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Association of Common Variants in *ERBB4* with Congenital Left Ventricular Outflow Tract Obstruction Defects

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Abstract

BACKGROUND—The left ventricular outflow tract (LVOT) defects aortic valve stenosis (AVS), coarctation of the aorta (COA), and hypoplastic left heart syndrome (HLHS) represent an embryologically related group of congenital cardiovascular malformations. They are common and cause substantial morbidity and mortality. Prior evidence suggests a strong genetic component in their causation.

METHODS—We selected *NRG1*, *ERBB3*, and *ERBB4* of the epidermal growth factor receptor (EGFR) signaling pathway as candidate genes for investigation of association with LVOT defects based on the importance of this pathway in cardiac development and the phenotypes in knockout mouse models. Single nucleotide polymorphism (SNP) genotyping was performed on 343 affected case-parent trios of European ancestry.

RESULTS—We identified a specific haplotype in intron 3 of *ERBB4* that was positively associated with the combined LVOT defects phenotype ($p = 0.0005$) and in each anatomic defect AVS, COA, and HLHS separately. Mutation screening of individuals with an LVOT defect failed to identify a coding sequence or splice site change in *ERBB4*. RT-PCR on lymphoblastoid cells from LVOT subjects did not show altered splice variant ratios among those homozygous for the associated haplotype.

CONCLUSION—These results suggest *ERBB4* is associated with LVOT defects. Further replication will be required in separate cohorts to confirm the consistency of the observed association.

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AUTHOR CONTRIBUTIONS K. L. McBride and J. W. Belmont conceived the design of the study, performed data analysis, and interpreted the data. J. A. Towbin contributed to study design and planning, and performed clinical phenotyping. S. M. Fitzgerald–Butt and S. D. Fernbach recruited subjects, collected clinical data, and managed clinical databases. M. Lewin also recruited subjects and contributed echocardiography data. K. L. McBride, G. A. Zapata, and N. J. Seagraves performed genotyping. K. L. McBride performed RT-PCR experiments. G. A. Zender performed cell culture work, DNA extraction, interpreted genotype data and performed data cleaning and quality control. G. A. Zender performed cell culture work and DNA extraction. K. L. McBride drafted the manuscript, J. W. Belmont provided additional intellectual input and revising, and all other authors read and helped revise the final version.

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Keywords

genetics of cardiovascular disease; heart defects; congenital; congenital abnormalities; cardiovascular abnormalities; analysis; genetic association

INTRODUCTION

Congenital cardiovascular malformations (CVMs) are common birth defects, occurring at a birth prevalence of 5 to 8 per 1000 live births (Hoffman and Kaplan, 2002). Despite recent medical and surgical advances, CVMs still cause significant mortality and morbidity in childhood (Centers for Disease Control and Prevention, 1998; Centers for Disease Control and Prevention, 2007).

Aortic valve stenosis (AVS), coarctation of the aorta (COA), hypoplastic left heart syndrome (HLHS), Shone complex, and interrupted aortic arch type A comprise a group of CVMs that collectively result in obstruction of the left ventricular outflow tract (LVOT). These defects cause 15 to 20% of medically significant CVMs (Hoffman and Kaplan, 2002; McBride et al., 2005a; Pradat et al., 2003). LVOT defects exhibit differential rates by both gender (males>females) and ethnicity (European ancestry>African American) (Ferencz et al., 1997; McBride et al., 2005a). They are responsible for a major portion of the mortality from CVMs, due in particular to the high mortality of HLHS (1 year survival of 60–70%) even with surgical palliation (Alsoufi et al., 2007).

Environmental and genetic etiologies have been described for LVOT defects (Jenkins et al., 2007; Pierpont et al., 2007). Exposure of the developing fetus to organic solvents or high levels of phenylalanine (as part of maternal phenylketonuria) have been associated with LVOT defects. These malformations may be seen in conjunction with chromosomal defects, such as 45,X Turner syndrome (Gøtzsche et al., 1994; Hou et al., 1993; Mazzanti and Cacciari, 1998) and del11q23 Jacobsen syndrome (Grossfeld et al., 2004). Overall, roughly 10 to 15% of individuals with an LVOT defect have a chromosome abnormality (Ferencz et al., 1989). Single gene defects causing LVOT defects have been described for only one gene, *NOTCH1*, in two families with bicuspid aortic valve (BAV) and calcific AVS (Garg et al., 2005), and sporadic cases of AVS, COA, and HLHS (McBride et al., 2008). The causes for most LVOT defects, however, remain unknown.

AVS, COA, HLHS, and BAV have been reported to recur within single families (Allan et al., 1986; Huntington et al., 1997; Menahem, 1990; Stoll et al., 1999; Van Egmond et al., 1976). The Baltimore Washington Infant Study was the first to observe a strong association between LVOT defects and familial CVMs, most of which were also LVOT defects. In addition, we and others (Lewin et al., 2004; Loffredo et al., 2004) have noted a much higher rate (~5.5-fold) of BAV among first degree relatives of children with severe LVOT defects compared to the general population, suggesting BAV may be a forme fruste of the more serious LVOT malformations.

Animal models have demonstrated the importance of intrinsic pattern formation through interacting molecular pathways and blood flow-directed remodeling in the formation of the ventricles, their outflow tracts, semilunar valves, and respective great arteries (Brand, 2003; Bruneau, 2008; Olson and Srivastava, 1996). The epidermal growth factor receptor (EGFR) signaling pathway seems particularly important in cardiogenesis through cell-cell interactions (Pentassuglia and Sawyer, 2009). Lack of ventricular trabeculation has been observed in mouse knockout models of ligand *NRG1* (Falls, 2003; Meyer and Birchmeier, 1995) and receptors *ERBB2* (Lee et al., 1995) and *ERBB4* (Gassmann et al., 1995) in this pathway. Semi-lunar valve abnormalities are observed in *NRG1* and *ERBB3* (Erickson et

al., 1997) knockout mice. A possible unifying mechanism leading to these defects is a disturbance of the vascular smooth muscle cell growth, proliferation, or maintenance or alterations in the developing endothelial and endocardial tissues (Fischer et al., 2007; Grego-Bessa et al., 2007; Timmerman et al., 2004).

Our initial efforts to characterize the genetic epidemiology of LVOT defects have been consistent with a large genetic component in their causation, which has been confirmed by others (Cripe et al., 2004; Hinton et al., 2007; McBride et al., 2005b). Heritability of BAV among first-degree relatives of individuals with AVS, COA, or HLHS was estimated at 50 to 90%, with relative risk for AVS, COA, or HLHS of approximately 39. Segregation and multiplex relative risk analysis was most consistent with a complex genetic inheritance involving from 2 to 6 interacting loci (McBride et al., 2005b). A model of autosomal dominant inheritance with reduced penetrance and locus heterogeneity was also consistent for some families. Supporting this, we have recently demonstrated suggestive linkage signals for LVOT defects on chromosomes 2p23, 10q21, and 16p12 (McBride et al., 2009).

The above information suggested LVOT defects share a common pathogenesis and have a strong genetic component. We hypothesized a genetic association study would be able to identify loci predisposing to LVOT defects. Given the animal data, we focused on the EGFR signaling pathway in a candidate gene approach, genotyping single nucleotide polymorphisms (SNPs) in *ERBB3* (12q13), *ERBB4* (2q33), and *NRG1* (8p12). We present here the results of the association study, which identified the association of these defects with the *ERBB4* gene.

METHODS

Subject Recruitment

Index cases and both of their parents (hereafter termed trios) were recruited from two pediatric tertiary care centers (Texas Children's Hospital, Houston, TX, and Nationwide Children's Hospital, Columbus, OH). Cohort 1 was collected from 2000 to 2003 (TX) and cohort 2 was collected from 2004 to 2008 (TX and OH). Informed consent was obtained with an institutional review board-approved protocol. In a few instances, nuclear families of more than three individuals were included. Proband with AVS, COA, HLHS, Shone complex, and interrupted aortic arch type A were ascertained and pedigrees obtained. Diagnosis of the cardiovascular malformation was confirmed by echocardiography, cardiac catheterization, or direct observation at surgery. We included individuals if their cardiac defect was isolated, or co-occurred with a BAV or ventricular septal defect. We excluded those individuals who had a complex cardiac defect (e.g., presence of LVOT defect and second major CVM), had a known chromosomal abnormality, or were diagnosed with a specific clinical syndrome. Detailed information on many families has been described previously (Lewin et al., 2004; McBride et al., 2005b). Subject characteristics are summarized in Table 1. Blood samples were collected, and lymphocytes were transformed to establish lymphoblastoid cell lines using standardized protocols (Gilbert, 2005). DNA was extracted from the transformed cells with the Gentra PureGene kit (Qiagen, Valencia, CA).

Single Nucleotide Polymorphism Selection and Genotyping

The genes *ERBB3*, *ERBB4*, and *NRG1* were selected for genotyping. Genotyping was performed in two stages, comprising a total of 146 SNPs. After data cleaning (see Results, below) 129 SNPs remained. First, 116 SNPs (100 after data cleaning) were genotyped using SNPs selected before the existence of the HapMap data, by molecular inversion probe technology. When the Hap-Map data became available, 46 SNPs were genotyped using

SNPlex technology. There were 41 SNPs available for analysis after data cleaning (12 overlapping the first set).

Initial SNP selection and genotyping was performed in 2003, before the existence of the HapMap phase I data. SNPs were selected from existing databases that had population allele frequencies or were present in two separate databases. The databases were queried by individual chromosome using the coordinates of the National Center for Biotechnology Information reference genome assembly (build 32) for the genome location for each candidate gene. Coordinates were used to identify SNPs between 10 kb 5' and 3' of the candidate gene. We performed genotyping using the molecular inversion probe (MIP) chemistry (Affymetrix, Santa Clara, CA). We used several quality controls in the genotyping. One external control was used in 10 replicates, whereas 10 randomly selected subjects (internal controls) were run in duplicate. Genotype calling was performed with ParAllele proprietary software as described elsewhere (Hardenbol et al., 2003).

When HapMap data became available, additional SNPs were selected and genotyped. The SNP genotype data for each gene was obtained from the HapMap project for the Centre d'Etude Polymorphisme Humain (CEPH) Utah residents with Northern and Western European ancestry (CEU) population. SNPs typed in the first round were also specifically included. Data were downloaded and analyzed in Haploview (Available at: <http://www.broadinstitute.org/haploview>) (Barrett et al., 2005). Tag SNPs were identified with the embedded Tagger algorithm in Haploview using 2 and 3 marker tagging and an r^2 threshold of 0.8.

The selected tag SNP markers were sent to Applied Bio-systems (Carlsbad, CA) for SNPlex assay design. The SNPlex genotyping was completed using the manufacturer's protocols. Genotypes were called with the GeneMapper software (Applied Biosystems) using the default quality control metrics. Genotyping was repeated for samples failing quality control. One external control Centre d'Etude Polymorphisme Humain (CEPH sample) and one internal control (a replicate sample from a randomly picked subject) were run on each 96-well plate. Markers that were considered significant were reviewed, and were re-genotyped using TaqMan chemistry (Applied Biosystems).

Statistical Analysis

Pedcheck (Available at: <http://watson.hgen.pitt.edu/register/docs/pedcheck>) (O'Connell and Weeks, 1998) was used to identify Mendelian inconsistencies. The Hardy-Weinberg equilibrium testing was performed in Haploview (Barrett et al., 2005) using $p < 0.001$ as the cutoff point. Single marker tests were performed in the FBAT program (Available at: <http://www.biostat.harvard.edu/~fbat/default.html>) (Horvath et al., 2001). Haplotype blocks were generated using the subject genotype data in Haploview, defined by the confidence interval method. Those haplotype blocks containing at least one individual SNP with a permuted $p < 0.05$ were used in haplotype testing by inputting the SNPs in the haplotype blocks into FBAT. We performed the analysis using the HBAT algorithm, with minimum haplotype frequency set at 0.01. Empiric p values were generated by permutation tests for individual SNPs and haplotypes, and results reported in the tables as uncorrected permuted p values. The Bonferroni method was used for correction of the p value for multiple testing. For individual marker tests (129 markers), using an alpha of 0.05, the corrected alpha was $p = 0.00039$. Similar correction for the haplotype testing (3 blocks of markers tested with 22 haplotypes analyzed) gives a corrected p value of 0.0023.

Bioinformatics

HapMap phase II data from the CEU population was used to construct linkage disequilibrium plots in Haploview. HapMap data was downloaded into Haploview and linkage disequilibrium (LD) plots were constructed using default parameters and the confidence interval method. Plots for visualizing cross species conservation, enhancer sites, miRNAs, and other genomic detail for *ERBB4* were obtained from the University of California Santa Cruz Genome browser (Available at: <http://genome.ucsc.edu/index.html>). Ensembl (Available at: <http://www.ensembl.org>) and Entrez Gene (Available at: <http://preview.ncbi.nlm.nih.gov/gene>) identification numbers with active links for all three genes are presented in the Supplemental Table. All chromosomal positions are based on the National Center for Biotechnology Information build 36.1.

Resequencing

Individual affected cases comprised of 20 cases each of AVS, CoA, and HLHS, were investigated for possible causative mutations or novel SNPs of all three genes by denaturing high performance liquid chromatography. We designed PCR primers for amplicons that met the best melting curve characteristics using the Navigator software (Transgenomics Laboratories, Omaha, NE) and ran the denatured products on the WAVE MD instrument (Transgenomic). Amplicons generating abnormal chromatograms were directly sequenced to identify the nucleotide change with dideoxy BigDye terminator chemistry on a capillary sequencer (ABI 3730). PCR primers and reaction details are available on request.

Quantitative PCR

Ebstein–Barr virus-derived lymphoblastoid cell lines were grown to 2×10^6 cells/mL, and then harvested. Total RNA was obtained with the RNeasy kit (Qiagen) and cDNA created with the High Capacity cDNA Reverse Transcriptase kit (Applied Biosystems). Real time PCR was performed to quantitate *ERBB4* isoforms JM-a, JM-b, CYT-1, and CYT-2 (using β -actin as internal control) on an Applied Biosystems 7500 real-time PCR system using primers, probes, and conditions as previously described (Junttila et al., 2003).

RESULTS

Genotyping

Genotyping by MIP was performed on 133 European ancestry trios. After data cleaning for poor call rates (<90%), monomorphic markers, or Mendelian inconsistencies, a total of 6 SNPs in *ERBB3*, 55 SNPs in *ERBB4*, and 39 SNPs in *NRG1* were available for analysis. The average call rate was 97.8%, and the repeatability was 99.97%. Estimated genotype error rate (from Mendel errors) was 0.06%.

SNPlex genotyping was performed on the first 133 trios, and an additional 210 European ancestry trios collected after the MIP genotyping was performed. Forty-one markers were available for analysis after data cleaning. Some markers were genotyped in both genotyping platforms, including two in *NRG1*, three in *ERBB3* and seven in *ERBB4*. The remaining markers genotyped in the MIP could be captured by the tag SNPs selected for the SNPlex genotyping. Genotype call rates for the SNPlex genotyping were 96.9%. Repeatability among replicate samples was 99.7%. Mendelian inconsistencies were found in 3.5% of trios, which were removed from analysis. No marker genotyped in either platform exhibited significant departure from Hardy–Weinberg equilibrium proportions using the unrelated parents as the sample. Single marker tests and haplotype tests were performed in FBAT.

Single marker tests of SNPs in *ERBB3* and *NRG1* did not show significant association with LVOT defects. A total of four markers for *ERBB4* achieved a nominal *p* value of < 0.05,

none of which remained significant after correction for multiple testing (Table 2 and Fig. 1). Based on these results, haplotype testing was performed only on SNPs in *ERBB4*.

A total of six haplotype blocks could be constructed from the subject genotype data for *ERBB4* (Supplemental Fig. 1). Haplotype analysis was performed on three haplotype blocks of the *ERBB4* gene in which one or more single SNPs had a permuted p value < 0.05 (blocks 2, 4, and 5 of Supplemental Fig. 1). Analysis of block 2 (containing SNP rs13030304) and block 4 (rs4223543) did not show a significant association. Haplotype testing of block 5 (rs1371203 and rs10203750) demonstrated an association with LVOT defects ($p = 0.0005$, corrected $p = 0.011$). Subanalysis defined one haplotype that seemed to confer increased risk and one that was protective. The haplotype conferring increased risk did so among all defects combined and for each of the defects AVS, COA, and HLHS independently (Table 3).

Haplotype block 5 encompasses a roughly 40 kb region in the 3' part of intron 3. This region contains an area conserved in vertebrates down to *Xenopus*, and also has several ENCODE H3K4Me1 enhancer and DNase hypersensitivity sites (Supplemental Fig. 2). This block is not in strong linkage disequilibrium with either of the neighboring exons 3 or 4 (Supplemental Fig. 3).

Resequencing

Sequencing of exons and intron/exon boundaries was performed on all three genes. No rare variants or pathogenic mutations were found.

ERBB4 Quantitative PCR

Ebstein–Barr virus-derived B-cell lymphoblastoid cells from nine individuals homozygous for the high-risk haplotype and five individuals homozygous for the low-risk haplotype were investigated. Low expression levels of *ERBB4* isoforms JM-a, CYT-1, and CYT-2 were identified. No JM-b could be identified in any individual. There was no difference in expression of any isoform between the two groups (data not shown).

DISCUSSION

This study focused on a small group of candidate genes from a signaling pathway important in cardiac development. We identified an association between *ERBB4*, a gene encoding a receptor tyrosine kinase of the EGFR family, and LVOT defects. The association with LVOT defects is noted not only for the whole group of defects, but also individually for AVS, COA, and HLHS.

The EGFR signaling pathway is important in cardiac development. *ERBB4* (Gassmann et al., 1995) and *NRG1* (Meyer and Birchmeier, 1995) mouse knockouts demonstrate decreased ventricular trabeculation, whereas *ERBB3* knockout animals have valve abnormalities (Erickson et al., 1997). *ERBB4* is a single pass transmembrane receptor tyrosine kinase (Fuller et al., 2008). It homodimerizes or heterodimerizes with other ERB receptors, and is activated by binding with a number of ligands, including Neuregulin1. The gene is located on chromosome 2, covering 1.16 Mb, and contains 28 exons coding for a 1308 amino acid protein. At least four splice variants are known (Junttila et al., 2003). Isoforms JM-a and JM-b are formed by the excision of exon 15 or exon 16, respectively. The CYT-1 isoform contains exon 26, whereas in CYT-2, this exon is spliced out. The presence of JM-a allows cleavage of the receptor and release of an intracellular domain, with subsequent degradation of the receptor. CYT-1 contains a PI3K docking site. Recently, a set of three SNPs around exon 3 were found to be associated with the amount of CYT-1 expression in the brain of patients with schizophrenia (Law et al., 2007).

The precise LVOT defect susceptibility variant in *ERBB4* is not yet known. No coding sequence changes were identified. The associated haplotype lies within intron 3 of the gene, thus a mutation in this area would not have been identified by our sequencing strategy. Investigation of altered splice variant ratios in lymphoblastoid cell lines from individuals with LVOT defects did not reveal any obvious changes. However, *ERBB4* is not well expressed in this cell type (Sei et al., 2007) and may not be indicative of gene expression patterns in the developing heart. Bioinformatics analysis of haplotype block 5 suggests this region may be important based on areas of conservation and presence of enhancer marks contained within the block region.

Recent work from our laboratories, along with the current report, suggests a possible endothelial/endocardial development defect in the pathogenesis of LVOT defects. We have found *NOTCH1* mutations among sporadic cases of AVS, COA, and HLHS, which seem to confer an increased susceptibility to these defects via altered signaling through the NOTCH pathway (McBride et al., 2008). *NOTCH1* is required for both endocardial (Timmerman et al., 2004) and myocardial differentiation (Grego-Bessa et al., 2007). Conditional knockouts of *NOTCH1* in the mouse cause abnormal endothelial to mesenchymal transformation in the developing heart and alter *NRG1* expression in the developing ventricular trabeculae (Grego-Bessa et al., 2007), highlighting the interaction between the NOTCH and EGFR pathways in heart development.

The involvement of this pathway may explain other phenomena observed in LVOT malformations. *NRG1* is becoming more appreciated in cardiovascular physiology through its role in cardiac sympathetic response (Pentassuglia and Sawyer, 2009). This may contribute to postrepair hypertension in individuals with COA (O'Sullivan et al., 2002). *ERBB2* and *ERBB4* seem to be cardioprotective, and dysregulation causes heart failure, particularly in the setting of drug-induced cardiomyopathy such as with herceptin for breast cancer therapy (Perez, 2008). Defects in this signaling cascade may thus contribute to poor response due to ventricular failure seen in many individuals between stages 1 and 2 for HLHS palliation (Simsic et al., 2005).

A possible weakness of our study is inherent to all candidate gene studies: success depends on selection of the correct genes. Although the candidates represented are known to be involved in cardiac development, it is estimated that there may be at least 500 genes critical for normal cardiac development. Our study was also small, with a higher risk of false-negative results.

In summary, we have identified an association between SNPs in the gene *ERBB4* and LVOT defects, collectively and by individual anatomic defect. *ERBB4* joins *NOTCH1* as a susceptibility gene for LVOT defects. Replication of these results in other cohorts of subjects will be required.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1.

Negative log p value of genotyped markers in the *ERBB4* gene plotted by genomic position on chromosome 2. Exon (*vertical bars*) and intron structure of *ERBB4* is indicated at the top of the figure. Single nucleotide polymorphism markers with a nominally significant result are labeled with their reference numbers. SNPs in Haplotype block 5 (position indicated by horizontal bar at top of plot) are represented by the 'X' symbol.

Table 1

Characteristics of the Proband, by Diagnosis, Cohort, and Gender

Diagnosis	COHORT 1	COHORT 2	Combined
AVS	36 (27.1)	63 (30.0)	99 (28.9)
COA	63 (47.4)	74 (35.2)	137 (40.0)
HLHS	32 (24.1)	61 (29.1)	93 (27.1)
Other	2 (1.5)	12 (5.7)	14 (4.1)
Gender			
Male	96 (72.1)	144 (68.6)	240 (70.0)
Female	37 (27.8)	66 (31.4)	103 (30.0)
TOTAL	133	210	343

Numbers reported are frequency (percentage) for each cohort.

Other = interrupted aortic arch type A, mitral stenosis.

AVS, aortic valve stenosis; COA, coarctation of the aorta; HLHS, hypoplastic left heart syndrome.

Table 2

Nominally significant FBAT single marker test results in *ERBB4* by cohort

Marker	Alleles	Position	MAF	COHORT 1		COHORT 2		Combined	
				S-E(S)	p value	S-E(S)	p value	S-E(S)	p value
rs13030304	C:A	211991391	0.207	13.33	0.0042	4	0.3883	17.33	0.0084
rs4423543	G:A	212244291	0.276	7.5	0.1037	10.5	0.0404	18	0.0090
rs1371203	T:C	212392191	0.199	10	0.0153	9.5	0.0562	19.5	0.0025
rs10203750	A:T	212394513	0.130	8	0.0237	6.5	0.0960	14.5	0.0059

Marker is the reference SNP identifier. Alleles are listed as Major:Minor alleles.

Position is in bp of build 36.2 reference assembly. Positive values indicate over transmission, and negative values under transmission of the marker. All *p* values reported are from permutation tests. MAF, Minor allele frequency; S, is the FBAT marker score; E(S), is the expected values of the marker score; T, number of individuals with transmitted risk alleles; U, untransmitted.

Table 3Haplotype analysis for block 5 of *ERBB4* gene, divided by cohort and diagnosis

By Cohort Haplotype (freq)	COHORT 1		COHORT 2		Combined	
	S-E(S)	p value	S-E(S)	p value	S-E(S)	p value
ATACGA (0.585)	12.83	0.012	14.9	0.0055	27.73	0.0001
ATAGTG (0.135)	-4.17	0.2479	-0.46	0.9271	-4.63	0.3488
ACTCGA (0.116)	-6	0.1016	-8.56	0.0079	-14.07	0.0016
ACACGA (0.076)	-3.5	0.1958	-1.87	0.6191	-5.39	0.2273
GTAGTG (0.072)	1.33	0.6869	-3.98	0.197	-2.65	0.5417

By anatomic defect Haplotype	AVS		COA		HLHS	
	S-E(S)	p value	S-E(S)	p value	S-E(S)	p value
ATACGA	7.63	0.0246	9.33	0.0415	8.5	0.0359
ATAGTG	-1.13	0.567	-3.17	0.2808	0	0.9282
ACTCGA	-3.5	0.1487	-6	0.0526	-3.5	0.1549
ACACGA	0.5	0.7236	-2	0.4541	-2	0.4147
GTAGTG	-3.37	0.082	2.33	0.2995	-2.5	0.36

Haplotypes are defined by genotypes of SNPs rs2118891, rs1371203, rs10203750, rs1439238, rs4673638, rs16847087, in that order. S is the FBAT marker score, and E(S) is the expected values of the marker score. Positive values indicate over transmission, and negative values under transmission of the marker. All *p* values reported are nominal values from permutation tests.

Whole haplotype permutation test chi square sum *p* = 0.0005.