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Variation in Folate Pathway Genes Contributes to Risk of Congenital Heart Defects Among Individuals With Down Syndrome

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Abstract

Cardiac abnormalities are one of the most common congenital defects observed in individuals with Down syndrome. Considerable research has implicated both folate deficiency and genetic variation in folate pathway genes with birth defects, including both congenital heart defects (CHD) and Down syndrome (DS). Here, we test variation in folate pathway genes for a role in the major DS-associated CHD atrioventricular septal defect (AVSD). In a group of 121 case families (mother, father, and proband with DS and AVSD) and 122 control families (mother, father, and proband with DS and no CHD), tag SNPs were genotyped in and around five folate pathway genes: 5,10-methylenetetrahyrdofolate reductase (*MTHFR*), methionine synthase (*MTR*), methionine synthase reductase (*MTRR*), cystathionine β-synthase (*CBS*), and the reduced folate carrier (*SLC19A1*, *RFC1*). *SLC19A1* was found to be associated with AVSD using a multilocus allele-sharing test. Individual SNP tests also showed nominally significant associations with odds ratios of between 1.34 and 3.78, depending on the SNP and genetic model. Interestingly, all marginally significant SNPs in *SLC19A1* are in strong linkage disequilibrium ($r^2 \geq 0.8$) with the nonsynonymous coding SNP rs1051266 (c.80A>G), which has previously been associated with nonsyndromic cases of CHD. In addition to *SLC19A1*, the known functional polymorphism *MTHFR* c.1298A was over-transmitted to cases with AVSD ($P = 0.05$) and under-transmitted to controls ($P = 0.02$). We conclude, therefore, that disruption of the folate pathway contributes to the incidence of AVSD among individuals with DS.

Keywords

Down syndrome; atrioventricular septal defect; folate; trisomy; congenital heart defects

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INTRODUCTION

Thirty years of research into folate metabolism has illustrated the crucial role folate plays in nearly all cellular processes. The folate metabolic pathway is integral in nucleotide synthesis (purines), amino acid synthesis (methionine and cysteine), and synthesis of s-adenosyl methionine the key substrate in protein, DNA, and lipid methylation reactions (Fig. 1). Understanding the role of folate deficiency, supplementation, and genetic variation has been of particular interest in the study of birth defects, where both case/control and epidemiological studies have revealed associations between folate deficiency and neural tube defects [Kirke et al., 2004], spontaneous abortions [George et al., 2002], chromosomal abnormalities [Patterson, 2008], oral-facial clefts [Boyles et al., 2008,2009], and congenital heart defects (CHD) [Bailey and Berry, 2005]. The strong association between folate deficiency and neural tube defects led to the 1992 recommendation from the U.S. Public Health Service [CDC, 1992] that all women capable of becoming pregnant take a folate-containing supplement and the 1998 FDA mandate for fortification of grains with folic acid.

Genetic variants in folate pathway genes are known to modulate function of this vital pathway (Fig. 1). Numerous studies have investigated the function of nonsynonymous coding variants in these genes: most commonly, c.677C>T (rs1801133) and c.1298A>C (rs1801131) variants in *MTHFR*; c.66A>G (rs1801394) in *MTRR*; and c.2756A>G (rs1805087) in *MTR*. The *MTHFR* c.677T and c.1298C alleles both result in an altered protein leading to increased thermolability and an approximately 50% decrease in function [Frosst et al., 1995;Weisberg et al., 1998].

DOWN SYNDROME AND CONGENITAL HEART DEFECTS

Trisomy 21, the cause of Down syndrome (DS), is among the most common human autosomal aneuploidies, observed in roughly 1 in 733 live births in the United States [Canfield et al., 2006]. Up to 80% of conceptuses with DS are lost prior to birth [Hassold and Jacobs, 1984]. DS is characterized by multiple clinical attributes including hypotonia, distinctive facial features, intellectual disabilities, as well as an increased risk of birth defects, such as CHD and gastrointestinal defects. CHDs occur in nearly half of individuals born with DS [Epstein, 1986; Freeman et al., 1998; Stoll et al., 1998]. Atrioventricular septal defects (AVSD) are particularly prevalent in people with DS occurring in 1 in 5 live births compared to 1 in 10,000 live births in the general population [Ferencz et al., 1997; Freeman et al., 2008].

Studies have associated CHDs with both folate deficiency and genetic variation in folate pathway genes (reviewed in Botto et al. [2003], Huhta and Hernandez-Robles [2005], and van Beynum et al. [2007]). Appropriately, these studies excluded cases of CHD associated with chromosomal abnormalities such as DS. Due to the relative rarity of many specific CHDs in the general population, studies have typically combined many different cardiac anomalies into a single “CHD” phenotype, despite evidence of heterogeneous molecular and developmental origins. In spite of the common occurrence of CHD among people with DS, little is known about their genetic origin. To test genetic variation in folate pathway genes as a potential risk factor for AVSD in people with DS, we compare a large, carefully phenotyped group of cases with DS and AVSD with a group of controls with DS and a structurally normal heart.

MATERIALS AND METHODS

ASCERTAINMENT

Subjects were ascertained from several sources, though all eligibility criteria and data collection methods were identical. Many participants included in this case/control study

were initially recruited through the population-based Atlanta Down Syndrome Project or National Down Syndrome Project, which have been described previously [Freeman et al., 1998, 2007]. Additional participants were identified and recruited through the Sibley Heart Center Cardiology (Atlanta, GA), Children's Healthcare of Atlanta, the Down Syndrome Clinic at Emory University (Atlanta, GA), the Kennedy Krieger Institute (Baltimore, MD), the Heart Center at Nationwide Children's Hospital (Columbus, OH), the California Birth Defects Monitoring Program, and through regional DS support and advocacy groups throughout the United States. All probands were born in 1989 or later.

ELIGIBILITY AND CASE DEFINITIONS

All case and control probands had trisomy 21 confirmed by karyotype or documented in medical records. Mosaic instances of trisomy 21 were excluded. Case probands had a complete, balanced AVSD with or without an additional CHD. Unbalanced AVSDs (those requiring a single ventricle repair) and partial AVSDs (inlet VSD only or primum ASD only) were also excluded. Control probands had a structurally normal heart as determined by an echocardiogram, no evidence of CHD in medical records, or by mother's report. Controls with a patent ductus artiosis or patent foramen ovale were allowed. One cardiologist (K.J.D.) reviewed all cardiac records for accuracy and consistency of the diagnosis prior to enrollment. The methods used in this study for the collection and abstraction of medical records were adopted from Freeman et al. [1998, 2007].

All participating mothers completed a detailed questionnaire administered by trained study personnel. From this questionnaire we obtained the race/ethnicity of the mother, father, and proband. They were required to have the same ethnicity for enrollment and only those with a reported race of black non-Hispanic or white non-Hispanic were included in the present analysis.

DNA SAMPLES

Blood samples were collected from all probands and participating parents. White blood cells were extracted to establish lymphoblastoid cell lines. DNA was extracted from buffy coat or lymphobast cells using the Puregene kit from Gentra (Minneapolis, MN). Ninety-two case and 97 control trios, 24 case and 15 control mother-proband pairs, 4 case and 10 control father-proband pairs, and 7 case and 4 control probands were enrolled and genotyped for this study.

GENE AND SNP SELECTION

Five genes encoding essential proteins in the transport, metabolism, and use of folate in basic cellular processes were studied: 5,10-methylenetetrahydrofolate reductase (*MTHFR*), methionine synthase (*MTR*), methionine sythase reductase (*MTRR*), cystathionine β-synthase (*CBS*), and the reduced folate carrier (*SLC19A1*, also known as *RFC1*). The genomic location, known nonsynonymous coding variants and the number of single nucleotide polymorphism (SNP) markers genotyped at each locus are shown in Figure 1. SNPs were selected to efficiently assay common variation in the genes of interest. The majority of our cases and controls self-reported as white, thus SNP selection was based on known SNP variation in parents of the Centre d'Etude du Polymorphisme Humain (CEPH) pedigrees using dbSNP build 123. Using the SeattleSNPs Program for Genomic Applications (PGA) Genome Variation Server (http://pga.gs.washington.edu) [SeattleSNPs, 2005], which implements the method of Carlson et al. [2004], we selected SNPs tagging common variation (MAF≥5%) at an r^2≥0.80 for each gene, including 5 kb up and downstream of the coding regions. Additionally, nonsynonymous coding variants identified using build 126 of dbSNP were also genotyped (http://www.ncbi.nlm.nih.gov/SNP/). Alleles

for each SNP are designated “A” for the major allele and “B” for the minor allele based on allele frequency data in dbSNP for the CEPH pedigrees [Sherry et al., 2001].

GENOTYPING

SNPs were genotyped on the Illumina BeadArray platform using the Golden Gate genotyping technology as part of a 384-SNP customized assay. Forty-five SNPs covered common variation in the five folate pathway genes of interest. The remaining SNPs were unrelated to the folate pathway and not included in this analysis. Genotyping was performed by the SeattleSNPs PGA through a service award. Parental genotypes and SNPs located on all chromosomes other than chromosome 21 were called using Illumina BeadStudio software, and confirmed with 100% concordance using the algorithm developed by Lin et al. [2008]. Genotypes for SNPs located on chromosome 21, where probands were expected to carry three alleles, were called only by the method of Lin et al. [2008]. Because genotyping initially failed on the Illumina platform, rs1801131 was genotyped by the Emory Biomarker Service Center (Emory University, GA) using the GenomeLab SNPStream 48-plex genotyping platform in white families only.

SNPs and trios were examined for Mendelian inconsistencies using HaploView (version 4, http://www.broad.mit.edu/haploview/) [Barrett et al., 2005]. Each disomic SNP was also tested for consistency with Hardy-Weinberg equilibrium.

STATISTICAL ANALYSES

SNP analyses must be handled separately and differently for genotypes from diploid sections of the genome and the triplicated chromosome 21 in probands. Some tests for trisomic data are logical extensions of traditional SNP analysis methods while others are novel adaptations specifically for instances of trisomy to account for the non-independent nature of alleles on the non-disjoined chromosomes. Methods for both disomic and trisomic SNP association analysis are described below.

Analysis of disomic SNPs—Among probands, we performed a gene-specific association analysis of multiple SNPs using a variation of the kernel-based approach of Kwee et al. [2008], extended to case/control data based on the algorithm in Liu et al. [2008]. Using all possible pairings of probands, this kernel approach tests whether pairwise genetic similarity across a region (here, defined as the average proportion of alleles shared identical-by-state (IBS) across the alleles in the gene of interest) correlates with pairwise phenotypic similarity. We fit this kernel approach using a logistic mixed model, where the SNPs within each gene were modeled as random effects whose covariance matrix is a function of the average identical-by-state sharing in the region. We then tested for association between the multiple SNPs within each gene and disease, using a score test that assesses whether the variance component of these genetic random effects significantly differs from zero. The kernel-based test was implemented in the R programming language. To further investigate associations between each individual SNP within a gene and disease, we tested individual disomic SNPs using the Armitage trend test implemented in logistic-regression using Statistical Analysis Software (SAS) version 9.1.

In addition to studying probands alone, we also performed family-based testing using the transmission disequilibrium test (TDT) and family-based association test (FBAT). The TDT detects alleles that are preferentially transmitted to affected offspring, while FBAT performs a combined test of association in both case and control trios adjusting for admixture [Horvath et al., 2001; Laird et al., 2000; Rabinowitz and Laird, 2000; Spielman et al., 1993]. The transmission disequilibrium test for preferentially transmitted alleles was performed using HaploView version 4 [Barrett et al., 2005].

Analysis of trisomic SNPs—We performed multi-SNP testing in genes using a trisomic version of the kernel-based approach of Kwee et al. [2008] extended to case/control data. To quantify pairwise genetic similarity, we calculated the number of alleles shared identical-by-state for all different proband pairs across all SNPs in the trisomic genes of interest.

We also assessed association between individual SNPs and disease using Armitage trend and genotype tests adapted for trisomic SNP data. The Armitage trend test for trisomic SNPs is a natural extension of the test for disomic SNPs that allows for an additional change in risk for the third allele. The genotype test regresses affection status on the separate effects of the four possible trisomic genotype categories (AAA, AAB, ABB, BBB). Within this genotype test, we treat the AAA genotype as baseline. We implemented both these trisomic SNP tests using logistic regression in SAS version 9.1.

As with the disomic SNPs, we also performed family-based tests of association using the trisomic TDT developed by Xu et al. [2004], to test for segregation distortion in the trisomic case. In case-parent trios, the trisomic TDT compares the likelihood of the genetic data under two models: a model of random segregation of alleles and one allowing for transmission distortion due to selection or trait effects. Calculation of the trisomic TDT requires prior knowledge of the parental origin and meiotic stage of the nondisjunction event for each parent-child trio. The test statistic is chi-squared distributed with three degrees of freedom.

Covariates and substructure—Previous epidemiological data on CHD in DS showed both race and sex of the proband significantly impact risk for AVSD [Freeman et al., 2008]. Sex was included as a covariate in all regression models. We performed two separate analyses of the data with respect to race. Primary analysis of individual SNPs included only cases and controls from self-reported white families. We also assessed individual SNP associations in a larger sample consisting of cases and controls from both white and black families. Analyses for this combined dataset included race as an additional covariate in the regression models.

To further identify and account for potential substructure in the combined study sample, we used a genomic control approach [Devlin and Roeder, 1999], by comparing allele frequencies of the case and control parents of both ethnic groups at 204 additional loci genotyped along with this study, yielding an inflation factor (λ) of 0.92. This result suggests no noticeable substructure in the distribution of allele frequencies between cases and controls, by and large, with the slight underdispersion likely a result of linkage disequilibrium between the markers used.

Consideration of multiple testing—Using the gene-level multilocus test, we tested five independent hypotheses, i.e., the pattern of variation in each candidate gene is associated with risk of AVSD. We performed a Bonferroni correction to control the global type I error rate at 0.05; therefore, 0.01 was set as the threshold for gene-level significance.

Determining a significance threshold for the individual SNP-level tests is less straightforward. SNPs within each gene are correlated and the tests performed, although based on different underlying genetic assumptions, are correlated as well, making a Bonferroni correction overly conservative. Moreover, the individual SNP tests were follow-up of the gene-level tests; thus, used as more exploratory analyses to understand the observed positive signal. Irrespective, to adjust for multiple testing for individual SNP tests, we performed 1,000 simulations of the dataset with label swapping of case/control status to determine an adjusted *P*-value for each SNP in the Armitage trend test and disomic TDT test.

Because of the small number of SNPs in this study, the focused nature of our unidirectional hypothesis, and past findings of association between heart defects and folate metabolism, all tests reaching an uncorrected *P*-value less than 0.05 are discussed.

RESULTS

Two hundred and fifty-three families (127 DS with AVSD cases and 126 DS with no CHD controls) were initially enrolled and genotyped for the study. Three case families and four controls families were removed due to failed genotyping of the proband, and three additional case families were dropped due to questionable sample identity. In addition, two control fathers, two control mothers, and one set of case parents were removed due to genotyping failure. As stated in the methods, two analyses of the data were performed; the first included only self-reported white cases and controls. After all quality control checks, this white-only sample consisted of 72 control trios, 78 case trios, 18 parent-control pairs, 10 parent-case pairs, and 4 proband-only control and 4 proband-only case families. The data set in the second analysis was comprised of a combined sample of self-reported white and black families, to determine whether associations in the white-only sample were also supported in the larger dataset and to test whether these findings were consistent with race-independent effects. This combined sample contained 89 control trios, 29 control parent-proband pairs, and 4 control proband-only families, as well as 85 case trios, 28 case parent-proband pairs, and 8 case proband-only families (Table I).

Twenty-three SNPs were genotyped on chromosome 21 in the *CBS* and *SLC19A1* genes. The genotype call rate was comparatively low for these trisomic SNPs compared to non-chromosome 21 SNPs, largely due to the difficulty of distinguishing all four genotype clusters (i.e., AAA, AAB, ABB, and BBB). Five *CBS* SNP assays failed quality control or did not produce distinguishable heterozygous clusters in trisomic probands. Of the remaining 18 trisomic SNPs, none had more than two Mendelian errors and all were in HWE in the parents.

In total, 22 SNPs were genotyped in the three non-chromosome 21 genes. Of these 22 SNPs, three failed to genotype on the Illumina platform, including the *MTHFR* nonsynonymous coding SNP c.1298A>C (rs1801131). Due to its known function and implication in other congenital anomalies, *MTHFR* c.1298A>C was genotyped separately using the SNPStream platform in the white families; the results are included in this analysis. One SNP was monomorphic in the study sample. No SNPs were significantly out of HWE when tested separately in the parents or probands.

We previously reported that both sex and race of the proband are significant risk factors for AVSD in infants with DS [Freeman et al., 2008]. Using logistic regression, we independently tested proband sex and race as potential risk factors in this study population. Consistent with our previous observation, females were at significantly increased risk for AVSD (OR 2.52, CI 1.50–4.22), and thus sex was included in all regression-based gene and SNP tests. Although race of the proband, as reported by the mother, was not a significant predictor of AVSD status in this study sample (OR 1.06, CI 0.59–1.91) because cases and controls were matched on race, race was included as a covariate in all analyses of the combined sample including both black and white families.

CHROMOSOME 21 CANDIDATE GENES

Gene-level testing of chromosome 21 candidate genes—the reduced folate carrier *SLC19A1* and the reducing enzyme of homocysteine *CBS*—was used to identify genes with increased allele sharing in AVSD cases compared to unaffected controls using all probands and adjusting for sex and race. Cases with AVSD shared significantly more alleles IBS across

SLC19A1 than expected ($P = 0.01$), suggesting an association between variation in this gene and AVSD.

Individual SNP tests were also consistent with association between variation in *SLC19A1* and AVSD. In analysis of whites only, two SNPs (rs3753019 and rs2330183) were nominally associated with AVSD in the trend test (Table IIA); however, the permuted *P*-values (0.680 and 0.563, respectively) were not significant. Using the genotype test, probands with a genotype at rs2330183 containing at least one C allele were at greater risk of AVSD in the sample of white probands. In combined analysis of white and black cases and controls, the same two SNPs, rs3753019 and rs2330183, as well as two additional SNPs, rs1051298 and rs12482346, reached nominal significance in the trend test, though again permutation-corrected *P*-values, 0.659, 0.747, 0.588, and 0.613, respectively, were not significant (Table IIB). The results of the genotype test also suggest a specific risk genotype for three of the SNPs (CTT for rs3753019, TTT for rs1051298, and TTT for rs12482346, respectively) (Table IIB).

For the family-based analyses, only 67 of 85 case families and 80 of 89 control families had chromosome nondisjunction data available. The sample size was further reduced due to relatively low levels of SNP heterozygosity. With this reduced sample, rs2838950 was the only SNP in *SLC19A1* significantly associated with AVSD based on the trisomic TDT (Table III). SNP rs2838950 showed no corresponding transmission distortion in control trios (data not shown).

The consistent association of several tag SNPs within *SLC19A1*, none of which has a known biological function, is suggestive of indirect association with an untested, functional polymorphism. Linkage disequilibrium patterns from the CEPH HapMap pedigrees indicate all significant tag SNPs are, in fact, in strong LD ($r^2 \geq 0.80$) with the untested SNP rs1051266 (Fig. 2) [2003]. SNP rs1051266 is a nonsynonymous variant (c.80A>G) that results in the replacement of a histidine codon (CAC) with an arginine codon (CGC) at amino acid 27 of the SLC19A1 protein (p. H27R). The risk allele associated with AVSD for all significant tag SNPs is found almost exclusively with the c.80G allele of rs1051266 in the CEPH population.

The gene-specific test of *CBS* showed no association with DS-associated AVSD ($P = 0.87$). Individual SNP analyses showed fewer significant *P*-values than expected by chance, with only two SNPs reaching nominal significance in any of the tests, one in the genotype test (GGT genotype of rs234715; Table IIA) and one in the trisomic TDT (rs706209; Table III). The same association in rs234715 was observed when analyzing the two ethnic groups together (Table IIB).

NON-CHROMOSOME 21 CANDIDATE GENES

The gene-level test for *MTHFR* did not show significant levels of allele sharing among individuals affected with AVSD ($P = 0.24$). In past studies, several nonsynonymous coding variants have been associated with CHD; therefore, SNPs were also tested individually for association with DS-associated AVSD. None of the individual SNPs reached significance in the trend test (Table IV), but the A allele of rs1801131 (c.1298A>C) was over-transmitted in cases ($P = 0.05$; Tables IV, V). Evaluation of control families for rs1801131 using the TDT allowed discrimination between selection effects and association with AVSD. In contrast to the cases, c.1298A allele was significantly under-transmitted in control families. Using FBAT, which performs a single test of association combining information from both case and control trios, the *MTHFR* c.1298A allele was significantly associated with AVSD risk under both dominant ($P = 0.03$) and additive ($P = 0.01$) models (Table V).

MTR ($P = 0.69$) and *MTRR* ($P = 0.67$) did not exhibit any significant patterns of allele sharing at the gene level among individuals affected with AVSD. No significant associations were detected in SNPs in *MTR*, using the trend test in either the white-only sample or in the combined analysis of all families (Table IV). The non-synonymous coding variant *MTR* c.2756G (rs1805087) was over-transmitted in cases ($P = 0.04$, permuted $P = 0.055$); however, there was no corresponding distortion in control trios ($P = 0.44$, permuted $P = 1.0$). In the combined FBAT test, *MTR* c.2756G was significantly associated with AVSD only under a recessive model ($P = 0.003$) (Table V).

All SNPs were also tested for interaction with the sex and race of the proband. No significant interactions were observed, though the power to detect potential interaction effects is limited given the small sample size.

DISCUSSION

The folate pathway and variation in the genes encoding its enzymes play a central role in the etiology of birth defects [Bailey and Berry, 2005; Boyles et al., 2009; Eskes, 2006; Patterson, 2008; van der Linden et al., 2006]. Maternal supplementation with folate in the periconceptional period protects against nonsyndromic CHDs [Pei et al., 2006; van Beynum et al., 2006]. Individuals with DS have abnormal folate metabolism; therefore, the potential role for altered DNA, or amino acid synthesis, or epigenetic effects in the etiology of DS-associated CHDs is intriguing [Chadefaux et al., 1985, 1988; Pogribna et al., 2001]. Also of interest, genes for two of the major components of the folate pathway, *CBS* and *SLC19A1*, are located on chromosome 21. *CBS* plays an integral role in regulating folate metabolism by converting homocysteine into cystathionine, while *SLC19A1* is the primary regulated transporter of 5-methyltetrahydrofolate into and out of the cytoplasm (Fig. 1). Overexpression of *CBS*, which occurs with trisomy 21, creates a functional folate deficiency [Pogribna et al., 2001]. Thus, cellular levels of many folate pathway components, such as homocysteine, methionine, SAM, and SAH, are altered in individuals with DS. Given the high risk for CHDs, particularly AVSDs, among individuals with DS, we tested SNPs in *MTHFR*, *MTRR*, *MTR*, *CBS*, and *SLC19A1* for association with cases of DS and AVSD compared to controls with DS and no CHD.

At the gene level, cases affected with AVSD showed a significantly increased proportion of alleles shared across *SLC19A1* than expected by chance ($P = 0.01$). Follow-up analysis of this association through individual SNP tests provided evidence consistent with association to a functional variant in or near *SLC19A1*. Based on the haplotype structure in CEPH pedigrees, these SNPs are in LD with rs1051266 (c.80A>G), a nonsynonymous coding variant in *SLC19A1*. We hypothesize that this variant may be the functional polymorphism, contributing to increased risk of AVSD in this population (Fig. 2).

Although the biochemical consequence of this *SLC19A1* coding variant (c.80A>G, p.27H>R) has not been established, the c.80A>G has been studied in conjunction with birth defects frequently associated with dietary and metabolic folate deficiency, including neural tube defects, orofacial clefts, and heart defects. An association between c.80G and spina bifida was observed only in conceptions where the mother did not supplement with folic acid, while there was no genetic effect from rs1051266 on orofacial clefts [Shaw et al., 2002, 2003]. Variants in *SLC19A1* have been associated with conotruncal defects independent of maternal supplementation status, but the effects were further exacerbated if the mother did not supplement with folic acid during fetal heart development [Shaw et al., 2003]. Similarly, Pei et al. [2006] observed that an offspring with a c.80G allele was at four times greater risk of any CHD, if the mother did not take a folate-containing supplement, an association further confirmed by family-based testing of the c.80G allele with CHD.

While the SNP and LD data are consistent with a functional role for *SLC19A1* in AVSD susceptibility, this region of LD extends into the 3′ region of *COL18A1* (Fig. 2). Fine mapping of the extended region, including genotyping of rs1051266, will help to determine whether the association with AVSD susceptibility is due to *SLC19A1*, *COL18A1*, or both.

The ancestral and fully enzymatically functional allele of *MTHFR*, c.1298A [Marini et al., 2008], also showed a unique pattern of association with DS-associated AVSD. The A allele was over-transmitted to cases ($P = 0.05$, permuted $P = 0.272$), under-transmitted to controls ($P = 0.02$, permuted $P = 0.495$; Tables IV and V), and significantly associated with AVSD in FBAT analysis under both a dominant and an additive model ($P = 0.03$ and $P = 0.01$, respectively; Table V). While these associations do not withstand correction for multiple testing, the opposing pattern of transmission between cases and controls is compelling and warrants further study.

Previous studies have reached conflicting conclusions on the role of the c.1298A>C variant in nonsyndromic CHDs. Van Driel et al. [2008] observed a preponderance of c.1298AC and c.1298CC genotypes in cases affected with various CHDs and their fathers. Hobbs et al. [2006], though, observed exactly the opposite—a significant under-transmission of the c. 1298C allele to offspring affected with septal, conotruncal, or left/right obstructive defects. Most studies of nonsyndromic CHDs, though, have more commonly identified the c.677T allele or c.677TT genotype as a risk factor [Junker et al., 2001; van Beynum et al., 2006, 2007; Wenstrom et al., 2001]. The T allele of c.677, similar to the C allele of c.1298, results in decreased enzymatic function due to increased thermolability [Frosst et al., 1995]. For example, van Beynum et al. [2007] observed a three-fold increased risk of having a child with a variety of CHD for mothers with the c.677CT genotype and six-fold increase for mothers with the c.677TT genotype. Consistent with these results, Botto et al. [2003] observed that the c.677T allele is more prevalent in the Hispanic population, a group that is particularly susceptible to CHD. The data presented here suggest that variation in *MTHFR* contributes less to the etiology of DS-associated cases of AVSD than nonsyndromic cases of CHD. These conclusions are complicated by the nonsyndromic studies investigating multiple and varied CHDs, while the present study looks specifically at cases of complete AVSD.

Given that our cases have both DS and AVSD and that folate polymorphisms have been associated with the occurrence of DS, we must be mindful that up to 80% of DS conceptuses are lost prior to birth [Hassold and Jacobs, 1984]. The highly selected nature of the sample population could lead to identification of alleles or genotypes associated with survival of the offspring to term rather than those associated with abnormal heart development. A variant associated with survival of a fetus with trisomy 21, regardless of CHD status, should not be detected by an association in this DS-case/DS-control comparison, but would show over-transmission in both case and controls trios. In contrast, a variant associated with disease susceptibility, or survival of the fetus with that specific disease, would show a different pattern: a significant association in a case/control comparison and over-transmission in DS-case trios, but not in DS-control trios [Kerstann et al., 2004]. The disproportionate over-transmission of the c.1298A allele to cases and the opposing under-transmission of c.1298A alleles to controls provide convincing evidence that c.1298A is associated with susceptibility to AVSD, not survival with trisomy 21. With diminished power in the trisomic TDT, we were unable to make as definitive an argument with respect to *SLC19A1* variants, although the significant association among cases compared with controls suggests *SLC19A1* variation contributes to AVSD susceptibility.

FUNCTIONAL IMPLICATIONS

Both *SLC19A1* and *MTHFR* affect the level of 5,10-methylenetetrahydrofolate available in cells. *SLC19A1* is a ubiquitously expressed transmembrane protein responsible for the regulated transport of 5-methyltetrahydrofolate, the physiologically active form of folate, into the cytoplasm [Chango et al., 2000; Taparia et al., 2007]. *MTHFR* converts 5,10-methylenetetrahydrofolate into 5-methyltetrahydrofolate, the substrate for the conversion of homocysteine into methionine. Chango et al. [2000] suggest that the c.80G allele of *SLC19A1* decreases the transport of folates into the cytoplasm, resulting in a functional folate deficiency. Conversely, the c.1298A allele of *MTHFR* (p. 429E) is more enzymatically active than the c.1298C allele (p.429A); the associated variants of these two enzymes both function to limit the amount of available 5,10-methylenetetrahydrofolate. In dividing cells, 5,10-methylenetetrahydrofolate is a key substrate for DNA and RNA synthesis, whereas the product of *MTHFR*, 5-methyltetrahydrofolate, is the methyl donor for generating methionine from homocysteine. Our data, suggesting diminished function of *SLC19A1* and proper function of *MTHFR*, support the hypothesis of Hobbs et al. [2006], wherein these polymorphisms result in a functional cellular folate deficiency that decreases efficient and accurate DNA and RNA synthesis. Diminished DNA and RNA synthesis could, thereby, impede the proper proliferation of cells in the developing heart. In support of this hypothesis, mice-fed folate-deficient diets where shown to have heart malformations resulting from defects in proliferation [Li and Rozen, 2006].

LIMITATIONS AND FUTURE STUDIES

Periconceptional folate supplementation has been recommended since associations with neural tube defects were confirmed in the mid-90s. A meta-analysis by Botto et al. [2003], combining a diverse array of study designs and cardiac defects, observed a decrease in the rate of CHD by up to 50% with periconceptional folate supplementation. Combining genotype and maternal dietary folate supplementation data would be a powerful way to assess the role that the folate pathway plays in DS-associated CHDs. Because families were recruited over an extended period of time, we did not have folate supplementation data for the majority of mothers participating in this study.

Although *SLC19A1* was significantly associated with AVSD at the gene level, after multiple test correction no individual SNP was significantly associated with AVSD. Thus, the significance of the gene-level test was a result of combined information from several of the tag SNPs across a large LD block. Other associations, such as the association of *MTR* c. 2756G with AVSD in the TDT, are less convincing, and could be a signal of selection effects on survival to term. Since folate pathway gene variants have been associated with CHD in past studies, we felt it important to discuss all nominally significant associations. We acknowledge that these results may be false positives and require replication in a larger population.

This is one of the largest studies of AVSD in people with DS; however, our conclusions are hindered by the small sample size, particularly in trisomy-specific statistical analyses (i.e., trisomic TDT) where power is comparatively low. Continuing efforts, with the benefit of larger cohorts, will replicate current results, examine a greater number of genetic variants, incorporate environmental factors such as folate supplementation, and explore gene-environment interactions to further study the causes of DS-associated AVSD.

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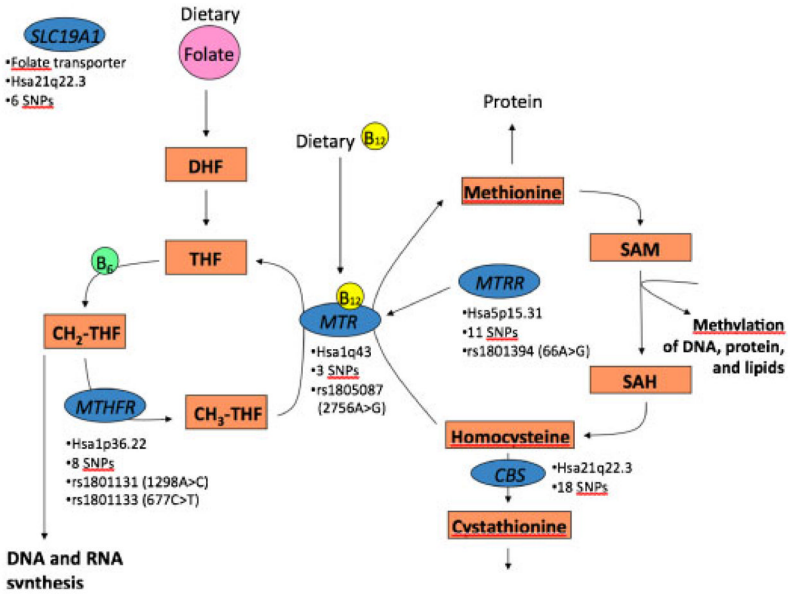

Fig. 1.

The folate pathway. Genes of interest are labeled in ovals with the genomic location, number of tag SNPs, and any nonsynonymous coding variants genotyped also listed.

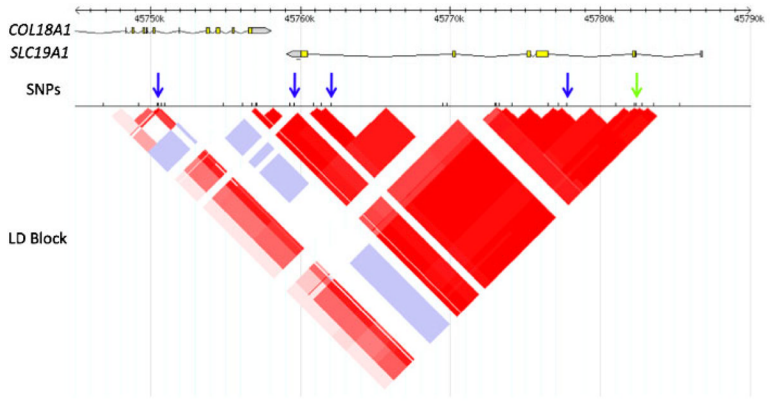

Fig. 2.

Genome view and LD structure around *SLC19A1*. This diagram (adapted from www.HapMap.org) shows the genomic region surrounding *SLC19A1*, including near-neighbor *COL18A1*. The four leftmost arrows indicate the locations of the four SNPs associated with AVSD (rs1051298, rs2330183, rs12482346, and rs3753019) and the rightmost arrow represents the untyped nonsynonymous coding SNP rs1051266 (c.80A>G) in *SLC19A1*. Note the large block of LD that each of the associated SNPs is tagging (based on CEPH HapMap data) covers nearly the entire length of *SLC19A1*, as well as the 3′ end of *COL18A1*.

TABLE I

Sample population with proband sex and racial demographics

		Sex		
Affection status	**Race**	**Female**	**Male**	**Total**
Control	White	27 trios	45 trios	122
		6 pairs	12 pairs	
		3 probands	1 proband	
	Black	3 trios	14 trios	
		5 pairs	6 pairs	
		0 probands	0 probands	
	Total	44	78	
Case	White	42 trios	36 trios	121
		6 pairs	4 pairs	
		3 probands	1 proband	
	Black	6 trios	1 trio	
		11 pairs	7 pairs	
		3 probands	1 proband	
	Total	71	50	

The breakdown of case and control samples by sex and race of the proband are shown, as well as the final breakdown of trios, parent-child pairs, and case/control-only samples included after all quality control checks.

TABLE II

Genotype counts and association test results for SNPs in *SLC19A1* and *CBS*

		Genotype counts (AAA/AAB/ABB/BBB)		Trend test	Genotype test		
SNP	**Alleles (A/B)**	**Cases**	**Controls**	**Odds ratio (CI)**	**AAB OR (CI)**	**ABB OR (CI)**	**BBB OR (CI)**
(A)							
SLC19A1							
rs10483080	C/G	61/23/8/0	63/26/4/1	1.11 (0.70–1.75)	0.88 (0.45–1.73)	2.32 (0.65–8.28)	NA
rs2838950	C/T	37/27/22/1	46/22/25/1	1.10 (0.77–1.55)	1.52 (0.74–3.13)	1.14 (0.55–2.37)	1.23 (0.07–21.00)
rs3753019	C/T	28/27/23/9	39/32/19/4	**1.40 (1.01–1.93)**	1.16 (0.57–2.37)	1.78 (0.81–3.93)	3.24 (0.89–11.76)
rs1051298	C/T	12/31/31/13	23/33/29/6	1.37 (0.99–1.90)	1.83 (0.77–4.35)	2.05 (0.86–4.91)	2.94 (0.96–8.97)
rs12482346	C/T	13/34/32/13	24/31/30/9	1.33 (0.97–1.83)	2.14 (0.92–4.99)	1.99 (0.85–4.68)	2.92 (0.97–8.83)
rs2330183	T/C	13/32/31/10	27/27/29/6	**1.46 (1.04–2.05)**	**2.40 (1.06–5.85)**	**2.39 (1.02–5.60)**	**3.78 (1.10–13.03)**
CBS							
rs706209	C/T	20/37/25/10	17/32/29/16	0.80 (0.59–1.10)	0.97 (0.43–2.19)	0.72 (0.31–1.68)	0.54 (0.19–1.51)
rs1051319	C/G	64/19/9/0	67/25/1/0	1.32 (0.78–2.21)	0.78 (0.39–1.56)	7.82 (0.95–64.36)	NA
rs6586282	C/T	53/25/6/3	57/24/11/2	0.99 (0.67–1.45)	1.23 (0.62–2.45)	0.59 (0.20–1.73)	1.75 (0.27–11.16)
rs234705	C/T	34/32/17/4	31/38/21/4	0.96 (0.68–1.36)	0.89 (0.44–1.79)	0.86 (0.38–1.95)	1.06 (0.24–4.70)
rs234706	G/A	35/31/23/3	32/36/22/4	1.02 (0.72–1.44)	0.92 (0.45–1.84)	1.11 (0.51–2.41)	0.85 (0.17–4.24)
rs2851391	C/T	13/35/31/12	24/31/28/11	1.25 (0.91–1.72)	2.07 (0.89–4.80)	2.14 (0.91–5.06)	2.03 (0.69–5.95)
rs234713	G/A	35/35/16/4	40/32/19/3	1.07 (0.76–1.51)	1.31 (0.67–2.57)	0.99 (0.43–2.23)	1.61 (0.33–7.88)
rs234715	G/T	45/37/6/2	53/26/14/1	1.06 (0.71–1.57)	**2.02 (1.03–3.94)**	0.53 (0.19–1.52)	2.19 (0.18–26.10)
rs234783	C/T	24/32/28/8	31/32/20/10	1.17 (0.86–1.59)	1.32 (0.63–2.77)	1.89 (0.85–4.20)	1.12 (0.38–3.33)
rs234785	C/G	30/33/24/5	22/41/27/4	0.83 (0.59–1.17)	0.54 (0.26–1.12)	0.60 (0.27–1.33)	0.68 (0.16–2.93)
rs2839632	G/A	35/35/15/7	36/29/22/7	0.98 (0.72–1.34)	1.32 (0.66–2.63)	0.80 (0.35–1.82)	1.18 (0.37–3.79)
rs1888523	G/A	19/33/23/15	17/33/24/20	0.93 (0.70–1.25)	0.85 (0.37–1.94)	0.93 (0.39–2.23)	0.74 (0.29–1.92)
(B)							
SLC19A1							
rs3753019	C/T	37/35/32/11	53/39/22/8	**1.34 (1.02–1.77)**	1.21 (0.64–2.29)	**2.15 (1.06–4.34)**	1.86 (0.67–5.22)
rs1051298	C/T	16/40/40/20	29/43/37/13	**1.37 (1.04–1.82)**	1.77 (0.82–3.80)	1.98 (0.91–4.30)	**2.96 (1.14–7.68)**
rs12482346	C/T	17/43/40/21	30/41/39/12	**1.36 (1.03–1.80)**	2.01 (0.95–4.29)	1.86 (0.87–3.99)	**3.31 (1.27–8.61)**
rs2330183	T/C	18/36/40/21	28/37/38/13	**1.34 (1.00–1.78)**	1.60 (0.74–3.47)	1.85 (0.86–4.00)	2.59 (0.98–6.84)
CBS							

		Genotype counts (AAA/AAB/ABB/BBB)		Trend test	Genotype test		
SNP	**Alleles (A/B)**	**Cases**	**Controls**	**Odds ratio (CI)**	**AAB OR (CI)**	**ABB OR (CI)**	**BBB OR (CI)**
rs234715	G/T	64/45/7/2	74/30/17/1	1.08 (0.75–1.56)	**2.21 (1.20–4.09)**	0.53 (0.20–1.39)	2.19 (0.18–26.81)

Genotype counts are shown for each of the four genotypes, subdivided by case/control status. The table presents a summary of logistic regression models, including odds ratios and 95% confidence intervals for the SNP variable(s), under both log-additive (trend) and model-free (genotype) tests for analysis of (A) the whites-only sample and (B) significant results from the combined study sample (including blacks and whites). All models also included proband sex (and race in combined analysis) as covariates. Nominally significant associations ($P \leq 0.05$) are highlighted in bold.

TABLE III

Trisomic TDT results for *SLC19A1* and *CBS*

	Trisomic TDT			
SNP	**P(Aff.) AAB**	**P(Aff.) ABB**	**P(Aff.) BBB**	***P*-value**
SLC19A1				
rs10483080	1.95	3.78	0.00	0.28
rs2838950	**0.59**	**1.63**	**0.29**	**0.03**
rs3753019	0.87	1.08	1.01	0.97
rs1051298	1.54	1.72	0.77	0.41
rs12482346	1.38	1.42	0.64	0.45
rs2330183	0.60	1.32	0.55	NA
CBS				
rs706209	**1.75**	**1.10**	**0.21**	**0.03**
rs1051319	1.71	0.97	0.61	0.77
rs6586282	1.02	1.08	5.75	0.53
rs234705	0.67	0.77	0.32	0.65
rs234706	1.02	0.36	0.27	0.59
rs2851391	2.73	3.92	3.05	0.19
rs234713	2.08	0.96	0.91	0.27
rs234715	1.30	0.59	0.73	0.67
rs234783	2.22	3.54	1.62	0.13
rs234785	1.29	0.67	0.32	0.40
rs2839632	1.79	1.55	1.85	0.55
rs1888523	1.84	1.39	1.97	0.47

For each SNP in *SLC19A1* and *CBS* the probability of being affected with AVSD given a genotype with one minor allele (AAB), two minor alleles (ABB), or three minor alleles (BBB) relative to the common homozygote genotype (AAA). The *P*-value of the likelihood ratio test statistic for each SNP is also included, with nominally significant associations ($P \leq 0.05$) marked in bold. This within-family analysis includes all case-parent trios regardless of race.

TABLE IV

Association test results for SNPs in *MTHFR, MTR*, and *MTRR*

		Genotype counts (AA/AB/BB)		Trend test	TDT
SNP	**Alleles (A/B)**	**Cases**	**Controls**	**Odds ratio (CI)**	**Transmitted/Untransmitted**
MTHFR					
rs3753584	A/G	65/27/0	57/36/1	0.64 (0.35–1.17)	17:30
rs2184226	A/G	73/16/1	85/7/1	2.05 (0.90–4.66)	14:10
rs1994798	T/C	32/47/13	25/51/18	0.77 (0.50–1.19)	33:49
rs1801131	A/C	42/39/6	30/49/9	0.65 (0.40–1.07)	**25:41**
rs1801133	C/T	38/39/14	49/37/8	1.41 (0.91–2.19)	40:32
rs17421511	G/A	70/19/3	61/30/3	0.71 (0.40–1.23)	15:23
MTR					
rs16834521	A/G	39/41/11	42/42/10	1.07 (0.69–1.66)	34:48
rs1266164	G/A	34/43/14	28/50/16	0.89 (0.57–1.37)	39:39
rs1805087	A/G	55/28/9	57/33/4	1.08 (0.67–1.74)	**34:19**
MTRR					
rs162036	A/G	72/17/3	64/29/1	0.70 (0.38–1.26)	20:14
rs162032	G/A	65/27/0	77/15/2	1.44 (0.75–2.77)	21:24
rs17267737	C/G	58/26/7	58/24/9	0.96 (0.61–1.51)	33:24
rs8659	A/T	36/46/7	41/40/10	0.96 (0.60–1.53)	39:33
rs162033	C/T	30/42/20	26/48/20	0.91 (0.60–1.37)	44:37
rs326121	T/C	49/33/8	55/35/4	1.15 (0.71–1.86)	31:33
rs327592	T/C	72/17/3	64/29/1	0.70 (0.38–1.26)	20:14
rs716537	C/T	40/40/12	38/43/13	0.95 (0.62–1.46)	37:43
rs1801394	A/G	15/50/27	17/46/31	0.99 (0.64–1.51)	48:41

Genotype counts are shown for each of the three genotypes, subdivided by case/control status. The table presents a summary of logistic regression models, including odds ratios and 95% confidence intervals for the SNP variables, under a log-additive model (Armitage trend test) for all SNPs in the whites-only study sample. All regression models also included proband sex as a covariate. TDT results represent transmission rates from all case-parent trios regardless of race. Nominally significant associations ($P \leq 0.05$) are highlighted in bold.

TABLE V

Case and control TDT results and FBAT results for SNPs significant in TDT test

			Case TDT		Control TDT		FBAT (*P*-values for each model)		
Gene	**SNP**	**Risk allele**	**Transmission ratio**	***P*-value**	**Transmission ratio**	***P*-value**	**Dominant**	**Additive**	**Recessive**
MTHFR	rs1801131 (c.1298A>C)	A	**0.62**	**0.05**	**0.35**	**0.02**	**0.03**	**0.01**	0.07
MTR	rs1805087 (c.2756A>G)	G	**0.64**	**0.04**	0.55	0.45	0.56	0.07	**0.003**

Separate case and control TDT tests and combined FBAT results (tested under additive, dominant, and recessive models) for *MTHFR* c.1298A>C and *MTR* c.2756A>G. These data represent all parent-child trios in the combined dataset. Nominal associations are highlighted in bold and with an asterisk.