Whole Exome Sequencing Reveals the Major Genetic Contributors to Nonsyndromic Tetralogy of Fallot

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Rationale: **Familial recurrence studies provide strong evidence for a genetic component to the predisposition to sporadic, nonsyndromic Tetralogy of Fallot (TOF), the most common cyanotic congenital heart disease phenotype. Rare genetic variants have been identified as important contributors to the risk of congenital heart disease, but relatively small numbers of TOF cases have been studied to date.**

Objective: **We used whole exome sequencing to assess the prevalence of unique, deleterious variants in the largest cohort of nonsyndromic TOF patients reported to date.**

Methods and Results: **Eight hundred twenty-nine TOF patients underwent whole exome sequencing. The presence of unique, deleterious variants was determined; defined by their absence in the Genome Aggregation Database and a scaled combined annotation-dependent depletion score of ≥20. The clustering of variants in 2 genes, *NOTCH1* and *FLT4*, surpassed thresholds for genome-wide significance (assigned as $P<5\times10^{-8}$) after correction for multiple comparisons. *NOTCH1* was most frequently found to harbor unique, deleterious variants. Thirty-one changes were observed in 37 probands (4.5%; 95% CI, 3.2%–6.1%) and included 7 loss-of-function variants 22 missense variants and 2 in-frame indels. Sanger sequencing of the unaffected parents of 7 cases identified 5 de novo variants. Three *NOTCH1* variants (p.G200R, p.C607Y, and p.N1875S) were subjected to functional evaluation, and 2 showed a reduction in Jagged1-induced NOTCH signaling. *FLT4* variants were found in 2.4% (95% CI, 1.6%–3.8%) of TOF patients, with 21 patients harboring 22 unique, deleterious variants. The variants identified were distinct to those that cause the congenital lymphoedema syndrome Milroy disease. In addition to *NOTCH1*, *FLT4* and the well-established TOF gene, *TBX1*, we identified potential association with variants in several other candidates, including *RYR1*, *ZFPM1*, *CAMTA2*, *DLX6*, and *PCM1*.**

Conclusions: **The *NOTCH1* locus is the most frequent site of genetic variants predisposing to nonsyndromic TOF, followed by *FLT4*. Together, variants in these genes are found in almost 7% of TOF patients. (*Circ Res*. 2019;124:553-563. DOI: 10.1161/CIRCRESAHA.118.313250.)**

Key Words: genes ■ genetic variation ■ heart diseases ■ Tetralogy of Fallot ■ whole exome sequencing

Congenital heart disease (CHD) is the most common type of birth defect, affecting 8/1000 live births.[1] CHD covers a large spectrum of heterogeneous cardiovascular phenotypes that range from single, localized defects to more complex structural abnormalities. Tetralogy of Fallot (TOF) is the most common complex, cyanotic CHD with a prevalence of 1/3000 births.[1,2] TOF is considered a malformation of the cardiac outflow tract which comprises 4 specific structural characteristics postnatally; a ventricular septal defect, anterocephalad deviation of the outflow septum with resultant overriding of the aorta, variable obstruction of the right ventricular outflow tract (pulmonary stenosis) and consequent hypertrophy of the right ventricle.[2,3] Surgical

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Original received April 19, 2018; revision received November 13, 2018; accepted November 26, 2018. In October 2018, the average time from submission to first decision for all original research papers submitted to *Circulation Research* was 14.86 days.

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The online-only Data Supplement is available with this article at https://www.ahajournals.org/doi/suppl/10.1161/CIRCRESAHA.118.313250.

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Novelty and Significance

What Is Known?

- Tetralogy of Fallot (TOF) is the most common cyanotic congenital heart disease.
- Nonsyndromic TOF is a genetically complex disease, with evidence for contributions from both common and rare variants.
- The major causative genes for nonsyndromic TOF have yet to be identified.

What New Information Does This Article Contribute?

- We performed whole exome sequencing in a large cohort of nonsyndromic TOF patients and identified rare deleterious variants in several genes.
- Variants in *NOTCH1* and *FLT4*, the most commonly observed genes, were found in 7% of TOF cases, indicating significant contributions from these genes to the population burden of disease.
- Functional analysis of *NOTCH1* variants found in patients with TOF confirmed a detrimental effect on the NOTCH signaling pathway.
- Identification of pathogenic variants in multiple genes in a substantial proportion of nonsyndromic TOF points to the utility of the whole exome sequencing approach in discovering the genetic basis of congenital heart disease in large cohorts of patients with homogeneous phenotypes.

Congenital heart disease occurs in almost 1% of live births. The most common severe cyanotic form, TOF, is well characterized phenotypically, but the genetic factors associated with nonsyndromic cases (80%) are mostly unknown. We performed whole exome sequencing on a large TOF cohort and found variants that were previously unobserved in the general population and were predicted to be highly damaging to protein function in 2 genes, NOTCH1 and FLT4, in 7% of cases. An in vitro activity assay showed that NOTCH1 variants observed in the patients disrupted NOTCH signaling. Significant (exome-wide $P<0.01$) excess of very rare deleterious variants were identified in 6 other genes; such variants were present in 15% of nonsyndromic TOF patients.

Nonstandard Abbreviations and Acronyms

AOS	Adams-Oliver syndrome
CHD	congenital heart disease
CNV	copy number variant
EGF	endothelial growth factor
JAG1	Jagged1
LOF	loss-of-function
NICD	NOTCH intracellular domain
RAM	RBPJ-associated molecule domain
TOF	Tetralogy of Fallot
VEGFR3	vascular endothelial growth factor receptor 3
WES	whole exome sequencing
WT	wild type

interventions during infancy mean that 85% to 90% of TOF patients now survive until at least 30 years of age.[1,4] However, this is not without consequence; event-free survival is just 25% after 40 years of age,[5] because resultant scar tissue from surgery and pulmonary regurgitation cause significant morbidity in adulthood.[6,7]

The cause of TOF is elusive and no single candidate gene can be held accountable for the disease phenotype. However, the genetic status of syndromic TOF sufferers has provided valuable insights into causative genes in some patients. Approximately 20% of cases are associated with a recognized syndrome or chromosomal anomaly.[2] Most significantly, ≈15% of TOF patients have 22q11.2 deletion syndrome, wherein the major causal gene is *TBX1*.[8,9] Approximately 80% of TOF cases are nonsyndromic, and there is generally no identifiable cause, largely because of their non-Mendelian patterns of inheritance.[10–13] Accordingly, a polygenic genetic architecture has been hypothesized and genome-wide approaches have been undertaken to provide insights into the complex genetic alterations responsible for TOF and other CHDs.[11,13–18]

Whole exome sequencing (WES) has been used successfully to identify new CHD candidate genes.[14,17,19,20] Many lines of evidence indicate a degree of phenotypic specificity of variants in particular genes. For example, the spectrum of phenotypes caused by 22q11.2 deletion or mutations in *TBX1* typically involves the outflow tract and great vessels,[9,21,22] while Down syndrome or mutations in *NKX2-5* typically cause septal defects.[23,24] To date, no WES study of CHD has included substantial numbers of any homogeneous phenotype, which should a priori have the highest power to identify causal variants.

Here, we present findings from WES of the largest cohort of nonsyndromic TOF patients reported to date. We performed WES in 829 TOF probands and identified the rarest and most deleterious protein-coding variants genome-wide. We sought evidence of pathological relevance for a subset of variants in the most significantly over-represented genes, based on the variants' de novo occurrence and functional consequences in cellular models.

Methods

Data can be accessed at the European Genome-phenome Archive (https://www.ebi.ac.uk/ega) using accession number EGAS00001003302.

Eight hundred twenty-nine TOF probands were subjected to WES, and unique (absent in the Genome Aggregation Database), deleterious (combined annotation-dependent depletion score of ≥20) variants were identified. Any variants observed in 1252 reference exome samples, that were analyzed using the same approach as our case data, were eliminated from further consideration. Clustering analysis within the cases was then used to identify genes in which significantly more variants were observed than expected given background levels of variation across all genes. De novo variants were identified by Sanger sequencing of proband and parent samples where possible. Immunoblotting and luciferase assays were used to assess the expression and signaling activity of selected variants in the most strongly supported candidate gene. Detailed methods can be found in the Online Data Supplement.

Results

Exome-Wide Analysis of Unique, Deleterious Variants Identifies the Highest Risk Loci for Nonsyndromic TOF

We assessed the incidence of unique, deleterious variants for 829 nonsyndromic TOF cases. Any variants observed in 1252 reference exomes were removed from consideration as potential

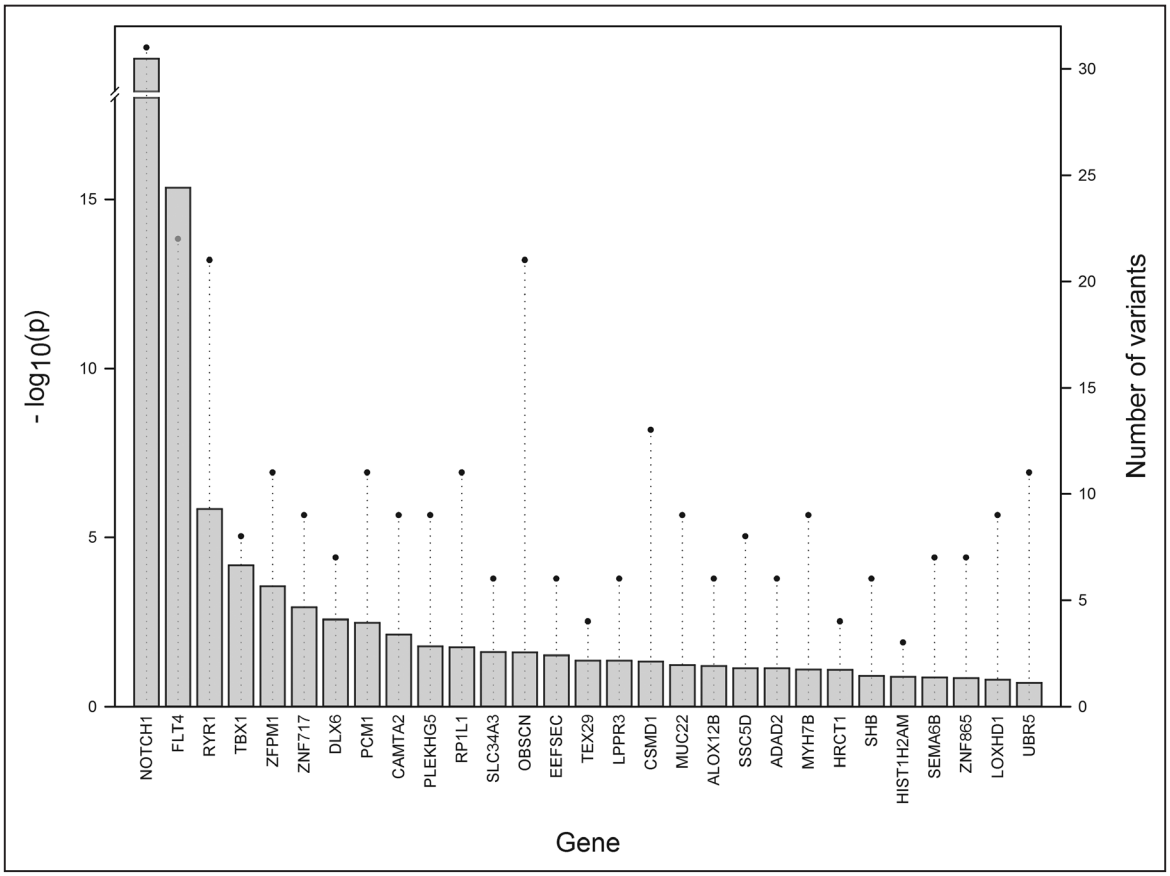

Figure 1. The top genes, in order of significance, in which nonsyndromic Tetralogy of Fallot (TOF) patients carry unique, deleterious variants. Bars indicate the respective significance levels of variant clustering for each gene, represented as –log *P* values. Circles represent the number of variants. The $-\log_{10}(P)$ column for NOTCH1 ($P<2.22\times10^{-16}$) goes towards infinity and is shown as arbitrarily high.

TOF susceptibility variants. The statistical significance of these findings was assessed for each gene using clustering analysis, which corrected for gene size (Online Table I). Two genes, *NOTCH1* and *FLT4*, surpassed the threshold for genome-wide significance (assessed as $P<5\times10^{-8}$; Figure 1) and the unique variants identified in these genes are likely to be contributors to the pathogenesis of TOF. Combined, variants in *NOTCH1* and *FLT4* account for 6.9% of our TOF cohort, with no overlap between probands with variants in these genes. Additionally, several other genes that harbor an excess of variant clustering are also of interest; including *RYR1* and *TBX1*, which have previously been implicated in CHD.[25,26] In particular, *TBX1* is a well-established TOF risk gene which is principally responsible for the cardiac manifestations of 22q11 deletion; additionally, deleterious single nucleotide variants and small functionally significant intragenic deletions in *TBX1* have been demonstrated in TOF patients.[9,21] A further 2 genes, *ZFPM1/FOG1* and *CAMTA2*, have roles in heart development and growth, respectively.[27] *DLX6* is negatively regulated by HAND2, a crucial transcription factor for heart morphogenesis[28] and *PCM1* is a regulator of ciliogenesis, a process strongly linked to CHD.[29] In addition, we specifically looked at the number of unique, deleterious variants in key cardiac transcription factors, including *NKX2.5*,[30] *GATA4*,[31] *HAND2*,[12] and *GATA6*,[32] because pathogenic variants have previously been identified in TOF cases, typically by targeted candidate gene sequencing. Variants in these genes account for just 1.2% of cases in our cohort. When considering the top 9 genes (or a *P* value cutoff of <0.01), 129 TOF cases had a unique, deleterious variant in 1 or more genes, accounting for over 16% of our patient cohort (Table 1). Just 8 samples had variants in >1 of the top 9 genes, highlighting the minimal overlap between probands with variants in these genes. Overall, *NOTCH1* and *FLT4* were found to be by far the most significant contributors to TOF; we, therefore, explored the variants in these 2 genes in greater detail.

Table 1. The Top Gene Candidates, Ordered by Levels of Significance, Following the Clustering Analysis of Unique, Deleterious Variants

Gene	Variants	P Value	Samples	Cumulative Sample Count
NOTCH1	31	$<2.22\times10^{-16}$	37	37
FLT4	22	4.44×10^{-16}	21	57
RYR1	21	1.43×10^{-06}	22	78
TBX1	8	6.50×10^{-05}	8	86
ZFPM1	11	0.000266817	12	98
ZNF717	9	0.001125519	10	106
DLX6	7	0.002583786	8	114
PCM1	11	0.003208801	11	123
CAMTA2	9	0.007243157	9	129

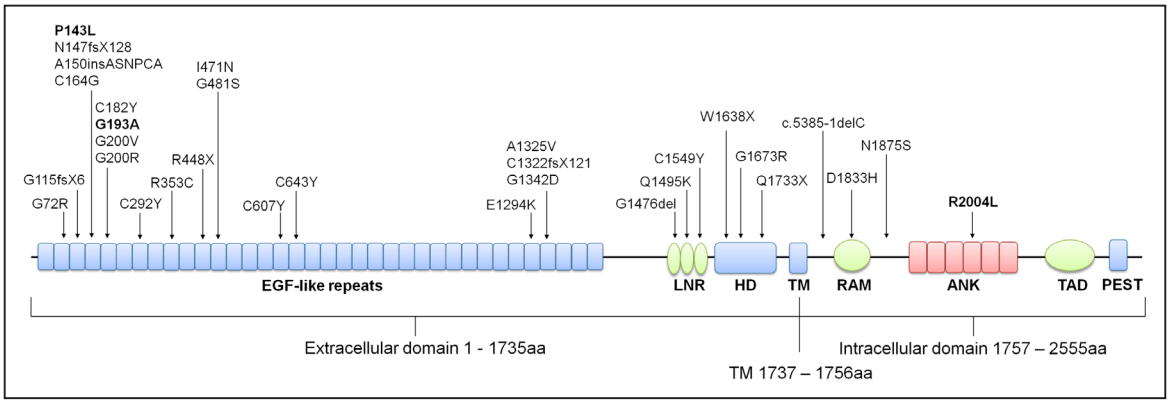

Figure 2. Unique, deleterious NOTCH1 variants in Tetralogy of Fallot (TOF) patients. Diagrammatic representation of the NOTCH1 protein with known protein domains indicated. The location of *NOTCH1* variants identified in our TOF cohort is shown. p.P143L, p.G193A, and p.R2004L discussed in the main text are indicated (bold). ANK indicates ankyrin repeats; EGF, epidermal growth factor; HD, heterodimerization domain; LBR, ligand binding region; LNR, Lin/Notch repeats; PEST, PEST domain; RAM, RBPJ-associated molecule domain; TAD, transactivation domain; and TM, transmembrane domain.

Variants in NOTCH1 Are the Most Commonly Present in Nonsyndromic TOF

The *NOTCH1* locus was the most frequently found to harbor a unique, deleterious variant among TOF patients ($P<2.22\times10^{-16}$), with 37 probands harboring 31 *NOTCH1* variants (Online Table II), accounting for 4.5% of our TOF patient cohort (95% CI, 3.2%–6.1%). Seven of the variants identified were loss-of-function (LOF), including 3 premature stop codons (p.R448X, p.W1638X, and p.Q1733X), 3 single base pair deletions resulting in frameshifts and eventual premature truncation (p.G115fsX6, p.N147fsX128, and p.C1322fsX121) and a single base pair deletion in a splice site consensus sequence (c.5385-1delC). Of the remaining 24 variants, 2 were in-frame indels and 22 were missense variants. *NOTCH1* is highly intolerant to LOF and missense variation, having a pLI of 1 and a missense *Z* score of 4.48 in the Exome Aggregation Consortium database. We mapped the distribution of the 31 variants to the various domains of NOTCH1 (Figure 2) and found the variants to be located throughout the protein with no significant clusters. The 3 frameshift mutations were located in the EGF (epidermal growth factor)-like repeats in addition to one truncating mutant, p.R448X, whereas the remaining 2 truncating variants were located in the heterodimerization domain. Of particular interest, one variant located in EGF-like repeat 5, p.G193A (Figure 2, bold), was identified in 5 unrelated patients and p.P143L (Figure 2, bold) located in EGF-like repeat 4 was identified in 3 unrelated patients. Together, these 2 variants account for almost 1% of our TOF patient cohort. Interestingly, a further 6 *NOTCH1* variants that map to the EGF-like repeats alter evolutionary conserved cysteine residues that contribute to disulphide bonds essential for maintaining the EGF structure.[33] Of the 4 intracellular domain mutants, a missense variant in the Ankyrin repeats region, p.R2004L is particularly notable (Figure 2, bold). R2004 is a surface exposed residue in Ankyrin domain 4 which is located in an interface region with the CSL transcription factor complex[34] and also located at an interface that binds the positive Notch regulator, Deltex.[35]

Deleterious mutations in other NOTCH pathway genes have been identified in patients with TOF, including *HEY2*[36] and *JAG1*.[37,38] For this reason, we compiled a list of NOTCH pathway genes using the MGI Gene Ontology Project and assessed the clustering of variants in these genes. Of 166 genes tested, only *NOTCH1* was found to have an excess of unique, deleterious variants (Online Table III). Hence, variants in other NOTCH pathway genes are not a major cause of TOF in our cohort.

Evidence of Pathological Consequences for NOTCH1 Variants

We investigated the occurrence rate of de novo variants in probands with *NOTCH1* variants. Of the 31 probands in our TOF patient cohort that harbored unique, deleterious variants in *NOTCH1*, samples from both parents were available for 7 probands and analyzed for variant inheritance. Following Sanger sequencing, 5 of the 7 *NOTCH1* variants tested were identified as de novo; 2 of these were truncating variants, whereas the remaining 3 de novo variants were missense (Table 2). These findings are in keeping with the results of previous WES experiments in CHD, where rare transmitted variants with strong bioinformatic support for functional impact, which are of presumed incomplete penetrance, have been uniformly encountered.[14,17,20]

The *NOTCH1* gene encodes an evolutionarily conserved transmembrane receptor that mediates cell-cell communication

Table 2. Sequencing of Parent Samples to Determine *NOTCH1* Variant Inheritance

Amino Acid Change	Ref	Alt	LOF	Impact	Inheritance Status
p.G200V	C	A	No	Missense variant	De novo
p.C292Y	C	T	No	Missense variant	From unaffected mother
p.R448X	G	A	Yes	Stop gained	De Novo
p.Q1495K	G	T	No	Missense variant	From unaffected father
p.C1549Y	C	T	No	Missense variant	De novo
p.W1638X	C	T	Yes	Stop gained	De novo
p.N1875S	T	C	No	Missense variant	De novo

Alt indicates alternate allele; LOF, loss of function; and Ref, reference allele.

to govern cell fate decisions during development.[39] S1 cleavage is an important step in the maturation of the NOTCH1 receptor. During this process, the 300 kDa translation product of NOTCH1 undergoes cleavage in the Golgi by a furin-like convertase to generate 2 polypeptides of 180 and 120 kDa.[40] To determine whether *NOTCH1* variants affect S1 cleavage, we assessed the expression of 3 NOTCH1 variants in comparison to wild-type (WT) NOTCH1 by immunoblotting. The variants assessed were p.G200R, p.C607Y, and p.N1875S (Figure 2); p.G200R is located in a conserved residue located within a β-hairpin turn within EGF5, and p.C607Y, located in EGF16, removes a conserved disulphide bond that normally would be expected to stabilize the EGF-domain conformation. p.N1875S is located in a residue that lies in a linker region between the RAM (RBPJ-associated molecule domain) and Ankyrin repeat regions of the Notch intracellular domain. As expected, we observed 2 bands at 300 kDa (P300) and 120 kDa (P120), representing full length and cleaved NOTCH1 protein[40]; the remaining 180 kDa product was not detectable because of the positioning of our FLAG-tag at the C terminus (Figure 3A). For WT NOTCH1, p.G200R and p.N1875S variants, we observe similar levels of both P300 and P120 (Figure 3A). However, the p.C607Y variant exhibited perturbed S1 cleavage. Indeed, quantification confirmed that 5±0.37% of NOTCH1 p.C607Y underwent cleavage in comparison to 57±3.96% of WT NOTCH1 (P=0.0002; Figure 3B). Hence, the p.C607Y variant affects S1 cleavage of NOTCH1, whereas the receptor is processed normally in the p.G200R and p.N1875S NOTCH1 variants.

Heterodimeric NOTCH1 is membrane-tethered and undergoes further cleavage by γ-secretase which releases the NICD (NOTCH intracellular domain). NICD subsequently translocates to the nucleus where it interacts with transcription factor RBPJ to activate NOTCH target genes.[39] To determine whether p.G200R, p.C607Y, and p.N1875S variants affect NOTCH1 canonical signaling function, we assessed NOTCH signaling through the RBPJ transcription factor-dependent pathway following stimulation with immobilized JAG1 (Jagged1) ligand. The variants were overexpressed in HeLa cells, and NOTCH1 signaling was assessed by RBPJ luciferase activity. Two of the 3 variants demonstrated reduced NOTCH signaling via RBPJ (Figure 3C). The p.C607Y variant, that exhibited perturbed cleavage, significantly reduced NOTCH signaling by 47±0.12% (P=0.008) compared to WT NOTCH1. Similarly,

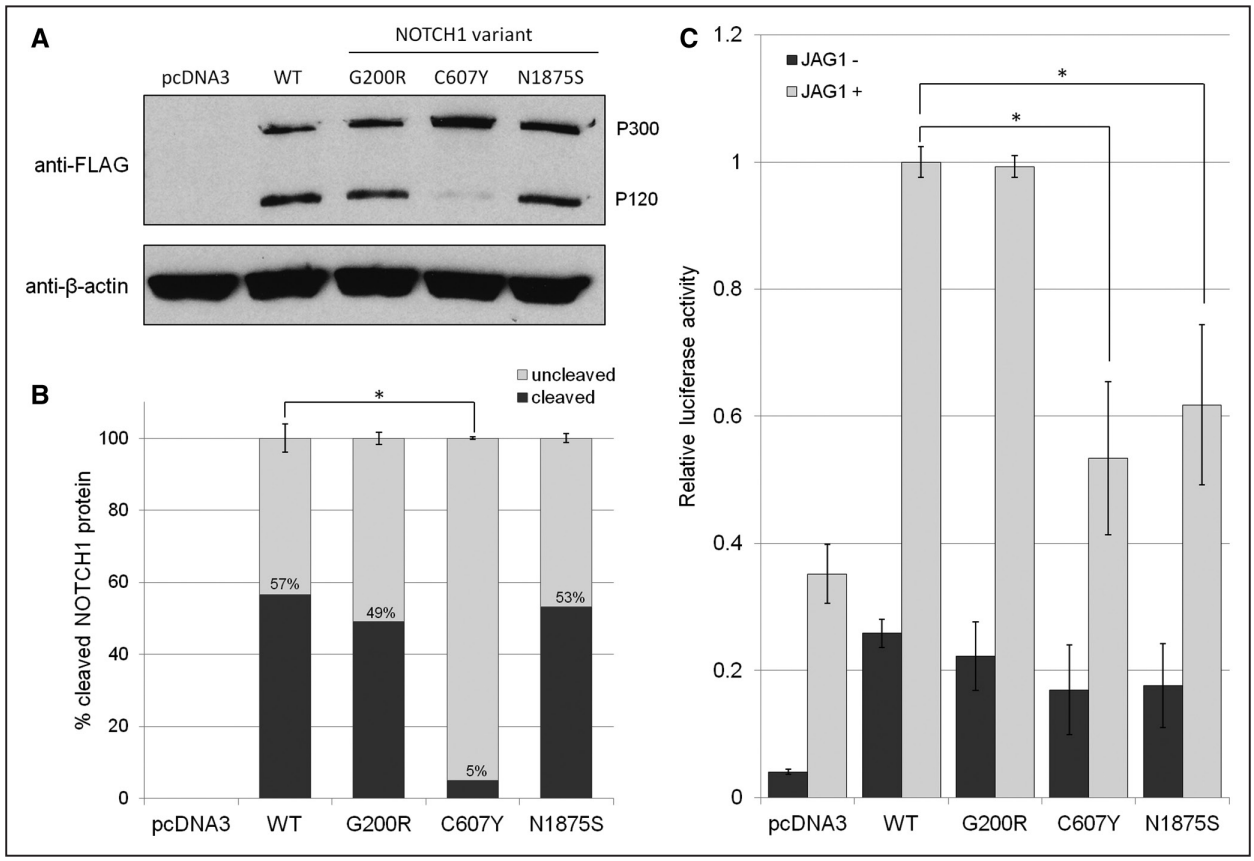

Figure 3. Impact of detected *NOTCH1* variants on NOTCH pathway signaling. **A**, Immunoblot for FLAG to determine the expression and S1 cleavage of NOTCH1 variants p.G200R, p.C607Y, and p.N1875S in comparison to wild-type (WT) NOTCH1 following overexpression in HeLa cells. The 2 bands at 300 kDa (P300) and 120 kDa (P120) represent the full length and the S1-cleaved NOTCH1 protein. β-actin was used as a loading control. **B**, Quantification of the percentage of S1 cleaved vs uncleaved NOTCH1 protein for WT NOTCH1 and NOTCH variants p.G200R, p.C607Y, and p.N1875S. Error bars: mean±SEM from 3 biological replicates and statistical significance was determined using 2-tailed paired *t* tests. **C**, The effect of rare, deleterious *NOTCH1* variants on Jagged-induced NOTCH signaling levels. NOTCH signaling activity was measured using a luciferase-based reporter system (RBPJ). HeLa cells were cultured with or without immobilized JAG1 ligand and cotransfected with RBPJ reporter constructs and WT NOTCH1, p.G200R, p.C607Y, or p.N1875S. Firefly luciferase readings were normalized to Renilla luciferase readings to control for transfection efficiency and cell number. RBPJ activity was expressed relative to WT NOTCH1 for comparison. Error bars: mean±SEM from 4 biological replicates, each with 3 technical replicates. Statistical significance was assessed using 2-tailed paired *t* tests and the Hochberg step-up procedure to control for family wise error rate.

de novo variant p.N1875S reduced NOTCH signaling by 38±0.13% (P=0.02). The p.G200R variant exhibited similar canonical NOTCH signaling to WT NOTCH1 (P=0.67; Figure 3C), yet mapping of this variant to the 3-dimensional NOTCH1 protein suggests structural implications (Online Figure II). Furthermore, p.G200R has also been reported in an independent study to segregate with CHD, supporting its pathogenicity.[41] No significant differences were observed between WT NOTCH1, p.G200R, p.C607Y, and p.N1875S variants in the absence of JAG1 ligand. In each transfection experiment, mRNA expression of WT NOTCH1 and the 3 *NOTCH1* variants was equal (Online Figure III), thus the differences in NOTCH1 signaling observed were not because of reduced mRNA expression of the variants. Hence, 2 variants identified in patients that were subjected to functional testing were shown to affect canonical NOTCH1 signaling.

FLT4 Variants Found in TOF Are Distinct From Those That Cause Milroy Disease

The second most frequent locus of variant clustering in our TOF cohort was *FLT4* (P=4.44×10^{-16}). *FLT4* encodes a receptor tyrosine kinase known as VEGFR3 (vascular endothelial growth factor 3). VEGFR3 is indispensable for lymphatic development, and *FLT4* mutations are a known cause of the hereditary lymphoedema, Milroy disease. Strikingly, all mutations reported for Milroy disease are missense variants or in-frame indels located in the VEGFR3 protein kinase domain (Figure 4). In our TOF cohort of 829 probands, we report 22 unique, deleterious *FLT4* variants in 21 TOF probands, accounting for 2.4% of cases (Online Table IV). Sixteen of the *FLT4* variants were LOF, including 6 premature stop codons (p.Y361X, p.Y369X, p.E896X, p.Q920X, p.R1031X, and p.Q1126X), 6 indels resulting in frameshifts and premature truncation (p.P363fsX25, p.Q423fsX3, p.L636fsX3, p.Y853fsX20, p.N905fsX20, and p.Y1337fsX19), and 4 splice variants (c.3002-1C >T, c.3002-2T >C, c.2300C >G, and c.2849del21). One premature stop codon, p.Y361X, was reported previously in a TOF proband and affected mother.[25] The remaining 6 variants were missense, all of which were located in the immunoglobulin domains of VEGFR3. *FLT4* is extremely intolerant to both LOF and missense variation, as demonstrated by a pLI of 1 and missense Z score of 3.73 on Exome Aggregation Consortium, respectively. In our 1252 reference exomes, no novel, LOF *FLT4* variants were identified. Parent DNA was available for 4 probands. Three of the variants (p.Q920X, p.Y853fsX20, and c.2300C>G) were inherited from unaffected parents indicating incomplete penetrance, and one missense variant, p.C51W, was de novo (Online Table V). Frameshift variant Y853fsX20 was identified in 2 siblings with TOF and was inherited from the mother who was unaffected. Crucially, no missense or in-frame variants were found in the kinase domain, a feature unique to Milroy disease (Figure 4). Our findings are in line with a recent publication by Jin et al[25] that reports LOF variants in *FLT4* in 2.3% of 426 TOF probands. Hence, we confirm this finding in the largest TOF cohort reported to date, approximately twice the size of previous studies, endorsing the importance of *FLT4* as a major contributor to the incidence of TOF.

Discussion

Despite TOF being the most common, severe cyanotic CHD, variants that could account for the high degree of genetic susceptibility, inferred from familial recurrence risk studies,[42] are as yet unidentified. This study represents the largest WES investigation of sporadic, nonsyndromic TOF performed to date. Using variant clustering analysis and stringent filtering, we identify 2 genes that reach genome-wide significance: *NOTCH1* and *FLT4*. As an additional safeguard against false positive results because of systematic methodological differences between our cohort and the studies which contributed to the Genome Aggregation Database database, we studied a set of over 1000 reference exomes in patients free from CHD; analyzed in the same fashion as the case exomes, stringently removing any variant that appeared even once in the reference exome set from consideration as a potential TOF susceptibility variant.

We identify *NOTCH1* as the major TOF susceptibility gene; 4.5% of patients carry heterozygous variants in *NOTCH1*, which based on Genome Aggregation Database allele frequency, bioinformatic in silico prediction, and functional characterization, we judged to be likely susceptibility alleles. With the exception of the 22q11 deletion, no single gene locus has been found to account for more TOF cases than *NOTCH1*. Seven of the variants were LOF, including truncating, frameshift, and splice variants, whereas the remaining 24 variants were missense or in-frame indels and anticipated to be pathogenic. Five out of 7 variants tested were de novo, adding to the evidence for pathogenicity; the remaining variants were transmitted from unaffected parents indicating incomplete penetrance. Previous sequencing studies of CHD have identified an association of *NOTCH1* variants in cardiac malformations, including bicuspid aortic valve, aortic valve stenosis, coarctation of the aorta and hypoplastic left heart syndrome, and TOF.[43–47] However, the extent of *NOTCH1* variant contribution to TOF has not been recognized until now. There are no clear distinctions between the type and location of *NOTCH1* variants identified in TOF compared with those reported in other isolated cardiovascular abnormalities. We, therefore, propose that genetic background and environmental influences may specify phenotypic expressivity.

A possible role for *NOTCH1* in nonsyndromic TOF has previously been suggested by copy number variant (CNV) analysis. A study of 34 infants with nonsyndromic TOF revealed 2 patients with CNVs encompassing the *NOTCH1* gene.[48] Additionally, a microdeletion including the *NOTCH1* locus in a patient with TOF was identified in a study of CNVs in 114 TOF patients.[49] A recent study that focused primarily on families with left-sided CHD also identified family members with TOF harboring pathogenic mutations in *NOTCH1*.[44] Further indirect evidence for *NOTCH1* contribution to TOF came from a study that analyzed the gene expression patterns in TOF patient right ventricles and found many genes from the *NOTCH* and *WNT* signaling pathways were significantly reduced. Interestingly, downregulation of *NOTCH* signaling components was also observed in TOF patients with a 22q11.2 deletion,[50] highlighting a common transcriptional signature between both syndromic and nonsyndromic TOF, initiated by different genetic events. More recently, exome sequencing of

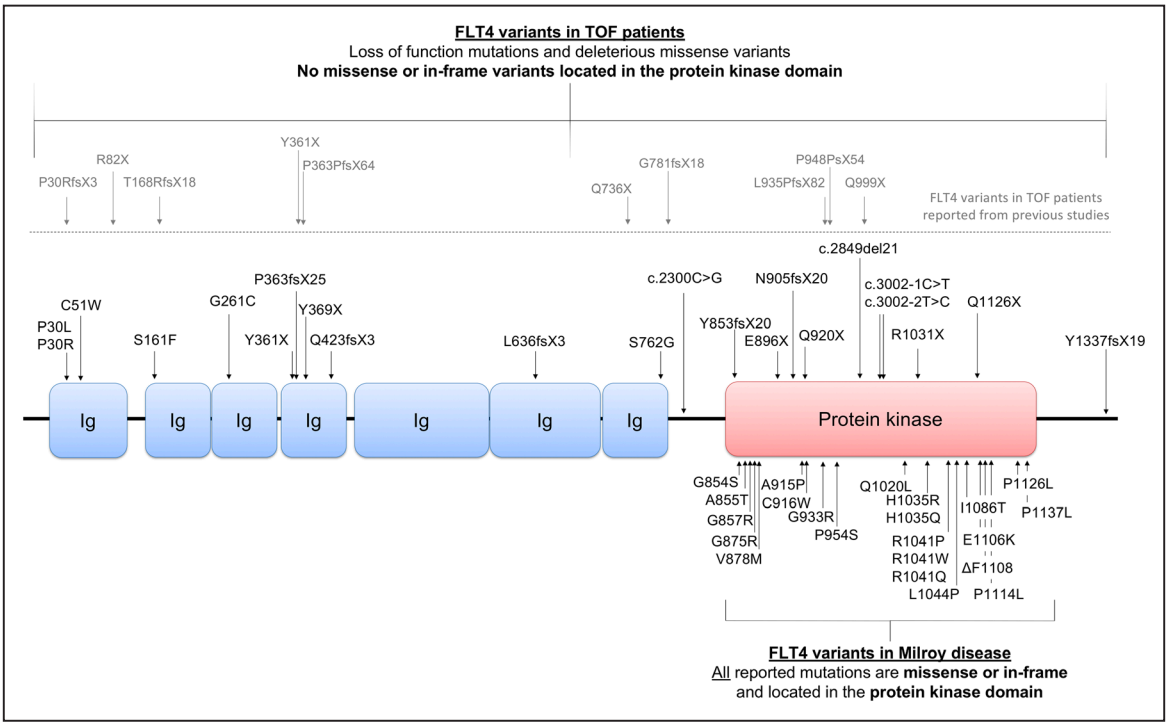

Figure 4. Unique, deleterious *FLT4* variants in Tetralogy of Fallot (TOF) patients. Schematic representation of a FLT4 structure with immunoglobulin (Ig) domains and protein kinase domain, indicated. **Top**: *FLT4* variants identified in our TOF cohort (black) and those previously reported (gray). **Bottom**: *FLT4* missense or in-frame mutations reported in Milroy disease, all located in the protein kinase domain.

426 TOF patients that focused solely on LOF heterozygous variants did not identify an enrichment of NOTCH1 mutations in TOF patients.[25] However, the present study involves, by a substantial margin, the largest TOF cohort studied by WES to date, including both LOF and damaging missense variants, hence providing the most accurate quantification thus far of the contribution of *NOTCH1* variants to TOF risk.

Autosomal dominant germ-line mutations in the *NOTCH1* gene are also one of the causes of Adams-Oliver syndrome (AOS) which is chiefly characterized by aplasia cutis congenita and terminal transverse limb defects. In addition to these features, around half of patients have congenital cardiac anomalies, including an atrial septal defect, ventricular septal defect, aortic valve stenosis, pulmonary valve stenosis, and TOF.[51,52] AOS is an extremely rare syndrome, with a prevalence of ≈1 in 225 000.[52] No patient in our cohort had diagnostic features of AOS. As with other CHDs associated with *NOTCH1* variants, there are no clear distinctions between the *NOTCH1* variants we have identified in TOF versus those that cause AOS, though no previously described AOS variant was present in our cases.[51,52] Interestingly, the extracardiac features of AOS have been suggested to occur because of early embryonic vascular abnormalities,[53] raising the possibility that AOS, TOF, and other cardiac anomalies that occur because of mutations in *NOTCH1* may be a spectrum of disorders. Other examples of syndromic genes that can cause isolated CHD, including TOF, are *PTPN11* (Noonan syndrome),[13,54] *TBX5* (Holt-Oram syndrome),[55] and *JAG1* (Alagille syndrome).[38] Determining the role of genetic background, environmental context, and the specific *NOTCH1* variants in determining the severity of the cardiac phenotype and the occurrence of extracardiac malformations requires further research.

The association of *NOTCH1* with a range of cardiac defects is consistent with the reported roles of NOTCH1 during heart development. Active NOTCH1 is observed in the trabecular endocardium and both global and endothelial-specific knockout of *Notch1* in mice results in abnormal ventricular trabeculae and abnormal cardiomyocyte patterning.[56] Relevant to TOF, Notch1 plays a role in the organization of the outflow tract, which requires the specification of cells from both the neural crest and secondary heart field.[57] Furthermore, Notch1 is important for endocardial epithelial-to-mesenchymal transition, a process that is essential for cardiac valve formation.[46,58] It should, however, be noted that all *NOTCH1* variants we report are heterozygous. There are numerous reports of global and tissue-specific *Notch1* heterozygous mutant mice that appear phenotypically normal, with no obvious cardiovascular pathologies,[59,60] although mice lacking endothelial/endocardial *Notch1* in various backgrounds do present with TOF-like characteristics, including septal defects and abnormal heart valves.[61,62] This suggests endothelial NOTCH1 may be partly responsible for the cardiac malformations associated with TOF, and again, emphasizing the importance of genetic background. In further support of this, *Notch1*$^{+/-}$ in a predominantly 129S6 background developed aortic root dilation whereas *Notch1*$^{+/-}$ in a mixed background did not.[63] Altogether, these reports highlight the importance of genetic background in disease expressivity and are consistent with the incomplete penetrance observed.

De novo mutations are a significant cause of early-onset genetic disorders, including CHD. Of the *NOTCH1* variants identified in this study where parents were available, 5 of 7 variants were found to be de novo. Similarly, we also found

de novo variation in *FLT4*. For both of our genome-wide significant TOF genes, variants were also found to be inherited from unaffected parents, confirming the role of incompletely penetrant variants observed for other CHD genes and phenotypes.[17,20] The incomplete penetrance is in keeping with the complex genetic causes of nonsyndromic TOF. Families segregating the condition in a Mendelian fashion are rarely encountered, and both genetic background and in utero environmental factors, can be inferred to play significant roles.

For a subset of *NOTCH1* variants, we provide evidence of functional impact by assessing canonical NOTCH1 signaling. The p.C607Y missense variant perturbed NOTCH1 receptor S1 cleavage by the calcium-dependent enzyme, furin-like convertase. The S1 cleavage site is located at amino acids 1651 to 1654, some distance away from the variant. A similar observation has been reported by McBride et al[64] where *NOTCH1* variant p.A683T, identified in 2 patients with left ventricular outflow tract malformations, also perturbed S1 cleavage by similar levels. In both cases, this led to a 50% reduction in RBPJ luciferase activity.[64] The mechanism by which such variants alter S1 cleavage to such an extent and reduce signaling by just 50% is unclear and requires further research. Furthermore, de novo variant p.N1875S was shown to have significantly reduced JAG1-induced NOTCH signaling relative to WT NOTCH1, providing further support as to the pathogenicity of de novo variants. p.G200R exhibited signaling levels similar to WT. However, in support of this variants pathogenicity, Blue et al[41] identified the same *NOTCH1* variant in an independent study; p.G200R segregated with disease in 2 cousins with right-sided CHD, including persistent truncus arteriosus, ventricular septal defect, pulmonary atresia, and major aortopulmonary collateral arteries. Furthermore, a case of TOF was also reported in the preceding generation, although sequencing analysis was not performed on this relative.

FLT4 was first associated with isolated TOF in a CNV analysis that identified a de novo duplication including *FLT4*, and a deletion of unknown inheritance upstream of *FLT4*.[18] Recent WES studies have also identified *FLT4* to be a significant contributor to the incidence of TOF. Jin et al[25] found 2.3% of TOF patients to have LOF *FLT4* mutations. Furthermore, Szot et al[65] also identified an *FLT4* variant in a family with TOF. Using our larger cohort, we confirm *FLT4* variants to be a significant contributor to the incidence of TOF, with 2.4% of our cohort exhibiting deleterious *FLT4* variants. In addition to LOF variants, we also identify a small number of pathogenic missense variants, including one variant that is de novo. The encoded product of *FLT4*, VEGFR3, has a well-established role in lymphatic development and in the adult, the VEGFR3 expression is almost entirely restricted to lymphatic vessels.[66,67] During embryonic development, VEGFR3 is also expressed in vascular endothelial cells and is crucial for blood vessel development. Loss of VEGFR3 in mice leads to lethality at E9.5 because of defects in blood vessel formation and cardiovascular failure.[68–70] This is before the emergence of lymphatics, suggesting VEGFR3 plays a unique role in cardiovascular development, independent of lymphangiogenesis. Importantly, patients with VEGFR3 variants causing Milroy disease are not reported to have congenital heart malformations. The distinction between the locations of the mutations in *FLT4* that cause Milroy disease in comparison to TOF may shed light on the evidently differing roles of the receptor in lymphatic versus heart development.

In addition to *NOTCH1* and *FLT4*, we also report an excess of clustering in several other genes of interest, including *RYR1, ZFPM1/FOG1, CAMTA2, DLX6, PCM1*, and known TOF gene, *TBX1*. A summary of in vivo and in vitro functional data currently available for these genes can be found in Online Table VII. Biallelic heterozygous mutations in *RYR1* have previously been linked to CHD, including TOF, in a small number of cases.[25,26] In addition, a mouse homozygous for the missense mutation I4895T, displayed notable delays in cardiogenesis including abnormal orientation, improper formation of the outflow tract and an atrial septal defect,[71] suggesting a role in early heart development. *ZFPM1*/FOG1 encodes a GATA cofactor previously implicated in heart development. *Fog1* null and endothelial lineage knockout mice develop heart malformations including a double outlet right ventricle and abnormal valve formation.[27] Morpholino knockdown of *fog1* also results in defective cardiac looping in zebrafish.[72] While in vivo models suggest a role for *FOG1* in heart development, we report a suggestive association of human *FOG1* mutations with CHD for the first time. *CAMTA2* interacts with *NKX2-5*, one of the core transcription factors controlling heart development. Together, *Camta2* and *Nkx2-5* promote cardiac hypertrophy in mice.[73] CAMTA2 was also identified as the likely candidate gene from a de novo CNV deletion at 17p13.2 in a patient with congenital pulmonary atresia.[74] *DLX6* encodes a homeobox protein involved with known role in cranial-facial morphogenesis. Interestingly in mice, Dlx6 is negatively regulated by Hand2,[28] a transcription factor crucial for cardiac morphogenesis. The significance of the relationship between HAND2 and DLX6 in the developing heart is not clear, although the formation of the great vessels and coronary arteries is reported to be independent of *Dlx6* in mice.[75] *PCM1* encodes Pericentriolar Material 1, which is essential for centrosomal proteins and microtubule organization. PCM1 also positively regulates ciliogenesis,[76] a process which has been strongly linked to the development of CHDs.[29] After validation in an independently ascertained cohort, investigations of the role these genes during heart development may be of interest. It should be mentioned that *ZNF717* also appears amongst our top TOF-associated genes. ZNF717 is a relatively small gene (<4 kb) yet of all genes, exhibits the highest frequency of nonsynonymous mutations per base pair in our reference exomes. For this reason, we do not consider *ZNF717* to be a TOF candidate gene.

In summary, our findings which, in addition to *NOTCH1* and *FLT4*, identified many potential novel TOF gene candidates, concur with previous studies about the marked locus heterogeneity of the condition. Among the genes that have been implicated in TOF thus far, our large study indicates that *NOTCH1* is the most commonly involved. The 2 most commonly involved genes (*NOTCH1* and *FLT4*) are also both crucial to angiogenesis, suggesting further investigation of common pathways between heart development and angiogenesis may be fruitful. In our top gene candidates, some mutations were de novo, but others were present in apparently asymptomatic individuals, indicating incomplete penetrance. Such incomplete penetrance has been frequently observed, for example, in Mendelian aortopathies, emphasizing the importance of genetic background

in structural cardiac and vascular diseases. Detailed phenotypic studies of mutation carriers who do not have overt CHD using advanced imaging may be of interest to delineate quantitative phenotypes potentially relevant to CHD.

Appendix

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Acknowledgments

This study makes use of the ICR1000 UK exome series data generated by Professor Nazneen Rahman's Team at The Institute of Cancer Research, London.[77] No other persons besides the authors have made substantial contributions to this article.

Sources of Funding

This work was supported by the British Heart Foundation Programme Grant RG/15/12/31616. B.D. Keavney and S. Bhattacharya hold BHF Personal Chairs. S. Bhattacharya was supported by the British Heart Foundation funded GOCHD study project grant. B. Mulder, C.R. Bezzina, and A.V. Postma were supported by the Netherlands Heart Foundation CVON project CONCOR-genes (CVON 2014–2018). The work in Nottingham/Leicester was funded by British Heart Foundation Programme Grant RG/13/10/30376.

Disclosures

None.

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