Camden, NJ). PCR amplifications were subsequently performed in a 384-well plate format with appropriate controls and processed according to the manufacturer's instructions (Applied Biosystems, Inc., Foster City, CA). Allelic discrimination results were graphed on a scatter plot and data transferred electronically for analysis.

Statistical Methods

The characteristics of the case individuals and their parents were summarized using counts and proportions. In addition, for each analyzed 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. These analyses were performed using SAS version 9.1 (SAS Institute, Inc, Cary, NC).

Log-linear analyses were used to assess the association between LSL and both the inherited (i.e. case) and maternal genotypes for each variant (Weinberg and others, 1998). For simplicity, the most common genotype for each variant was designated as the referent category. The risk of LSL in cases and the risk of having a child with a LSL in mothers of cases with the heterozygous or rare homozygous genotypes, relative to cases and mothers with the common homozygous genotype were estimated along with associated 95% confidence intervals. The significance of the inherited and maternal genetic effects was determined using the likelihood ratio test to compare the log-linear model that included terms for both the inherited and maternal genotypes, with reduced models that included terms for only the inherited or only the maternal genotype. In general, an unrestricted model, which allowed the relative risks associated with the heterozygous and rare homozygous genotypes to vary independently, was fitted to the data. However, when the number of cases or mothers with the rare homozygous genotype was small ($N \leq 10$), a dominant model of inheritance was fitted to the data. These analyses were run using LEM (Vermunt, 1997), a program for log-linear analysis with missing data that allows data from triads that have not been completely genotyped to be included in the analysis.

The association between LSL and haplotypes formed by the two *MTHFR* variants were evaluated using an extension of the log-linear model that provides estimates of single- and double-dose haplotype effects (Gjessing and Lie, 2006). The haplotype analyses were conducted using HAPLIN version 2.1.1 running under R Version 2.5.1 for Windows.

In all log-linear analyses, likelihood ratio tests with uncorrected p-values of <0.05 were considered to be of interest. However, given that multiple tests were performed (i.e. N=18, tests of inherited and maternal genetic effects for nine variants) the approach of Benjamini and Hochberg (Benjamini and Hochberg, 1995) was used to control the false discovery rate at 0.05.

The log-linear analyses were conducted using data from all triads, and in a subset of the data that excluded triads in which the mother reported that she was diabetic or used insulin, was epileptic or used seizure medications, or took the drug Paxil during her pregnancy. Triads in which at least one parent was not non-Hispanic Caucasian were also omitted from this subgroup to minimize the potential for biased assessment of the maternal genotype effects that can result when parents are from different racial/ethnic groups (Mitchell and Weinberg, 2005).

Results

The study sample included 386 triads in which the case had a left sided cardiac defect and was not diagnosed with a recognized genetic syndrome or chromosomal abnormality (Table 1). The most common diagnoses among the cases were hypoplastic left heart syndrome (45.9%), coarctation of the aorta (32.6%) and aortic valve stenosis (19.9%). There was a predominance of males among the cases (62.9%), as is seen in the general population of cardiac patients (Ferencz and others, 1997) and, in the majority, both parents were reported to be non-Hispanic Caucasian. Maternal diabetes (pre-pregnancy or gestational) or the use of insulin (N=6), epilepsy or the use of a seizure medication (N=2), or use of Paxil (N=1) was reported in 2.3% of the triads. The majority of cases (62.5%) were born in 1999 or later and thus, would have been conceived following mandatory folic acid fortification of the food supply in the United States.

Members of the study triads (n=979 DNA samples) were genotyped for nine variants (Table 2). The genotype call rates ranged from 94% – 97%. 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 0.0% to 2.3%. All genotype data from families that included at least one genotype combination that was incompatible with Mendelian inheritance were omitted from all analyses (N=63 samples from 21 triads). In addition, all genotype data from individual samples that failed or provided discrepant results on repeat genotyping for four or more of the genotyped variants were omitted from all analyses (N=21 samples). The number of useable genotypes for each of the variants ranged from 855 – 891. The observed genotype distributions in case individuals and their mothers and fathers are presented in Supplementary Table 1.

Log-linear analyses of individual variants and haplotypes formed by the two *MTHFR* variants were performed using data from all triads and a subset of triads selected to minimize heterogeneity and bias (see Methods). However, as the results obtained from these two sets of analyses were similar, only the results of the analyses using the full dataset are presented. The distribution of the analyzed triads, by genotypes of the mother, father and case, for each variant are provided in Supplementary Table 2.

Estimates of relative risk of LSL and 95% confidence intervals for the inherited and maternal genotypes, the likelihood ratio test statistics and associated p-values for the model comparisons for each variant are summarized in Table 3. A borderline significant association was observed between LSL and case genotype for the *BHMT* G742A variant (unadjusted p=0.06). Among cases, individuals with *BHMT 742 GA and AA* genotypes were at decreased risk relative to cases with the GG genotype ($RR_{GA\ vs.\ GG}$ = 0.91, 95% CI 0.67–1.25; $RR_{AA\ vs.\ GG}$ = 0.50, 95% CI 0.27–0.93). A single association with an unadjusted p-value <0.05 was also observed between LSL and maternal genotype. Specifically, the risk of having a child with a LSL was decreased among women with the *MTR* AG and GG genotypes as compared to those with the AA genotype ($RR_{AG\ or\ GG\ vs.\ AA}$ = 0.66, 95% CI 0.48–0.93, p=0.01. A dominant model was used for this variant due to the small number of mothers of cases (n=4) with the GG genotype). However, neither of these associations achieved significance when the false discovery rate was controlled at 0.05 (case *BHMT* G742A, p=0.06>0.003; maternal *MTR* A2756G, p=0.01>0.003). Analyses of the haplotypes formed by the two *MTHFR* variants provided no evidence on an affect of either the case or maternal haplotype on the risk of LSL.

Discussion

The most extensively studied folate variant, *MTHFR* C677T, has been inconsistently associated with the risk of CHD (van Beynum and others, 2007). The results of our study, as well as those of the single previous study of LSL and *MTHFR* C677T (McBride and others, 2004), indicate that this variant is not strongly related to the risk of LSL.

The *MTHFR* A1298C variant has also been inconsistently associated with the risk of CHD. While this variant was not strongly related to the risk of LSL in this study, the two largest studies of this variant and CHD risk identified an association with conotruncal malformations (Goldmuntz and others, 2008) and with a group of CHD including septal, conotruncal and LSL (Hobbs and others, 2006). Hence, the *MTHFR* A1298C variant may only be associated with specific subsets of CHD and, therefore, difficult to detect in study samples that include heterogeneous CHD phenotypes.

Our analyses of these data also provide little evidence of an association between LSL and either the inherited or maternal genotype for *MTRR* A66G, *MCP1* (-A2518G), *TCN2* C777G, *SHMT* C1420T, or *NOS3* G894T. Of these variants, only *MTRR* A66G, *TCN2* C776G and *NOS3* G894T have previously been assessed as risk factors for CHD and none of these variants have previously been evaluated in a sample restricted to include only LSL. Consistent with the results of our analyses, previously published studies provide no compelling evidence that either the *MTRR* A66G (van Beynum and others, 2006; Verkleij-Hagoort and others, 2008), or the *TCN2* C777G (Verkleij-Hagoort and others, 2008) variant has a significant, independent effect on the risk of CHD. The two previous studies of *NOS3* variants and CHD risk provided inconsistent results (Shaw and others, 2005; van Beynum and others, 2008).

Our analyses provided some evidence that the *MTR* A2756G variant may influence the risk of LSL via the maternal genotype and suggested that the 2756G allele acts in a dominant manner such that women with the AG or GG genotypes have a reduced risk of having a child affected with a left sided cardiac defect as compared to women with the AA genotype. The maternal *MTR* A2756G genotype has been associated with the risk of several other birth defects including conotruncal heart defects (Goldmuntz and others, 2008), neural tube defects (Doolin and others, 2002) and facial clefts (Mostowska and others, 2006). However, in contrast to our finding for LSLs, the maternal *MTR* AG and/or GG genotypes appear to be associated with increased risk of these malformations among offspring. While it is possible

that this variant is associated with increased risk of some birth defects and decreased risk of others, it seems more likely that the observed association with LSL represents a false-positive finding, as suggested by the failure of this association to achieve statistical significance when the false discovery rate was controlled at 0.05.

Our analyses also provided some evidence that the *BHMT* G742A variant may influence the risk of LSL via the inherited genotype, and suggested that the 742AA genotype was associated with decreased risk of LSL compared to the GG genotype. In a large study of conotruncal heart defects, no association with this variant was observed (Goldmuntz and others, 2008) and studies assessing the association of *BHMT* variants and the risk of neural tube defects have provided conflicting results (Boyles and others, 2006; Morin and others, 2003; Zhu and others, 2005). While it is possible that this variant is associated with the risk of LSL and not conotruncal heart defects, it seems likely that this association also represents a false-positive finding.

This report summarizes the largest and most comprehensive study of the relationship between folate-related genes and LSL to date. Our analyses provide little evidence that the risk of LSL is influenced by the effects of the evaluated folate-related gene variants. However, the possibility that other folate-related gene variants confer risk, or that folate-related gene variants exert small effects on risk and/or effects that are mediated through other risk factors (e.g. gene-gene, gene-environment interactions) cannot be excluded based on our analyses. Analyses based on larger study samples with carefully defined cardiac phenotypes and additional folate-related gene variants are clearly warranted to define the relationship between folate and CHD risk. Furthermore, studies large enough to permit well powered analyses of gene-gene interaction will be of great value given the inter-relationship between folate pathway gene members. Such studies are critical given the potential to identify an at-risk CHD population that might benefit from simple, targeted intervention.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Table 1

Characteristics of LSL cases and their parents

Characteristic	N (%)
Case sex	
Male	243 (63.0)
Female	143 (37.0)
Cases' year of birth[1]	
Prior to 1999	144 (37.5)
1999 or later	240 (62.5)
Race/Ethnicity (parental mating pairs)[2]	
White	301 (79.0)
Black	26 (6.8)
Hispanic	9 (2.4)
Asian	3 (0.8)
Mixed	42 (11.0)
Primary diagnosis	
Hypoplastic left heart syndrome	177 (45.9)
Coarctation of the aorta[3]	126 (32.6)
Aortic valve stenosis	77 (19.9)
Other[4]	6 (1.6)

[1] Information on year of birth was missing for two cases

[2] Information on parental race was missing for five cases

[3] Includes 8 with ventricular septal defect.

[4] Includes isolated mitral valve anomalies (n=3), supravalvar aortic stenosis (n=2), subaortic membrane (n=1).

Table 2

Genotyping assays for selected variants in eight folate-related genes

Gene Name	Gene Abbreviation	Polymorphism	dbSNP-rs#	AA*-position	Assay	Forward/Reverse Primer/Vic-reporter or AOD Reference Number*
5,10-methylene tetrahydrofolate reductase	*MTHFR*	A1298C	1801131	E429A	ABD	GGAGGAGCTGCTGAAGATGTG CCCGAGAGGTAAAGAACAAAGACTT ACCAGTGAAGAAAGTGT
5,10-methylene tetrahydrofolate reductase	*MTHFR*	C677T	1801133	A222V	AOD‡	C____1202883_20*
Methionine synthase	*MS/MTR*	A2756G	1805087	D919G	AOD	C____12005959_10*
Methionine synthase reductase	*MTRR*	A66G	1801394	I22M	AOD	C____3068176_10*
Monocyte chemotactic protein-1	*MCP1*	(-A2518G)	1024611	UTR	ABD	TTCTTGACAGAGCAGAAGTGG GCCTTTGCATATATCAGACAGTA AGACAGCTGTCACTTTC
Betaine-homocysteine methyltransferase	*BHMT*	G742A	3733890	R239Q	ABD†	CTCATGAAGGAGGGCTTGGA CCAAGGGCTGGCTCATCAG CTTTCAGTTGGGCAGC
Transcobalamin II	*TCN2*	C777G	1801198	P259R	AOD	C____325467_10*
Serine Hydroxymethyltransferase	*SHMT*	C1420T	1979277	F474L	ABD	CTCCGGGAGGAGGTTGAGA GCCCGCTCCTTTAGAAGTCA CTTCGCCTCTCTCTTC
Endothelial nitric oxide syntthase	*NOS3*	G894T	1799983	E298D	EPOCH	ACGGCTGGACCCCAGGAAA ACCAGCTCGGG*GGGCAGAA CCCCAGATGATCC

*AA, amino acid;

†ABD, assay by design;

‡AOD, assay on demand;

§EPOCH

Table 3

Summary of log-linear modeling results for case-parent triads based on full group

	MTHFR A1298C	MTHFR C677T	MS/MTR A2756G	MTRR A66G	MCP1 (-A2518G)	BHMT G742A	TCN2 C777G	SHMT C1420T	NOS3 G894T
Trios/Dyads/Mono(N)	235/80/12	239/75/13	228/84/14	224/83/17	226/88/13	234/79/14	217/89/20	231/81/16	232/86/10
General Model									
R1 (95% CI)	1.06 (0.77–1.45)	1.08 (0.78–1.50)	0.89 (0.65–1.24)	1.14 (0.77–1.69)	0.88 (0.64–1.20)	0.91 (0.67–1.25)	0.69 (0.49–0.98)	1.12 (0.81–1.54)	0.88 (0.65–1.20)
R2 (95% CI)	1.28 (0.73–2.25)	1.18 (0.70–2.01)	1.75 (0.85–3.59)	1.09 (0.67–1.79)	0.98 (0.51–1.89)	0.50 (0.27–0.93)	0.65 (0.38–1.09)	1.10 (0.60–2.00)	1.22 (0.71–2.10)
S1 (95% CI)	1.14 (0.81–1.60)	1.01 (0.70–1.44)	NA	1.03 (0.66–1.61)	0.96 (0.69–1.35)	0.83 (0.57–1.20)	1.17 (0.80–1.71)	0.81 (0.57–1.16)	1.17 (0.83–1.64)
S2 (95% CI)	1.09 (0.59–2.01)	1.18 (0.66–2.09)	0.66 * (0.48–0.93)	0.95 (0.59–1.53)	0.96 (0.50–1.83)	0.90 (0.47–1.70)	0.83 (0.51–1.34)	1.07 (0.55–2.07)	1.33 (0.75–2.37)
LRT (2df) for offspring effects (p value)	0.75 (0.69)	0.42 (0.81)	3.75 (0.15)	0.45 (0.80)	0.75 (0.69)	5.49 (0.06)	4.04 (0.13)	0.45 (0.80)	2.03 (0.36)
LRT (2df) for maternal effects (p value)	0.57 (0.75)	0.33 (0.85)	5.95 * (0.01)	0.16 (0.92)	0.06 (0.97)	1.00 (0.61)	1.68 (0.43)	1.49 (0.48)	1.52 (0.47)

* Estimated by dominant model in which S1=S2.