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NAT1, NOS3, and TYMS Genotypes and the Risk of Conotruncal Cardiac Defects

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Abstract

Background—Although congenital heart defects (CHDs) are a common and serious group of birth defects, relatively little is known about the causes of these conditions, and there are no established prevention strategies. There is, however, evidence suggesting that the risk of CHDs in general, and conotruncal and related defects (CTRDs) in particular, may be associated with maternal folate status and genetic variants of folate-related genes. Although several folate-related genes have been studied as they relate to CHDs and CTRDs (e.g., *MTHFR*), others have not been adequately assessed.

Methods—Case-parent triads were examined using log-linear analyses to assess the associations between CTRDs and both the genotype inherited by the case and the maternal genotype for the following variants: *NAT1* 1095C>A, *NOS3* 894G>T, and *TYMS* 1494del6. Subgroup analyses were also conducted among cases with classic conotruncal defects and cases with normally related great arteries.

Results—The results provided little evidence that CTRD risk was associated with the genotype inherited by the case for any of the analyzed variants. However, our results suggest that CTRD risk may be associated with the maternal genotype for *NOS3* 894G>T ($p = 0.024$ in the subgroup with normally related great arteries) and *TYMS* 1494del6 ($p = 0.048$ in the subgroup with classic conotruncal defects). However, these results were not significant after correcting for multiple comparisons.

Conclusion—This study provides further evidence that CTRD risk may be related to variation within folate-pathway genes and suggests that these associations are, at least in part, mediated through the maternal genotype.

Keywords

folate; *NAT1* gene; *NOS3* gene; *TYMS* gene; conotruncal cardiac defects

Introduction

Congenital heart defects (CHDs) are the most common type of structural malformation, occurring in approximately 1 in 200 live births, and are associated with significant morbidity and mortality (Botto et al., 2007). Despite considerable effort to determine the causes of CHDs, few risk factors have been consistently identified (Jenkins et al., 2007). This relative lack of success can be attributed to several factors, including the potential for etiologic heterogeneity among the many and varied CHD phenotypes (Botto et al., 2007). Hence, etiologic studies often focus on subgroups of CHDs that appear to share common embryologic, genetic, and epidemiologic features (e.g., conotruncal and left-sided heart defects) (Shaw et al., 2005; Goldmuntz et al., 2008; Shaw et al., 2009; Mitchell et al., 2010).

There is evidence that the risk of having a child with a CHD, and, in particular, a child with a conotruncal or related heart defect (CTRD), is associated with maternal folate status (Jenkins et al., 2007). Moreover, there is evidence that this risk is also influenced by variation in genes that are involved in folate-related processes (Shaw et al., 2005; Hobbs et al., 2006; Pei et al., 2006; Goldmuntz et al., 2008; Lupo et al., 2010). For instance, the genotype inherited by the case for the *MTHFR* A1298C variant appears to be associated with the risk of having a CTRD (Hobbs et al., 2006). However, there are many other folate-related genes that have yet to be adequately studied as potential CTRD risk factors.

The present study extends our previous analyses of CTRDs and folate-related genes (Goldmuntz et al., 2008) to include functional variants in three additional genes: *NAT1*, *NOS3*, and *TYMS*. The variants selected for analysis are functional polymorphisms that have been associated with changes in plasma folate levels and other birth defects (Stanley et al., 1998; Brown et al., 2003; Volcik et al., 2003; Lammer et al., 2004; Shaw et al., 2009). As in our previous analyses, the present study includes the assessment of associations between CTRDs and the genotypes inherited by the case as well as the maternal genotypes.

Methods

Case-parent triads ($n = 733$) were enrolled from the Cardiac Center at The Children's Hospital of Philadelphia from 1997 to 2007 in accordance with a protocol approved by the Institutional Review Board for the Protection of Human Subjects. Cases included individuals with a classic conotruncal defect: tetralogy of Fallot (TOF), D-transposition of the great arteries (D-TGA), double outlet right ventricle (DORV), truncus arteriosus (TA), or interrupted aortic arch (IAA). Individuals with conoventricular or posterior malalignment type ventricular septal defects (VSDs), or isolated aortic arch anomalies (AAAs), which appear to share common genetic underpinnings with the classic conotruncal defects (McElhinney et al., 2001; McElhinney et al., 2003), were also included as cases. Additional details of this family-based, case-parent triad study have been provided previously (Goldmuntz et al., 2008).

Blood samples were collected from cases, and blood, buccal, or saliva samples were collected from each available parent. Standard methods were used to extract DNA from whole blood, buccal swabs, or lymphoblastoid cell lines (Puregene DNA isolation kit;

Gentra Systems, Minneapolis, MN). Genotyping of single nucleotide polymorphisms was conducted in the High-Throughput Genotyping Core Laboratory at the Molecular Diagnosis and Genotyping Facility at the University of Pennsylvania. The ABI 7900HT Sequence Detection System (Applied Biosystems, Foster City, CA) was used to genotype *NAT1* 1095C>A (rs15561) and *NOS3* 894G>T (rs1799983). Duplicate samples were included both within and across plates such that 5% of the samples were genotyped three times. For *TYMS* 1494del6, we designed and optimized forward (forward primer: 5'-TTC ACA AGC TAT TCC CTC AAA TC-3') and reverse (reverse primer: 5'-CTG AGC AGA TAA ETG ECA ETA CAG-3') primer pairs containing the ins/del polymorphism. Polymerase chain reaction was performed, and amplified products were separated by gel electrophoresis and visualized by staining with ethidium bromide.

Genotype data from individuals and families that previously failed the genotype quality control checks for 10 variants in nine folate-related genes were excluded from this study (Goldmuntz et al., 2008). Further, for each of the three variants genotyped for the present study, the proportion of samples that could not be genotyped, the proportion of samples with discrepant results on repeated genotypes and the proportion of triads that had genotype combinations incompatible with Mendelian inheritance were determined. These analyses were performed using SAS version 9.1 (SAS Institute, Cary, NC).

Log-linear models were used to assess the association between CTRDs and both the inherited (i.e., case) and maternal genotypes for each variant (Kistner and Weinberg, 2004; Weinberg et al., 1998). In these analyses, a log-additive model of inheritance was used for the genotype being assessed (e.g., the maternal genotype), and an unrestricted model of inheritance was used for the other genotype (e.g., the inherited genotype). This approach provides a one-degree of freedom test for the effect under study, while fully accounting for the effect of the other genotype. Genotype relative risks were estimated along with 95% confidence interval. In addition, *p* values for inherited and maternal genetic effects were 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. Given the exploratory nature of these analyses, both unadjusted and Bonferroni corrected *p* values were assessed. Adjusted *p* values accounted for the SNPs in our current analyses, as well as the 10 folate-related SNPs included in our previous analysis (Goldmuntz et al., 2008). These analyses were run using the LEM program, which allows for the inclusion of individuals with missing genotype information (Vermunt, 1997; Weinberg, 1999).

Analyses were conducted using data from all triads (i.e., full group) and in two, overlapping subgroups: classic conotruncal defects (TOF, D-TGA, DORV, TA, or IAA) and defects with normally related great arteries (TOF, VSD, AAA, TA, or IAA). These subgroups were assessed to determine whether the results obtained using data from all triads were influenced by heterogeneity within the full group. Finally, because of concerns about population stratification bias, the analyses of the full CTRD group were repeated in the subset of triads in which both parents were non-Hispanic white (Weinberg et al., 1998). However, this was not done in the two subgroups due to small numbers.

Results

The distribution of CTRD phenotypes among cases was: TOF (0.38), D-TGA (0.21), VSD (0.20), DORV (0.10), AAA (0.05), TA (0.03), and IAA (0.03). As previously reported, there was a predominance of males among the cases (0.59), and the majority of parents were non-Hispanic white (0.72). Additional details of the study sample have been provided elsewhere (Goldmuntz et al., 2008).

Genotyping was performed on DNA samples derived from 733 case-parent triads. Based on quality control checks from our previous study, 225 samples from 75 families with at least one Mendelian inconsistency were excluded, and 55 samples were omitted based on samples that failed (i.e., no genotype called) or provided discrepant results on repeat genotyping (Goldmuntz et al., 2008). Based on the genotyping results for *NAT1* 1095C>A, *NOS3* 894G>T, and *TYMS* 1494del6, an additional 34 samples from 13 families with a Mendelian inconsistency were excluded, and 54 samples were omitted based on samples that failed or provided discrepant results on repeat genotyping. Based on these quality control measures, at least 97% of the samples for each variant were available for analyses; therefore the genotypes were considered to be of sufficiently high quality to include in the present study.

After all exclusions, there were 1416, 1435, and 1706 useable genotypes for *NAT1* 1095C>A, *NOS3* 894G>T, and *TYMS* 1494del6, respectively. The counts of each triad type, defined by the genotypes of the father, mother, and case, used in the analyses for each genetic variant are provided in Table 1.

Estimates of the relative risks (RR, heterozygote vs. common homozygote) of CTRDs and 95% confidence intervals (CIs) for the inherited and maternal genotypes, as well as the likelihood ratio test statistics and associated *p* values for the model comparisons for each variant are summarized in Table 2. The inherited genotypes for *NAT1* 1095 C>A, *NOS3* 895 G>T, and *TYMS* 1496del6 were not significantly associated with CTRDs, or either of the CTRD subgroups. In addition, the maternal *NAT1* 1095 C>A genotype was not significantly associated with CTRD or either subgroup. However, there was evidence of an association between the maternal *NOS3* 894 G>T genotype and all analysis groups, although the effect was not statistically significant in the classic conotruncal defect group. Specifically, the maternal *NOS3* 894 GT genotype was significantly associated with an increased risk, relative to the GG genotype, of CTRDs (RR, 1.25; 95% CI, 1.00–1.57; *p* = 0.046), and the subgroup of CTRDs with normally related great arteries (RR, 1.36; 95% CI, 1.04–1.79; *p* = 0.024) but was not significantly associated with the subgroup including only the classic conotruncal defects (RR, 1.21; 95% CI, 0.93–1.57; *p* = 0.160). In addition, the maternal *TYMS* 1494del6 genotype was associated with a significantly increased risk in the classic conotruncal defect group (RR, 1.22; 95% CI, 1.00–1.48; *p* = 0.048), but not in the full group (RR, 1.17; 95% CI, 0.99–1.39; *p* = 0.066) or in the subgroup of CTRDs with normally related great arteries (RR, 1.05; 95% CI, 0.86–1.29; *p* = 0.605).

Although several associations were significant at *p* < 0.05, these associations were not significant after applying a Bonferroni correction for multiple tests (*N* = 26 comparisons) based on the number of comparisons in this and our previous analysis (Goldmuntz et al.,

2008). However, the estimates of relative risk are suggestive of an association between the maternal *NOS3* 894G>T genotype and all CTRDs as well as the subgroup with normally related great arteries, and between the maternal *TYMS* 1494del6 genotype and classic conotruncal defects. As results were similar when analyses were restricted to non-Hispanic whites in the full group, only the results for the full CTRD group, based on all races and ethnicities, are presented.

Discussion

Several lines of evidence suggest that maternal folate and homocysteine metabolism is associated with CTRD risk. These include reduced risks of conotruncal defects in infants whose mothers take folic acid vitamin supplements periconceptionally (Jenkins et al., 2007), the influence of increased homocysteine levels on CHD phenotypes (Rosenquist et al., 1996), and the association between folate metabolic pathway genes and CTRDs and other birth defects (Brown et al., 2004; Lammer et al., 2004; Shaw et al., 2005; Goldmuntz et al., 2008).

The three variants evaluated in the present study were selected as potential CTRD risk factors because they are within genes that are involved in folate-related processes (Minchin, 1995; Brown et al., 2003; Kealey et al., 2005). *NAT1* encodes arylamine N-acetyltransferase 1, which plays a central role in the catabolism of folate (Minchin, 1995) and is expressed in folate-dependent neural crest cells (Stanley et al., 1998). Further, polymorphisms in this gene have been associated with both oral clefts (Lammer et al., 2004) and spina bifida (Jensen et al., 2006). However, this is the first study to assess the *NAT1* 1095C>A genotype as a risk factor for CTRDs, and these analyses provide little evidence of an association between CTRDs and either the inherited or maternal genotype for this polymorphism.

Endothelial nitric oxide synthase 3 (*NOS3*) is responsible for the production of nitric oxide, which modulates homocysteine concentrations by inhibition of 5-methyltetrahydrofolate-homocysteine methyltransferase, the enzyme that synthesizes methionine from homocysteine and 5-methyltetrahydrofolate (Brown et al., 2003). In this study there was evidence that the maternal, but not the inherited, *NOS3* 894G>T genotype is associated with CTRDs. The two previous studies that have assessed the association between *NOS3* variants and CTRD risk (Shaw et al., 2005; Shaw et al., 2009) also found no evidence of an association with inherited *NOS3* genotypes, but neither of these studies assessed maternal genetic effects. However, van Beynum et al. (van Beynum et al., 2008) reported that among women who smoke, the maternal *NOS3* 895G>T genotype is associated with the risk of CHDs. As the enzyme encoded by the 894T allele has lower activity than the enzyme encoded by the 894G allele, women who carry the T allele are expected to have higher nitric oxide levels (Wang et al., 2000; Brown et al., 2003) than women with the GG genotype. In turn, such women, may have elevated homocysteine levels (Brown et al., 2003), which have been shown to be teratogenic in animal models (Rosenquist et al., 1996).

Thymidylate synthase, encoded by *TYMS*, catalyzes the reductive methylation of deoxyuridylate to thymidylate, which is required for DNA synthesis and repair. This process also requires 5,10-methylenetetrahydrofolate (Liu et al., 2002), a key metabolite in folate

metabolism (Volcik et al., 2003; Shaw et al., 2009). In this study, there was evidence that the maternal *TYMS* 1494del6 genotype is associated with the classic conotruncal defect subgroup, but not the full CTRD group (although $p=0.066$) or the subgroup with normally related great arteries. The only previous study to assess the association between *TYMS* variants and CTRD risk (Shaw et al., 2009) also found no evidence of an association with inherited *NOS3* genotypes, but did not assess maternal genetic effects. As the *TYMS* 6-bp deletion polymorphism is thought to affect mRNA stability (Mandola et al., 2004), the maternal genotype for this variant could impact on the risk of CTRDs, via inhibition of DNA synthesis (Ulrich et al., 2002) or elevated homocysteine levels (Trinh et al., 2002).

Our analyses were conducted in one of the largest study populations available to assess inherited and maternal genetic effects in a case-parent triad context, which is robust against confounding due to population stratification (Weinberg et al., 1998). However, as none of the reported associations remained significant after accounting for multiple comparisons, the findings from this study should be considered preliminary and interpreted with appropriate caution. Nonetheless, we believe that this study provides additional evidence that the risk of CTRDs may be influenced by folate-related genes. Further, these results lay the groundwork for more comprehensive studies that consider diagnostic subgroup analyses, the maternal effect of additional folate-pathway genes and variants within those genes, as well as indicators of maternal folate status (e.g., maternal dietary folate intake, biomarkers) and new methods for assessing the complexity of these associations (e.g., pathway-based analyses) with CTRD risk.

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Table 1

Distribution of Triads by NAT1, NOS3, and TYMS Genotypes

NAT1 1095C>A				NOS3 894G>T				TYMS 1494del6			
Mother	Father	Case	N	Mother	Father	Case	N	Mother	Father	Case	N
CC	CC	CC	79	GG	GG	GG	102	II	II	II	83
CC	AC	CC	34	GG	GT	GG	31	II	ID	II	36
CC	AC	AC	30	GG	GT	GT	28	II	ID	ID	44
CC	AA	AC	13	GG	TT	GT	8	II	DD	ID	21
AC	CC	CC	40	GT	GG	GG	40	ID	II	II	35
AC	CC	AC	43	GT	GG	GT	38	ID	II	ID	45
AC	AC	CC	10	GT	GT	GG	12	ID	ID	II	15
AC	AC	AC	41	GT	GT	GT	35	ID	ID	ID	43
AC	AC	AA	14	GT	GT	TT	18	ID	ID	DD	19
AC	AA	AC	6	GT	TT	GT	10	ID	DD	ID	14
AC	AA	AA	9	GT	TT	TT	6	ID	DD	DD	18
AA	CC	AC	11	TT	GG	GT	9	DD	II	ID	32
AA	AC	AC	6	TT	GT	GT	5	DD	ID	ID	12
AA	AC	AA	7	TT	GT	TT	10	DD	ID	DD	13
AA	AA	AA	9	TT	TT	TT	1	DD	DD	DD	10
CC	CC	CC	46	GG		GG	45	II		II	38
CC	AC	AC	19	GG		GT	14	II		ID	8
AC	CC	CC	15	GT		GG	16	ID		II	20
AC	AC	AC	21	GT		GT	35	ID		ID	30
AC	AA	AA	4	GT		TT	7	ID		DD	14
AA	AC	AC	8	TT		GT	7	DD		ID	16
AA	AA	AA	5	TT		TT	5	DD		DD	10
	CC	CC	13		GG	GG	15		II	II	15
	CC	AC	7		GT	GG	3		II	ID	5
	AC	CC	4		GG	GT	7		ID	II	4
	AC	AC	11		GT	GT	7		ID	ID	6
	AC	AA	1		GT	TT	4		ID	DD	2

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NAT1 1095C>A				NOS3 894G>T				TYMS 1494del6			
Mother	Father	Case	N	Mother	Father	Case	N	Mother	Father	Case	N
	AA	AC	3		TT	GT	2		DD	ID	2
CC	CC		7		TT	TT	1		DD	DD	4
CC	AC		2	GG	GG		6	II	II		1
AC	CC		2	GG	GT		1	II	ID		1
AC	AC		2	GT	GG		1	ID	II		2
AC			2	GT	GT		1	DD	ID		1
AA			1	TT	GT		1	II			4
	CC		1	GT			1	ID			1
	AC		1	GG			2	DD			2
	AA		2	TT			1		DD		2
		CC	5			TT	1			II	11
		AC	8			GG	7			ID	6
						GT	8			DD	2

Table 2
**Log-Linear^a Results for the Association between CTRD Phenotypes and *NAT1*, *NOS3*,
and *TYMS* Variants**

	<i>NAT1</i> 1095C>A	<i>NOS3</i> 894G>T	<i>TYMS</i> 1494del6
CTRD ^b			
Number of triads	542	551	647
Inherited RR (95% CI)	1.05 (0.86–1.28)	1.11 (0.91–1.36)	1.11 (0.93–1.34)
Maternal RR (95% CI)	1.05 (0.86–1.29)	1.25 (1.00–1.57)	1.17 (0.99–1.39)
<i>p</i> value LRT offspring effects	0.654	0.280	0.245
<i>p</i> value LRT maternal effects	0.616	0.046	0.066
Classic conotruncal defects ^c			
Number of triads	414	406	481
Inherited RR (95% CI)	1.06 (0.84–1.33)	1.15 (0.92–1.44)	1.08 (0.88–1.34)
Maternal RR (95% CI)	1.04 (0.82–1.30)	1.21 (0.93–1.57)	1.22 (1.00–1.48)
<i>p</i> value LRT offspring effects	0.644	0.228	0.451
<i>p</i> value LRT maternal effects	0.766	0.160	0.048
Defects with normally related great arteries ^d			
Number of triads	378	373	447
Inherited RR (95% CI)	1.09 (0.86–1.39)	1.16 (0.92–1.48)	1.11 (0.89–1.38)
Maternal RR (95% CI)	1.22 (0.95–1.58)	1.36 (1.04–1.79)	1.05 (0.86–1.29)
<i>p</i> value LRT offspring effects	0.462	0.214	0.357
<i>p</i> value LRT maternal effects	0.122	0.024	0.605

^aLog-linear results are based on an additive model (i.e., the risk of being heterozygote vs. the common homozygote).

^bConotruncal and related defects.

^cClassic conotruncal defects: TOF, D-TGA, DORV, TA, or IAA.

^dDefects with normally related great arteries: TOF, VSD, AAA, TA, or IAA.