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A Common Variant in the *PTPN11* Gene Contributes to the Risk of Tetralogy of Fallot

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Abstract

Background—Tetralogy of Fallot (TOF) is the commonest cyanotic form of congenital heart disease. In 80% of cases, TOF behaves as a complex genetic condition exhibiting significant heritability. As yet, no common genetic variants influencing TOF risk have been robustly identified.

Methods and Results—Two hundred and seven haplotype-tagging single nucleotide polymorphisms in 22 candidate genes were genotyped in a test cohort comprising 362 nonsyndromic British white patients with TOF together with 717 unaffected parents of patients and 183 unrelated healthy controls. Single nucleotide polymorphisms with suggestive evidence of association in the test cohort ($P<0.01$) were taken forward for genotyping in an independent replication cohort comprising 392 cases of TOF, 218 unaffected parents of patients, and 1319 controls. Significant association was observed for 1 single nucleotide polymorphism, rs11066320 in the *PTPN11* gene, in both the test and the replication cohort. Genotype at rs11066320 was associated with a per-allele odds ratio of 1.34 (95% confidence interval [CI], 1.19 to 1.52; $P=2.9\times10^{-6}$) in the total cohort of TOF cases and controls; this remained highly significant after

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Disclosures
None.

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Bonferroni correction for 207 analyses (corrected *P*=0.00061). Genotype at rs11066320 was responsible for a population-attributable risk of TOF of approximately 10%.

Conclusions—Common variation in the linkage disequilibrium block including the *PTPN11* gene contributes to the risk of nonsyndromic TOF. Rare mutations in *PTPN11* are known to cause the autosomal dominant condition Noonan syndrome, which includes congenital heart disease, by upregulating Ras/mitogen-activated protein kinase (MAPK) signaling. Our results suggest a role for milder perturbations in *PTPN11* function in sporadic, nonsyndromic congenital heart disease.

Keywords

congenital; genetics; tetralogy of Fallot; PTPN11; association studies

Congenital heart disease (CHD) affects approximately 1% of live births and is a major source of morbidity and mortality in childhood. Among CHD phenotypes, Tetralogy of Fallot (TOF) is the most common cyanotic defect, affecting approximately 3 per 10 000 newborns.[1] TOF is characterized by the presence of a ventricular septal defect between the anterior and posterior limbs of the trabecular septal band, overriding of the aortic valve due to anterocephalad deviation of the outlet septum, right ventricular outflow tract obstruction, and right ventricular hypertrophy. TOF is considered a malformation of the cardiac outflow tract. Although most cases of TOF now undergo complete repair in infancy, there is substantial late morbidity, in particular, from pulmonary valvular insufficiency and atrial arrhythmia.

Approximately 20% of postnatally diagnosed TOF occurs in the setting of chromosomal conditions (notably 22q11 deletion syndrome), or other multisystem malformation syndromes (eg, Alagille syndrome).[2] Recurrence risk studies in the families of the remaining ≈80% of sporadic cases indicate a significant complex genetic component to the risk of TOF.[3,4] Rare variants in cardiac transcription factors such as Nkx2.5 and Tbx1 have been shown in previous studies to account for small proportions of the population-attributable risk of TOF.[5,6] As yet, there is minimal evidence of association between common variation in any candidate gene and TOF risk.

We carried out a genetic association study in nonsyndromic cases of TOF to investigate the effects of common variation in 22 candidate genes on disease risk. During cardiac development, cells are added to the arterial pole of the primary heart tube from the anteriorly situated second heart field; in the fully developed heart, the progeny of cells derived from the primary heart field are essentially restricted to the left ventricle while other structures, including the outflow tract, are derived from the second heart field.[7] We selected candidate genes because of previous evidence (from transcriptional studies, syndromic forms of CHD or mouse models), indicating their potential importance in the second heart field during cardiac development.[8]

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Methods

Patient and Control Recruitment

White patients of British ancestry (adults or children) diagnosed with tetralogy of Fallot (TOF) were recruited from congenital heart disease units in Bristol, Leeds, Leicester, Liverpool, Newcastle, Oxford, and London, United Kingdom. Appropriate ethical committees in the recruiting centers approved the study. All patients (or their parents, if the patient was a child too young to provide consent) gave informed consent. Patients with recognized syndromes associated with CHD (such as 22q11 deletion, Noonan syndrome [NS], or Down syndrome) were excluded. When possible, healthy parents of the patients were also recruited for use in a family-based association approach. When more than 1 member of the family was affected with TOF, we attempted to collect all the affected individuals in the family, but such families were very rare. There were no families recruited in whom TOF or other CHD appeared to be segregating as a Mendelian trait.

In addition to review of the clinical records, all patient samples entered into this study underwent screening for 22q11 deletion using a commercially available Multiplex Ligation-dependent Probe Amplification (MLPA) kit (MRC-Holland) before genotyping was carried out, and the sample was excluded from analysis if a deletion was confirmed. Since complete trios (a case and both parents) were only available for about one third of cases, additional healthy British white controls, recruited as previously described,[9,10] were genotyped. Although controls did not undergo echocardiography or clinical assessment for CHD, any misclassification due to undiagnosed TOF in the controls would be extremely unlikely to have occurred. The total population comprised 754 cases, of which approximately one third had both parents available, and 1502 additional unrelated controls. The population was randomly subdivided into approximately equal-sized discovery and replication cohorts. To provide additional security that allele frequencies in our control population were representative of the healthy UK population, we also obtained genotypes for any single nucleotide polymorphisms (SNPs) showing significant association with TOF from 5376 common controls used in the Wellcome Trust Case-Control Consortium 2 project (www.wtccc.org.uk) for comparison.

Genotyping

Two hundred and seven tagSNPs were identified within 22 candidate genes for TOF. Candidate genes and numbers of SNPs typed are shown in Table 1; a full list of SNPs is provided in online-only Data Supplement Table 1. TagSNPs were identified in the genic region plus 15 Kb upstream and downstream of each gene, using the Phase II SNP data from the HapMap CEU samples of Northern and Western European ancestry (www.hapmap.org). The Tagger utility in the Haploview package was used to generate a list of tagSNPs capturing the common variation at each locus, using a pairwise approach, aiming to capture all SNPs with a minor allele frequency of >0.05 with r^2>0.8.

Genotyping was carried out using 3 platforms. The majority of SNPs were typed using a SEQUENOM MALDI-TOF instrument, as previously described.[11] The optimal plex-level for each genotyping reaction and the forward, reverse, and extension primers for each SNP

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in these reactions were determined using RealSNP software (www.sequenom.com). SNPs were typed in a discovery cohort comprising 213 complete trios, 149 nontrio cases, and 183 unrelated controls. Seven SNPs not typable using SEQUENOM (rs13262643, rs9986272, rs1441642, rs3735816, rs7673205, rs186233, rs2970899) were typed using a proprietary system involving competitive allele-specific PCR (KASPar, KBioScience). SNP rs750472 in the *FoxH1* gene was typed using an Applied Biosystems 7900HT Real-Time PCR System with Sequence Detection System software version 2.3 and predesigned ABI TaqMan probes. Primers and conditions for SNP typing are available on request. SNPs that were associated with TOF risk at the level $P<0.01$ in the discovery cohort were genotyped in a replication cohort, including 70 case-parent trios and 322 nontrio cases together with 1319 unrelated controls.

Statistical Analysis

Quality control of genotypes in the discovery cohort was carried out using PLINK software.[12] Association analysis was carried out using a likelihood-based approach implemented in the UNPHASED program, as this program has the capacity to incorporate data from complete and incomplete trio families, unrelated cases, and unrelated controls to give an overall probability value for association.[13,14] We examined the multiplicative (additive on the log scale) model and present our results as per allele odds ratios with 95% confidence intervals.[15] To make a conservative allowance for multiple testing, we subjected the *P* values for the SNPs genotyped in the entire cohort (N=6) to a Bonferroni correction for 207 analyses (the number of SNPs genotyped at the screening stage); we accepted $P<0.05$ after Bonferroni correction to indicate significant association. We calculated the population-attributable risk of TOF for any significantly associated SNP using the formula

$$\mathrm{PAR}_{\mathrm{allele}}=\frac{[\mathrm{Freq}_{\mathrm{allele}}\times(\mathrm{OR}-1)]}{[1+(\mathrm{Freq}_{\mathrm{allele}}\times(\mathrm{OR}-1))]}$$

Results

Demographics of the population are summarized in Table 2. At the screening stage, 12 SNPs were excluded from analysis for having a minor allele frequency <0.05 in our population, 18 SNPs were excluded for missing <10% of genotypes, and 39 individuals were excluded for missing >10% of genotypes. Among the remaining 188 SNPs, none failed Hardy-Weinberg equilibrium at a threshold of $P<0.001$. Q/Q plots for the SNP association tests showed no overall departure from the expected distribution. (See online-only Data Supplement Figure 1.) All SNPs passing quality control had frequencies in our control population that were concordant with HapMap data from the CEU population. Six SNPs showed suggestive association with TOF risk at a threshold of $P<0.01$ in the discovery cohort and were typed in the replication cohort.

Among those 6 SNPs, there was significant association with TOF risk at $P<0.01$ in the replication cohort for 1 SNP, rs11066320, which is in intron 6 of the *PTPN11* gene (online-only Data Supplement Figure 2). In the entire population, rs11066320 was associated with an odds ratio for TOF of 1.34 (95% confidence interval [CI], 1.19 to 1.52; $P=2.9\times10^{-6}$) per

copy of the minor allele (Table 3). There was no deviation from Hardy-Weinberg equilibrium at this SNP (at $P<0.05$ threshold) in cases, parents, or unrelated controls. The minor allele frequency at rs11066320 was 0.42 in our control population, which corresponded precisely with the frequency in the WTCC2 control cohort of healthy British subjects genotyped using genechip technology, providing additional security that the result was not due to systematic genotyping error. Allele and genotype frequencies at rs11066320 in the entire population are shown in Table 4. The point estimate of the odds ratio (OR) suggests that the rs11066320 genotype was responsible for a population-attributable risk of TOF of ≈10%. A maximally conservative estimate (to allow for the winner's curse phenomenon), using the lower 95% CI of the OR, suggests a PAR of at least 5%. There was no evidence for association of any other SNP with the risk of TOF at the $P<0.05$ significance level (after Bonferroni correction), in the combined test and replication cohorts.

Discussion

This gene-focused association study of common variants in 22 genes of importance in outflow tract development shows evidence for association between the rs11066320 SNP in the *PTPN11* gene and risk of TOF. In our total cohort of 754 cases and 1502 controls, rs11066320 genotype was associated with a per allele relative risk of 1.34 (95% CI, 1.19 to 1.52; $P=2.9\times10^{-6}$). The *P* value for association remained highly significant after application of a Bonferroni correction (acknowledged to be a conservative approach to multiple testing). This study is the first to provide robust evidence that common genetic variation influences the risk of TOF; we calculate that genotype at rs11066320 accounts for at least 5% of the population-attributable risk of the condition.

Most previous genetic studies investigating sporadic (rather than syndromic or Mendelian) CHD have focused on the detection of rare variants through sequencing. Such studies have provided evidence that rare coding sequence mutations in several genes including *Nkx2.5, GATA4, Tbx1*, and *SMAD6* can be found in small numbers of patients (typically <1%) with different CHD phenotypes.[5,6,16-18] Rare copy number variants at several loci in the genome have also been shown to affect the risk of nonsyndromic TOF.[19,20] Before the modern surgical era, the adverse consequences of a diagnosis of CHD are likely to have selected strongly against genetic variants conferring even a small additional risk of CHD. Moreover, since severe CHD is an uncommon condition, the acquisition of sufficiently large cohorts of patients with homogeneous phenotypes to confer adequate power to detect the low odds ratios typically associated with common SNPs is challenging. These considerations may account for the limited number of studies of common variation and CHD risk in the literature to date. Several studies have investigated the C677T SNP at the *MTHFR* gene that is associated with lower plasma folate levels and a higher risk of neural tube defect, but results remain inconclusive.[21] A previous investigation of common variation in the *ISL1* gene showed evidence for association of 2 different haplotypes with CHD risk in a cohort with mixed phenotypes[22]; we were unable to confirm this association in our cohort of patients with TOF, possibly reflecting the different phenotypic composition of the cohorts.

The rs11066320 SNP has not been previously associated with developmental diseases, and its function is unknown. The A allele at rs11066320 tags a long-range (1.6 Mb) haplotype at

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chromosome 12q24 that is associated with blood platelet count and with the risks of myocardial infarction, hypertension, and a variety of autoimmune diseases, including celiac disease, type I diabetes, multiple sclerosis, and systemic lupus erythematosus.[23-28] The risk haplotype for all these conditions, including TOF, shows evidence of having been subject to positive selection in Europeans that occurred some 3600 years ago; this has been hypothesized to be owing to enhanced infectious disease resistance.[23] Our result adds to the already remarkable disease pleiotropy associated with the 12q24 chromosomal region.

PTPN11 is the strongest candidate gene for TOF among the 15 located within the 1.6-Mb haplotype tagged by rs11066320 (listed in online-only Data Supplement Table 1). *PTPN11* is a nontransmembrane member of the protein-tyrosine phosphatase family. These proteins function in intracellular signaling cascades by modulating the phosphotyrosine content of their target molecules.[29] *PTPN11* consists of 2 tandem SRC homology 2 (SH2) domains, which facilitate binding of SHP2, the protein encoded by *PTPN11*, to phosphotyrosine residues on its targets and a carboxy-terminal PTPase catalytic domain. The N-terminal SH2 domain regulates SHP2 activity through conformational changes that occur when phosphopeptides are encountered.[30]

PTPN11 missense mutations that result in gain of function are present in >50% of patients with NS (OMIM 163950).[31] NS is an autosomal-dominant dysmorphic syndrome, with an estimated incidence of between 1/1000 and 1/2500. It has variable phenotypic expression, involving a characteristic facial appearance, short stature, variable cognitive defects, and cardiac malformation. Seven genes, of which *PTPN11* is the most frequently mutated, have been shown to be causative of NS; mutations in these genes cause upregulation of RAS-MAPK signaling, which is thought to be the common pathway leading to the NS phenotype.[32] The characteristic cardiac abnormalities observed in NS are pulmonary stenosis (1 of the component features of TOF) and hypertrophic cardiomyopathy, although other defects, including TOF (reported in 4% of patients in 1 study), also occur.[33] Lentigines, ECG abnormalities, Ocular hypertelorism, Pulmonary stenosis, retARDation of growth and deafness (LEOPARD) is a much rarer distinct syndrome, with an outflow tract cardiac phenotype that also results from mutations in *PTPN11* that increase downstream RAS-MAPK signaling (OMIM 151100). By contrast with these 2 syndromes, inactivating mutations in PTPN11 (frameshift and nonsense) cause the autosomal dominant bone disease metachondromatosis (OMIM 156250), a condition which is not characterized by CHD.[34] We therefore hypothesize that the common associated variant we describe upregulates SHP2 activity (either directly or through linkage disequilibrium with a causative SNP), to a lesser degree than mutations that cause NS, and thereby leads to a moderate increase in TOF susceptibility. SHP2 is expressed at high levels in neuromuscular tissues in postnatal life but only at modest levels in readily accessible sources of RNA such as blood (http://www.proteinatlas.org/ENSG00000179295/normal); further tissue-based studies will therefore be required to confirm this hypothesis.

It is highly unlikely that our result is artifactual, owing to the inclusion of substantial numbers of unrecognized patients with NS in our TOF cohort. The most common NS mutation, c.922A>G, has an estimated frequency in the population of around 0.0001, with all other mutations at least an order of magnitude less common; by contrast, the frequency of

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the associated allele at rs11066320 was 0.42 in the control population, which indicates that even if complete linkage disequilibrium (LD) (measured as D′) were present, the correlation (r^2) between rs11066320 and any NS-causative mutation would be negligible. Additionally, TOF is not the typical presentation of NS, only occurring in around 4% of patients. Finally, patients in this study had been clinically classified as nonsyndromic and were from families without evidence of Mendelian segregation of CHD.

Some limitations of our study merit comment. TOF is not an entirely homogeneous phenotype; for example, there is heterogeneity regarding aortic arch position and presence of aberrant subclavian vessels. Our study did not have sufficient power to examine whether the genes we studied were responsible for particular subtypes of TOF. Based on animal model data and evidence from human single-gene disease, *PTPN11* is the most likely gene influencing TOF risk among the 15 present in the 1.6-Mb region of 12q24 tagged by rs11066320; however, further functional assessment will be required to determine precisely how the haplotype affects TOF risk. Finally, since the biology of the second heart field (SHF) remains rather sparsely characterized, our candidate genes cannot be considered to have captured all potential for common variation in SHF-expressed genes to affect TOF risk.

With respect to the clinical implications of our work, genotype at rs11066320 accounted for an insufficiently large relative risk to suggest a role for genotyping the SNP as an adjunct to genetic counseling in families in which a case of TOF has occurred. Moreover, further studies involving much larger numbers of cases will be necessary to determine whether rs11066320 genotype interacts with other genetic factors (such as rare copy number variants or de novo mutations) to affect TOF risk. It will also be of interest to establish whether there is a relationship between *PTPN11* genotypes and other common CHD phenotypes. Our demonstration that a common genetic variant affects TOF risk suggests that GWAS approaches in larger cohorts may result in the identification of additional loci.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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CLINICAL PERSPECTIVE

Known genetic syndromes such as Down and DiGeorge syndromes explain approximately 20% of cases of congenital heart disease. There remains a significant familial predisposition to congenital heart disease among the remaining ~80% of cases that are sporadic; 5% to 10% of these can be attributed to rare copy number variants in the genome, but other genetic risk factors remain largely unknown. No common genetic risk factors for congenital heart disease (defined as alleles with >5% frequency in the population) have as yet been robustly identified. We carried out a candidate-gene association study of tetralogy of Fallot (TOF), the most common cyanotic congenital heart disease phenotype. We found strong evidence of association between a common single nucleotide polymorphism in the *PTPN11* gene (rs11066320) and TOF risk, which we replicated in a second independently ascertained cohort. Each copy of the risk allele increased the risk of TOF by ~30%. Sixty-four percent of the population carries 1 or 2 copies of the risk allele. Rare gain-of-function mutations in the *PTPN11* gene cause Noonan syndrome, which is a multisystem malformation syndrome in which pulmonary stenosis and TOF both occur. The long-range haplotype where the risk allele occurs, which spans *PTPN11* and 14 other genes on chromosome 12q21, has been shown by others to be also associated with coronary artery disease, hypertension, blood platelet count, and a variety of autoimmune diseases. Although the relative risk of TOF conferred by rs11066320 alone is too small for genotype to be useful in risk profiling, larger studies incorporating genomewide data may discover additional loci.

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Table 1

Twenty-Two Candidate Genes for Tetralogy of Fallot Investigated in this Study

Gene	Location (Based on NCBI36/hg18)	No. of Exons	Region Investigated (Gene, +/– 15 kb)	No. of SNPs Genotyped
ACVR2A	Chr2: 148 319 040 to 148 404 862	11	115.82	4
BOP (SMYD1)	Chr2: 88148 497 to 88 194 015	10	75.52	27
CITED2	Chr6: 139 735 092 to 139 737 478	2	32.87	4
CRKL	Chr22: 19 601 714 to 19 637 889	3	66.18	5
DHCR7	Chr11: 70 823 105 to 70 837 125	9	44.02	5
FGF10	Chr5: 44 340 854 to 44 424 541	3	113.69	1
FOLH1	Chr11: 49124 764 to 49 186 798	20	92.03	5
FOXH1	Chr8: 145 670 317 to 145 672 526	3	32.21	4
GATA4	Chr8: 11 599 162 to 11 654 918	7	85.76	51
HAND1	Chr5: 153 834 726 to 153 838 017	2	33.29	6
HAND2	Chr4: 174 684 228 to 174 687 953	2	33.726	21
HEY2	Chr6: 126 112 425 to 126 124 107	5	41.68	3
ISL1	Chr5: 50 714 715 to 50 726 314	6	41.6	5
MEF2C	Chr5: 88 051 922 to 88 214 780	12	192.9	29
NKX2-5	Chr5: 172 591 744 to 172 594 868	2	33.125	3
PDGFRA	Chr4: 54 790 204 to 54 859 168	23	98.97	1
PTPN11	Chr12: 111 340 919 to 111 432 099	16	121.18	5
SOX4	Chr6: 21 701 951 to 21 706 826	1	34.876	3
TBX1	Chr22: 18 124 226 to 18 151 110	10	56.89	1
TBX20	Chr7: 35 208 568 to 35 259 767	8	81.2	9
UFD1L	Chr22: 17 817 702 to 17 846 726	12	59.02	7
VANGL2	Chr1: 158 636 991 to 158 665 088	8	58.1	8

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Table 2

Case and Control Populations Genotyped

	Case Trio Families	Non-Trio Cases	Total Cases (Trios Plus Non-Trios)	Unrelated Controls
Discovery cohort (207 SNPs)	213	149	362	183
Replication cohort (6 SNPs)	70	322	392	1319

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Table 3

Association of 6 SNPs With mAF >0.05 in Case and Control Populations and *P*<0.01 in the Discovery Cohort, in the Discovery and Replication Cohorts

SNP	Gene	Discovery Cohort *P* Value	Replication Cohort *P* Value	Entire Population *P* Value	Bonferroni Corrected *P* Value (207 Comparisons)	Odds Ratio (95% CI), Entire Population
rs11066320	PTPN11	0.004	0.0002	2.9×10^{-6}	6.1×10^{-4}	1.34 (1.19–1.52)
rs9385353	HEY2	0.001	0.7	0.08	NS	1.12 (0.99–1.27)
rs3095870	NKX2.5	0.005	0.76	0.14	NS	1.10 (0.97–1.25)
rs723166	FGF10	0.006	0.97	0.04	NS	1.16 (1.01–1.34)
rs121004	CRKL	0.01	0.71	0.08	NS	1.25 (0.97–1.60)
rs3729848	GATA4	0.01	0.97	0.16	NS	1.14 (0.95–1.37)

NS indicates not significant at $P<0.05$ after Bonferroni correction.

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Table 4
Genotype and Allele Frequencies for PTPN11 rs11066320

	Genotypes				Allele Frequencies	
	GG	**AG**	**AA**	**Total Samples**	**G**	**A**
Cases	181	368	154	703	0.5192	0.4808
Parents	318	434	183	935	0.5722	0.4278
Unrelateds	498	698	252	1148	0.5849	0.4151