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Dominant-Negative ALK2 Allele Associates With Congenital Heart Defects

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Background—Serious congenital heart defects occur as a result of improper atrioventricular septum (AVS) development during embryogenesis. Despite extensive knowledge of the genetic control of AVS development, few genetic lesions have been identified that are responsible for AVS-associated congenital heart defects.

Methods and Results—We sequenced 32 genes known to be important in AVS development in patients with AVS defects and identified 11 novel coding single-nucleotide polymorphisms that are predicted to impair protein function. We focused on variants identified in the bone morphogenetic protein receptor, ALK2, and subjected 2 identified variants to functional analysis. The coding single-nucleotide polymorphisms R307L and L343P are heterozygous missense substitutions and were each identified in single individuals. The L343P allele had impaired functional activity as measured by in vitro kinase and bone morphogenetic protein–specific transcriptional response assays and dominant-interfering activity in vivo. In vivo analysis of zebrafish embryos injected with ALK2 L343P RNA revealed improper atrioventricular canal formation.

Conclusion—These data identify the dominant-negative allele ALK2 L343P in a patient with AVS defects. (Circulation. 2009;119:3062-3069.)

Key Words: ALK2 protein, human ▪ genes ▪ heart defects, congenital ▪ screening ▪ signal transduction

The primitive heart tube of vertebrates consists of an inner layer of endothelial cells, the endocardium, and an outer muscular layer of myocardial cells. After formation of the heart tube, endothelial cells delaminate from the endocardium and migrate into an extracellular matrix, called cardiac jelly, which resides between the endocardium and myocardium. These invading endothelial cells undergo an endothelium-to-mesenchyme transition (for detailed review, see elsewhere) and give rise to swellings known as endocardial cushions (ECs). ECs contribute to the valves and septa of the heart, and disruptions in their formation result in valvular and septal defects. A number of signaling pathways, including vascular endothelial growth factor signaling, Notch, Wnt/β-catenin, bone morphogenetic protein (BMP)/transforming growth factor-β signaling have been implicated in atrioventricular septum (AVS) development either in vitro or in vivo. This extensive knowledge of the genetic control of AVS development has yet to be translated into a broader clinical knowledge of the genetic determinants of congenital heart defects (CHDs). This is due largely to the complex pathogenesis of CHD and the scarcity of large families with multiple affected individuals suitable for conventional genetic analyses. More recently, candidate screening approaches have been used to circumvent this limitation. Such approaches, when coupled with kindred linkage and/or detailed functional analyses, can identify novel causative mutations in genes previously suspected to function in AVS development.

In an effort to identify genetic lesions that may cause CHD, we used a candidate approach and sequenced the coding regions of 32 candidate genes in DNA from patients with defects associated with improper EC development. We focused on the functional characterization of a number of coding single-nucleotide polymorphisms (cSNPs) in the ALK2 gene. ALK2 is a BMP receptor, and roles for BMP signaling in EC development have been well documented. Compound mutants of BMP ligands (including BMP6/BMP7 and BMP5/BMP7) have been shown to result in defective EC development in mice. Moreover, the genetic ablation of...
ALK2 in the endocardium of developing mouse embryos results in hypoplastic ECs.\(^{11}\) This role for ALK2 in EC development is also conserved evolutionarily whereby an absence of ALK2 in the ALK2/\(^{-}\) zebrafish mutant, lost-a-fin, results in reduced EC development (manuscript in preparation). Despite the well-characterized role of ALK2 in AVS development, examples of aberrant BMP signaling in patients with CHDs have yet to be reported. Here, we report the identification of 11 genetic lesions in 10 different genes predicted to affect protein function. Furthermore, we report a detailed characterization of 2 genetic lesions in the BMP receptor gene, ALK2.

### Methods

#### SNP/Mutation Discovery

cSNPs in selected genes were determined by dideoxy resequencing of polymerase chain reaction–amplified exonic fragments. Polymerase chain reaction primers (sequences available on request) for amplification were designed with an in-house–developed management system (http://www.limsttl.nl), which also was used for the semi-automated identification and classification of mutations.\(^{12}\)

#### Clinical Evaluation, Sample Collection, and Genotyping

DNA material from the Netherlands national congenital heart disease registry, CONCOR, was obtained as previously described.\(^{13}\) DNA material from patients with non–cardiac-related diseases (Fragile X \(n=170\), male infertility \(n=90\), and Rett syndrome \(n=90\)) was used for controls. Controls were sourced from the Dutch population, which comprises 80% of individuals of European ancestry (according to the Central Bureau of Statistics, the Netherlands, for 2008). The 2 probands harboring the ALK2 variants (R307L and L343P) were of European ancestry, and the remaining 10 mutations were identified in patients from the Dutch population. After identification of variant alleles, patients and kindred were examined and samples were collected in accordance with the local research ethics committee of the University Medical Center Utrecht. Clinical evaluation was performed by history and physical examination; positive carriers were identified by transthoracic echocardiographic examination. Genotyping was performed on DNA from peripheral blood, oral epithelium, hair follicles, or tissue biopsy by polymerase chain reaction amplification of exon 6, followed by sequencing with Big Dye Terminator chemistry.

#### Cell Lines and Transfections

Bovine aortic endothelial cells\(^{14}\) were used for luciferase assays, and cos7 cells were used for construct expression and kinase assay experiments. All transfections were performed with Lipofectamine (Invitrogen, Carlsbad, Calif) according to the manufacturer’s instructions.

#### Constructs, Luciferase Assay, and Kinase Activity Assays

Expression constructs for full-length human ALK2 have been previously described,\(^{15}\) and mutant variants were generated by site-directed mutagenesis with a QuikChange kit (Stratagene, La Jolla, Calif). Inserts were fully sequenced and recloned into the original parental vector or the pCS2+ vector (for mRNA synthesis). Luciferase assay was performed as previously described,\(^{16}\) in combination with a short hairpin RNA (shRNA) targeting bovine ALK2 construct. The shRNA targeting construct was made by cloning complementary oligonucleotides into the pSuper vector (5’-gatcccg-GATGAGAAGTCGTGGTTAttcaagagaTAAACCACGACTTCTC-ATCttttgaaa-3’) to knock down endogenous bovine ALK2 but not vector-based human ALK2. Kinase assay was performed previously described\(^{17}\) with \(\gamma\)-ATP (Perkin Elmer, Waltham, Mass).

### Protein Isolation and Western Blot Analysis

Protein for construct expression analysis was isolated from transfected cells by direct lysis in Laemmli buffer. For embryos lysates, lysesates were collected as previously described.\(^{18}\) Western blotting was performed as previously described\(^{18}\) with an HA antibody (12CA5; Roche, Basel, Switzerland) at 1:1000 and rabbit anti–phospho-Smad1,5,8 (Cell Signaling Technology, Danvers, Mass) at a concentration of 1:1000.

#### Fish Lines, mRNA Synthesis, Injections, In Situ Hybridization, and Immunofluorescence

Wild-type and \(Tg(Tie2:EGFP)^{9007\text{b}}\) fish were kept under standard conditions.\(^{20}\) pCS2+ ALK2 constructs were linearized with NotI enzyme and capped mRNA prepared with the Message Machine kit (Ambion Corp, Naugatuck, Conn). mRNA was diluted in nucleasfree water, and 1 nL per embryo was injected at the 1- to 2-cell stage. In situ hybridization was carried out as previously described.\(^{20}\) Embryos were cleared in methanol and mounted in benzylbenzoate/ benzylalcohol (2:1) before pictures were taken. Immunohistochemistry was performed as previously described.\(^{21}\) Mouse anti–tropomyosin (Sigma, St Louis, Mo) and mouse anti–green fluorescent protein (GFP) (Santa Cruz Biotechnology, Inc, Santa Cruz, Calif) were applied at 1:200.

### Results

#### Identification of cSNPs in Patients With CHDs

In an effort to identify SNPs that may cause CHDs, the coding regions of 32 candidate genes (selected on the basis of relevant phenotypes from model organisms) were analyzed in DNA from 190 patients with AVS defects. Seven genes showed no variation in their coding sequences, whereas 418 variants were identified in the other 25 genes, including 199 of 200 known SNPs that were present in the database of SNPs at that time, indicating high sensitivity for SNP detection. A total of 86 SNPs were identified, resulting in nonsynonymous substitutions or cSNPs, of which 43 cSNPs were unique to the patient group and did not occur in any of the control DNA samples derived from a Dutch population with noncardiac diseases (see Methods for details; see also Table I of the online-only Data Supplement). The potential functional effect of all cSNPs was analyzed with the SIFT\(^{22}\) and PolyPhen\(^{23}\) programs (Table I of the online-only Data Supplement), showing that 18 cSNPs are predicted to affect protein function by both algorithms. Interestingly, only 7 were found in the class of cSNPs not unique to the patient group, whereas 11 cSNPs that are predicted to affect protein function were found in the patient-specific class of cSNPs and may thus contribute to the disease phenotype in these patients (the Table).

We focused on the functional characterization of a number of cSNPs in the ALK2 gene. ALK2 was found to contain 3 cSNPs: Ala15Gly (A15G; located in the predicted signal peptide), Arg307Leu (R307L), and Leu343Pro (L343P) (these last 2 located in the kinase domain) (Figure 1a through 1d). Of the 350 control individuals screened, 9 possessed the A15G variant (allele frequency, 1.3%), indicating that this
cSNP is common to the general population. No controls (n=350) were found to possess the R307L or L343P variant. Only L343P was predicted to affect protein function.

Analysis of the crystal structure of transforming growth factor-β receptor I, which is highly homologous to the cytoplasmic domain of the ALK2 receptor, suggests that R307 is solvent exposed, whereas L343 is part of a β sheet that shields the nucleotide binding site. Interestingly, the side chain of L343 is in van der Waals interaction distance of the base of the nucleotide. The L343P substitution would interrupt this interaction and is likely to disrupt the β sheet, thereby destabilizing the overall structure of the kinase domain (Figure 1e).

### Table. List of Patient-Specific cSNPs Predicted to Be Damaging to Protein Function

<table>
<thead>
<tr>
<th>No.</th>
<th>Gene and Resultant Substitution</th>
<th>Patients, n</th>
<th>Sift Prediction</th>
<th>Polyphen Prediction</th>
</tr>
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<tr>
<td>1</td>
<td>ALK2 L343P</td>
<td>1</td>
<td>Affects, 0.00</td>
<td>Probably damaging</td>
</tr>
<tr>
<td>2</td>
<td>ALK3 R443C</td>
<td>1</td>
<td>Affects, 0.0</td>
<td>Possibly damaging</td>
</tr>
<tr>
<td>3</td>
<td>APC N1217T</td>
<td>1</td>
<td>Affects, 0.01*</td>
<td>Possibly damaging</td>
</tr>
<tr>
<td>4</td>
<td>ECE2 Y417C</td>
<td>1</td>
<td>Affects, 0.01</td>
<td>Probably damaging</td>
</tr>
<tr>
<td>5</td>
<td>EGFR D1152H</td>
<td>1</td>
<td>Affects, 0.03</td>
<td>Possibly damaging</td>
</tr>
<tr>
<td>6</td>
<td>EGFR P848L</td>
<td>2</td>
<td>Affects, 0.00</td>
<td>Probably damaging</td>
</tr>
<tr>
<td>7</td>
<td>ERBB3 P1283R</td>
<td>1</td>
<td>Affects 0.00*</td>
<td>Probably damaging</td>
</tr>
<tr>
<td>8</td>
<td>FOXP1 P604L</td>
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<td>Affects, 0.04</td>
<td>Possibly damaging</td>
</tr>
<tr>
<td>9</td>
<td>GATA4 R285C</td>
<td>1</td>
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<td>Probably damaging</td>
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<tr>
<td>10</td>
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<td>Affects, 0.00</td>
<td>Probably damaging</td>
</tr>
<tr>
<td>11</td>
<td>UGDH R141C</td>
<td>2</td>
<td>Affects, 0.02</td>
<td>Probably damaging</td>
</tr>
</tbody>
</table>

*Low confidence for this prediction because of a low sequence number for comparison.

Figure 1. Identification of ALK2 variations in patients with CHDs. a, Schematic representation of the ALK2 protein and structural domains: signal peptide (SP), ligand binding domain (LBD), transmembrane domain (TM), GS domain (GS), and kinase domain (kDa). b through d, Sequencing chromatograms showing heterozygous cSNPs A15G (b), R307L (c), and L343P (d) in genomic DNA from affected individuals with accompanying protein alignments below (aligned by ClustalW). The protein alignment shows the poorly conserved nature of the A15 and R307 residues and strong conservation for the L343 residue. e, Transforming growth factor-β receptor I (pdb entry 1PY5) as a model for ALK2, showing R307 and L343 in red, ADP in blue, and the β sheet, in which L343 resides in yellow. f, g, Echocardiograms of probands carrying variations R307L (f; individual III:2) and L343P (g; individual III:4). f, Echocardiogram of the R307L proband (after surgery, 4-chamber view). Corrected common atrium, cleft of anterior MV, and interruption of inferior caval vein showing a thickened anterior MV leaflet (white arrow), thickened portion of atrial septum (yellow arrow), and an enlarged right chamber as a result of pulmonary hypertension. g, Echocardiogram of L343P proband (short-axis parasternal) showing a cleft MV (arrows) located in the middle of the anterior MV leaflet. Doppler examination showed mild MV regurgitation. RV indicates right ventricle; LV, left ventricle; RA, right atrium; and LA, left atrium.
Pedigree Analysis of Patients Carrying cSNPs in ALK2

The proband carrying the R307L allele (individual III:2; see Figure 1a of the online-only Data Supplement) was diagnosed soon after birth with a complex congenital defect that included a common atrium, a cleft of the anterior mitral valve (MV) leaflet, interruption of the inferior caval vein (Figure 1f), and a partial abdominal situs inversus. The proband experienced a miscarriage in early pregnancy (IV:1). At physical examination, no evidence for other congenital malformations or other dysmorphic features was found. Pedigree analysis revealed that the proband’s father (II:3) carried the R307L allele; however, ultrasound and examination revealed no CHD in this individual. No other living relatives were positive for the variant or had cardiac complaints.

The proband carrying the L343P variant (III:4; Figure Ib of the online-only Data Supplement) was born with a primum-type atrial septal defect with a cleft anterior MV leaflet and a small left-to-right shunt (Figure 1g). The MV showed mild regurgitation, and no other cardiac defects were found. There was no evidence of other congenital malformations or other dysmorphic features, and his blood cell count was within normal parameters. His father (II:5) had a cardiac murmur since he was 14 years of age, and DNA sequencing demonstrated that he was a carrier of the L343P allele. There was no evidence of mosaicism in the father in DNA derived from skin fibroblasts, hair roots, blood, and oral epithelium. Additionally, equal amounts of the wt and L343P allele were found to be expressed in skin fibroblast, as determined by sequencing of cloned reverse-transcription polymerase chain reaction products (data not shown). Ultrasound examination showed calcification of the anulus of the posterior MV leaflet and prolapse of both leaflets. No other relatives carried the mutation or had cardiac complaints.

ALK2 L343P Has Reduced Transcriptional and Kinase Activity In Vitro

To determine whether the cSNPs affect ALK2 function, the various alleles were tested for their capacity to induce BMP-specific transcriptional reporter activity16 (Figure 2a). There was no significant difference in the activity of either the A15G or the R307L allele compared with wtALK2 using this assay, indicating that in vitro receptor activity is not altered by these substitutions. Strikingly, however, expressing the L343P variant resulted in significantly lower luciferase units (RLUs) without (dark gray) and with (light gray) induction by BMP6 protein. Significantly higher RLUs were observed for ALK2 R307L without induction, whereas significantly lower RLUs were observed for ALK2 DN with and without BMP6 induction. No significant differences were observed for ALK2 A15G or ALK2 R307L without or with induction compared with wtALK2. Significantly lower RLUs were observed for ALK2 L343P both without and with induction compared with wtALK2. No statistically significant interaction was detected between BMP6 treatments and the various ALK2 alleles (as determined by 2-way ANOVA; P=0.140). RLUs were measured as luciferase activity/renilla activity and expressed relative to unstimulated wtALK2 activity. RLUs are depicted as mean±SEM; n=3 to 8 experiments (3 replicates per experiment). Data were transformed to normality with equal variance by rank transformation. Statistical significance was determined by 2-way ANOVA. All pairwise comparisons were made with the Holm-Sidak method, and alleles significantly different from wtALK2 are indicated by *P<0.05. b, Construct expression was approximately equal for all constructs when transfected into cos7 cells, as determined by Western blot for the HA tag harbored by each ALK2 construct. c, Kinase activity assay showing kinase activity present for wtALK2, ALK2 A15G, and ALK2 R307L but no detectable activity for ALK2 DN or ALK2 L343P alleles (autoradiogram). Western blotting for HA-tagged protein shows that the protein was expressed for each construct approximately equally (middle blot) and that the total loading was approximately equal for all samples (bottom blot).
Figure 3. ALK2 L343P acts in a dominant-negative fashion in vivo. Zebrafish embryos uninjected and injected with variant RNA at the single-cell stage. a, Graphical depiction of the dorsoventral phenotype of embryos uninjected, injected with wtALK2 or ALK2 DN alone, coinjected with ALK2 or ALK2 L343P alone, or coinjected with wtALK2 at the single-cell stage and scored at 28 hours post fertilization. b through d, Images of wtALK2- (b), ALK2 L343P–(c), and wtALK2 and ALK2 L343P– (d) injected embryos at 28 hpf. a, d, wtALK2 RNA resulted in a wt phenotype in the majority of embryos (91% of 292 embryos), whereas the ALK2 DN RNA resulted in the majority of embryos with severe C5 dorsalization (86% of 149 embryos). ALK2 L343P RNA also severely dorsalized embryos to C5 (c; 57% in 150 embryos) although not as potently as ALK2 DN RNA. Coinjection of wtALK2 with ALK2 L343P RNA partially rescued the dorsalization phenotype, resulting in a more intermediate C2 phenotype in the majority of embryos (d; 50% in 70 embryos). A similar effect was observed after coinjection of wtALK2 with ALK2 DN RNA. e, f, pSMAD levels in wt embryos injected with ALK2 variant RNA. e, Representative Western blots of pSMAD (top) and total SMAD (bottom) proteins. f, Graphical representation of 4 independent experiments showing significantly lower pSMAD signaling in embryos injected with ALK2 L343P RNA. Bars are mean±SEM and represent the percentage of SMAD phosphorylation relative to uninjected embryos. The percentage of pSMAD was determined as a ratio of optical density of the pSMAD band compared with the total SMAD band. Statistical significance was determined by t test (*P<0.05).

Overexpression of ALK2 L343P in Zebrafish Embryos Disrupts Atrioventricular Canal Formation

Finally, we investigated heart morphology of embryos injected with ALK2 L343P RNA (Figure 4). In situ hybridization analysis for cmhc2, a cardiac myosin marker, showed that the overall morphology of the heart tube was disrupted in ALK2 L343P–injected embryos compared with uninjected and wtALK2-injected embryos, similar to that observed in lafalk2/8 mutant.29 tbx2b, anf/nppa, and has2, markers of the atrioventricular canal (AVC) myocardium, the atrial and ventricular chambers, and the ECs, respectively, revealed a loss of AVC identity. tbx2b expression was lost or reduced in ALK2 L343P–injected embryos compared with uninjected and wtALK2-injected embryos. anf expression, which is characteristically absent in the AVC, was continuous throughout the heart tube in ALK2 L343P–injected embryos compared with controls, and has2 expression was lost in ALK2 L343P–injected embryos compared with control embryos, reminiscent of mouse mutants with reduced BMP signaling, which also exhibit a loss of tbx2b and has2 expression.9 Loss of these markers was not due to a disruption in cardiac looping because these markers were patterned normally in a nonlooping mutant (HU119). Finally, we visualized the endocardial cells of the AVC cushions by immunofluorescent staining of uninjected and wtALK2- and ALK2 L343P RNA–injected embryos. Using the transgenic line Tg(Tie2:EGFP), which has enriched expression of GFP in ECs, we counterstained the myocardium with antitropomyosin antibody. We observed reduced expression of GFP in ECs and a loss of ECs in ALK2 L343P RNA–injected embryos. These data demonstrate that reduced ALK2 signaling in the zebrafish, via injection of L343P RNA, disrupts normal heart morphology and formation of the AVC.
Discussion

CHDs occur in >1 of every 100 live births, yet only a limited number of determinants responsible for this set of diseases have been uncovered. The number of causative mutations described to date has been restricted to a subset of genes, including GATA4, NKX2.5, and TBX20, which are essential for normal cardiac development. Most of the identified lesions, however, have been identified by conventional genetic approaches using large families with multiple affected individuals. The incidence of such large families with an inheritance pattern of CHDs is rare. The rarity of such families has been attributed to the multifactorial nature of CHD and/or low penetrance of the causative lesion. In recent years, a number of genetic lesions have been described using candidate approaches, yielding a vast number of putative mutations. However, the inherently biased approach of this method requires further validation of these mutations by either pedigree analyses and/or functional characterization of the gene products.

We have identified 11 novel cSNPs predicted to be damaging to protein function in patients with AVS defects. These putative mutations were identified in 10 genes shown through animal and cell culture experimentation to be important in EC formation and, to the best of our knowledge, represent the largest collection of cSNPs for this disease. Surprisingly, of the 11 novel cSNPs predicted to be damaging to protein function, we identified only 1 patient with a cSNP in GATA4 and none in NKX2-5 or TBX20. Our systematic analysis of a large group of patients with defects associated with improper EC development demonstrates that mutations in GATA4, NKX2-5, or TBX20 have a very small contribution to such CHDs in the Netherlands.

We have identified 43 cSNPs that are unique to the group of 190 patients. Among this collection of cSNPs, we identified 2 variants in the BMP receptor gene, ALK2. This equates to 1% of the CHD patients screened (2 of 190). Pedigree analysis showed a lack of segregation of the R307L allele with a CHD. Furthermore, no functional differences were observed between the wt and R307L alleles either in the BMP-specific transcriptional response assays or by phenotypic evaluation of RNA-injected embryos. These findings do not preclude this allele from contributing to the R307L proband phenotype, but this is difficult to determine with the current data. For the L343P variant, it was not possible to establish a pattern of inheritance for the variant with a cardiac phenotype. Although the atypical site of the MV prolapse in the proband’s father and the historical nature of the cardiac murmur from childhood favor a congenital origin for the defect, a degenerative cause for this phenotype could not be ruled out. The ambiguous nature of the heart defect was uninformative and, though not contradicting an inheritance pattern for this allele, could not support cosegregation either.

Structural modeling of the kinase domain of the ALK2 receptor in combination with kinase assay suggested that the L343P variation interferes with kinase activity of the ALK2 receptor, and transcriptional assay results demonstrate a
loss of BMP-responsive receptor activity. Interestingly, a dominant-negative effect was observed in RNA-injected embryos, suggesting that the L343P variant is capable of participating in the receptor complex and interfering with BMP signaling. Whether this variant acts at the level of ligand binding, thus acting as a ligand sink, or by occupying binding sites of coreceptors was not addressed here. What is clear from the coinjection experiment of wt and L343P RNA, however, is that the L343P allele is capable of interfering with wt ALK2 signaling when present in equimolar amounts. Furthermore, the downstream effector of ALK2 signaling, pSmad1, was activated at lower levels in L343P RNA–injected embryos. Finally, we show that the AVC in embryos injected with L343P RNA is disrupted. The overall morphology of the heart in L343P-RNA–injected embryos was disturbed as characterized by a loss of cardiac looping. Importantly, however, a mutant with cardiac looping defects exhibited restricted expression patterns for markers of the AVC, cardiac cushions, and cardiac chambers, similar to wt control embryos, demonstrating that normal cardiac looping is not required for patterning of these markers. This loss of AVC identity is therefore attributed to a specific disruption in BMP signaling.

To the best of our knowledge, this is the first specific report linking variations in ALK2 with CHDs. A database study examining the chromosomal regions most frequently altered in patients with CHDs failed to identify patients with disruptions in the region harboring the ALK2 gene (region 2q24.1). A current investigation of the Database of Chromosomal Imbalance and Phenotype in Humans Using Ensembl Resources (Decipher) reveals 6 patients in possession of a deletion inclusive of the ALK2 gene. Although there is only limited clinical information for these patients, none are listed as possessing a CHD. In contrast, examination of the European Cytogeneticists Association Register of Unbalanced Chromosome Aberrations (Ecaruca) database identifies >10 patients with deletions that map to this genomic region and possess CHDs manifesting as atrial septal defects or ventricular septal defects. Although these diseases are consistent with improper EC formation, it is difficult to determine whether these defects are the direct result of ALK2 hemizygosity or the surrounding genes that are also absent because of the deletion. Given the lack of phenotype in ALK2 homozygous null mice, however, it is unlikely that a loss of 1 copy of ALK2 would be sufficient to be causative.

The requirement for ALK2 in EC formation has been well documented. Chick AV cushion explants fail to undergo epithelial-mesenchymal transition when exposed to ALK2-neutralizing antibodies. Furthermore, endothelium-specific deletion of ALK2 in mice results in hypoplastic EC from a loss of epithelial-mesenchymal transition. Therefore, it is not surprising that individuals carrying a dominant-negative allele of ALK2 such as the L343P variant also possess EC-associated defects. What is surprising, however, is the viability of such individuals. ALK2-deficient mice die during gastrulation, demonstrating the necessity for ALK2-mediated signaling during early stages of embryonic development. That these individuals survive through gastrulation to develop an EC-associated cardiac defect suggests that a minimal amount of ALK2-mediated signaling still occurs in these individuals. It is also possible that, although equally expressed transcriptionally, the wt protein is more highly represented by virtue of a less stable L343P protein. Alternatively, other type I BMP receptors such as ALK3 may be playing a compensatory role. Further studies are necessary to tease out the details of how these individuals escape the devastating effect that this allele is expected to exert during gastrulation.

**Conclusion**

Using a candidate sequencing approach, we have screened the coding sequences of 33 genes known to be involved in AVS development in a DNA database derived from a host of AVS defect patients. We identified 11 putative mutations that are predicted to affect protein function and thus potentially contribute to CHD in the screened patients. In this study, we focus on the functional characterization of the cSNPs identified in ALK2, the BMP receptor. We identified 2 novel heterozygous missense mutations in ALK2, one of which, the L343P variant, showed reduced signaling capacity in vitro and dominant-negative activity in vivo assays. The dominant-negative activity of ALK2 L343P interferes with normal formation of the AVC and the development of ECs in developing zebrafish embryos. Finally, the ALK2 L343P allele was demonstrated in a father and his son, who both have a structural cardiac defect. However, whereas the son displayed a primum-type atrial septal defect with cleft MV, the father had an anomaly that could not be unambiguously classified as a CHD and was clinically nonpenetrant, consistent with the notion that CHDs are usually complex diseases. Taken together, our data suggest that mutations in ALK2 may be causative for AVS defects and report a significant resource in the identification of 11 cSNPs potentially causative of AVS defect.

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**Disclosures**

None.

**References**


Clinical Perspective

In an attempt to identify genetic mutations that may cause atrioventricular septal defects, a large-scale candidate screen of genes involved in atrioventricular valve and septum (AVS) formation has been performed in patients with atrioventricular septal defects. By sequencing 32 candidate genes (selected for their role in AVS development from animal and cell culture studies), we identified 11 putative deleterious mutations in 10 different genes in patients with atrioventricular septal defects. Focusing on variations identified in the bone morphogenetic protein receptor, ALK2, we performed in vitro and in vivo functional analyses and demonstrated that a dominant-negative allele of ALK2 exists in a patient with a defect originating in the AVS. The functional evidence suggests that individuals possessing this dominant-negative allele have reduced bone morphogenetic protein signaling capacity, and considering the role of bone morphogenetic protein signaling in AVS development, it is likely to be deleterious to AVS formation. These findings may have implications for screening these genes, particularly ALK2, in instances when atrioventricular septal defects are suspected.
Supplementary Table 2. Phenotype of zebrafish embryos at 28 hpf after injected at the one-cell stage with various ALK2 construct.

<table>
<thead>
<tr>
<th>RNA</th>
<th>Concentration</th>
<th>Genotype</th>
<th>Number</th>
<th>V4</th>
<th>V3</th>
<th>V2</th>
<th>V1</th>
<th>wt</th>
<th>C1</th>
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* 10ng/uL of each RNA
+ Phenotypic classification based on^{23, 24}

# All constructs are of human sequence

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Supplementary Figure 1. Family pedigrees of patients (identified by asterisk) with identified mutations. **a.** The family carrying the R307L variant exhibited no obvious inheritance pattern. **b.** The L343P pedigree was uninformative for inheritance of the mutation with CHD. Individual II:5 had a cardiac murmur since he was 14 years of age and ultrasound examination showed calcification of the annulus of the posterior MV leaflet and prolapse of both leaflets. Despite this history of cardiac symptoms, the phenotype could not be ruled out as degenerative and thus could not unambiguously be classified with a CHD. Carriers or non-carriers of variants are depicted with a red or green outlines, respectively, and individuals with or without CHDs are shaded black or white, respectively. The ambiguous phenotype is represented by hatching. Grey colouration indicates the genotype or phenotype was unable to be determined. The diamond indicates a miscarriage and deceased individuals are annotated by a diagonal strike-through.
Supplementary Figure 2. DV patterning markers are altered in embryos injected with ALK2 L343P. DV patterning defects in uninjected embryos and embryos injected with wt ALK2 and ALK2 L343P RNA at the single cell stage. The ventral ISH markers, *eve1* and *gata2*, demonstrate a dramatic reduction of ventral tissue in ALK2 L343P RNA injected embryos and, conversely, the dorsal ISH marker, *chordin*, demonstrates the clear expansion of dorsal cell fate in ALK2 L343P RNA injected embryos. *myoD* and *krox20* staining, which mark the developing somites and hindbrain rhombomeres, respectively, show the sever dorsalisation of ALK2 L343P RNA injected embryos. An expansion of these structures around the entire embryo is reminiscent of the classical severely dorsalised embryo.

<table>
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<tr>
<th>eve1</th>
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<th>chordin</th>
<th>myoD</th>
<th>krox20</th>
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<td><img src="image4" alt="myoD" /></td>
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1

![Figures](image6)