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VEGFA polymorphisms and cardiovascular anomalies in 22q11 microdeletion syndrome: a case-control and family-based study

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ABSTRACT

Microdeletion 22q11 in humans causes velocardiofacial and DiGeorge syndromes. Most patients share a common 3Mb deletion, but the clinical manifestations are very heterogeneous. Congenital heart disease is present in 50-80% of patients and is a significant cause of morbidity and mortality. The phenotypic variability suggests the presence of modifiers. Polymorphisms in the VEGFA gene, coding for the vascular endothelial growth factor A, have been associated with non-syndromic congenital heart disease, as well as with the presence of cardiovascular anomalies in patients with microdeletion 22q11. We evaluated the association of VEGFA polymorphisms c.-2578C>A (rs699947), c.-1154G>A (rs1570360) and c.-634C>G (rs2010963) with congenital heart disease in Chilean patients with microdeletion 22q11. The study was performed using case-control and family-based association designs. We evaluated 122 patients with microdeletion 22q11 and known anatomy of the heart and great vessels, and their parents. Half the patients had congenital heart disease. We obtained no evidence of association by either method of analysis. Our results provide further evidence of the incomplete penetrance of the cardiovascular phenotype of microdeletion 22q11, but do not support association between VEGFA promoter polymorphisms and the presence of congenital heart disease in Chilean patients with this syndrome.

Key terms: congenital heart disease, DiGeorge syndrome, genetic modifiers, VEGFA, velocardiofacial syndrome

INTRODUCTION

Microdeletion 22q11 syndrome (del22q11) is the most frequent known microdeletion syndrome in humans with an estimated incidence of 1/4,000-1/10,000 live births (reviewed in Kobrynski and Sullivan 2007). The microdeletion causes of approximately 80-90% of cases of DiGeorge syndrome and 95-100% of cases of velocardiofacial (VCFS) and conotruncal anomaly face syndromes. The clinical manifestations of del22q11 are highly variable, and over 180 clinical features have been described. The most frequent findings include congenital heart disease (CHD), seen in 50-80% of patients, palatal abnormalities in 70-100%, endocrine defects such as hypoparathyoidism, hypothyroidism and growth hormone deficiency in 15-50%, immune deficiencies in 10%, learning disabilities in 50-80% and psychiatric

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disorders in 6-30% of affected individuals (Kobrynski and Sullivan 2007; McDonald-McGinn et al., 1999; Ryan et al., 1997; Vantrappen et al., 1999, Repetto et al., 2009). The majority of patients share a common 3Mb deletion, encompassing more than 30 genes. Despite this molecular similarity among patients, the clinical manifestations can vary widely. Presentation of patients with the same deletion can range from a severely ill newborn with DiGeorge syndrome, having congenital heart disease, thymic hypoplasia, hypoparathyoidism and a cleft palate, to a school age child with learning disabilities, diagnosed on the basis of characteristic facial features and hypernasal speech due to velopharyngeal insufficiency.

A major role for haploinsufficiency of the TBX1 gene in the etiology of the congenital anomalies of the syndrome has been proposed. TBX1 is located within the common deletion region and encodes for a transcription factor, T-box 1, which has a relevant role in the formation of the pharyngeal arches and pouches, precursors of the cardiac outflow tract and the craniofacial and cervical structures affected in the syndrome. TBX1-haploinsufficient mice show a reduction in thickness or an absence of the 4th branchial arteries in 20-40% of embryos. Homozygous mutant mice all have cardiac and aortic abnormalities, as well as craniofacial and cervical anomalies. A bacterial artificial chromosome (BAC) containing TBX1 rescues the cardiovascular anomalies in mice hemizygous for the deletion. Varying frequencies of heart defects are seen in mice with hypomorphic or null TBX1 alleles (Jerome and Papaioannou 2001; Liao et al., 2004; Lindsay et al., 2001; Merscher et al., 2001). In addition, a small number of non-deleted patients with VCFS phenotype have been found to harbor mutations in TBX1 (Yagi et al., 2003).

The studies in animal models have also shown that the phenotype of TBX1 haploinsufficient mice varies depending on the strain. Specifically, the penetrance of the cardiovascular anomalies is different in inbred mice compared to mixed genetic background ones, although the types of anomalies are comparable (Jerome and Papaioannou 2001; Taddei et al., 2001). These observations suggest a role for genetic modifiers of the primary effect of the deletion, which may account for the phenotypic variability. In mouse models, candidate genes include Fgf8, encoding for fibroblast growth factor 8 (Vitelli et al., 2002), VEGFA, encoding for vascular endothelial growth factor A (Stalmans et al., 2003), Pitx2, encoding for the paired-like homeodomain transcription factor 2 (Nowotschin et al., 2006) and Crkl, encoding for V-CRK avian sarcoma virus CT10 oncogene homologue-like (Moon et al., 2006).

The role of these and other factors as potential modifiers of the phenotype in humans with del22q11 has not been clearly delineated. VEGFA, located in human chromosome region 6p12, encodes for the vascular endothelial growth factor A that has a crucial role in angiogenesis. In addition to this function, animal models bearing hypomorphic variants of VEGFA and association studies in patients with nonsyndromic CHD have implicated it in structural cardiac anomalies, suggesting a role for VEGFA in heart formation (Lambrechts and Carmeliet 2004; Lambrechts et al., 2005; Stalmans et al., 2003; Vannay et al., 2006; Xie et al., 2007). Stalmans et al. (2003) studied VEGFA polymorphisms c.-2578C>A (rs699947), c.-1154G>A (rs1570360) in the promoter region and c.-634C>G (rs2010963) in the 5' untranslated region (5'UTR) and the presence of congenital heart disease (CHD) in a case-control study of Flemish patients with del22q11 and provided evidence of association with the latter 2 SNPs (Stalmans et al., 2003). These polymorphisms are known to downregulate VEGFA expression (Awata et al., 2002; Lambrechts et al., 2005; Shahbazi et al., 2002). Given the relevance of validation of these studies in independent populations, and the benefits of using test for association that reduce potential population stratification bias (Ott 2004; Lewis 2002), we evaluated the association of these 3 polymorphisms in the VEGFA promoter with the presence of CHD in Chilean

patients with del22q11, using case-control and family-based designs.

METHODS

Patients with del22q11 syndrome, confirmed by fluorescence in situ hybridization (FISH) testing with TUPLE1 probe (Abbott Molecular, IL, USA), and known cardiac and great vessel anatomy by cardiac ultrasound, angio-computed tomography or catheterization, were invited to participate. The study was approved by the Ethics Committee at each participating institution, and all participants and/or their parents gave written informed consent.

Patients had been diagnosed with del22q11 and were followed in 5 centers in Santiago and one in Viña del Mar, Chile. Clinical information was collected by a standard questionnaire, and peripheral blood samples for DNA extraction were obtained from each patient and his/her parents. The patients are part of the cohort described by Repetto et al (2009). Patients were classified as with or without congenital heart based on their imaging studies. Patients with normal intracardiac anatomy, but with abnormalities of the great vessels such as right sided aortic arch or aberrant subclavian artery, were classified as having CHD. Only the primary or most significant defect was considered for the analysis. For the purpose of this study, patients with intracardiac or great vessel anomaly were considered cases, and those with normal anatomy, were considered controls. Data was collected and statistical analysis performed using SPSS v12.0 (SPSS Inc, Chicago, IL, USA). Comparisons of clinical and demographic features between cases and controls were performed by X^2 or Fisher's exact test for categorical variables, and Student's t test for quantitative variables.

VEGFA polymorphisms c.-2578C>A (rs699947), c.-1154G>A (rs1570360), c.-634C>G (rs2010963) were analyzed by polymerase chain reaction followed by restriction enzyme assay as previously described (Xie et al., 2007) and confirmed by capillary sequencing. Allelic and genotypic frequencies of these 3 polymorphisms were compared between patients with del22q11 with CHD (cases) and without CHD (controls) by X^2 testing. Transmission disequilibrium testing (TDT) was performed to evaluate the transmission of alleles from parents to offspring with and without CHD. This method eliminates population stratification bias and does not require genotypes of additional unaffected relatives. Analysis in families of individuals with and without CHD was performed using the family-based association test (FBAT) method (Horvath et al., 2001) (available at http: // www.biostat.harvard.edu/~fbat/fbat.htm)

Haplotypes for the 3 polymorphisms in VEGFA were constructed from the patients' and parents' genotypes using Haploview (Barrett et al., 2005) (available at http: // www.broad.mit.edu/haploview/haploview-downloads). The haplotype frequencies were compared between patients with and without CHD by X^2 test. A p-value of 0.05 or less was considered evidence of statistically significant difference.

RESULTS

One hundred and twenty two patients with 22q11del were included in the study. The mean age (±1 SD) at diagnosis of the syndrome was 6.3 ± 8.1 years, ranging from newborn to 37.9 years of age. Sixty one cases (50.0%) had CHD. Forty-four percent were male and no gender difference was observed between patients with and without CHD. Patients with CHD were diagnosed at a significantly earlier age than those without CHD. A higher frequency of palatal abnormalities was observed in patients without CHD. Eight cases (6.6%) were inherited, all of them from an affected mother. Only one of the affected mothers had CHD. Relevant clinical features of the patients and types and relative frequencies of CHD are summarized in Tables I and II, respectively.

Samples from 110 mothers and 100 fathers were obtained, resulting in 99 complete trios for TDT analysis, 49% of them from patients with CHD. Allelic and

TABLE I

Clinical characterization of patients with del22q11

	With CHD (n=61)	Without CHD (n=61)	p value
n Males/Females	28/33	26/35	0.85*
Age at diagnosis in years (mean ± 1 standard deviation)	3.9±5.3	8.6±9.4	0.001**
Median age at diagnosis in years	1.1	6.6	
Palatal abnormalities, n affected patients/ n with available data (%)	31/50 (62.0)	48/56 (86.0)	0.005**
Deceased, n patients	2	0	0.49***

* X^2 test **Student's t test ***Fisher's exact test

TABLE II

Results of cardiovascular evaluation

Diagnosis	N patients	%
Normal	61	50
Tetralogy of Fallot	24	19.7
Ventricular septal defect	13	10.7
Interrupted aortic arch type b	7	5.7
Coarctation of the aorta	4	3.3
Atrial septal defect	4	3.3
Truncus arteriosus	3	2.5
Other anomalies	6	4.8

genotypic frequencies of VEGFA promoter polymorphisms in the parents were in Hardy-Weinberg equilibrium. Minor allele frequencies were 0.47 for -2578A, 0.27 for -1154A, and 0.37 for - 634C. These figures are similar to the healthy control population described by Stalmans et al. (2003), of 0.44, 0.31 and 0.32, respectively.

Allelic and genotypic frequencies in patients with del22q11 are summarized in Table III. We observed no statistically significant differences in frequencies between patients with and without CHD.

Linkage disequilibrium (LD) metrics were D'= 0.822 and r^2=0.289 between SNPs -2578 and -1154, D' = 0.857 and r^2 =0.176 between -1154 and -634, and D'=0.644 and r^2= 0.233 between -2578 and -634. VEGFA promoter haplotype frequencies showed no statistically significant difference in patients with or without CHD (Table IV). TDT results are summarized in Table V. We observed no evidence of statistically significant over-transmission of any of the lower activity VEGFA SNPs to patients either with or without CHD.

DISCUSSION

CHD is a frequent manifestation of del22q11, often requiring complex surgical and intensive care management (Jatana et al., 2007). In addition, a higher immediate postoperative mortality has been seen for CHD repair in children with del22q11, compared to children with other syndromes and non-syndromic CHD (Anaclerio et al., 2004). Kyburz et al.(2008) described a 1-year survival of 85% and a 5-year survival of 80% in del22q11 patients with CHD. Given the relatively high frequency of the deletion, the consequences of CHD in morbidity and mortality, the increased survival of the patients and its autosomal dominant transmission, it is relevant to identify the factors that contribute to the risk of having CHD in this group of individuals.

TABLE III

Allelic and genotypic frequencies of VEGFA promoter polymorphisms in patients with del22q11, with and without CHD

Polymorphism	Alleles or genotypes	Patients with CHD, n alleles or genotypes (frequency)	Patients without CHD, n alleles or genotype (frequency)	Total	p value, uncorrected*
VEGFA -2578	C	60 (0.49)	65 (0.53)	125	
rs 699947	A	62 (0.51)	57 (0.47)	119	
	subtotal	122	122	244	0.52
	CC	14(0.23)	16 (0.26)	30	
	CA	32(0.52)	33(0.54)	65	
	AA	15(0.25)	12 (0.20)	27	
	subtotal	61	61	122	0.79
VEGFA -1154	G	84 (0.69)	93 (0.76)	177	
rs1570360	A	38 (0.31)	29 (0.24)	67	
	subtotal	122	122	244	0.20
	GG	29 (0.48)	37 (0.61)	66	
	GA	26 (0.43)	19 (0.31)	45	
	AA	6 (0.09)	5 (0. 08)	11	
	subtotal	61	61	122	0.34
VEGFA -634	C	40 (0.33)	42 (0.34)	82	
rs2010963	G	82 (0.67)	80 (0.66)	162	
	subtotal	122	122	244	0.79
	CC	6 (0.10)	8 (0.13)	14	
	CG	28 (0.46)	26 (0.43)	54	
	GG	27 (0.44)	27 (0.44)	54	
	subtotal	61	61	122	0.84

* X^2 test

TABLE IV

VEGFA promoter haplotype analysis. Haplotypes are expressed with alleles in the following order: c.-2578C>A (rs699947), c.-1154G>A (rs1570360), c.-634C>G (rs2010963)

	Patients with CHD		Patients without CHD		p value*
Haplotype	n	frequency	n	frequency	
CGC	18	0.3	18	0.3	1
AAG	18	0.3	13	0.22	0.69
CGG	11	0.17	15	0.24	0.50
AGG	11	0.17	11	0.18	1
AGC	2	0.04	4	0.06	0.68
CAG	1	0.02	0	0	1

* X^2 test

TABLE V

Transmission disequilibrium testing of VEGFA hypomorphic promoter polymorphisms in families of patients with del22q11, with or without CHD

SNP	Clinical diagnosis	n informative parents	n transmitted alleles	n non-transmitted alleles	T value*	p value
-2578A(rs699947)	With	49	27	22	0.33	0.57
	Without CHD	50	28	22	0.5	0.47
-1154A(rs1570360)	With CHD	43	28	15	3.35	0.06
	Without CHD	35	14	21	1.03	0.32
-634G(rs2010963)	With CHD	36	15	21	0.69	0.41
	Without CHD	41	26	15	2.44	0.11

* McNemar test

This series shows a lower frequency of CHD compared to other large series of reported patients (McDonald-McGinn et al., 1999; Ryan et al., 1997). This may be due to regional or ethnic differences in phenotype or to increased awareness of this syndrome in patients presenting palatal anomalies or cognitive deficits. As described by Oskarsdottir et al., (2005), diagnosis of del22q11 was made significantly later in patients without CHD, since facial and other features may be subtle and not obviously recognized in the neonatal or early infancy periods.

This study evaluated VEGFA promoter polymorphisms and the presence or absence of CHD in a large number of Chilean patients with 22q11 deletion, and found no evidence of association, analyzing the results by case-control design or by TDT, a more robust association test used to avoid population stratification bias. The results contrast with the findings published by Stalmans et al. (2003) in a Flemish group of del22q11 patients that showed a higher frequency of VEGFA promoter SNPs -2578A and -1154A in patients with CHD. As has been described for many other allelic association studies (Ott 2004), these findings were not replicated in our study. This may be due to ethnic differences among the 2 populations, leading perhaps to association with different VEGFA polymorphisms in each of them, or to lack of association, at least in Chilean patients. Alternatively, the sample size may have been insufficient to detect smaller effects, though the study by Stalmans et al. (2003) included a smaller sample size (58 patients with CHD and 33 without) than the present study. Moreover, a recent study of TDT in over 700 cases of non-syndromic CHD and meta-analysis of VEGFA polymorphism in another 500 cases showed no evidence of association, questioning the role of this gene in non-syndromic CHD susceptibility (Griffin et al., 2009).

Similar apparently contradictory findings for another important phenotypic feature of del22q11, psychiatric disease, and COMT gene polymorphism have been published. COMT encodes for cathecol-O-methyltransferase and is located within the deletion region and is therefore haploinsufficient in del22q11. Studies have reported association between COMT 158 Met, a low activity allele and bipolar spectrum disorder (Lachman et al., 1996) or clinically significant behavior problems (Bearden et al., 2005) in patients with del22q11 but others (Baker et al., 2005; Murphy et al., 1999) have found no association with psychotic symptoms or schizophrenia. These differences may be due to factors related to study design that limits their comparison, or to spurious association.

The factors involved in the incomplete penetrance of the cardiovascular abnormalities in patients with del22q11 remain unknown. Additional studies of recently identified downstream targets of

TBX1 (Liao et al., 2008), genes relevant in the embryonic development of the structures affected in del22q11, or analysis of other genes identified by genome-wide association studies, will be relevant to improve our understanding of the pathogenesis and variability of this common genetic syndrome.

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