HHS Public Access

Author manuscript

Hum Genet. Author manuscript; available in PMC 2016 June 07.

Published in final edited form as:

Hum Genet. 2016 March ; 135(3): 273–285. doi:10.1007/s00439-015-1623-9.

Rare copy number variants and congenital heart defects in the 22q11.2 deletion syndrome

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Electronic supplementary material The online version of this article (doi:10.1007/s00439-015-1623-9) contains supplementary material, which is available to authorized users.

Web Resources

The URLs for data presented herein are as follows:

dbVAR, http://www.ncbi.nlm.nih.gov/dbvar

Gene Ontology, http://www.geneontology.org

MetaCore, http://thomsonreuters.com/en/products-services/pharma-life-sciences/pharmaceutical-research/metacore.html

Mouse Genome Informatics Resource, http://www.informatics.jax.org

Online Mendelian Inheritance in Man (OMIM), http://omim.org

Primer3, http://bioinfo.ut.ee/primer3

ReactomeFIViz, http://wiki.reactome.org/index.php/Reactome_FI_Cytoscape_Plugin_4#

UCSC genome database, http://genome.ucsc.edu

UCSC in silico PCR, http://genome.ucsc.edu/cgi-bin/hgPcr?command=start

Ethical approval All procedures performed in studies involving human participants were in accordance with the ethical standards of the institution and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

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Abstract

The 22q11.2 deletion syndrome (22q11DS; velocardiofacial/DiGeorge syndrome; VCFS/DGS; MIM #192430; 188400) is the most common microdeletion syndrome. The phenotypic presentation of 22q11DS is highly variable; approximately 60–75 % of 22q11DS patients have been reported to have a congenital heart defect (CHD), mostly of the conotruncal type, and/or aortic arch defect. The etiology of the cardiac phenotypic variability is not currently known for the majority of patients. We hypothesized that rare copy number variants (CNVs) outside the 22q11.2 deleted region may modify the risk of being born with a CHD in this sensitized population. Rare CNV analysis was performed using Affymetrix SNP Array 6.0 data from 946 22q11DS subjects with CHDs (n = 607) or with normal cardiac anatomy (n = 339). Although there was no significant difference in the overall burden of rare CNVs, an overabundance of CNVs affecting cardiac-related genes was detected in 22q11DS individuals with CHDs. When the rare CNVs were examined with regard to gene interactions, specific cardiac networks, such as Wnt signaling, appear to be overrepresented in 22q11DS CHD cases but not 22q11DS controls with a normal heart. Collectively, these data suggest that CNVs outside the 22q11.2 region may contain genes that modify risk for CHDs in some 22q11DS patients.

Introduction

The 22q11.2 deletion syndrome (22q11DS; velo-cardio-facial syndrome; DiGeorge syndrome, VCFS/DGS; MIM #192430; 188400) affects approximately 1 in 2000–4000 live births and is the most common microdeletion syndrome (Burn and Goodship 1996; Robin and Shprintzen 2005). The majority of individuals with 22q11DS carry the typical 3 million base pair (3 Mb) deletion on one chromosome 22 homolog, however, smaller nested 1.5–2 Mb deletions are seen, albeit in <10 % of individuals (Carlson et al. 1997; Emanuel 2008).

The typical 3 Mb deletion and the smaller nested interstitial deletions are the result of non-allelic homologous recombination events between low copy repeats that punctuate the 22q11.2 region (Edelmann et al. 1999; Shaikh et al. 2000). The clinical features attributed to the hemizygous 22q11.2 deletion are highly variable and include congenital heart defects (CHDs), dysmorphic facial features, palatal anomalies, immune deficiencies, hypocalcemia, a variety of neuropsychiatric disorders and cognitive impairment (McDonald-McGinn and Sullivan 2011).

Various CHDs and/or aortic arch defects have been reported in approximately 60–75 % of individuals with 22q11DS (McDonald-McGinn and Sullivan 2011; Ryan et al. 1997). The etiology of this cardiac phenotypic variability is currently unknown, but it does not appear to correlate with sex, race, 22q11.2 deletion size, or parent of origin of the deletion (Goldmuntz et al. 2009; Sandrin-Garcia et al. 2007; Swaby et al. 2011). The reduced penetrance of CHDs and variable expressivity within the 22q11DS population is influenced in part by genetic factors, since 22q11DS patients with a CHD are more likely to have an unaffected relative with an isolated CHD than 22q11DS patients with normal cardiac anatomy (Swaby et al. 2011). These findings are not explained by the inheritance of the non-deleted chromosome 22, suggesting that variants outside of the 22q11.2 region may influence the development of CHDs in these families (Swaby et al. 2011). Therefore, we hypothesized that structural variants, possibly in the form of rare CNVs, may increase the risk of intracardiac and/or aortic arch malformations in individuals already sensitized by the 22q11.2 deletion.

Large genic CNVs that are rare in the general population have been identified as pathogenic in a variety of human diseases and disorders. Rare CNVs have also been associated with congenital defects, such as CHDs. Recent non-syndromic CHD studies have identified causative rare CNVs at recurrent loci, such as 1q21.1 and 8p23.1 (Glessner et al. 2014; Greenway et al. 2009; Silversides et al. 2012; Soemedi et al. 2012b; Tomita-Mitchell et al. 2012). A common CNV, the duplication of *SLC2A3*, has been associated with an increased risk for CHDs in 22q11DS (Mlynarski et al. 2015). However, rare CNVs have not been examined in 22q11DS, and it is not currently known if rare CNVs are involved in the etiology of CHDs in 22q11DS.

In this manuscript, we present the first genome-wide analysis of rare CNVs in 22q11DS. Rare CNV analysis was performed on a cohort of 946 22q11DS subjects in order to determine whether rare CNVs, the genes they contain and the pathways or networks in which those genes reside, are potential genetic modifiers of the highly variable cardiac phenotype in 22q11DS.

Materials and methods

22q11DS cohort, Affymetrix genotyping and CNV detection

In compliance with IRB protocols (Internal Review Board, 1999–201, Albert Einstein College of Medicine, NY; 07-005352_CR2 CHOP IRB) and with informed consent, blood or saliva samples were obtained from 22q11DS subjects. A detailed description of the 22q11DS cohort's recruitment criteria, cardiac phenotypic information, genotyping procedures using Affymetrix SNP6.0 arrays, and the quality control metrics used in vetting

the cohort has been reported (Mlynarski et al. 2015). Three subjects from the original cohort were removed based on updated clinical information. A total of 946 22q11DS samples, 607 cases with CHDs and 339 controls with normal cardiac anatomy, passed all quality control metrics and were included in our analysis of 22q11DS rare CNVs. The specific cardiac defects observed in these subjects and the distribution of 22q11.2 deletion sizes in the 22q11DS cohort have been previously reported (Mlynarski et al. 2015).

CNV detection using PennCNV (Wang et al. 2007) and CNV workshop (Gai et al. 2010) was performed as was previously described (Mlynarski et al. 2015). Briefly, the PennCNV-Affymetrix tool was used to extract the signal intensity data from the raw. CEL files (Wang et al. 2007) and the log2ratios generated by PennCNV were used in CNV Workshop to produce CNV calls using circular binary segmentation (Gai et al. 2010). The BAF and LRR plots were subsequently visualized using Chromosome Analysis Suite to support CNV calls.

Rare CNV analysis

A list of autosomal CNVs detected by >10 contiguous probes for deletions and 20 probes for duplications was generated; the LogR ratio deviation for deletions is easier to detect than that of duplications (Wang et al. 2007), therefore a more stringent threshold of 20 contiguous probes was used for duplications in order to prevent detecting false positives. In addition, only CNVs detected by both PennCNV and CNV Workshop were included in the analysis, as these CNVs are less likely to be false positives due to variation in algorithms. CNV boundaries were determined by averaging the breakpoint locations predicted by PennCNV and CNV Workshop. Any CNVs with a 50 % or greater overlap with centromere, telomere, immunoglobulin regions, olfactory receptor genes and/or segmental duplications were excluded. In addition, CNVs within 10 kb of one another were merged and considered as possible single contiguous events. We adopted a stringent definition of rare CNVs in which CNVs that share ≥50 % non-reciprocal overlap with CNVs found in ≥1.0 % of a previously published control population [dbVaR accession nstd100 (Coe et al. 2014)] were categorized as common and discarded; only the remaining CNVs were designated as rare and included in this analysis. CNV detection was performed using the GRCh36/hg18 build and the CNV coordinates were converted to the GRCh37/hg19 build using the UCSC Genome Browser LiftOver tool; all subsequent analyses were performed using the GRCh37/hg19 build. The genomic coordinates presented in figures and tables herein are based on the February 2009 Human Genome Build (GRCh37/hg19).

CNV validation by qPCR

Selected CNVs were validated by SYBR Green quantitative PCR (qPCR) on an ABI SDS-7500 Fast Real-Time PCR system (Applied Biosystems, Carlsbad, CA, USA) as described (Mlynarski et al. 2015). Briefly, primers were designed to target a minimum of two amplicons within the deleted/duplicated region and at least one amplicon in a flanking region with normal copy number for each CNV. Each qPCR run included amplification of a previously reported endogenous control with known copy number [*RPPH1*, (Mlynarski et al. 2015)], and two control DNA samples with normal copy number.

CNV burden analysis using the CNV Map (Zarrei et al. 2015)

CNVs detected in the 22q11DS cohort were compared to the comprehensive CNV map of the human genome (Zarrei et al. 2015). The coordinates for the inclusive CNV map copy number variable regions (CNVRs) and stringent CNV map CNVRs were obtained from Zarrei et al.'s Tables S9 and S10 (2015). The 22q11DS deletions and duplications were evaluated separately in the various CNV map analyses. If ≥1 bp of a 22q11DS CNV overlapped with a CNVR, the 22q11DS CNV was considered to be "inside" the CNV map. A 22q11DS CNV was categorized as "outside" of the CNV map only if it did not overlap with any CNVRs. 22q11DS CNVs that were both outside of the CNV map and rare (frequency <1.0 %) in the published control population [nstd100 (Coe et al. 2014)] were classified as "ultra rare". The "ultra rare" CNVs were evaluated further to assess CNV burden in CHD cases versus controls.

The 22q11DS CNVs were first analyzed by CN type using the inclusive CNV map. The 22q11DS duplications and deletions were compared separately to the inclusive CNVR gains and CNVR losses, respectively [Zarrei et al. Table S9 "Gains (inclusive)" and "Losses (inclusive)" (Zarrei et al. 2015)]. Next, the 22q11DS duplications and deletions were compared to all variants in the inclusive CNV map with the CNVR gains and CNVR losses combined [Zarrei et al. Table S9 "Gains + Losses (inclusive)" (Zarrei et al. 2015)]. Both of the analyses were subsequently repeated using the stringent CNV map (Zarrei et al. 2015). The four analyses performed using the CNV maps will hereafter be referred to as (1) inclusive by CN type, (2) inclusive all CNVRs, (3) stringent by CN type and (4) stringent all CNVRs.

In silico analysis of gene function

CNVs in 22q11DS subjects that passed all selection criteria were annotated using the RefSeq gene set downloaded from the UCSC Table browser of the GRCh37/hg19 build (April 2015). The Mammalian Phenotype (MP) Ontology term annotations for each RefSeq gene were retrieved from the Mouse Genome Informatics (MGI) database (July 2014 available version). Gene Ontology (GO) annotations were obtained from the Ensembl database (July 2014 available version). The curated KEGG, BioCarta and Reactome gene sets were downloaded from the Molecular Signatures Database (MSigDB) v4.0 (July 2013 available version) (Subramanian et al. 2005). Previously published analytical methods were employed to expand the annotation of the Mammalian Phenotype ontology terms, Gene Ontology terms and the MSigDB gene sets (White et al. 2014). For each functional term (MP and GO) and MSigDB gene set, we directly compared the frequency of occurrence between cases and controls using Fisher's exact test. Due to high correlations between functional terms, the Benjamini–Hochberg false discovery rate (FDR) estimation procedure was then applied as previously described (Benjamini and Hochberg 1995). Duplication and deletion events were evaluated separately, and the genes contained within the 22q11.2 deleted region were excluded from these analyses.

Cardiac gene sets

Three sets of cardiac-related genes were acquired from different published sources. The "Lage" list is the manually curated set of cardiac developmental genes from Lage et al.;

there is functional evidence for each of the 254 genes in the “Lage” set, as targeted mutation of these cardiac developmental genes has been shown to cause heart phenotypes in mouse models (Lage et al. 2010). The “MetaCore” list (n = 310; Supplementary appendix 1) was derived from four canonical maps specific for cardiac development from MetaCore from Thomson Reuters: (1) “Cardiac development BMP TGF beta signaling”, (2) “Cardiac development FGF ErbB signaling”, (3) “Cardiac development Role of NADPH oxidase and ROS”, and (4) “Cardiac development Wnt beta catenin Notch, VEGF IP3 and integrin signaling”. The “HHE” list of high heart expression genes contains the top quartile of genes (n = 4171) expressed in the developing mouse heart at day E14.5 (Zaidi et al. 2013); genes were ranked by expression level, and the top 25 % of genes with the highest expression were included in the “HHE” list.

Mouse gene expression profiling of developing heart and pharyngeal arches (PA) at day E9.5 was performed as described to generate the “Heart_High” and “PA_High” gene lists (Racedo et al., manuscript in submission; see Supplementary Information). Briefly, RNA was extracted from micro-dissected pharyngeal arches and heart tubes from wild-type mouse embryos at E9.5. cDNA was generated and hybridized to Affymetrix Mouse GeneST 1.0 expression arrays following the manufacturer’s instructions. The resulting microarray expression data were normalized, and the mouse transcripts were compiled and converted to human gene designations for each tissue type. Genes were then ranked by expression level and the top 25 % of genes with the highest expression were included in each list. The “Heart_High” list contains the top quartile of genes expressed in the developing mouse heart at E9.5 (n = 3872; Supplementary appendix 2). The “PA_High” list contains the top quartile of genes expressed in the pharyngeal apparatus at E9.5 (n = 3873; Supplementary appendix 3).

Analysis of cardiac genes in rare CNVs

For each of the cardiac-related gene sets described above, we determined how many individuals carried rare CNVs that encompassed cardiac-related genes, and also computed the number of cardiac genes detected per subject. The 22q11DS CHD cases and controls were analyzed separately using the five cardiac-related gene lists. The genes contained within the rare deletions, rare duplications and all rare CNVs were evaluated independently in each analysis. The cardiac-related gene analyses were then repeated using the subset of genes unique to CHD cases or controls.

Rare CNV genes that were only observed in CHD cases, or only in controls, were considered to be unique. Since duplications and deletions are mechanistically distinct genetic lesions with disparate phenotypic consequences, genes that were impacted by different CN event types in CHD cases than in controls were also considered unique. For example, if a gene was affected by rare deletions in CHD cases and by rare duplications in controls (or vice versa), then it was labeled as a unique gene. In total, four iterations of the cardiac-related gene analysis were performed in order to identify and evaluate cardiac genes contained within rare CNVs carried by 22q11DS CHD cases and controls: (1) all rare CNV genes detected in 22q11DS CHD cases, (2) all rare CNV genes detected in 22q11DS

controls, (3) rare CNV genes unique to 22q11DS CHD cases, and (4) rare CNV genes unique to 22q11DS controls.

Functional interaction network analysis of rare CNV cardiac genes

A single combined set of cardiac-related genes was constructed from the union of the five cardiac genes lists ("HHE", "Heart_High", "PA_High", "Lage" and "MetaCore"), such that the combined set contained every gene in each list without any duplicates ($n = 5986$; Supplementary appendix 4). The frequency of cardiac-related genes in the 22q11DS cohort was assessed to determine how many individuals had rare CNVs that encompassed each specific cardiac-related gene. This was compiled into a list of cardiac genes observed in subjects with CHD or in controls and the corresponding sample count for each gene. The 22q11DS CHD cases and controls were analyzed separately and the resulting cardiac gene/sample count lists were the input files used to create the gene interaction networks.

Gene interaction network analysis of the 22q11DS cardiac-related genes was performed using the Cytoscape 3 (Shannon et al. 2003) ReactomeFIViz App [Reactome Functional Interaction (Wu et al. 2014)]. The 22q11DS CHD cases and controls were analyzed separately using all cardiac-related genes in rare CNVs. The standard ReactomeFIViz "Gene Set/Mutation Analysis" was implemented for the CHD cases and again for controls following the standard protocol as described in the ReactomeFIViz user guide documentation (Wu et al. 2014). The interaction networks were generated from a "gene/sample count" input file using the Reactome FI Network 2014 version and default parameters; linker genes were not included and the sample cutoff was set to 1. The networks were partitioned into modules based on Reactome gene pathway annotations with the ReactomeFIViz built-in "cluster FI network" tool. A pathway enrichment analysis was run for each individual network module using the ReactomeFIViz built-in "Analyze module functions" tool. Pathways and modules were then filtered using an FDR cutoff of 0.05 and module size of 4. Only genes from statistically significant pathways were included in each module, and modules without statistically significant pathways (FDR ≥ 0.05) were excluded.

The gene interaction network analyses were then repeated using the cardiac-related genes unique to CHD cases or controls. A total of four gene interaction network analyses were performed with the Cytoscape 3 ReactomeFIViz App using different "gene/sample count" input files: (1) all cardiac-related genes in rare CNVs from 22q11DS CHD cases, (2) all cardiac-related genes in rare CNVs from 22q11DS controls, (3) rare CNV cardiac-related genes unique to 22q11DS CHD cases, and (4) rare CNV cardiac-related genes unique to 22q11DS controls.

Statistical analysis

The two-tailed Fisher exact test was used for the gene enrichment analyses. The Wilcoxon rank sum test was used in the CNV burden analyses. Multiple test correction was performed using the Benjamini–Hochberg false discovery rate estimation procedure as previously described (Benjamini and Hochberg 1995).

Results and discussion

To identify rare CNVs that might alter risk for development of CHDs, CNV analysis was performed using PennCNV and CNV workshop on Affymetrix SNP6.0 array data from 946 22q11DS subjects, of which 607 were CHD cases with intracardiac defects and/or aortic arch anomalies and 339 were controls with a normal heart. A total of 13,310 autosomal CNVs outside of the 22q11.2 deleted region were detected by both algorithms in the 946 22q11DS individuals. These CNVs are unlikely to be false positives as they were identified by two methods and passed the probe cutoffs that were chosen based on extensive validation testing, during which CNVs that met these criteria had a 100 % validation success rate (Mlynarski et al. 2015). The previous study determined there was no significant difference in CNV distribution with regard to gender, size of the 22q11.2 deletion or type of cardiac defect (Mlynarski et al. 2015). Therefore, this study focusses specifically on the rare CNVs detected in the 22q11DS cohort, and investigates possible associations between rare structural variants and CHDs in 22q11DS individuals.

A previously published control cohort of 11,256 phenotypically normal individuals [dbVaR accession nstd100 (Coe et al. 2014)] was used to determine the frequency of the 22q11DS CNVs in the general population. Of the 13,310 autosomal CNVs (12,095 deletions and 1215 duplications) detected outside the 22q11.2 deleted region, 7217 CNVs (54.22 %) occurred at a frequency <1.0 % in the control population (Coe et al. 2014), and were categorized as rare. A greater number of rare deletions ($n = 6489$) were identified than rare duplications ($n = 728$). The disparity between the number of deletions and duplications may be due to the more stringent threshold of 20 contiguous probes that was used to identify duplications in order to prevent detection of false positives, whereas 10 contiguous probes were used to identify deletions. Figure 1 depicts the genomic distribution of rare CNVs identified in 22q11DS CHD cases and controls. The rare CNVs are distributed relatively evenly across the genome with a slight clustering at 1q, 3p, 4q, 7p, 8p, 9p, 16p, 16q and 19q (Fig. 1). Perhaps this represents a tendency for higher CNV density in certain regions of the genome more susceptible to CN variation, especially at telomeres.

Rare CNV burden

An increased burden of rare CNVs has been associated with a variety of diseases and disorders (Girirajan et al. 2013; Keller et al. 2014; Wheeler et al. 2013), including non-syndromic CHDs (Glessner et al. 2014; Soemedi et al. 2012b). We therefore hypothesized that 22q11DS individuals with CHDs, defined as cases, may carry a higher rare CNV burden than 22q11DS individuals with normal hearts, and perhaps the greater number of rare CNVs may have altered their risk for developing heart defects. In order to assess rare CNV burden in 22q11DS, the mean number and size of rare CNVs carried in each subject was evaluated. The analysis revealed that there was no significant difference in the number of rare CNVs detected (7.65 ± 3.80 vs. 7.58 ± 3.59, $p = 0.99$), or the average size of rare CNVs (47.21 ± 71.81 vs. 45.43 ± 65.91 kb, $p = 0.66$), between subjects with CHDs including aortic arch anomalies and those with a normal heart (Table 1). Furthermore, there was no discernible difference in the number or size of rare deletions (Table 2) and rare duplications (Table 3) carried in 22q11DS CHD cases versus controls with 22q11DS. Rare CNV burden was also

examined using more stringent CNV frequency threshold of <0.1 % (Table S1; see Supplementary Information). The results of the <0.1 % rare CNV analyses showed that CNV burden, both in terms of number and size, was equivalent in CHD cases and controls with 22q11DS.

Since there was no difference in burden at either CNV frequency, the recently published CNV map (Zarrei et al. 2015) was then utilized to further assess rare CNV burden in 22q11DS (Figs. S1–S4; Tables S2–S5; see Supplementary Information). 22q11DS CNVs that were absent from the CNV map (i.e., did not overlap with known CNV regions in the CNV map) and rare in the published population control cohort, were classified as "ultra rare". These CNVs were of particular interest and additional analyses were performed to investigate whether 22q11DS individuals with CHDs carried a greater burden of "ultra rare" CNVs. The burden analyses revealed that there was no significant difference in the number or mean size of "ultra rare" CNVs detected in 22q11DS individuals with CHDs compared to those with normal heart anatomy (Tables S6–S9). The CNV map "ultra rare" CNV findings were consistent with the other various CNV burden analyses, which indicates that regardless of how rare CNVs are defined, the burden of rare CNVs is equivalent in 22q11DS CHD cases and controls. Although the overall rare CNV load is not associated with CHDs in 22q11DS, perhaps rare CNVs of certain loci or cardiac-specific genes may increase the risk of being born with a heart defect.

Previously identified rare CNVs associated with non-syndromic CHDs

Recent studies have suggested that recurrent, rare CNVs may contribute to the pathogenesis of non-syndromic CHDs (Glessner et al. 2014; Greenway et al. 2009; Silversides et al. 2012). The 22q11DS cohort was assessed for the rare CNVs reported in these non-syndromic CHD studies. The most commonly reported "rare" recurrent rearrangement in non-syndromic CHDs is a gain (Erdogan et al. 2008; Geng et al. 2014; Glessner et al. 2014; Greenway et al. 2009; Lalani et al. 2013; Serra-Juhe et al. 2012; Silversides et al. 2012; Soemedi et al. 2012a; Tomita-Mitchell et al. 2012; Xie et al. 2014) or loss (Christiansen et al. 2004; Greenway et al. 2009; Soemedi et al. 2012a) of 1q21.1; interestingly, this 1q21.1 CNV was not detected in any of the 22q11DS subjects. One 22q11DS subject with TOF carried a deletion at 16p13.11 that encompassed *MYH11* (MIM 160745) (Fig. S5; Table S10), a known CHD risk gene (Zhu et al. 2006). CNVs impacting *MYH11* have been observed in several non-syndromic CHD studies (Lalani et al. 2013; Soemedi et al. 2012b; Tomita-Mitchell et al. 2012; Xie et al. 2014). The largest nonrecurrent rare CNV identified in the 22q11DS cohorts was a rare 5.3 Mb deletion at 18q11.2–12.1 that overlaps a 202 kb deletion reported by Soemedi et al. (2012b); the heterozygous deletion included the gene *CDH2* (MIM 114020), which is involved in Wnt-signaling (Fig. S6; Table S10). The previously reported 3p25 duplication (Greenway et al. 2009), which included the genes *RAF1* (MIM 164760) and *TMEM40*, was detected in a subject with a ventricular septal defect and interrupted aortic arch type B (Fig. S7). A duplication of *PTPN11* [MIM 176876] at 12q24.13 was also identified in the same 22q11DS individual (Fig. S7). This exact 12q24.13 duplication has not been reported in the non-syndromic CHD literature, but variants in *PTPN11* have been associated with non-syndromic TOF (Cordell et al. 2013; Goodship et al. 2012). Furthermore, *PTPN11* mutations are known to cause a spectrum of

cardiac developmental defects, and are observed frequently in patients with Noonan syndrome and LEOPARD syndrome (Lauriol et al. 2015; Sznajer et al. 2007).

A variety of other CNVs identified in non-syndromic CHD studies were observed in the 22q11DS cohort and validated by qPCR (Table S10); however, these CNVs occurred as singletons or at low frequencies and were not specifically associated with CHDs. For example, the previously identified duplication of *LTBP1* (MIM 150390) at 2p22.3 (Erdogan et al. 2008), a gene that is essential for cardiac outflow tract septation and remodeling (Todorovic et al. 2007), was detected in two 22q11DS individuals with CHDs and in three 22q11DS individuals with normal heart anatomy (Fig. S8; Table S10). Since none of the non-syndromic CHD CNVs were significantly enriched in our CHD subjects, various in silico analyses were performed to investigate whether the genes contained within these CNVs converged upon pathways or gene networks relevant to cardiac development.

In silico analysis of gene function

Based on our prior analysis of common CNVs (Mlynarski et al. 2015), it is not surprising that overall rare CNV burden and non-syndromic CHD CNVs were not associated with CHDs in 22q11DS. As such, we hypothesized that 22q11DS individuals with CHDs may carry a greater burden of rare CNVs that specifically impact genes and pathways important for proper heart development. To address this possibility and determine the relevance of genes affected by rare CNVs to cardiac development, we used phenotype data from Mouse Genome Informatics Resource, Gene Ontology, and MSigDB as previously described (Gai et al. 2012; Subramanian et al. 2005). The genes deleted in 22q11DS were excluded from this analysis.

Mammalian phenotype (MP)—MP analysis was performed on the rare CNVs to investigate the various phenotypes associated with genes impacted by CNVs in 22q11DS CHD cases versus controls. The results from the MP analyses are listed in Tables S11–S13. None of the MP terms were statistically significant after FDR correction and only the top 25 terms are shown (Tables S11–S13).

Although none of the terms passed the strict significance cutoff, some of the most enriched MP terms in subjects with CHDs were cardiac related such as “decreased ventricle muscle contractility” (Table S11). More MP terms relevant to cardiac function and heart morphology were associated with the rare duplications detected in CHD cases (Table S13) than were found in the rare deletions (Table S12) or in MP analysis of all rare CNV events combined (Table S11). Included in the top 25 MP terms associated with CHDs in the rare duplication analysis were: “abnormal heart size”, “abnormal heart left ventricle morphology”, “decreased cardiac muscle contractility” and “enlarged heart” (Table S13). In addition to the cardiac-specific terms, several terms involved in muscle morphology and function were enriched in the rare duplications such as “abnormal muscle physiology”, “impaired muscle contractility” and “abnormal muscle fiber morphology” (Table S13). These data suggest that 22q11DS patients with a CHD may be carrying additional CNVs that affect cardiac morphology and muscle function, thereby altering their risk of developing a heart defect.

Gene ontology (GO)—GO analysis was performed on the rare CNVs in order to examine the annotated biological processes, cellular components and/or molecular functions of genes impacted by CNVs in CHD cases versus controls. The results from the GO analyses are listed in Tables S14–S16. Despite suggestive Fisher's exact test p values (<0.05), none of the GO terms passed the strict significance cutoff and only the top 25 terms are shown (Table S14–S16).

Several of the GO terms associated with CHDs (albeit not significantly) pertain to biological processes that are involved in or directly impact early heart development. The top three terms in the rare deletion GO analysis were "negative regulation of cell migration", "negative regulation of cellular component movement" and "negative regulation of cell motility" (Table S15). These GO terms were also enriched in the analysis of all rare CNVs (Table S14). "Cell migration" was also overrepresented in individuals with CHDs in the rare duplication GO analysis (Table S16). Together the GO analyses indicate that genes involved in cell migration are affected by CNVs much more frequently in 22q11DS individuals with CHDs than in those with normal heart anatomy.

These findings are intriguing since migration of cardiac progenitor cells during embryogenesis is a carefully orchestrated process that is tightly regulated and essential for proper heart development (Buckingham et al. 2005). Causal mutations in several genes related to cell migration have been previously identified in patients with non-syndromic CHDs (Buckingham et al. 2005; Di Felice and Zummo 2009; Silversides et al. 2012). Therefore, the overabundance of genes involved in cell migration impacted by CNVs in 22q11DS individuals with CHDs, may have contributed to their risk of being born with a CHD by altering cardiac progenitor cell migration during cardiogenesis.

MSigDB gene set enrichment analysis—The KEGG, BioCarta and Reactome gene network sets obtained from MSigDB were used to analyze the rare CNVs to examine the networks and pathways associated with genes impacted by CNVs in CHD cases versus controls. The results from the MSigDB gene set analyses are listed in Tables S17–S19. None of the gene sets were statistically significant after FDR correction and only the top 25 pathways are shown (Tables S17–S19). The "KEGG_WNT_SIGNALING_PATHWAY", which plays an essential role in cardiac development (Buikema 2014), was detected in the rare deletion analysis (Table S18). Another known cardiac pathway was identified in the rare duplication analysis: "KEGG_ARRHYTHMOGENIC_RIGHT_VENTRICULAR_CARDIOMYOPATHY_ARVC" (Table S19). Several ErbB signaling pathways that are involved in regulating heart morphogenesis and important during cardiogenesis (Sanchez-Soria and Camenisch 2010) were also detected (Tables S17–S19).

The MP, GO and MSigDB analyses determined that genes and pathways involved in cardiogenesis were enriched in the 22q11DS CHD cases but the associations did not meet the strict threshold for statistical significance, likely because the analyses were performed using all genes contained within rare CNVs. Therefore, we narrowed the focus of the investigation and examined only the rare CNV genes that were relevant to cardiac development.

Cardiac genes in rare CNVs

Even though the associations were not statistically significant, the in silico gene function analyses indicated that several pathways related to cardiac development were over represented amongst CNVs in 22q11DS subjects with CHDs. To further address the hypothesis that genes involved in cardiac development might be impacted by rare CNVs more frequently in 22q11DS individuals with CHDs than those with normal cardiac anatomy, five sets of cardiac-related genes were examined. Zaidi et al. (2013) recently demonstrated that genes involved in CHDs are often expressed in the developing heart; therefore, we investigated genes that are highly expressed at different stages during cardiogenesis. The “HHE” list contains genes highly expressed in the mid-gestational, E14.5, mouse embryonic heart (Zaidi et al. 2013). Many key genes are expressed earlier in development, so genes that are highly expressed in the mouse heart tube at E9.5 were also included (the “Heart_High” list; Racedo et al., manuscript in submission). Genes that are responsible for cardiac outflow tract development are expressed in the pharyngeal apparatus and not the heart at mouse stage E9.5. Since most of the anomalies observed in 22q11DS derive from defects in the remodeling of the cardiac outflow tract, we therefore evaluated genes expressed in the pharyngeal apparatus (the “PA_High” list; Racedo et al., manuscript in submission). We also examined the curated set of cardiac developmental genes from Lage et al. (2010), referred to as the “Lage” list. In addition, the MetaCore software package from Thomson Reuters was used to generate the vetted “Metacore” list of genes involved cardiogenesis.

For each gene set, we investigated how many 22q11DS individuals with CHDs or with normal heart anatomy had rare CNVs that impacted cardiac-related genes. Although some of the gene sets had suggestive associations, there was not a statistically significant difference in the number of 22q11DS CHD cases versus controls when all rare CNV genes were included in the analysis (Table 4). Some of the cardiac-related genes were detected in both 22q11DS individuals with CHDs and individuals with normal heart anatomy. Since genes found in cases and controls are less likely to cause or contribute to heart defects in 22q11DS, these genes were removed and the analyses were repeated using the subset of genes that were found only in CHD cases or in controls but not both (Table 5). The “HHE” and “Heart_High” gene sets were significantly overrepresented amongst CHD cases in the unique gene analyses (Table 5: all CNVs and duplications). Genes from the “Lage” list were also disproportionately abundant in CHD cases as indicated by the high odds ratios (Table 5: deletions), but the gene list was not large enough for a statistically significant enrichment. Together these analyses suggest that genes involved in cardiac development may be affected by rare CNVs at a higher frequency in 22q11DS individuals with CHDs compared to those with normal cardiac anatomy.

Functional interaction network analysis of rare CNV cardiac genes

The cardiac gene set analysis revealed that cardiac-related genes were statistically enriched in 22q11DS CHD cases. However, 22q11DS individuals with normal heart anatomy also carried rare CNVs that affected genes involved in cardiac development. Since cardiac-related genes were observed in both CHD cases and controls, we wanted to examine the genes in more detail. Therefore, gene network analyses were performed in order to investigate the

specific pathways and biological functions of the cardiac-related genes found in 22q11DS CHD cases compared to 22q11DS controls.

Gene network analyses were performed separately for the 22q11DS CHD cases and controls using all cardiac-related genes impacted by rare CNVs. The functional interaction networks were constructed with the Cytoscape 3 (Shannon et al. 2003) ReactomeFIViz App (Wu et al. 2014). The network genes were clustered into modules based on gene annotation using the ReactomeFIViz "Cluster FI network" tool. The modules were filtered by size and pathway enrichment; only statistically significant modules (FDR < 0.05) that contained four or more genes were retained.

The results of the CHD case gene interaction network analysis is shown in Fig. 2. Several of the top modules with pathways that are significantly enriched in CHD cases pertain to pathways that are important for heart development, which may not be surprising as the interaction network was constructed using the cardiac-related genes detected in rare CNVs. However, the striking contrast between the CHD case and control functional interaction networks was unexpected. The CHD cases and controls were analyzed using the same FDR and size cutoffs; the CHD case network contained multiple modules with many significantly enriched pathways, whereas none of the control modules passed the cutoff criteria. As a result, the module size cutoff was lowered to three for the control network analysis. Even with the decreased stringency, the control network only contained two modules: nectin adhesion pathway and EGF receptor signaling pathway (Fig. 3).

Some of the cardiac-related genes were affected by rare CNVs in both CHD cases and controls, and thus were unlikely to cause CHDs in 22q11DS. Therefore, the network analyses were repeated using the subset of unique genes found only in CHD cases or only in controls but not both. The unique gene networks were smaller and contained fewer genes (Figs. 4, 5), but the results were consistent with the previous analyses. The interaction network of unique genes in controls still contained only two modules, neither of which was directly relevant to heart development: nectin adhesion pathway and inflammation mediated by chemokine and cytokine signaling pathway (Fig. 5). The same pathways important for cardiac development were also significantly enriched in the case unique gene network, further demonstrating that the enrichment of cardiac pathways was specific to CHD cases.

The statistically significant enrichment of the Wnt signaling and assembly of primary cilium pathways in both of the CHD case network analyses was particularly intriguing (Figs. 2, 4). The involvement of Wnt signaling pathways in cell specification and differentiation during early cardiogenesis has been well established (Buikema 2014). The canonical Wnt/β-catenin pathway is essential for regulating proper proliferation of second heart field cells that give rise to the outflow tract and right ventricle (Tzahor 2007). Furthermore, perturbations in Wnt/β-catenin signaling have been shown to cause OFT defects (Tzahor 2007), such as those observed in patients with 22q11DS. The assembly of primary cilium module was an especially interesting finding, because recent discoveries in mice determined that the cilium has a critical role in CHD pathogenesis (Li et al. 2015) and ciliary genes have been identified in rare CNV studies of patients with non-syndromic CHDs (Silversides et al.

2012). The network analyses indicate that cilia may also contribute to the etiology of CHDs in 22q11DS as well, but further investigations will be necessary to confirm this finding.

Additional pathways relevant to cardiogenesis were also significantly enriched in the 22q11DS CHD cases. The largest module contained growth factor signaling and ErbB signaling pathways (Figs. 2, 4), which have essential roles in embryogenesis and heart development (Perrimon et al. 2012). Signaling by the Robo receptor was also highly overrepresented in CHD cases (Figs. 2, 4). Robo receptor signaling regulates cell migration (Giovannone et al. 2012); cell migration is an essential process in cardiogenesis and defects in cell migration can lead to CHDs (Buckingham et al. 2005; Di Felice and Zummo 2009; Silversides et al. 2012). The other significant pathways, sumoylation and non-sense mediated decay, were not directly involved in heart development but rather pertained to more basic biological processes important for cell function. Together the gene interaction network analyses revealed that pathways important during cardiogenesis and essential for proper heart development are significantly enriched in CHD cases but not in the controls.

We originally hypothesized that individuals with 22q11DS and CHDs have rare CNVs that affect their risk of being born with intracardiac and/or aortic arch malformations, and undertook this study looking for genetic modifiers involved in the variable 22q11DS cardiac phenotype. Although no particular gene or gene family shows a striking number of hits within rare CNVs, the fact that pathways relevant to cardiogenesis seem to converge in 22q11DS CHD cases and not controls is thought-provoking and warrants further investigation. In the future, WES or WGS studies may shed additional light on this intriguing finding.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

Thanks to Harold (Reed) Salmons, Brenna Lilley, Colleen Franconi, Meghan McNamara, Daniel McGinn and Petra Warner for technical assistance. Special thanks go to the subjects and their families for their willing participation. We also thank the Molecular Cytogenetics and Genomics Cores at Einstein for preparing DNA and genotyping with Affymetrix 6.0 arrays. This work was funded by NIH (HL84410, HD070454 and HD026979) and in part by NIH/NCATS (National Center for Advancing Translational Sciences), Grant UL1TR000003.

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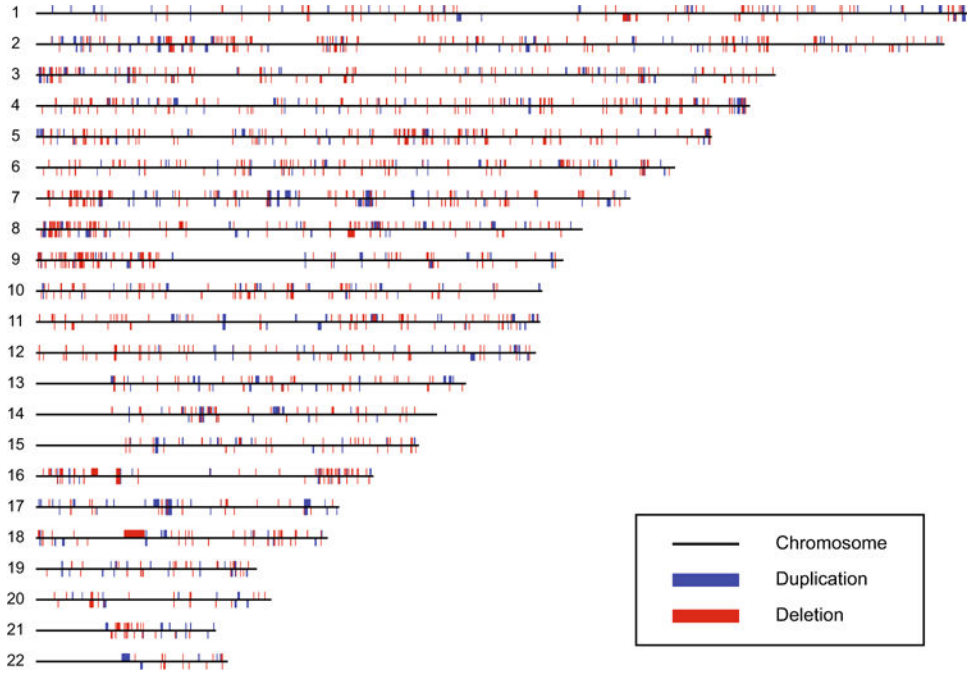

Fig. 1.

Genomic distribution of 7217 rare CNVs identified in 22q11DS subjects. Each autosome is depicted as a *black horizontal line*. The CNVs identified in 22q11DS CHD cases are shown above the line and CNVs identified in 22q11DS controls appear below. Deletions are shown in *red* and duplications are shown in *blue*

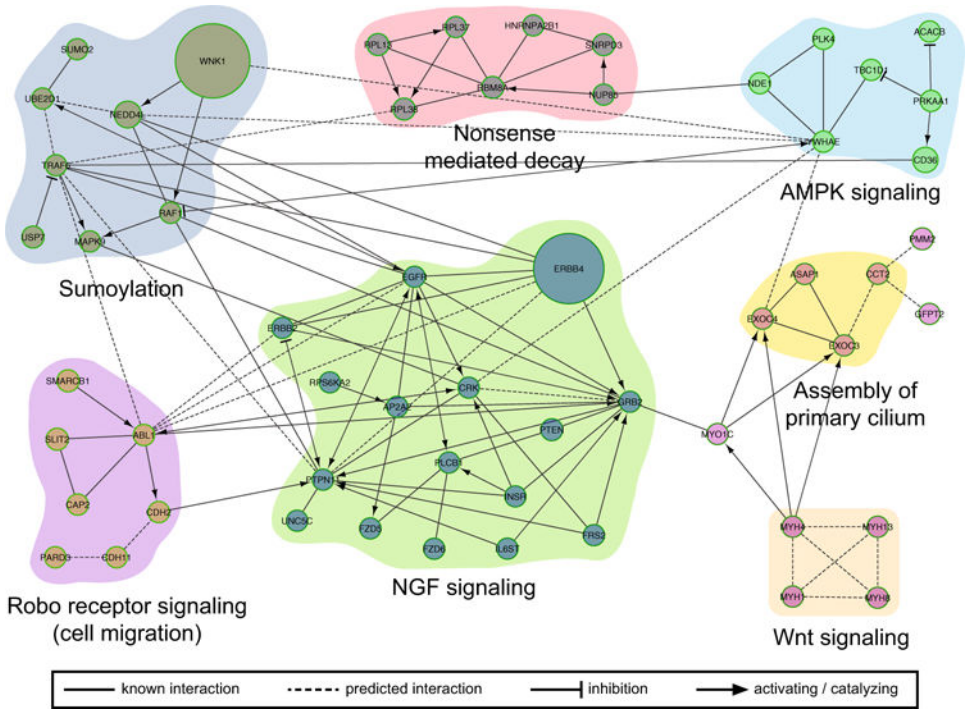

Fig. 2.

Network of all cardiac-related genes found in 22q11DS CHD cases. The functional interaction network was constructed with the Cytoscape 3 (Shannon et al. 2003) ReactomeFIViz App (Wu et al. 2014). Each node depicts a cardiac-related gene detected in the 22q11DS CHD cases. The size of each node represents the number of subjects with rare CNVs containing that gene. Genes are clustered into modules based on pathway interactions, and modules are labeled with the most significantly enriched pathway it contains

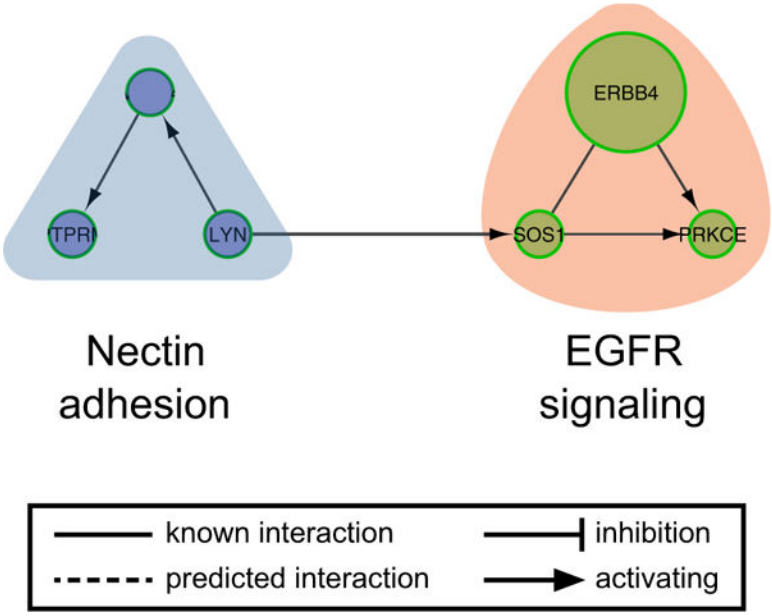

Fig. 3.

Network of all cardiac-related genes found in 22q11DS controls. The functional interaction network was constructed with the Cytoscape 3 (Shannon et al. 2003) ReactomeFIViz App (Wu et al. 2014). Each node depicts a cardiac-related gene detected in the 22q11DS controls. The size of each node represents the number of subjects with rare CNVs containing that gene. Genes are clustered into modules based on pathway interactions, and modules are labeled with the most significantly enriched pathway it contains

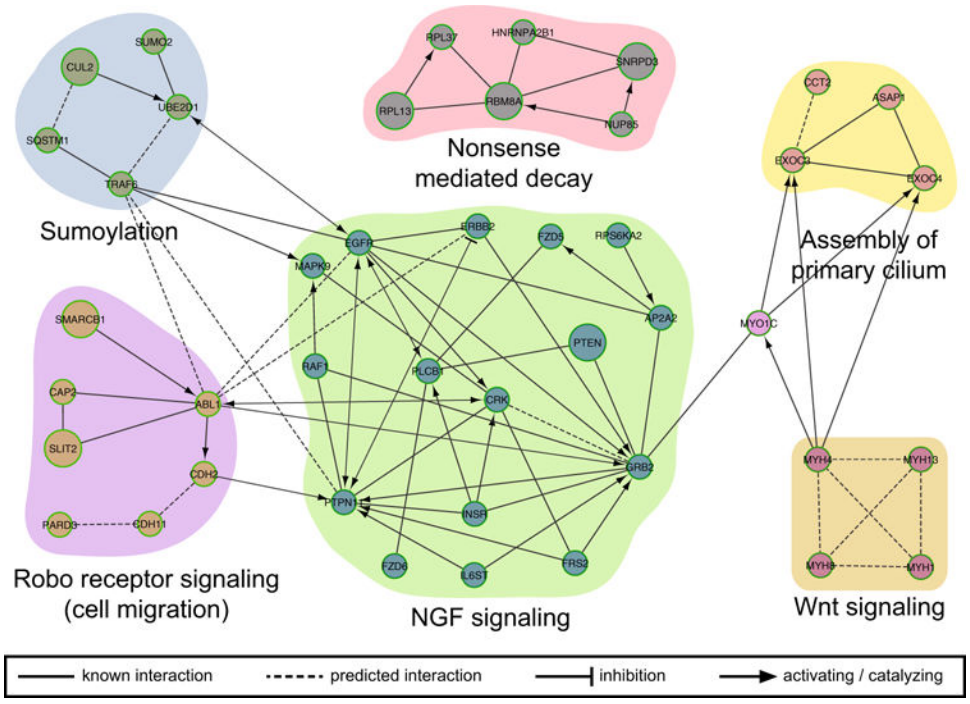

Fig. 4.

Network of cardiac-related genes unique to 22q11DS CHD cases. The functional interaction network was constructed with the Cytoscape 3 (Shannon et al. 2003) ReactomeFIViz App (Wu et al. 2014). Each node depicts a cardiac-related gene detected in the 22q11DS CHD cases. The size of each node represents the number of subjects with rare CNVs containing that gene. Genes are clustered into modules based on pathway interactions, and modules are labeled with the most significantly enriched pathway it contains

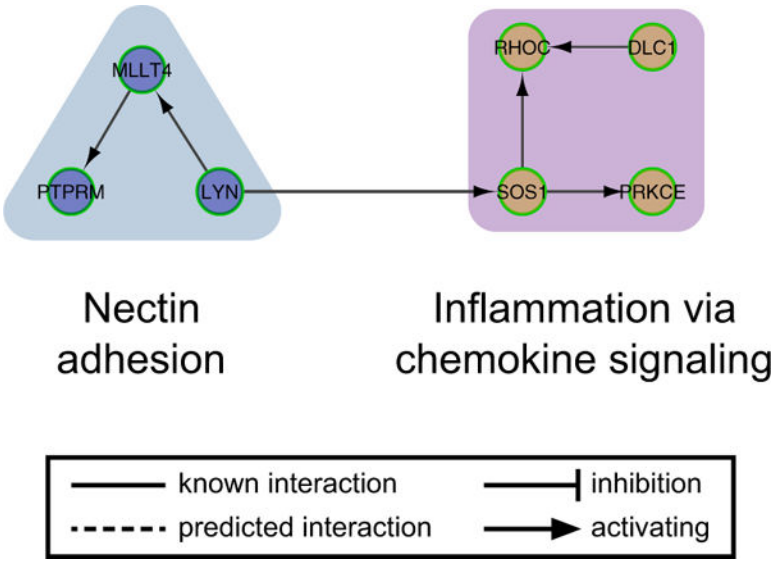

Fig. 5.

Network of cardiac-related genes unique to 22q11DS controls. The functional interaction network was constructed with the Cytoscape 3 (Shannon et al. 2003) ReactomeFIViz App (Wu et al. 2014). Each node depicts a cardiac-related gene detected in the 22q11DS controls. The size of each node represents the number of subjects with rare CNVs containing that gene. Genes are clustered into modules based on pathway interactions, and modules are labeled with the most significantly enriched pathway it contains

Table 1

Rare CNV burden: all rare CNVs (<1.0 %)

	CHD	No CHD	*p* value
# of subjects with rare CNVs	604	339	0.56[a]
% with rare CNVs	99.51 %	100.00 %	
Mean # of rare CNVs per subject	7.65	7.58	0.99[b]
±StdDev	±3.80	±3.59	
Mean rare CNV length (kb) per subject	47.21	45.43	0.66[b]
±StdDev (kb)	±71.81	±65.91	
# with ≥1 rare CNV ≥500 kb	60	36	0.74[a]

CHD $n = 607$; no CHD $n = 339$

[a]Two-tailed Fisher's exact test

[b]Wilcoxon rank sum

Table 2

Rare CNV burden: rare deletions (<1.0 %)

	CHD	No CHD	*p* value
# of subjects with rare CNVs	602	338	0.43[a]
% with rare CNVs	99.18 %	99.71 %	
Mean # of rare CNVs per subject	6.86	6.85	0.87[b]
±StdDev	±3.55	±3.37	
Mean rare CNV length (kb) per subject	26.87	27.57	0.82[b]
±StdDev (kb)	±43.49	±40.91	
# with ≥1 rare CNV ≥500 kb	6	8	0.16[a]

CHD $n = 607$; no CHD $n = 339$

[a] Two-tailed Fisher's exact test

[b] Wilcoxon rank sum

Table 3

Rare CNV burden: rare duplications (<1.0 %)

	CHD	No CHD	*p* value
# of subjects with rare CNVs	305	166	0.73[a]
% with rare CNVs	50.25 %	48.97 %	
Mean # of rare CNVs per subject	0.79	0.73	0.48[b]
±StdDev	±0.96	±0.93	
Mean rare CNV length (kb) per subject	108.89	93.30	0.52[b]
±StdDev (kb)	±219.49	±171.56	
# with ≥1 rare CNV ≥500 kb	54	28	0.81[a]

CHD $n = 607$; no CHD $n = 339$

[a]Two-tailed Fisher's exact test

[b]Wilcoxon rank sum

Table 4

22q11DS subjects with cardiac-related gene(s) in rare CNVs: all genes in CHD cases and controls

CNV type	Gene list	CHD	No CHD	*p* value[a]	Odds ratio
All CNVs	HHE	242	136	0.945	0.990
	Heart_High	188	87	0.087	1.300
	PA_High	116	58	0.484	1.145
	Lage	73	31	0.194	1.358
	MetaCore	21	7	0.317	1.700
Deletions	HHE	190	112	0.611	0.923
	Heart_High	128	60	0.234	1.243
	PA_High	52	28	0.904	1.041
	Lage	68	29	0.220	1.349
	MetaCore	9	3	0.553	1.686
Duplications	HHE	75	31	0.162	1.401
	Heart_High	76	32	0.167	1.373
	PA_High	70	33	0.447	1.209
	Lage	5	2	1.000	1.400
	MetaCore	12	5	0.799	1.347

CHD $n = 607$; no CHD $n = 339$

[a]Two-tailed Fisher's exact test

Table 5

22q11DS subjects with cardiac-related gene(s) in rare CNVs: only genes UNIQUE to CHD cases or controls

CNV type	Gene list	CHD	No CHD	*p* value[a]	Odds ratio
All CNVs	HHE	102	39	0.029	1.554
	Heart_High	106	40	0.024	1.582
	PA_High	98	44	0.217	1.291
	Lage	11	3	0.400	2.067
	MetaCore	21	7	0.317	1.700
Deletions	HHE	38	17	0.472	1.265
	Heart_High	42	17	0.265	1.408
	PA_High	39	20	0.781	1.095
	Lage	6	1	0.432	3.374
	MetaCore	9	3	0.553	1.686
Duplications	HHE	68	23	0.029	1.733
	Heart_High	69	24	0.040	1.683
	PA_High	62	26	0.243	1.370
	Lage	5	2	1.000	1.400
	MetaCore	12	5	0.799	1.347

CHD $n = 607$; no CHD $n = 339$

[a]Two-tailed Fisher's exact test