The human achaete-scute homologue 2 (ASCL2, HASH2) maps to chromosome 11p15.5, close to IGF2 and is expressed in extravillus trophoblasts


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Identification of a complex congenital heart defect susceptibility locus by using DNA pooling and shared segment analysis

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The identification of genetic loci involved in most forms of congenital heart disease has been hampered by the complex inheritance patterns of these disorders. Atrioventricular canal defects (AVCDs) are most commonly associated with Down syndrome, although non-syndromic cases also occur. Non-syndromic AVCDs have been attributed to multifactorial inheritance. However, the occurrence of a few kindreds with multiple affected individuals has suggested that a major genetic locus can account for the disorder in some families. We have used a combination of DNA pooling and shared segment analysis to perform a high density screen of the entire autosomal human genome in an extended kindred. In so doing, we have identified a genetic locus on chromosome 1 shared by all affected individuals. Our data demonstrate the existence of a congenital heart defect susceptibility gene, inherited as an autosomal dominant with incomplete penetrance, involved in AVCD. Furthermore, our data demonstrate the power of using key isolated kindreds in combination with high density genomic screens to identify loci involved in complex disorders such as congenital heart defects.

INTRODUCTION

The genetic basis of most forms of congenital heart disease remains unknown. Due to the paucity of large kindreds segregating congenital heart disease, few candidate loci for such disorders have been identified. One form of congenital heart disease, atrioventricular canal defects (AVCDs), have been shown to be frequently associated with trisomy 21 (Down syndrome) in humans (1–3) and trisomy 16 in mice (4). In addition, AVCDs have been shown to be associated with partial deletions of human chromosome 8 (5–7). Non-syndromic AVCDs occur in ~1/1000 births (8). Most non-syndromic cases have been considered to be sporadic or the result of multifactorial inheritance (9). The existence of several reported families with multiple affected individuals suggests that some cases of AVCDs may result from a major susceptibility gene displaying dominant inheritance (10–12). However, even in these kindreds, the AVCDs appear to require environmental factors or additional genetic loci due to the observation of non-penetrance and variable expressivity. In 1985, Desegni et al. (12) reported a nuclear family with four affected individuals with a complete or partial AVCD. Both parents were found to be normal by echocardiography. Since the initial report, evaluation of the extended family has resulted in the identification of ten additional affected individuals. The disorder in this kindred presents as a range of phenotypes from complete AVCD to isolated cleft mitral valve. Chromosome 21 was excluded from carrying the genetic mutation resulting in AVCDs in this family (13) and in an unrelated family with AVCDs (14). We now report the findings of a genome-wide screen with highly informative short tandem repeat polymorphic markers (STRPs) to search for a genetic locus involved in AVCDs. In order to facilitate the linkage search, we utilized a DNA pooling strategy (in which DNA samples from affected individuals are pooled together and used as the template for DNA amplification) (15–19), in combination with shared segment analysis (20,21) to identify a region of the genome shared by affected family members.

RESULTS

We hypothesized the existence of a major locus necessary (but not sufficient) for the development of AVCDs in a large kindred with 14 affected individuals (Fig. 1), and devised a strategy to perform a genome-wide search for this locus. If the hypothesis is correct, affected individuals should share a segment of the genome containing the disease susceptibility gene. An efficient method for searching for the shared segment would be to genotype a few key affected individuals with densely spaced STRPs across the genome. Such an approach would be expected to yield numerous markers for which an allele is identical by state in a few distantly related affected individuals, but few markers for which alleles are identical by descent in the affected individuals. Those markers that are identical by descent can be recognized by examining haplotypes of markers in a given region.

The proband (IV-3) and three affected relatives (IV-4, V-1 and V-4) were selected for genotyping with STRPs distributed across
Figure 1. Pedigree of the kindred with AVCD. Open symbols = unaffected individuals; solid symbols = affected individuals and hatched symbols = obligate carriers based on having an affected offspring. Genotype data for five STRPs are given below each symbol. Individuals IV-4, V-3 and V-4 had complete AVCDs. Individual IV-2 had an isolated cleft mitral valve with mitral insufficiency. All other affected individuals had ostium primum atrial septal defects and cleft mitral valves. Individuals III-3, III-12, IV-18 and IV-19 carry the complete disease haplotype, but are clinically unaffected (non-penetrant). Unaffected individuals III-8 and III-20 are recombinant within the disease interval and cannot be excluded from carrying the disease gene. Genotypic data on individual III-19 was derived from genotyping her spouse and four offspring.

the human genome. In addition, a pool of DNA from 13 of the 14 affected individuals and a DNA pool from 40 unrelated normal individuals were also genotyped with each marker. DNA from one affected individual (III-19) was not available for study. STRPs which shared an allele in at least three of the four AVCD patients and in which the shared allele was a predominant allele in the affected DNA pool, based on visualization of the allelic bands on the gel by two observers, were considered to be of interest. Approximately 60 STRPs gave evidence of a shared allele in all four affected individuals. However, for only a third of these STRPs was the shared allele a predominant allele in the affected DNA pool, compared with a control pool of unaffected individuals. We next considered whether there existed a cluster of such markers anywhere in the genome. A cluster of four contiguous markers (D1S1665, D1S1728, D1S551 and D1S1588) which displayed a shared allele in the four AVCD patients and predominance in the affected DNA pool was observed on chromosome 1. No other region of the genome showed more than two contiguous markers with these features. Genotyping of these markers in all available members of the kindred allowed us to establish phase for each marker, and indicated that 13 of the 14 affected individuals shared the same haplotype. The genotypes of affected individual III-19 could be derived from genotyping her spouse and four offspring. Genotyping of additional markers between and flanking these STRPs identified a region shared by all 14 affected individuals.

LOD score analysis using only affected individuals in the kindred demonstrated support for linkage at LOD >4 (Table 1). The highest lod score was observed with marker D1S406 (LOD = 4.01, θ = 0). This marker is completely informative and completely linked. A summary of the two-point linkage data is shown in Table 1. The narrowest interval is determined by recombination events in individual IV-4 with markers proximal to and including D1S3471 (GATA65B07), and in individual IV-9 with markers distal to and including D1S1587 (ATA1D01) (Fig. 2). These data define the current interval to be between markers D1S3471 and D1S1587, a distance of ∼12 cM (Fig. 3).

The availability of DNA and reliable phenotypic information from multiple members of the kindred allowed us to investigate the degree of penetrance of AVCD in this kindred. All unaffected first degree relatives for which there existed reliable clinical
Figure 3. The location of linked markers on chromosome 1 defining the AVCD locus. Recombination distances are shown in centimorgans. Markers D1S3471 and D1S1587 are flanking markers.

Table 1. Pairwise linkage data between AVCD susceptibility locus and chromosome 1 markers using affected individuals only

<table>
<thead>
<tr>
<th>Marker</th>
<th>0</th>
<th>0.05</th>
<th>0.1</th>
<th>0.2</th>
<th>0.3</th>
<th>0.4</th>
<th>θ_{max}</th>
<th>Z_{max}</th>
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<tr>
<td>GGAA10G11</td>
<td>–</td>
<td>∞</td>
<td>2.22</td>
<td>2.19</td>
<td>1.80</td>
<td>1.28</td>
<td>0.68</td>
<td>0.065</td>
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<tr>
<td>GATA6S8B07</td>
<td>2.77</td>
<td>2.51</td>
<td>2.24</td>
<td>1.71</td>
<td>1.16</td>
<td>0.60</td>
<td>0</td>
<td>0.060</td>
</tr>
<tr>
<td>DIS406</td>
<td>4.01</td>
<td>3.64</td>
<td>3.27</td>
<td>2.50</td>
<td>1.70</td>
<td>0.88</td>
<td>0</td>
<td>4.01</td>
</tr>
<tr>
<td>ATAI1D01</td>
<td>–</td>
<td>2.48</td>
<td>2.42</td>
<td>1.97</td>
<td>1.36</td>
<td>0.70</td>
<td>0.062</td>
<td>2.48</td>
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<tr>
<td>GATA124C08</td>
<td>2.11</td>
<td>2.08</td>
<td>1.69</td>
<td>1.16</td>
<td>0.59</td>
<td>0.065</td>
<td>2.13</td>
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Table 2. Pairwise linkage analysis at various penetrance values between DIS406 and AVCD using all available pedigree members

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<th>Penetrance</th>
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<th>LOD score</th>
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<td>1.000</td>
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</table>

DISCUSSION

We report the identification of a chromosome 1 locus involved in the development of atrioventricular canal defects. Linkage to this locus is supported by a positive LOD score of 4.01 at θ = 0, and by the common haplotype across the interval shared by all affected individuals (Fig. 3). Based on the narrowest shared interval in affected individuals, the AVCD susceptibility gene mapped in this study lies between flanking markers D1S3471 and D1S1587, an interval of ~12 cM (Fig. 3).

Our data demonstrate that AVCD is a complex disorder with a major genetic component. In the kindred used in this study, the disorder displays both incomplete penetrance and variable expressivity, indicating the involvement of additional genetic and/or environmental factors. The phenotype in this kindred ranges from an isolated cleft mitral valve to the complete AVCD. The most common phenotype observed in 10 individuals is the presence of an ostium primum atrial septal defect (ASD) and a cleft mitral valve. Three individuals were found to have complete AVCDs, and one individual had an isolated cleft mitral valve. The prevalence of AVCDs seen in patients with trisomy 21 suggests that a gene or genes on chromosome 21 also contribute to AVCDs (1–3). In addition, several individuals with partial deletion of chromosome 8 have the AVCD phenotype, indicating another
susceptibility gene on chromosome 8 (5–7). The current study demonstrates the existence of a gene involved in non-syndromic AVCD. It is intriguing to speculate that variants of the chromosome 1 AVCD susceptibility gene could modify the severity of the AVCD phenotype seen in patients with trisomy 21. In order for this hypothesis to be correct, alleles of this gene displaying a lower penetrance and milder effect than the allele segregating in this family would need to exist. Similarly, other loci (i.e. chromosome 21 or chromosome 8 loci) could modify the phenotype resulting from a defect in the AVCD susceptibility gene on chromosome 1.

A number of candidate genes lie within the AVCD interval. Because of the relatively large size of this interval and the imprecise mapping of some genes assigned to chromosome 1, the list of candidates remains quite large. However, it is interesting that the genes for two important extracellular matrix molecules, collagen VI and collagen XI, map to the AVCD critical regions of chromosome 21 and chromosome 1, respectively. We are currently attempting to refine the genetic interval of AVCDs on chromosome 1 in order to narrow the search for the disease gene.

Pooling of DNA samples from inbred pedigrees has been a successful approach for mapping recessive diseases (15–19,22,23). This approach greatly facilitates the mapping of genetic loci by vastly decreasing the number of genotypes necessary for linkage mapping. However, this approach has not been previously utilized to map dominant or multifactorial disorders. Shared segment analysis utilizes extensive genotyping of a few individuals to identify loci inherited by descent. This method has been successfully used to identify loci involved in recurrent intrahepatic cholestasis (20), Hirschsprung disease (24) and hereditary ataxia (25). We now demonstrate the use of a combination of DNA pooling and shared segment analysis to map a disorder showing both incomplete penetrance and variable expressivity. We genotyped four affected individuals, in addition to a pool of DNA from all affected individuals. The genotyping data from the four individuals served to indicate the putative disease associated allele for a given marker, whereas the pooled DNA sample allowed for rapid evaluation as to whether the disease associated allele was likely to be shared by most of the affected individuals. Using this approach, we were able to rapidly identify a region of the genome shared in common by all affected individuals.

Several factors played a role in the successful search for an AVCD locus in this study. A set of highly polymorphic STRPs distributed across the genome was essential. The marker set utilized in this study consisted mostly of tri- and tetranucleotide STRPs generated by the Cooperative Human Linkage Center (CHLC; 26). We have found that the use of tri- and tetranucleotide STRPs greatly facilitates the analysis of pooled DNA samples due to their low background and good allele separation. The successful mapping also relied on the correctness of the hypothesis that AVCD in this population resulted from the inheritance of a major susceptibility gene from a common founder. This assumption was felt to be correct due to the fact that all affected individuals were descended from a common ancestor based on reliable genealogical information and the fact that AVCD is a relatively rare disorder. The application of this approach to disease mapping is less likely to be successful for more common disorders where the susceptibility locus could be inherited from several sources within the family. However, the approach used in this study could be applied to more common disorders when genetically isolated populations are used.

**MATERIALS AND METHODS**

**Clinical evaluation**

The congenital heart disease found in affected individuals was diagnosed by echocardiography, cardiac catheterization, surgery and/or post-mortem examination. All living obligate carriers (based on having an affected offspring), spouses and first degree relatives of affected individuals were evaluated by electrocardiography and echocardiography. No individuals considered unaffected for the purposes of the study had an abnormal echocardiogram, left-axis deviation on electrocardiogram, or evidence of mitral insufficiency on color-flow doppler studies. None of the spouses had a family history of congenital heart defects, and there was no consanguinity. Karyotype analysis was normal for all affected individuals.

**Genotyping**

DNA was prepared from peripheral blood using standard protocols (27). DNA concentrations were determined by spectrophotometric readings at OD260. Pooled DNA samples were made by combining equal molar concentrations of DNA from selected individuals into a single tube. The affected DNA pool consisted of DNA from 13 affected individuals. The control DNA pool consisted of DNA from 40 unrelated CEPH parents.

Polymorphic markers used in this study were tri- and tetranucleotide STRPs developed by the Cooperative Human Linkage Center (CHLC; 26). The panel of markers used was screening set 5 (28) modified by replacing all dinucleotide repeat markers with tri- or tetranucleotide repeat markers recently developed by CHLC (26). A total of 360 STRPs were used to screen the entire autosomal genome. Primers for these STRPs were synthesized by and purchased from Research Genetics (Huntsville, AL). Amplification of STRPs was performed with 40 ng of genomic DNA in an 8.4 µl PCR containing: 1.25 µl PCR buffer (100 mM Tris-HCl pH 8.8, 500 mM KCl, 15 mM MgCl2, 0.01% w/v gelatin), 200 µM each of dATP, dCTP, dGTP and dTTP, 2.5 pmol of each primer and 0.25 U Taq polymerase. Samples were subjected to 40 cycles of 94°C for 30 s, 55°C for 30 s and 72°C for 30 s, and analyzed on 6% denaturing polyacrylamide gels (7.7 M urea). The polyacrylamide gels were silver stained using the method of Bassam et al. (29). The same procedure was used for both pooled and individual DNA samples.

Gels were scored separately by two individuals. Initial genotyping was performed on DNA samples from only four affected individuals, a pooled DNA sample from 13 affected individuals, and a control DNA pool from 40 CEPH parents. Special attention was paid to the shared allele for markers which shared an allele in at least three of the four affected individuals. For such markers, the allele frequency of the shared allele was estimated by visual inspection. STRPs in which the shared allele appeared to be a predominant allele (allele frequency of 0.50 or greater) in the affected DNA pool were scored as potential disease candidate markers. Candidate markers were used to genotype the entire kindred.

**Statistical analysis**

Linkage was tested using the LOD score method (30). Analysis was performed using the MENDEL program (31). Equal allele frequencies for each STRP were used in the analysis. A
population disease allele frequency of 0.0001 was used. The mode of inheritance was assumed to be autosomal dominant with incomplete penetrance. The reference genetic map used for this analysis was generated by the CHLC (32). The genetic maps and primer sequences for the tri- and tetranucleotide markers used in this study are available electronically via anonymous FTP at FTP.CHLC.ORG or via the World Wide Web at WWW.CHLC.ORG.

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