ORIGINAL RESEARCH ARTICLE | Genetics inMedicine

Contribution of copy-number variation to Down syndrome–associated atrioventricular septal defects

Dhanya Ramachandran, PhD[1], Jennifer G. Mulle, MPH, PhD[1,2], Adam E. Locke, PhD[3,9], Lora J.H. Bean, PhD[1], Tracie C. Rosser, PhD[1], Promita Bose, MS[1], Kenneth J. Dooley, MD[4], Clifford L. Cua, MD[5], George T. Capone, MD[6], Roger H. Reeves, PhD[7], Cheryl L. Maslen, PhD[8], David J. Cutler, PhD[1], Stephanie L. Sherman, PhD[1] and Michael E. Zwick, PhD[1]

Purpose: The goal of this study was to identify the contribution of large copy-number variants to Down syndrome–associated atrioventricular septal defects, the risk for which in the trisomic population is 2,000-fold more as compared with that of the general disomic population.

Methods: Genome-wide copy-number variant analysis was performed on 452 individuals with Down syndrome (210 cases with complete atrioventricular septal defects; 242 controls with structurally normal hearts) using Affymetrix SNP 6.0 arrays, making this the largest heart study conducted to date on a trisomic background.

Results: Large, common copy-number variants with substantial effect sizes (OR > 2.0) do not account for the increased risk observed in Down syndrome–associated atrioventricular septal defects. By contrast, cases had a greater burden of large, rare deletions ($P < 0.01$) and intersected more genes ($P < 0.007$) as compared with controls. We also observed a suggestive enrichment of deletions intersecting ciliome genes in cases as compared with controls.

Conclusion: Our data provide strong evidence that large, rare deletions increase the risk of Down syndrome–associated atrioventricular septal defects, whereas large, common copy-number variants do not appear to increase the risk of Down syndrome–associated atrioventricular septal defects. The genetic architecture of atrioventricular septal defects is complex and multifactorial in nature.

Genet Med advance online publication 23 October 2014

Key Words: atrioventricular septal defects; ciliome; congenital heart defect; copy-number variation; Down syndrome

INTRODUCTION

Congenital heart defects (CHDs) are the most prevalent and serious of all recognized structural birth defects, occurring in 8–10 of every 1,000 live births in the United States.[1] Infant death rates due to CHD are high, with 44.0 deaths per 100,000 infants whose parents report having European ancestry and 56.2 deaths per 100,000 infants whose parents report having African ancestry. Even with improved treatments, affected infants who survive birth require multiple surgeries with lengthy hospitalizations. Many of these individuals experience lifelong disabilities, such as cognitive impairment, depression, anxiety disorder, and behavioral problems, as well as reduced quality of life. Affected individuals also are at increased risk for sudden cardiac death in later childhood and adulthood. In total, CHDs account for more than 2.6 billion health-care dollars spent each year in United States.

CHDs encompass a large, heterogeneous group of structural and functional abnormalities that arise during embryogenesis. As with most complex disorders, the underlying etiology reflects the combined influence of both genetic susceptibility and environmental exposures.[2,3] In humans, studies of the genetic architecture of CHD have implicated multiple classes of genetic variants, including both rare[3–5] and common single-nucleotide variants[6] and rare structural variants.[7–10] Genes identified as the cause of rare familial forms of CHD have been found to play a role in simplex forms of CHD as well. Recently, using an exome sequencing approach and filtering for de novo events, CHD susceptibility variants have been identified in histone-modifying genes.[11]

One of the most important contributors to CHD is chromosome aneuploidy.[3] In the current study we have focused on trisomy 21, or Down syndrome (DS (OMIM 190685)),[12] because it is the most common autosomal aneuploidy condition that survives to term. The prevalence of DS is ~1 in 700 live births in the United States, rendering it the most commonly identified form of intellectual and development disability and a leading cause of birth defects. CHD occurs in 40–50% of individuals with DS, with the majority being septal defects. The most

[1]Department of Human Genetics, Emory University, Atlanta, Georgia, USA; [2]Department of Epidemiology, Rollins School of Public Health, Emory University, Atlanta, Georgia, USA; [3]Genetics and Molecular Biology Graduate Program, Graduate Division of Biological and Biomedical Sciences, Laney Graduate School, Emory University, Atlanta, Georgia, USA; [4]Sibley Heart Center Cardiology, Department of Pediatrics, Children's Hospital of Atlanta, Emory University, Atlanta, Georgia, USA; [5]Heart Center, Nationwide Children's Hospital, Columbus, Ohio, USA; [6]Down Syndrome Clinic and Research Center, Kennedy Krieger Institute, Baltimore, Maryland, USA; [7]Department of Physiology and McKusick Nathans Institute for Genetic Medicine, School of Medicine, Johns Hopkins University, Baltimore, Maryland, USA; [8]Knight Cardiovascular Institute, and Department of Molecular and Medical Genetics, Oregon Health & Science University, Portland, Oregon, USA; [9]Current address: Center for Statistical Genetics and Department of Biostatistics, University of Michigan School of Public Health, Ann Arbor, Michigan, USA. Correspondence: Michael E. Zwick (mzwick@emory.edu)

Submitted 26 June 2014; accepted 11 September 2014; advance online publication 23 October 2014. doi:10.1038/gim.2014.144

striking and severe form of DS-associated CHD is atrioventricular septal defect (AVSD (OMIM 606215)),[12] also known as atrioventricular canal defect. Although the incidence of nonsyndromic AVSD in the general population is very low, 0.83 in 10,000 live births,[13] ~20% of individuals with DS have complete AVSD, corresponding to a 2,000-fold increased risk. More than 65% of all AVSD cases occur in children with DS.[14] This condition typically requires surgery during the first year of life. Thus, the costs and burdens of CHD are clearly amplified among those with DS and their families.

Although trisomy 21 dramatically increases the risk of CHD and AVSD, nearly 80% of children with DS do not have an AVSD, and 50% of children with DS are born with structurally normal hearts. Therefore, the increased dosage of genes on chromosome 21 explains only part of the increased risk for CHD in DS and suggests that additional variants throughout the genome may play a role. Thus, individuals with DS represent a "sensitized" population in whom genetic studies might reveal novel factors contributing to the risk for development of CHD, and any genetic mechanisms revealed may be relevant to both trisomic and disomic individuals. Indeed, our studies of the genetic risk factor, *CRELD1* (OMIM 607170),[12] have shown that the contributing missense mutations are found in both simplex euploid and DS-associated AVSD,[15] and that mutation of *CRELD1* increases risk for CHD when expressed on a trisomic mouse background similar to trisomy 21.[16] This approach, i.e., using a carefully phenotyped group of individuals with a known genetic susceptibility factor for CHD, is the human equivalent of a sensitized screen used in model organisms. This strategy can be successful in revealing variation and identifying novel members of pathways affecting developmental or functional processes.[17] Recent findings from genome-wide and chromosome 21–specific single-nucleotide polymorphism (SNP) and copy-number variant (CNV) association studies on a relatively smaller set of individuals with DS and other heart forms highlight the complex etiology seen in DS-associated CHD.[2,4,5,18–20]

Here we report the largest genetic study to date of a carefully phenotyped collection of individuals with DS and AVSD (cases, $n = 210$) compared with individuals who have DS and documented structurally normal hearts (controls, $n = 242$). The cases and controls in this study were selected from the extreme ends of the observed phenotypic distribution of heart development in children with DS. We sought to test two specific hypotheses. First, we hypothesized that common (>0.01 frequency) CNVs of large effect contribute to the dramatically increased risk of AVSD in the DS population. Second, we hypothesized that the genome-wide burden of rare (<0.01 frequency) CNVs increases the risk of AVSD in the DS population, much as they do for other CHDs in the disomic population. We found no support for the common variant hypothesis, but we did find a significant increase in burden for rare deletions in cases. We also found a suggestive enrichment of genes involved in the ciliome pathway, echoing a previous finding from a gene expression study of a similar collection of cases and controls.[21] Taken together, these results suggest the genetic architecture of AVSD is multifactorial and complex, even in the DS population.

MATERIALS AND METHODS

Sample ascertainment

Details regarding the recruitment and enrollment have been documented previously.[18,22] Briefly, participants with a diagnosis of full trisomy 21 were enrolled, with the vast majority confirmed by karyotype. Individuals with partial or mosaic trisomy 21 were not enrolled. Study participants were recruited through multiple centers across the United States. Protocols were approved by institutional review boards at each participating center, and informed consent was obtained from a custodial parent for each participant. To minimize phenotypic heterogeneity of cases, we focused on AVSD as the most severe heart phenotype seen associated with DS. A single cardiologist (K.J.D.) reviewed medical records and classified cases as individuals with DS who had a complete, balanced AVSD documented most often by echocardiogram or surgical reports (DS+AVSD). Controls were those with a structurally normal heart, patent foramen ovale, or patent ductus arteriosus (DS+NH). Medical records and echocardiography were used to document normal heart status. Only participants whose mothers reported being of non-Hispanic European ancestry were included in the current study. This information was obtained from a questionnaire administered to the mothers of the children with DS by a trained interviewer. The data files are available at the Gene Expression Omnibus repository (accession number GSE60607).

Genotyping and CNV detection

Genomic DNA was isolated from lymphoblastoid cell lines using the Puregene DNA purification kit according to the manufacturer's instructions (Qiagen, Valencia, CA). The quantity and quality of the extracted DNA were determined on the Nanodrop ND-1000 spectrophotometer (Nanodrop Technologies, Wilmington, DE), and the integrity of the genomic DNA was assessed on 0.8% agarose gel stained with ethidium bromide. Genotyping and CNV detection were performed using the Affymetrix Genome-Wide Human SNP Array 6.0 at Emory University per the manufacturer's protocol. Genotype calling was performed using the Birdseed algorithm (version 2), as implemented in the Affymetrix Power Tools software (APT 1.12.0). Individual arrays with <86% call rate, <0.4 contrast quality control, and mismatched gender concordance were excluded from downstream analyses. In addition, samples were removed if the heterozygosity was more than 3 SD above the mean and the total call rate was less than 94.5% (outliers with >3 SD below the total call rate), indicating potential sample mixture. Samples with inconsistent family structure based on genotype calls were also excluded from downstream analysis. SNPs with completion rate <95%, as well as those that deviated from Hardy-Weinberg equilibrium, were also excluded from analysis. A total of 773,359 autosomal SNPs were retained, whereas 133,301 autosomal SNPs were excluded.

From the total 471 genotyped samples, 9 were excluded because of poor data completeness or inconsistent family structure (130 cases had parental genotype information). Matching of cases and controls was evaluated by principal component analysis using Eigenstrat software v4 (**Supplementary Figure S1** online).[23] Seven outlier samples were excluded based on principal component analysis. We also excluded three samples with excess number of CNVs (>3 SD) called from downstream analyses because these likely result from experimental artifacts. The final data set consisted of 452 subjects, of which 210 were cases and 242 were controls.

For CNV analysis, data normalization and log2 ratios for autosomes were calculated using APT 1.12.0. We used stringent criteria to filter CNVs as previously described.[24] To minimize false-positive findings, we first used three algorithms, GLAD, GADA, and BEAST, to identify CNVs.[24,25] CNVs called by only a single algorithm were removed from analysis. When algorithms disagreed on break point determination, the smallest start point and the largest end point (largest interval) were considered. As a further filter, we required each CNV to include at least 20 SNPs. For deletions, we required that the genotypes be homozygous at 90% of sites. For duplications, we required that the percentage of heterozygotes exceed the genome-wide average heterozygosity of 27% reported for SNPs genotyped on the microarray. Finally, we visually inspected the log2 ratio plots and excluded eight large deletions that we judged to be cell line artifacts.

Statistical analysis

Before conducting association tests, we excluded CNVs that overlapped centromeric regions (**Supplementary Table S1** online) because these regions are highly repetitive with few SNP markers on commercial arrays, making CNV calls intersecting these regions unreliable. Global burden of CNVs in cases as compared with controls was determined using PLINK.[26] Deletions and duplications were analyzed separately. We also performed region-based and position-based association tests for CNVs among the case–control group. Region-based association analyses were conducted to see whether any specific genomic region was associated with case CNVs as compared with control CNVs. For this, we used the hg19 UCSC gene list as the candidate genomic region. Position-based analyses tested for association of individual CNVs and determined whether any single genomic position was associated with cases as compared with controls. We performed the burden and association analyses first by using all deletions/duplications, and then by filtering deletions/duplications by their frequency and then by size (large was considered to be >100 kb). The frequency-based filtering was based on frequency among cases and controls combined and classified variants as rare (<0.01) or common (>0.01). Empirical *P* values from 1,000,000 random permutations were reported for each test.

We performed gene set enrichment analyses using the CNV enrichment test implemented in PLINK v1.07.[27] The list of genes we used for enrichment analyses is provided in **Supplementary Table S2** online and is primarily based on the candidate gene lists published elsewhere, with modifications.[11,21] For the genes in the ciliome pathway, we excluded any mouse genome–based genes for which a corresponding human ortholog (hg19) could not be found using the UCSC genome browser. Because our CNV analyses were focused on autosomes, we also excluded candidate genes falling on sex chromosomes. For comparison, we used the same hg19 gene list mentioned above. Enrichment analyses were conducted for all deletions, rare deletions (<0.01), and all duplications. Similar to burden tests, empirical *P* values from 1,000,000 random permutations were reported.

Validation of CNVs

We attempted to validate 31 CNVs (27 deletions and 4 duplications) in 25 cases and 33 CNVs (32 deletions and 1 duplication) in 25 controls (**Supplementary Table S3** online). Selection criteria included CNVs intersecting ciliome genes, CNVs from the chromosome 14q32.22 (the region corresponding to our most significant finding from region-wise analysis), and a random, genome-wide selection of CNVs in cases and controls not found in the Database of Genomic Variants (DGV). We genotyped these samples with the Illumina HumanOmni2.5–8 (Omni2.5) bead chip to validate CNVs located genome-wide (Illumina, San Diego, CA). CNVs were called with CNV partition using Illumina Genotyping module v1.8.4. To confirm, the logR plot of each call was visually inspected using Illumina Karyostudio 1.3. Deletions on chromosome 21 were validated using a custom-made high-density chromosome 21 array comparative genomic hybridization platform (Agilent Technologies, Santa Clara, CA). Among the 59 deletions chosen for validation, 23 were previously reported in the DGV. Among the five duplications chosen for validation, four were previously reported in the DGV. We successfully validated 91.5% of deletions (54 of 59 attempted) and 100% of duplications (five of five attempted), demonstrating that our data quality is high. All nonvalidated CNVs were excluded from final analyses.

RESULTS

Copy-number variation was assessed in a collection of 210 cases and 242 controls of European ancestry using Affymetrix Genome-wide SNP 6.0 arrays with rigorous quality control and validation, as described in the Methods. We identified 541 deletions (253 in cases, 288 in controls) and 383 duplications (177 in cases, 206 in controls) genome wide. Among the 541 deletions, 400 (194 in cases and 206 in controls) were previously reported in the DGV and 141 (59 in cases and 82 in controls) were unreported. Of the 383 duplication events, 310 (147 in cases and 163 in controls) were previously reported in the DGV and 73 were novel (30 in cases and 43 in controls). The complete list of CNVs identified, along with the DGV status, is provided in **Supplementary Tables S4–S7** online.

Contribution of common CNVs to DS-associated AVSD

We first tested the hypothesis that an increased burden of common CNVs might account for the 2,000-fold increased risk of

AVSD among individuals with DS. We did not identify any significant difference in the number or size of common deletions or duplications between cases and controls. Burden analyses of large common deletions and duplications also failed to identify any significant differences between our cases and controls (**Table 1**). Region-based association analyses did not identify any specific gene to be significantly associated with case CNVs after correcting for multiple testing. None of the common CNVs showed a significant association after correction for multiple testing. Under an additive model, using 210 cases and 242 controls, we had 80% power to find any common marker that explained more than 4% of the variance or that had an odds ratio greater than ~2.0 at a $P < 0.00019$ (i.e., 0.05 multitested for 263 tests, corresponding to the total number of common CNVs identified among cases and controls). Our findings provide strong evidence that common CNVs do not explain the increased risk of AVSD in children with DS.

Contribution of rare CNVs to DS-associated AVSD

We next asked whether an increased burden of rare CNVs might explain the increased risk of AVSD in DS. We observed an elevated frequency of large, rare deletions in cases as compared with controls ($P < 0.01$; **Table 2**). Furthermore, these large, rare deletions in cases spanned genes more often than did those in controls ($P < 0.007$; **Table 2**). By contrast, we did not observe any statistically significant differences in large, rare duplications between cases and controls.

Gene enrichment analysis

Because the burden analyses revealed an excess of large, rare deletions spanning genes in cases, we next asked whether specific pathways were differentially affected in cases as compared with controls. We performed a gene-set enrichment analysis of different pathways that contribute to normal heart development and/or function. These include Notch, WNT, Jak-Stat, Hedgehog, epithelial-to-mesenchymal transition, angiogenesis, ciliome, and histone-modifying genes.[11,21,28] We observed a marginally significant trend for enrichment of all deletions and of rare deletions affecting the ciliome pathway in cases as compared with controls (deletions intersecting ciliome genes are provided in **Supplementary Table S8** online; complete list of genes is provided in **Supplementary Table S2** online). Interestingly, this finding parallels the results of a recent gene expression study that identified a statistically significant enrichment of differentially expressed ciliome genes in DS+AVSD cases as compared with DS controls with a structurally normal heart (**Table 3**).[21] None of the other pathways tested showed a significant enrichment in our cases as compared with controls. No statistically significant enrichment was observed for duplications in any of the pathways tested.

Overlap of case-associated CNVs with previous non-DS, disomic heart studies

To understand whether the observed CNV burden among our cases overlapped with that identified in studies of CHD among non-DS cases, we compared our results with published studies of all types of CHD (**Table 4**).[5,8–10,29–31] We found three rare deletions in our cases at 2q13, 8p23.1, and 22q11.2, which overlapped with CNVs identified among isolated nonsyndromic CHD patients.[9,29,32] Although we did not see an increased burden of rare duplications in cases as compared with controls, we did find two large duplications at 8p23.1 and one at 16p13.11 in cases that overlapped rare CNVs observed in other CHD phenotypes (**Table 4**).[9,10,30] Among the rare CNVs observed in our cases, only one of the euploid patients with an identified CNV had an AVSD as a part of their phenotypic spectrum (**Table 4**). In addition, we observed a number of CNVs predominantly in DS+NH controls that have been previously reported in disomic CHD patients (**Supplementary Table S9** online).

Table 1 Results of burden analyses of common (>0.01) copy-number variants > 100 kb

Test	Cases	Controls	*P* value[a]
Number of deletions	98	140	—
Deletion rate per person	0.46	0.56	0.96
Proportion of individuals with at least one deletion	0.41	0.47	0.91
Number of genes spanned by deletions	2.70	3.63	0.98
Number of deletions with at least one gene	0.37	0.44	0.95
Number of duplications	78	111	—
Duplication rate per person	0.37	0.45	0.92
Proportion of individuals with at least one duplication	0.33	0.38	0.90
Number of genes spanned by duplications	2.03	2.24	0.69
Number of duplications with at least one gene	0.28	0.34	0.94

[a]*P* values in each category were estimated from 1 million permutations.

Table 2 Results of burden analysis of rare (<0.01) copy-number variants > 100 kb in size

Test	Cases	Controls	*P* value[a]
Number of deletions	94	75	—
Deletion rate per person	0.45	0.30	0.01
Proportion of individuals with at least one deletion	0.32	0.26	0.10
Number of genes spanned by deletions	0.87	0.37	0.007
Number of deletions with at least one gene	0.19	0.17	0.25
Number of duplications	67	76	—
Duplication rate per person	0.32	0.32	0.49
Proportion of individuals with at least one duplication	0.26	0.24	0.33
Number of genes spanned by duplications	1.02	1.23	0.71
Number of duplications with at least one gene	0.22	0.21	0.46

[a]*P* values in each category were estimated from 1 million permutations.

Table 3 Results of gene set enrichment analyses (*P* values based on 1 million permutation tests)

Pathway tested	Notch	Wnt	Jak-Stat	Hedgehog	Angiogenesis/ cardiogenesis	Ciliome	Epithelial-to-mesenchymal	Histone-modifying genes
Deletions	0.60	0.26	1.0	0.95	0.73	0.10	1.0	0.91
Deletions (<0.01)	0.64	0.36	1.0	0.96	0.89	0.13	1.0	0.93
Duplications	0.68	0.23	0.70	1.0	1.0	0.75	1.0	0.81
DE genes in AVSD[a]	0.62	0.28	0.26	0.91	0.70	0.00017	0.67	ND

AVSD, atrioventricular septal defect; DE, differentially expressed; ND, not determined.

[a]Included here are results from Ripoll et al.[21] that indicate the same pattern of enrichment of DE genes among their case and control groups.

Table 4 Rare CNVs observed only among cases, that overlap with CNVs identified among non-DS CHD cohorts published elsewhere

Genomic location in DS+AVSD cases (size of CNV)	DS+AVSD CNV	Count in DS+AVSD cases	Non-DS CHD CNV	Non-DS CHD phenotypes observed	Genes intersected[a]
2q13 (583.3–559.3 kb)	del	2	del	CoA, cardiovascular[9,32]	*MIR4267*
8p23.1 (79.1 kb)	del	1	del	TOF, AVSD, cardiovascular[9,32]	*XKR5*, *DEFB1*
22q11.21 (957.8 kb)	del	1	dup	VSD, triscuspid valve dysplasia, right-ventricular hypoplasia[29]	*PPM1F*, *TOP3B*
2p21 (584.5 kb)	dup	1	dup	DORV, cardiovascular[9,32]	*LINC01121*, *SRBD1, PRKCE*
2q12.3 (784.7 kb)	dup	1	del	LS-CHD[31]	*LIMS1*
8p23.1 (251.2–524.7 kb)	dup	2	dup	BAV, VSD[9]	*CTSB*, *DEFB134, DEFB136*, *DEFB135*
15q13.3 (514.7 kb)	dup	1	del	IL, PDA, PS, cardiovascular[9,32]	*CHRNA7*
16p13.11 (1.54 Mb)	dup	1	del, dup	ASD-SEC, HLHS, TOF, CoA [9,10,30]	*MPV17L*, *C16orf45*, *KIAA0430*, *NDE1*, *MIR484*, *MYH11*, *ABCC1*, *ABCC6*

ASD-SEC, atrial septal defect secundum; AVSD, atrioventricular septal defect; BAV, bicuspid aortic valve; CNV, copy-number variant; CHD, congenital heart defect; CoA, coarctation of the aorta; del, deletion; DORV, double-outlet right ventricle; DS, Down syndrome; dup, duplication; HLHS, hypoplastic left-heart syndrome; IL, left isomerism; LS-CHD, congenital left-sided heart defect; PDA, patent ductus arteriosus; PS, pulmonary valve stenosis; TOF, tetralogy of Fallot; VSD, ventricular septal defect. [a]Genes that are commonly intersected by the current study and the corresponding references.

DISCUSSION

Approximately 20% of children with DS are born with a life-threatening AVSD, reflecting a 2,000-fold increased risk of AVSDs as compared with the disomic population. Our goal was to uncover genetic variants that distinguish children with DS and an AVSD from those with DS and a structurally normal heart. This is the largest study of this condition to date, surpassing the 1989 Baltimore–Washington heart study, which considered 190 cases (DS+AVSD).[14] Here we genetically characterized a carefully phenotyped collection of 210 DS+AVSD cases and 242 DS+NH controls. Our study had sufficient power to detect common variants that explained more than 4% of the variance or that had an odds ratio greater than ~2. Our findings provide strong evidence that common CNVs do not account for the increased risk of AVSD in children with DS.

Recently, Sailani et al.[20] conducted a case–control SNP and CNV association study of a smaller collection of individuals with DS and a variety of CHD phenotypes (cases, $n = 187$; controls, $n = 151$). Their genome-wide association and the SNP-based interaction studies failed to detect a significant association in any of the CHD groups. Interestingly, a CNV association analysis using 55 DS cases with AVSD and 53 DS individuals without CHD (controls) identified two small CNVs (<5 kb) enriched in cases as compared with controls (relative risk ~2.2). Both the CNVs were located on previously known CHD loci on chromosome 21 and were confirmed in a replication cohort. The lower marker density of our genome-wide genotyping platform precluded us from replicating these findings. Our strict filtering criteria were designed to ensure accurate detection of large CNVs; therefore, we cannot exclude the possibility that small, common CNVs that contribute to the risk of AVSD remain undiscovered. We intend to present a replication of the SNP and CNV association study reported by Sailani et al. in a future paper.

Our genome-wide analysis of CNVs revealed a significant burden of large, rare deletions in cases as compared with controls. Moreover, the case deletions were more likely to include genes as compared with those of controls. These findings are consistent with those of Ackerman et al.,[4] who conducted targeted sequencing of 26 candidate genes in a subset of samples analyzed here. They found a significant excess of deleterious missense variants in 19% of cases as compared with 2.6% of controls ($P < 0.0001$). Many of the potentially damaging variants occurred within vascular endothelial growth factor-A pathway genes. Functional studies demonstrating a potentially pathogenic allelic interaction between the AVSD-associated gene *CRELD1* and vascular endothelial growth factor-A genotype further substantiate the complex interactions among different genetic modifiers of AVSD pathology.[15,33,34] Together, these data suggest an etiology whereby the enormous increase in risk of AVSD in children with DS is multifactorial and is influenced by many different genes and environmental exposures in addition to the substantial risk from trisomy 21.[2,22]

We performed pathway analysis to determine the nature of pathways affected by the large, rare deletions observed in our cases. Previous reports from both DS populations[18,21] and disomic populations[8–10] have described the contribution of different signaling pathways to CHD pathogenesis. Our genetic analysis revealed marginally significant twofold enrichment of deletions in ciliome genes in cases (5.2%) as compared with controls (2.9%). Of note, our strongest genetic finding (ciliome) was identical to the most significant gene expression finding reported by Ripoll et al.[21] Using differential gene expression analyses on a subset of DS patients, they identified a significant enrichment for cilia genes in their AVSD and atrial and ventricular septal (ASD (OMIM 108800), VSD (OMIM 614429))[12] CHD groups. This is intriguing because the association between CHD and defective cilia is well established in the euploid population, particularly for laterality defects.[35] In fact, there is a 200-fold higher prevalence of CHD among patients with primary ciliary dyskinesia (OMIM 244400)[12] as compared with the general population, emphasizing the significance of intact cilia for normal heart development.[36] An intact cilium is required for Hedgehog (Hh) signaling for normal embryonic development in vertebrates.[37] Recent developmental studies demonstrated that the cilia-based Hh signaling in the second heart field is required for development of the dorsal mesenchymal protrusion, which is a critical component of complete atrioventricular septation. Disruption of Hh signaling could result in AVSD-associated clinical features.[38,39]

Recent studies on mouse models implicate attenuated Hh signaling as a significant underlying factor behind many DS-associated phenotypes. For example, trisomic granule cell precursors in the cerebellum are less responsive to Hh signaling than their euploid counterparts. This deficit can be rescued using a sonic hedgehog agonist, suggesting the importance of Hh signaling in DS-associated pathology as well as a possible candidate for therapeutic intervention.[40] Our results complement these studies and suggest an important role for ciliome/hedgehog genes in the elevated CHD risk in a trisomic background.

We also identified large, rare CNVs in our cases at genomic loci previously associated with nonsyndromic CHD (**Table 4**).[9,10,30,32] These previous reports indicate variable penetrance of CHD phenotypes, although the specific defects differ from those seen most often in the DS population. However, the overlap validates the idea that regions identified in DS are relevant to CHD in euploid population. These variations may be easier to observe in the sensitized DS background.

In summary, our data provide strong evidence that large, rare deletions increase the risk of DS-associated AVSD, whereas large, common CNVs do not account for the 2,000-fold elevated risk of DS-associated AVSD. This is the single largest genetic study of a well-defined homogeneous cardiac phenotype (extreme ends of the phenotypic spectrum) in ethnically matched individuals with DS. The stringent filtering we performed to reduce false-positive results supports the accurate detection of large CNVs but may miss smaller CNVs, thus underestimating the total impact of CNVs on DS-associated AVSD. Replication studies with larger sample sizes and more detailed genomic analyses, such as whole-exome or whole-genome sequencing, would be required to get a true assessment of the contribution of rare variants and the intersected genes we have identified to CHD pathology. The launch of DS-Connect (https://dsconnect.nih.gov/), the new DS registry, may facilitate DS-associated research in this regard. Given that the prevalence of AVSD, like other forms of CHD, varies by sex and ethnic/racial background, a complementary approach would be to test a population at higher risk, such as individuals with DS who are of African-American ancestry.[22] To confirm the association of ciliome genes with AVSD pathology, large genetic studies and direct functional studies, such as in a mouse model of DS, will be required. A more comprehensive approach to elucidate how the diverse genetic, epigenetic, and environmental factors converge at a functional level to create distinct heart phenotypes is of paramount importance to identify potential therapeutic targets and can be expected to influence the lifelong medical treatment of individuals with DS.

SUPPLEMENTARY MATERIAL

Supplementary material is linked to the online version of the paper at http://www.nature.com/gim.

ACKNOWLEDGMENTS

This work is a collaborative effort with the DS Heart Project (R.H.R.) and was supported by R01 HL092981-01A1 (M.E.Z.) and R01 HL083300 (R.H.R.) from the National Institutes of Health (NIH)/National Heart, Lung, and Blood Institute, R01 HD38979 (S.L.S. and E. Feingold) from NIH/National Institute of Child Health and Human Development, the Training Program in Human Disease Genetics 1T32MH087977 (D.R.), and the American Heart Association Predoctoral Fellowship (A.E.L.). Additional support was provided by the Oregon Clinical and Translational Research Institute (UL1 RR024140) from the National Center for Research Resources, a component of the NIH, and the NIH Roadmap for Medical Research.

We gratefully acknowledge the individuals with DS and their families for participating in this research. Our thanks go to Katy Lesowski and Jessica Hunter (Oregon Health & Science University), Kay Taylor and Valerie Deleon (John Hopkins University/Kennedy Krieger Institute), Lindsay Kehoe (Children's National Medical Center), and Helen Smith and Elizabeth Sablon (Emory University), who engaged and collaborated with the families to gather the needed information. CNV validation experiments were performed by Emory Integrated Genomics Core. We also thank Shoshona Lee, Weiya He, and Ashima Amin (Emory University) for technical assistance.

DISCLOSURE

The authors declare no conflict of interest.

REFERENCES

1. Hoffman JI, Kaplan S. The incidence of congenital heart disease. *J Am Coll Cardiol* 2002;39:1890–1900.
2. Bean LJ, Allen EG, Tinker SW, et al. Lack of maternal folic acid supplementation is associated with heart defects in Down syndrome: a report from the National Down Syndrome Project. *Birth Defects Res A Clin Mol Teratol* 2011;91:885–893.

3. Fahed AC, Gelb BD, Seidman JG, Seidman CE. Genetics of congenital heart disease: the glass half empty. *Circ Res* 2013;112:707–720.
4. Ackerman C, Locke AE, Feingold E, et al. An excess of deleterious variants in VEGF-A pathway genes in Down-syndrome-associated atrioventricular septal defects. *Am J Hum Genet* 2012;91:646–659.
5. Al Turki S, Manickaraj AK, Mercer CL, et al.; UK10K Consortium. Rare variants in NR2F2 cause congenital heart defects in humans. *Am J Hum Genet* 2014;94:574–585.
6. Cordell HJ, Bentham J, Topf A, et al. Genome-wide association study of multiple congenital heart disease phenotypes identifies a susceptibility locus for atrial septal defect at chromosome 4p16. *Nat Genet* 2013;45:822–824.
7. Greenway SC, Pereira AC, Lin JC, et al. De novo copy number variants identify new genes and loci in isolated sporadic tetralogy of Fallot. *Nat Genet* 2009;41:931–935.
8. Silversides CK, Lionel AC, Costain G, et al. Rare copy number variations in adults with tetralogy of Fallot implicate novel risk gene pathways. *PLoS Genet* 2012;8:e1002843.
9. Soemedi R, Wilson IJ, Bentham J, et al. Contribution of global rare copy-number variants to the risk of sporadic congenital heart disease. *Am J Hum Genet* 2012;91:489–501.
10. Tomita-Mitchell A, Mahnke DK, Struble CA, et al. Human gene copy number spectra analysis in congenital heart malformations. *Physiol Genomics* 2012;44:518–541.
11. Zaidi S, Choi M, Wakimoto H, et al. De novo mutations in histone-modifying genes in congenital heart disease. *Nature* 2013;498:220–223.
12. OMIM (Online Mendelian Inheritance in Man). Johns Hopkins University, Center for Medical Genetics: Baltimore, MD, 1996. http://www.ncbi.nlm.nih.gov/omim. Accessed March, 2014.
13. Hartman RJ, Rasmussen SA, Botto LD, et al. The contribution of chromosomal abnormalities to congenital heart defects: a population-based study. *Pediatr Cardiol* 2011;32:1147–1157.
14. Ferencz C, Neill CA, Boughman JA, Rubin JD, Brenner JI, Perry LW. Congenital cardiovascular malformations associated with chromosome abnormalities: an epidemiologic study. *J Pediatr* 1989;114:79–86.
15. Maslen CL, Babcock D, Robinson SW, et al. CRELD1 mutations contribute to the occurrence of cardiac atrioventricular septal defects in Down syndrome. *Am J Med Genet A* 2006;140:2501–2505.
16. Li H, Cherry S, Klinedinst D, et al. Genetic modifiers predisposing to congenital heart disease in the sensitized Down syndrome population. *Circ Cardiovasc Genet* 2012;5:301–308.
17. Zwick ME, Salstrom JL, Langley CH. Genetic variation in rates of nondisjunction: association of two naturally occurring polymorphisms in the chromokinesin nod with increased rates of nondisjunction in *Drosophila melanogaster*. *Genetics* 1999;152:1605–1614.
18. Locke AE, Dooley KJ, Tinker SW, et al. Variation in folate pathway genes contributes to risk of congenital heart defects among individuals with Down syndrome. *Genet Epidemiol* 2010;34:613–623.
19. Priest JR, Girirajan S, Vu TH, Olson A, Eichler EE, Portman MA. Rare copy number variants in isolated sporadic and syndromic atrioventricular septal defects. *Am J Med Genet A* 2012;158A:1279–1284.
20. Sailani MR, Makrythanasis P, Valsesia A, et al. The complex SNP and CNV genetic architecture of the increased risk of congenital heart defects in Down syndrome. *Genome Res* 2013;23:1410–1421.
21. Ripoll C, Rivals I, Ait Yahya-Graison E, et al. Molecular signatures of cardiac defects in Down syndrome lymphoblastoid cell lines suggest altered ciliome and Hedgehog pathways. *PLoS One* 2012;7:e41616.
22. Freeman SB, Bean LH, Allen EG, et al. Ethnicity, sex, and the incidence of congenital heart defects: a report from the National Down Syndrome Project. *Genet Med* 2008;10:173–180.
23. Price AL, Patterson NJ, Plenge RM, Weinblatt ME, Shadick NA, Reich D. Principal components analysis corrects for stratification in genome-wide association studies. *Nat Genet* 2006;38:904–909.
24. Mulle JG, Dodd AF, McGrath JA, et al. Microdeletions of 3q29 confer high risk for schizophrenia. *Am J Hum Genet* 2010;87:229–236.
25. Satten GA, Allen AS, Ikeda M, Mulle JG, Warren ST. Robust regression analysis of copy number variation data based on a univariate score. *PLoS One* 2014;9:e86272.
26. Purcell S, Neale B, Todd-Brown K, et al. PLINK: a tool set for whole-genome association and population-based linkage analyses. *Am J Hum Genet* 2007;81:559–575.
27. Raychaudhuri S, Korn JM, McCarroll SA, et al.; International Schizophrenia Consortium. Accurately assessing the risk of schizophrenia conferred by rare copy-number variation affecting genes with brain function. *PLoS Genet* 2010;6:e1001097.
28. Rochais F, Mesbah K, Kelly RG. Signaling pathways controlling second heart field development. *Circ Res* 2009;104:933–942.
29. Erdogan F, Larsen LA, Zhang L, et al. High frequency of submicroscopic genomic aberrations detected by tiling path array comparative genome hybridisation in patients with isolated congenital heart disease. *J Med Genet* 2008;45:704–709.
30. Iascone M, Ciccone R, Galletti L, et al. Identification of de novo mutations and rare variants in hypoplastic left heart syndrome. *Clin Genet* 2012;81:542–554.
31. Hitz MP, Lemieux-Perreault LP, Marshall C, et al. Rare copy number variants contribute to congenital left-sided heart disease. *PLoS Genet* 2012;8:e1002903.
32. Cooper GM, Coe BP, Girirajan S, et al. A copy number variation morbidity map of developmental delay. *Nat Genet* 2011;43:838–846.
33. Zhian S, Belmont J, Maslen CL. Specific association of missense mutations in CRELD1 with cardiac atrioventricular septal defects in heterotaxy syndrome. *Am J Med Genet A* 2012;158A:2047–2049.
34. Redig JK, Fouad GT, Babcock D et al. Allelic interaction between CRELD1 and VEGFA in the pathogenesis of cardiac atrioventricular septal defects. *AIMS Genet*, 2014;1:1–19.
35. Baker K, Beales PL. Making sense of cilia in disease: the human ciliopathies. *Am J Med Genet C Semin Med Genet* 2009;151C:281–295.
36. Kennedy MP, Omran H, Leigh MW, et al. Congenital heart disease and other heterotaxic defects in a large cohort of patients with primary ciliary dyskinesia. *Circulation* 2007;115:2814–2821.
37. Goetz SC, Anderson KV. The primary cilium: a signalling centre during vertebrate development. *Nat Rev Genet* 2010;11:331–344.
38. Goddeeris MM, Rho S, Petiet A, et al. Intracardiac septation requires hedgehog-dependent cellular contributions from outside the heart. *Development* 2008;135:1887–1895.
39. Briggs LE, Phelps AL, Brown E, et al. Expression of the BMP receptor Alk3 in the second heart field is essential for development of the dorsal mesenchymal protrusion and atrioventricular septation. *Circ Res* 2013;112:1420–1432.
40. Haydar TF, Reeves RH. Trisomy 21 and early brain development. *Trends Neurosci* 2012;35:81–91.