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Clinical Genetic Risk Variants Inform a Functional Protein Interaction Network for Tetralogy of Fallot

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Supplemental Materials:
Supplemental methods
Supplemental Tables I-VIII (V provided as a separate file)
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References[46–82]

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Abstract

Background -—Tetralogy of Fallot (TOF), the most common cyanotic heart defect in newborns, has evidence of multiple genetic contributing factors. Identifying variants that are clinically relevant is essential to understand patient-specific disease susceptibility and outcomes, and could contribute to delineating pathomechanisms.

Methods -—Using a clinically-driven strategy, we re-analyzed exome sequencing data from 811 probands with TOF, to identify rare loss-of-function and other likely pathogenic variants in genes associated with congenital heart disease (CHD).

Results -—We confirmed a major contribution of likely pathogenic variants in *FLT4* (VEGFR3; n=14) and *NOTCH1* (n=10), and identified 1–3 variants in each of 21 other genes, including *ATRX*, *DLL4*, *EP300*, *GATA6*, *JAG1*, *NF1*, *PIK3CA*, *RAF1*, *RASA1*, *SMAD2*, and *TBX1*. In addition, multiple loss-of-function variants provided support for three emerging CHD/TOF candidate genes: *KDR* (n=4), *IQGAP1* (n=3), and *GDF1* (n=8). In total, these variants were identified in 63 probands (7.8%). Using the 26 composite genes in a STRING protein interaction enrichment analysis revealed a biologically relevant network (p-value 3.3e-16), with VEGFR2 (*KDR*) and NOTCH1 representing central nodes. Variants associated with arrhythmias/sudden death and/or heart failure indicated factors that could influence long-term outcomes.

Conclusions -—The results are relevant to precision medicine for TOF. They suggest considerable clinical yield from genome-wide sequencing, with further evidence for *KDR* (VEGFR2) as a CHD/TOF gene, and for vascular endothelial growth factor (VEGF) and Notch signaling as mechanisms in human disease. Harnessing the genetic heterogeneity of single gene defects could inform etiopathogenesis and help prioritize novel candidate genes for TOF.

Journal Subject Terms:

Genetics; Congenital Heart Disease; Developmental biology

Keywords

congenital heart disease; genetic variation; tetralogy of Fallot; vascular endothelial growth factor receptor; genomics

Introduction

Tetralogy of Fallot (TOF) affects about one in 3000 live births, and is the most common cyanotic heart defect in newborns[1, 2]. Initially described in 1671 by Danish anatomist Niels Stensen, the four components (pulmonary outflow tract obstruction, aorta overriding both ventricles, ventricular septal defect, and hypertrophy of the right ventricle) represent a single developmental anomaly[3, 4]. Further detailed anatomical documentation by Arthur Fallot, Maude Abbott, and others, laid the foundation first for palliative, and later corrective, surgical procedures[4, 5]. Advances in imaging, medical management, and surgeries across the lifespan have transformed TOF from a usually fatal pediatric condition to a chronic disease that is more prevalent in adults than children, with life expectancy into the seventh decade and beyond[6–9].

The pursuit of determining the genetic underpinnings and recognizing how these may affect late outcomes in TOF, has proceeded in parallel with these clinical advances[10–13]. This research began with the recognition of multi-system syndromes in approximately 20% of patients, most commonly caused by 22q11.2 microdeletions, other copy number variants, or aneuploidies[14], along with some single-gene defects[15]. Availability of newer genomic technologies, particularly genome-wide sequencing, has expanded gene discovery studies. The cumulative genetic evidence indicates a pattern of molecular etiology for TOF that is characterized by genetic heterogeneity and some distinction from congenital heart disease (CHD) as a whole[14, 16–21]. However, there has been relatively limited consideration of the clinical pathogenicity of genetic variants for TOF and translation of findings into the clinic[22, 23].

Here, we re-analyzed data from the largest genome-wide sequencing dataset for TOF available, where an initial study had reported that deleterious variants in *NOTCH1* and *FLT4* surpassed statistical thresholds for genome-wide significance[20, 24]. By design, genome-wide variant burden analyses generally include abundant variants of uncertain significance, and lack power to detect the rare gene-disease associations which comprise the genetic heterogeneity of a disease and form the basis for defining clinically reportable genetic variants.

The objective of our study was to improve understanding of precisely such variants, i.e., those that are of greatest relevance to clinical practice for patients with TOF. We re-analyzed and re-annotated the raw sequence data files, and used American College of Medical Genetics (ACMG) interpretation guidelines[25] to adjudicate variants in genes that were considered relevant for the congenital cardiac phenotype. This re-analysis identified 63 (7.8%) of 811 pediatric TOF probands to have pathogenic/likely pathogenic variants in known CHD genes (n = 49), or loss-of-function variants in emerging CHD/TOF candidate genes (n = 15; Figure 1). As expected given the difference in approach and methodology, few of these variants and genes were reported in the previous study[20]. The implicated genes encode proteins that functionally interact, indicating that the heterogeneous genetic architecture could inform mechanisms. Other pathogenic/likely pathogenic variants identified add to potential genetic implications for cardiovascular outcomes of TOF.

Methods

Exome sequencing raw data (bam files) have been accessed through the European Genome-phenome Archive (EGA; https://www.ebi.ac.uk/ega), and can be made available to qualified researchers through accession number EGAS00001003302. As described here[20], ethical approval had been obtained from the local institutional review boards at each participating centre prior to blood or saliva sample collection, and informed consent had been obtained from all subjects or their parents/legal guardians. The downloaded dataset was analyzed at The Centre for Applied Genomics (TCAG, The Hospital for Sick Children, Toronto, Canada) under a research protocol of the Hospital for Sick Children (REB# 0019980189). Full methods and quality metrics (Supplemental Table I) are available as supplemental data.

Results

Re-analyzing the exome sequencing data from 811 probands with TOF, we identified 48 probands with at least one pathogenic/likely pathogenic variant in a CHD-associated gene (48/811; 5.9%). Five CHD-associated genes had multiple pathogenic/likely pathogenic variants[25] (Figure 1). These included 14 likely pathogenic loss-of-function variants in *FLT4* (all loss-of-function), and 10 in *NOTCH1* (6 loss-of-function, 4 missense) (Supplemental Table II). The prevalence for variants in these two genes was collectively 24/811 (3.0%). We also identified three likely pathogenic variants in *JAG1* (OMIM-P 187500, 118450), and two each in *TBX1* (OMIM-P 187500) and *GATA6* (OMIM-P 187500, 600001) (Supplemental Table II). Consistent with the genetic heterogeneity of TOF, we identified 16 other individuals to have one pathogenic/likely pathogenic variant (11 loss-of-function, 5 missense) in 16 CHD genes: *ARHGAP31*, *ATRX*, *CACNA1C*, *CHD7*, *CSNK2A1*, *DLL4*, *EP300*, *GATAD2B*, *KAT6A*, *LZTR1*, *NF1*, *NODAL*, *PIK3CA*, *RAF1*, *RASA1*, and *SMAD2*, and one individual with loss-of-function variants in two genes, *ASXL1* and *PSMD12* (Supplemental Table II).

In a further 16 individuals in this TOF cohort, we identified loss-of-function variants in three emerging CHD candidate genes (*KDR*, *IQGAP1*, and *GDF1*), i.e., genes with substantial research evidence but as yet insufficient to clinically deem variants “likely pathogenic” (Figure 1, Supplemental Table III). For *KDR* (encoding VEGFR2), four individuals with TOF had high-confidence loss-of-function variants in *KDR*, compared with none in 6,201 controls from the 1000 genomes and MSSNG projects (Fisher’s exact test (FET): p = 1.8E-4), and compared with 9 in 76,156 controls from gnomAD (Chi-squared test with Yates’ correction (X^2) = 83.46; p < 1E-5; observed/expected loss-of-function constraint score (o/e LOF) = 0.15). The significant findings in this large TOF cohort add to results of several recent studies, reporting rare loss-of-function variants in *KDR* in independent cohorts with TOF or other conotruncal defects[18, 21, 26], which collectively provide evidence for KDR as a TOF/CHD gene (Figure 2).

For another vascular endothelial growth factor (VEGF) related gene, *IQGAP1*, with previous reports of loss-of-function variants in independent cohorts of TOF and other CHD[18, 27, 28], we identified three individuals with loss-of-function variants. This was significantly enriched compared to 1000 genomes control data (3/811 vs. 1/2,504; FET: p = 4.8E-2), and to

gnomAD (23/76,156; X^2 = 18.29; p < 1E-5; o/e LOF = 0.19), but not when compared to control (parental) data from an autism cohort (3/811 vs. 5/3,697; FET: p = 1.6E-1). For *GDF1*, the pathogenicity of biallelic (homozygous or compound heterozygous) variants is well established [OMIM-P 208530][21], and heterozygous variants have also been suggested as risk factors for CHD[29] [OMIM-P 613854]. In the current exome data, the prevalence of *GDF1* heterozygous loss-of-function variants is likely to be underestimated due to insufficient coverage of this locus. Nonetheless, we identified 8 individuals to have such a variant, including an identical, heterozygous *GDF1* stopgain variant p.Cys227* in 7 of the 706 unrelated probands having samples with adequate read depths (≥10x). In comparison, the carrier frequency of this variant was 6 of 3,697 European controls from MSSNG (FET: p = 1.9E-3) and 64 of 33,079 controls from gnomAD (X^2 = 17.36; p < 1E-5), indicating significant enrichment of heterozygous loss-of-function variants in *GDF1* in the TOF cohort studied.

Taken together, we identified 63 probands (7.8%) with n = 49 pathogenic/likely pathogenic variants in 23 known CHD genes (Supplemental Table II) or n = 15 loss-of-function variants in 3 emerging CHD/TOF candidate genes (Supplemental Table III) in these TOF exome data. We note that the majority of these variants (40/64, 62.5%) and genes (23/26, 88.5%) were not previously reported for this cohort[20] (Supplemental Tables II, III).

We next considered the 23 established TOF genes and 3 candidate genes with significant findings as a group, in an *in silico* functional interaction analysis. This showed evidence for a highly interactive network of the encoded proteins (STRING interaction enrichment p value = 3.3E-16). VEGFR2 (*KDR*) and NOTCH1 were central nodes within this network map, each connecting directly with 11 other proteins. In all, 23 of the 26 genes (88.5%) form an interactive network, related to VEGFR2 (*KDR*) or NOTCH1 by ≤2 edges (Figure 3). Pathogenic/likely pathogenic loss-of-function variants from an independent cohort of 424 children with TOF[21] supported, and slightly extended, this network, identifying NOTCH2 as an additional protein with multiple (five) interactions (Supplemental Table IV, Supplemental Figure I). All of the composite genes are expressed in human hearts (https://www.proteinatlas.org/), but at varying levels (Figure 3, Supplemental Figure I). A pathway enrichment map for the respective proteins is provided as supplemental material (Supplemental Figure II, Supplemental Table V).

Notably, we additionally identified rare nonsynonymous or predicted splice-altering variants of uncertain significance, according to ACMG interpretation guidelines[25]. This re-analysis classified the majority of *NOTCH1* rare nonsynonymous missense and in-frame variants that were previously reported for this sample[20] as were variants of uncertain significance (n = 21; Supplemental Table VI). Three of these variants – p.(Glu1294Lys), p.(Gly200Arg)[30], and p.(Pro143Leu) – were identified in probands with other likely pathogenic variants: *NOTCH1* p.(Gln1733*), *NF1* c.5206-1G>C, and *EP300* p.(Phe1595Val), respectively (Supplemental Table VI). There were other CHD-relevant genes or candidate genes with variants of uncertain significance, including loss-of-function variants in *CHD4*, *ECE1*, *SMAD6*, *ZFPM1*, *PRKD1* and *VEGFA* (Supplemental Table VI). In some cases, clinical data or knowledge about the parental genotypes, which were inaccessible for this study, could have informed more accurate variant classifications.

We also report pathogenic/likely pathogenic variants for childhood-onset disorders, but with less established evidence at this time for clinical relevance to TOF/CHD (Supplemental Table VII). These include loss-of-function variants in *POLR1A* (2x), *TCF12* (2x), *APC*, and *GLI2* (Supplemental Table VII).

To further investigate the potential clinical utility of exome sequencing, we also interrogated the dataset for rare variants with potential clinical implications for cardiovascular management and outcome. In 16 (2.0%) of the 811 probands, we identified pathogenic/likely pathogenic variants meeting these criteria. There were 11 variants associated with cardiac hypertrophy, arrhythmia and sudden cardiac death: hypertrophic cardiomyopathy (*MYBPC3* (3x), *MYH7*, *MYL2*, *TNNI3*), arrhythmogenic right ventricular dysplasia (*DSP* (2x), *DSC2*), Brugada syndrome (*SCN5A*), and dilated cardiomyopathy (*DMD*) (Supplemental Table VIII). There were also five variants in genes (*LZTR1*, *RAF1*, *CACNA1C*, *NF1*, *RASA1*) implicated in other conditions, including e.g., Noonan and Timothy syndromes[31, 32], that in addition have been reported to be associated with CHD, thus were also considered in the above etiologic variant analysis (Supplemental Tables II, VIII).

Discussion

Precision medicine is an emerging concept that involves health management based on individual characteristics, including genetic disease susceptibilities and pharmacogenomics[33, 34]. Delineation of disease-causing variants and genes and their functional networks will advance both precision health initiatives and our understanding of the relevant molecular mechanisms. The rationale for re-analyzing the exome sequencing data from these 811 individuals with TOF was to identify sequence variants with sufficient evidence for pathogenicity according to consensus clinical guidelines[25], that could thereby inform disease etiologies of TOF, and to detect rare and emerging gene-disease associations, unlikely to be identified in the previous study of these data[20]. We identified 49 pathogenic/likely pathogenic variants for TOF, plus 15 loss-of-function variants in emerging CHD/TOF candidate genes. The majority of variants (40/64, 62.5%) and genes (23/26, 88.5%) were not previously reported for this cohort[20] (Supplemental Tables II, III). This demonstrates the novelty of our findings using a stringent clinical approach to variant assessment, and the benefits of re-analysis. Collectively, the results document both genetic and allelic heterogeneity in the pathogenesis of TOF across this large cohort. The findings may also help to define minimal clinical gene panels for TOF.

Novel results from this study also provide further evidence that haploinsufficiency of *KDR* (VEGFR2) contributes to risk for TOF. Rare *KDR* loss-of-function variants were previously reported in several cohorts with TOF and other conotruncal defects[18, 21, 26] (Figure 2), and our results document highly significant enrichment in TOF compared to controls. None of these *KDR* variants were previously reported for this cohort[20]. Other recent studies report evidence that similar loss-of-function variants in *KDR* are also risk factors for pulmonary arterial hypertension, independent of any heart malformations[35, 36]. As for many CHD genes, this suggests pleiotropy for *KDR* requiring further study. There

may also be clinical implications with respect to pulmonary hypertension in individuals with *KDR*-related TOF[37].

For *IQGAP1*, loss-of-function variants were identified in this and in other CHD cohorts[18, 27, 28] (including several *de novo* variants). However, we found a similar prevalence of variants in parents of probands with autism, used here as a control sample. Besides its essential role in VEGF receptor signaling, IQGAP1 regulates and integrates other cellular processes, including neuronal functions[38]. We consider *IQGAP1* a promising candidate gene for CHD, but further statistical support and phenotypic characterization will be needed.

Taken together, pathogenic/likely pathogenic variants in known CHD genes and variants in candidate genes were identified in a total of 26 genes: *ARHGAP31*, *ASXL1*, *ATRX*, *CACNA1C*, *CHD7*, *CSNK2A1*, *DLL4*, *EP300*, *FLT4*, *GATAD2B*, *GATA6*, *GDF1*, *JAG1*, *IQGAP1*, *KAT6A*, *KDR*, *LZTR1*, *NF1*, *NODAL*, *NOTCH1*, *PIK3CA*, *PSMD12*, *RAF1*, *RASA1*, *SMAD2*, and *TBX1*. The results further indicated that the encoded proteins form highly inter-connected networks of functional interaction. This suggests that the genetic heterogeneity identified through human disease studies may help to inform overlapping or unifying molecular pathomechanisms for TOF. Notably, VEGFR2 (encoded by *KDR*) and NOTCH1 form central nodes in this interaction network, supporting and extending evidence that the developing right outflow tract is vulnerable to VEGF/Notch dysregulation[39–42]. VEGF signaling was recently reported to be the top canonical pathway associated with *de novo* variants in conotruncal defects[28], and low VEGF expression was linked to TOF risk in a historical family study[43]. Delineating the relevant protein networks and associated pathomechanisms will help to rank novel candidate genes and could inform potential therapeutic targets. For example, loss-of-function variants in *TCF12* (Supplemental Table IV) were recently reported in multiple individuals with unexplained CHD[26], and the encoded protein functionally interacts with three confirmed TOF-associated proteins (NOTCH1, SMAD2, EP300).

Even with overlapping molecular functions, however, the phenotypic spectrum (pleiotropy) can vary largely not only from one gene to another, and one variant to another, but for individuals with the same variant within and between families. Most of the identified pathogenic/likely pathogenic variants were in CHD genes associated with multisystemic genetic disorders (Supplemental Table II), as may be expected for confirmed genes in early stages of clinical interpretation. Clinical genetic testing results may thus, in certain cases, flag the potential for an increased risk for comorbidities including neurodevelopmental delays. On the other hand, pathogenic variants historically identified through syndromic phenotypes may have cardiac phenotypes without classic extracardiac expression[14]. For most individual genetic predispositions however, the extent of the disease spectrum is yet unknown. Delineating genotype-associated clinical traits, and understanding their penetrance, will be essential for genetic counselling, familial risk assessments, and informing outcome.

Identifying the genetic etiologies of TOF can improve clinical management, by providing information on outcomes and risks related to the variant, in addition to those related to the

cardiac anatomical severity and other clinical parameters[13, 44, 45]. After the surgical repair of TOF, heart failure and arrhythmias are leading causes of morbidity, impaired quality of life, and mortality. Genetic factors that can affect the molecular and structural properties of the heart and vasculature may play a role. In this study, 16 (2.0%) of 811 probands were identified to have pathogenic/likely pathogenic variants that could affect cardiac surveillance and management recommendations (Supplemental Table VIII). Longitudinal clinical data will be needed to characterize adverse or favourable outcomes of patient populations that include such genetic variant data, in order to identify predictive markers and to inform preventive and therapeutic interventions, as part of precision medicine.

Advantages and limitations

We analyzed genetic risk variants in the largest available exome dataset of individuals with TOF. We prioritized variants with sufficient evidence for pathogenicity, according to consensus clinical guidelines[25], in order to increase the “signal-to-noise ratio” in the reporting of single gene defects. In contrast to primarily statistical approaches, such as that previously applied to this cohort[20], this clinical approach enabled us to capitalize on the expected genetic and allelic heterogeneity of pathogenic variants in TOF. For example, 18 genes were identified with only one pathogenic/likely pathogenic variant each in this cohort. The lack of variant segregation data and the inaccessibility of individual clinical information, such as anatomical subtypes, disease progression, associated features, or age for this cohort[20], however limited the interpretation of the findings. Our analyses were also restricted to rare small sequence variants and insertions/deletions in regions targeted by exome sequencing, typically involving ~95% of exonic regions. The exome data available did not allow us to assess structural aberrations, such as rare copy number variants, despite their established contribution to CHD[14] (individuals with typical 22q11.2 deletions were however excluded[20]). All variants reported here were identified in the heterozygous state, passed internal quality metrics, and were visually validated in the aligned sequencing reads, however we could not confirm their accuracy by Sanger sequencing.

Conclusions

We studied the largest published exome sequencing dataset of patients with TOF, interpreting variants from the perspective of clinical pathogenicity. The identified genetic results add evidence for a major contribution of VEGF/Notch dysregulation, including *KDR*/VEGFR2, and provide novel findings for functionally interacting protein networks relevant to the pathomechanism of TOF. We anticipate that clinical genomic sequencing, especially where capability of detecting structural variants is included, will become an essential component for assessing risks and outcomes in patients with CHD[22]. Re-analysis of existing datasets is warranted, and valuable, especially as our knowledge to identify and interpret disease-related variants continuously evolves.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments:

Sequence data has been accessed through the European Genome-phenome Archive, which is hosted by the EBI and the CRG, under accession number EGAS00001003302 (https://ega-archive.org). We thank Page et al.[20] for allowing us access to the data, and the patients and their families for participation in this research effort. We thank the staff at The Centre for Applied Genomics (TCAG), a node of CGEn, for support in data analysis.

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Nonstandard Abbreviations and Acronyms:

TOF	Tetralogy of Fallot
CHD	Congenital heart disease
ACMG	American College of Medical Genetics
EGA	European Genome-phenome Archive

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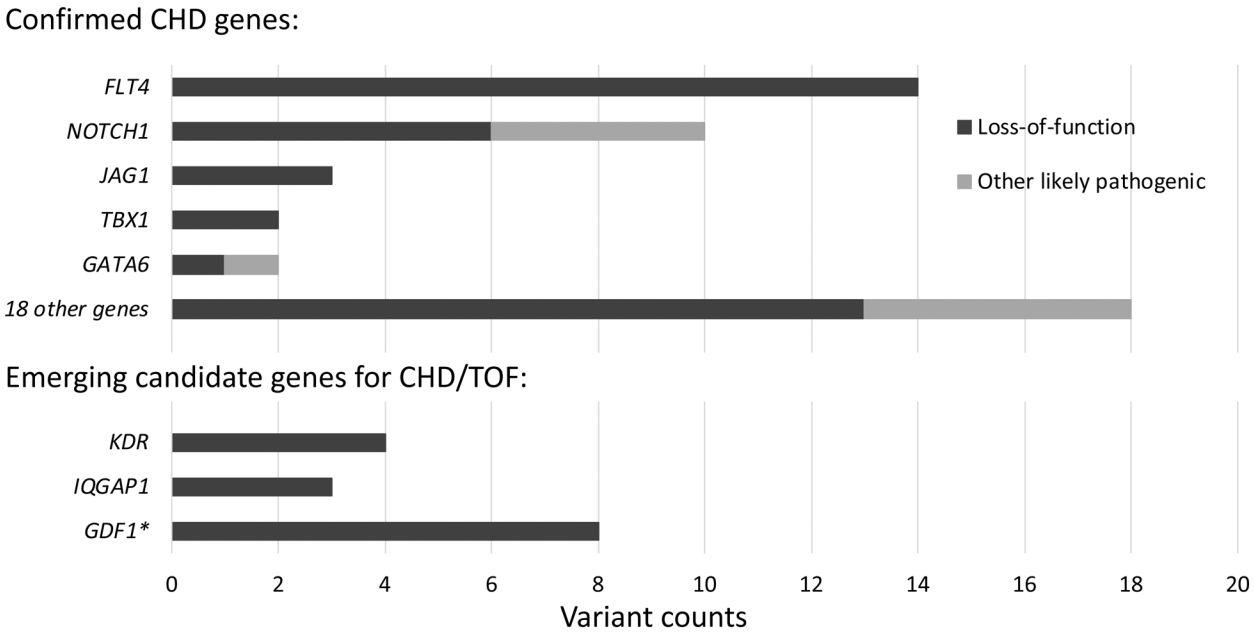

Figure 1:

Clinically relevant variants associated with congenital heart disease (CHD), identified in exome sequencing data of n = 811 probands with tetralogy of Fallot (TOF). Likely pathogenic loss-of-function and missense variants meeting ACMG criteria for clinical relevance (pathogenic or likely pathogenic, n = 49) in 23 recognized CHD genes were identified in 48 probands. Loss-of-function variants (n = 15) in 3 emerging candidate genes for CHD/TOF were identified in another 15 probands. Variant and gene details are provided in Supplemental Tables II and III, respectively, and Figure 2 provides details of *KDR* variants.

* Note: Comprehensive assessment of gene *GDF1* was not possible due to insufficient coverage of this locus with the exome sequencing data available.

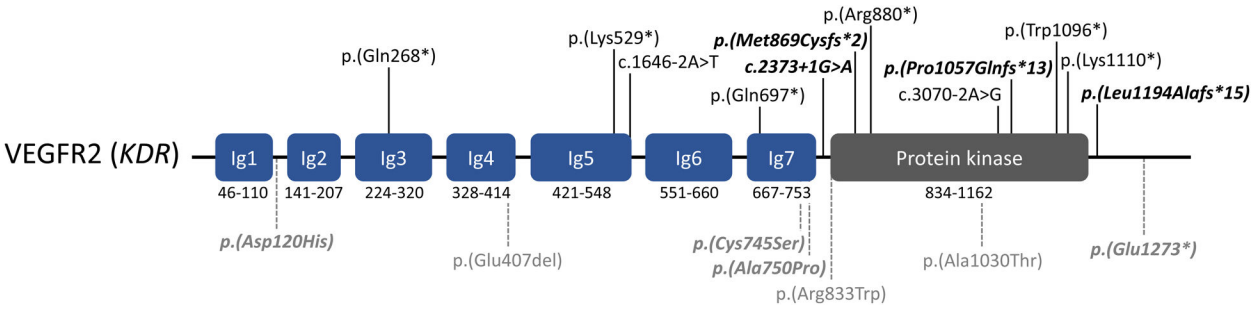

Figure 2:

Variants identified in vascular endothelial growth factor receptor 2 (VEGFR2; *KDR*: NM_002253.2), in patients with TOF or other conotruncal defects. Rare loss-of-function variants (n = 12) are shown in black font (above, solid vertical lines). Rare variants of uncertain significance (in-frame deletion, missense variants, and stopgain variant in penultimate exon) are shown in gray font (below, dashed vertical lines). Variants from this study (Supplemental Tables III and VI) are in ***bold/italics***. Variants reported in Jin et al.[21]: p.(Lys529*), c.1646-2A>T. Variants reported in Reuter et al.[18], Figure 1: p.(Arg880*), p.(Trp1096*), p.(Glu407del), p.(Arg833Trp), p.(Ala1030Thr). Variants reported in Morton et al.[26] (Note: some probands may overlap those reported by Jin et al.): p.(Gln268*), p.(Lys529*), c.1646-2A>T, p.(Gln697*), c.3070-2A>G, p.(Lys1110*).

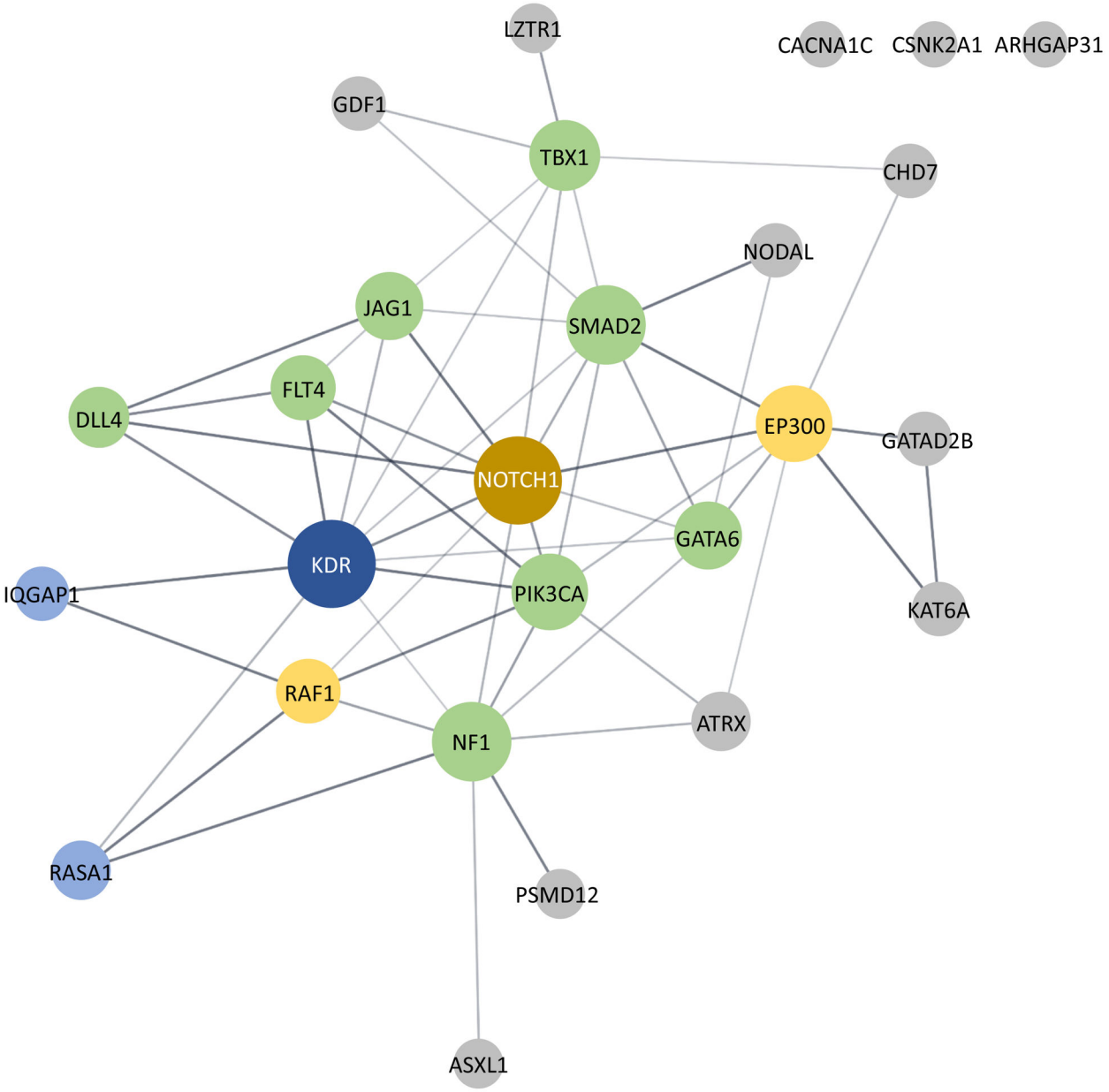

Figure 3:

Functionally interacting proteins encoded by confirmed genes (n = 23) and emerging candidate genes (n = 3) for CHD/TOF identified in n = 811 individuals with TOF. Network analysis was performed using Cytoscape, STRING and the 26 genes identified from exome sequencing of this cohort (STRING interaction enrichment *p* value 3.3E-16; see text for details). Node sizes (circles) represent the connectivity (numbers of edges to other proteins). Node colors represent the interaction with VEGFR2/KDR (blue), NOTCH1 (yellow), or both (green). Edge widths represent the confidence (strength of data support for functional and physical protein associations, including textmining, experiments, databases,

and co-expression). 23 of the 26 genes form an interactive network with VEGFR2 (gene *KDR*) and NOTCH1 as central nodes, each connecting directly with 11 other proteins.