

Low expression *VEGF* haplotype increases the risk for tetralogy of Fallot: a family based association study

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Congenital heart disease (CHD) presents a huge medical problem, as it affects between two and eight newborn children per 100 live births.¹ Risk factors include alcohol and drug consumption as well as genetic defects. However, chromosomal and single gene defects cause only a relatively minor proportion of cases and, thus, most CHD is considered to be multi-factorial in origin, with various genes interacting with each other or with environmental factors to determine disease liability.² To date, none of the CHD genetic susceptibility factors have been discovered.

Tetralogy of Fallot (TOF) is a common form of CHD, characterised by a subaortic ventricular septum defect (VSD), an overriding aorta, a right ventricular outflow tract obstruction, and right ventricular hypertrophy. TOF occurs in 4.21 of every 10 000 births and is the most common type of CHD with cyanosis after 1 year of life.¹ TOF may occur as part of the DiGeorge syndrome (DGS) which is caused by deletions of chromosome *22q11* and characterised by conotruncal cardiac, craniofacial, thymic, and parathyroid anomalies. However, in most cases (in 2.65 per 10 000 children) TOF occurs as an isolated defect. Mutations in the *JAGGED1* or *NKX2.5* genes have been found in only a few percentages of cases with isolated, non-syndromic TOF and thus, the genetic etiology in the large majority of these cases remains entirely unknown.² By using a multi-genetic approach, we recently discovered that *VEGF* is a modifier of DGS.³ We therefore assessed here whether *VEGF* might be a modifier of the cardiac birth defects in subjects with isolated, non-syndromic TOF.

METHODS

Study design and participants

To examine whether *VEGF* gene variations are associated with TOF, we used the transmission disequilibrium test (TDT) to analyse linkage disequilibrium of single nucleotide polymorphisms (SNPs) in the *VEGF* gene in trios of parents and their proband affected with TOF. A total of 148 probands (84 males, 64 females; 73 Caucasians, 58 Caucasians of Flemish origin, four Afro-Americans, two Hispanics, and 11 trios of unknown or mixed ethnic origin) and their parents were studied. Ninety trios were recruited by the Children's Hospital in Philadelphia, USA, and 58 trios by the University Hospital in Leuven, Belgium. Criteria for diagnosis of TOF included the presence of a subaortic VSD, an overriding aorta, a right ventricular outflow tract obstruction (infundibular, valvar, supravalvar, or a combination), and right ventricular hypertrophy. Subjects with a *22q11* deletion were excluded from the analysis. To determine whether the association was specific for TOF, we also studied probands with transposition of the great arteries (TGA). A total of 40 probands (27 males and 13 females; 29 Caucasians, one Asian, one Hispanic, three Afro-Americans, and six of mixed or unknown ethnic origin) and their parents were recruited at the Children's Hospital, Philadelphia. Thirty seven patients were diagnosed

Key points

- Tetralogy of Fallot (TOF), one of the most common forms of congenital heart disease, occurs as part of the DiGeorge syndrome (DGS). However, in most cases, TOF is not caused by chromosomal or single gene defects, but presumably results from genetic variations of several susceptibility factors.
- Here, we report that common polymorphisms in the *VEGF* promoter (–2578A, –1154A) and leader sequence (–634G), known to lower vascular endothelial growth factor (VEGF) levels, increase the risk for TOF. Genotyping of 148 families with isolated, non-syndromic TOF revealed that the low-*VEGF* haplotype –2578A/–1154A/–634G (AAG) was overtransmitted to affected children ($p=0.008$). Moreover, meta-analysis of patients with isolated, non-syndromic TOF and DGS patients with TOF revealed that the AAG haplotype increased the risk for TOF 1.8-fold ($p=0.0008$).
- *VEGF* is the first modifier gene identified for TOF.

with a D-TGA, which is the more common form of TGA and consists of a complete inversion of the great vessels, so that the aorta arises from the right ventricle and the pulmonary artery from the left ventricle, while three patients were diagnosed with an L-TGA, in which the ventricles are inverted. The study was approved by the Institutional Review Board for the Protection of Human Subjects and the Research Ethics Board in Philadelphia and Leuven, respectively, and written informed consent to participate in the study was obtained from all subjects or their legal representatives.

SNP genotyping

Three SNPs in the *VEGF* gene regulatory regions were studied: the first two SNPs (–2578C/A and –1154G/A; positions relative to the *VEGF* translation start site) are located in the *VEGF* promoter and the third SNP (–634G/C) is located in the *VEGF* leader sequence. Characterisation of the *VEGF* genotypes was performed by TaqMan minor groove binding probes and real time allelic discrimination of *VEGF* SNPs. The following forward and reverse primers were used for the –1154G/A SNP: 5'-CCGCTACCAGCCGACTTTT-3' and

Abbreviations: CHD, congenital heart disease; DGS, DiGeorge syndrome; SNP, single nucleotide polymorphism; TDT, transmission disequilibrium test; TGA, transposition of the great arteries; TOF, tetralogy of Fallot; VEGF, vascular endothelial growth factor; VSD, ventricular septum defect

Table 1 Transmission of *VEGF* alleles from heterozygous parents to TOF affected children

Gene variation	Risk allele	Allele frequency (%)	Transmitted risk alleles	Untransmitted risk alleles	χ^2	p Value
-2578 C/A	-2578A	0.49	87 (0.59)	61 (0.41)	4.6	0.016
-1154 G/A	-1154A	0.32	73 (0.59)	50 (0.41)	4.3	0.019
-634 G/C	-634G	0.66	77 (0.59)	54 (0.41)	4.1	0.022

The exact number of transmitted and untransmitted risk alleles with their relative frequency between brackets is indicated.

5'-TGGGTAATTTTCAGGCTGTGAAC-3'; and for the -634G/C SNP: 5'-CAGAGACAAGTCGAGGAAGAGAGAGA-3' and 5'-CCCCAAAAGCAGGTCCTCA-3'. The following labelled probes were used for the -1154G/A SNP: 5'-CCTCAGCCCTTCC-VIC-3' and 5'-CCTCAGCCCCTCC-FAM-3'; and for the -634G/C SNP: 5'-CTGTCGCTTTCGCTGCTGCTCGCA-FAM-3' and 5'-TGCCCTGTCCCTTGGCTGC-TET-3'. The -2578C/A SNP was genotyped by pyrosequencing as described previously.⁴

Statistical analysis

The GeneHunter transmission disequilibrium command was used to calculate the non-parametric linkage statistic for the *VEGF* alleles and haplotypes.⁵ Because previously obtained biological data suggested which low-*VEGF* alleles or haplotypes might increase the risk for TOF, we knew in advance the direction of the test.^{3,4} We therefore calculated one sided p values by halving the two sided probabilities generated by GeneHunter for the AAG haplotype and for the *VEGF* alleles in the direction of their appearance in the AAG haplotype. For the meta-analysis, we compared the number of AAG haplotypes in cases and controls from the DGS study (replication study) and calculated the crude odds ratio and 95% confidence interval (CI) by χ^2 analysis with 1 degree of freedom.³ The odds ratio for the TDT study (present study) was calculated by treating the number of transmissions of the AAG haplotype as the number of occurrences of that haplotype in cases and considering the controls to be a very large population with equal numbers of the haplotype (to reflect the expected 50:50 transmission ratio).⁶ As both studies measure a different true effect (that is, the risk in non-syndromic TOF or syndromic TOF), we calculated the average effect or pooled odds ratio for the AAG haplotype under a random effects model.⁷ To take into account the possibility of heterogeneity across both studies, a statistical test for heterogeneity was performed based on the Q statistic, for which a p value < 0.05 indicates a lack of heterogeneity.⁸

RESULTS

We previously established that the -2578A and -1154A alleles, which are located in the *VEGF* promoter, lower *VEGF* gene transcription, while the -634G allele, which is located in the *VEGF* 5'UTR, reduces internal ribosome entry site mediated *VEGF* expression and translation of the large L-*VEGF* isoform.⁴ Genotyping of the *VEGF* SNPs in trios of parents and their proband affected with TOF revealed that the -2578A, -1154A, and -634G alleles were transmitted to

patients with TOF more frequently than expected by mendelian inheritance, that is in 59% of all transmissions (table 1). Thus, alleles associated with low levels of *VEGF* were significantly overtransmitted towards patients with TOF. In European populations, the -2578, -1154, and -634 *VEGF* SNPs are in linkage disequilibrium and only four *VEGF* haplotypes are commonly observed, that is AAG, AGG, CGG, and CGC (each letter refers to the SNP of the -2578, -1154, and -634 allele, respectively; see table 2 for more explanation).⁴ Despite the genetic heterogeneity of the American population, the same four haplotypes (that is, AAG, AGG, CGG, and CGC) present in European populations also predominated in the American population.⁴ Haplotypes could therefore be converted into genotypes and the parent to offspring transmission of haplotypes unambiguously determined for all families. The AAG haplotype, which lowers *VEGF* expression,⁴ was transmitted in 61% of all transmissions (p = 0.008; table 2). The AGG and CGG haplotypes were not significantly overtransmitted to affected children, though there was a tendency for the AGG haplotype to be overtransmitted (table 2).

To further assess whether the association of *VEGF* with isolated, non-syndromic TOF was not false-positive, we performed a meta-analysis by calculating the pooled odds ratio for the association of the common AAG haplotype with isolated, non-syndromic forms of TOF (present study) and with TOF associated with DGS (replication study³). By meta-analysis, the AAG haplotype was found to increase the risk 1.8-fold under a random effect model (CI = 1.3 to 2.5; p = 0.0008; test of homogeneity Cochran Q, p = 0.15). Thus, the low-*VEGF* AAG haplotype conferred an increased risk for TOF, both when occurring as an isolated, non-syndromic defect as well as in the context of DGS.

To determine whether this association was specific for TOF, we also studied probands with TGA and their parents. None of the *VEGF* alleles or haplotypes were transmitted significantly more often than expected by normal mendelian inheritance (table 3). This suggests that the association between the low-*VEGF* alleles and TOF was specific and that low-*VEGF* alleles do not simply predispose to CHD. A negligible role for *VEGF* in TGA may not be surprising, as this lesion does not belong to the typical spectrum of conotruncal defects in DGS patients and mouse models.⁹⁻¹¹ In addition, there is little evidence for a genetic predisposition for TGA, as only very few familial cases have been reported to date¹² and a recent study even failed to identify any recurrence among offspring of adults with TGA.²

Table 2 Transmission of *VEGF* haplotypes from heterozygous parents to TOF affected children

Haplotype	Haplotype frequency (%)	Transmitted haplotypes	Untransmitted haplotypes	χ^2	p Value
AAG	0.33	72 (0.61)	46 (0.39)	5.8	0.008
AGG	0.17	44 (0.55)	36 (0.45)	0.8	0.19
CGC	0.33	52 (0.41)	74 (0.59)	3.2	0.04
CGG	0.17	43 (0.47)	49 (0.53)	0.4	0.53

The *VEGF* haplotypes and their frequencies are presented. The exact number of transmitted and untransmitted haplotypes with their relative frequency between brackets is indicated. AAG: -2578A/-1154A/-634G haplotype; AGG: -2578A/-1154G/-634G haplotype; CGC: -2578C/-1154G/-634C haplotype; CGG: -2578C/-1154G/-634G haplotype.

Table 3 Transmission of *VEGF* alleles from heterozygous parents to TGA affected children

Gene variation	Risk allele	Allele frequency (%)	Transmitted risk alleles	Untransmitted risk alleles	χ^2	p Value
-2578 C/A	-2578A	0.48	18 (0.53)	16 (0.47)	0.1	0.73
-1154 G/A	-1154A	0.34	16 (0.48)	17 (0.52)	0.03	0.86
-634 G/C	-634G	0.73	16 (0.55)	13 (0.45)	0.3	0.58

The exact number of transmitted and untransmitted risk alleles with their relative frequency between brackets is indicated. Two sided p values are presented.

DISCUSSION

Both environmental and genetic risk factors are generally considered to contribute to CHD. However, single gene defects, such as mutations in *TBX1*, *TBX5*, *GATA4*, and *NKX2.5*, explain only very few CHD cases (<1–4%) and, even then, their clinical spectrum is often quite variable.^{13–15} It has, therefore, often been hypothesised, but never proven, that susceptibility to and the expressivity of most cases of CHD is in fact caused by genetic variations and several interacting susceptibility factors.^{12 16 17} The most significant finding of this study is that *VEGF* may well be such a modifier gene for TOF. Indeed, a low-*VEGF* haplotype (AAG), commonly occurring in up to a third of European and American populations, increased the risk for the development of an isolated, non-syndromic form as well as a syndromic form of TOF. To the best of our knowledge, this is the first evidence for an association between a modifier gene and any type of CHD.

How does VEGF modify the clinical spectrum of CHD and, in particular, of TOF? In mice *Vegf* expression in the developing heart occurs in a highly specific spatio-temporal pattern. At the onset of chamber septation, *Vegf* is up regulated in two diametrically opposed regions of the myocardium, which precisely mark the sites of atrioventricular canal formation.^{18 19} *Vegf* expression hotspots are also present in the fourth and sixth pharyngeal arches, preceding remodelling of the corresponding arch arteries.³ Thus, VEGF is expressed at sites known to play a critical role in the formation of the atrioventricular canal and the remodelling of the large thoracic arteries. That VEGF is also functionally involved and that VEGF levels must be tightly regulated in cardiac morphogenesis is evidenced by various genetic mouse models. For instance, lack of even a single *Vegf* allele disregulates embryonic heart formation,^{20 21} while newborn mice lacking the most critical *Vegf*¹⁶⁴ isoform die of DGS-like cardiac birth defects—several of them due to TOF.³ All these findings thus indicate that precise control of *VEGF* expression is required for cardiac morphogenesis, even though the downstream pathways of VEGF involved in this process remain to be elucidated. VEGF was once considered to be the most endothelial cell-specific growth factor, but numerous studies now indicate that this growth factor also affects neural, epithelial, and mesenchymal cells.^{22 23} Whether VEGF thus affects neural crest cells, epithelial arch endoderm cells, or other cell types involved in cardiac morphogenesis is an outstanding but intriguing question. Whatever the mechanism, the role of VEGF must be specific, as this growth factor is not associated with the transposition of the great arteries.

In conclusion, the present human genetic evidence that the AAG haplotype of the *VEGF* gene increases the risk for DGS associated and non-syndromic TOF, together with our previous functional in vivo data in genetic mouse and zebra fish models and with the in vitro findings that the “at risk” AAG haplotype lowers *VEGF* expression,⁴ suggests that *VEGF* is indeed a modifier of TOF.

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