

ARTICLE

ALK2 mutation in a patient with Down's syndrome and a congenital heart defect

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Down's syndrome (DS), resulting from an additional copy of chromosome 21 (trisomy 21), is frequently associated with congenital heart defects (CHDs). Although the increased dosage of chromosome 21 sequences is likely to be part of the etiology of cardiac defects, only a proportion of DS patients exhibit a congenital heart defect (birth prevalence 40–60%). Through a large-candidate gene-sequencing screen in patients with atrioventricular septal defects, substitutions were identified in bone morphogenetic protein (BMP) type I receptor *ALK2* and two other genes in a patient with DS and a primum-type atrial septal defect. Structural modeling of the cytoplasmic domain of the *ALK2* receptor suggests that H286 is in close proximity to the nucleotide-binding site of the kinase domain. We investigated whether this p.His286Asp substitution altered *ALK2* function by using both *in vitro* as well as *in vivo* assays. The p.His286Asp variant demonstrated impaired functional activity as measured by BMP-specific transcriptional response assays. Furthermore, mild dominant-interfering activity was observed *in vivo* compared with wild-type *ALK2* as determined by RNA injection into zebrafish embryos. These data indicate that in the context of a DS background, *ALK2*-mediated reduction of BMP signaling may contribute to CHDs.

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INTRODUCTION

Down's syndrome (DS) is the most frequent cause of mental retardation, most often caused by trisomy 21 (in approximately 95% of cases). DS occurs in about one in 700–1000 live births.^{1–3} The syndrome is characterized by well-defined and distinctive phenotypic features including characteristic facies, minor limb anomalies, hypotonia and growth retardation.⁴ Although all individuals with DS manifest these phenotypes, there is significant phenotypic variability between such individuals.⁵ In conjunction with these phenotypic traits, DS individuals suffer from a higher incidence of several conditions, including CHDs.⁵

The (birth) prevalence of CHDs in DS is about 40–60%, most frequently atrioventricular septal defects and ventricular septal defects.^{6–8} Efforts have been made to determine the critical chromosomal region for specific phenotypic features of DS by deletion mapping and characterization of patients with partial trisomy 21.^{4,9,10} One potential candidate gene for these trisomy 21-related CHDs is collagen type VI. However, the evidence for the involvement of collagen type VI has, to date, been restricted to correlative expression pattern analysis.^{11,12}

Although this susceptibility and variability of the DS phenotype is widely accepted, the factors contributing to this variability have not

been established. It is postulated that variations in gene dosage of chromosome 21, environmental factors and genetic modifications not linked to chromosome 21 account for this variability, and it is likely that contributions from each of these elements are involved. Genetic imbalance caused by the presence of an extra copy of chromosome 21 will seriously disrupt one or more developmental pathways. In addition, the presence of non-chromosome 21 loci variation may predispose for a heart defect. To date, *CRELD1* (3p25.1) is the only non-chromosome 21 gene that has been reported to associate with DS-related CHDs.¹³

We have previously reported mutations in *ALK2*, a type I receptor for BMPs, in patients with endocardial cushion-associated CHDs. One of these variants identified has a dominant-interfering effect on BMP signaling and is associated with primum-type atrial septal defect (ASD) type I.¹⁴ Here we report an additional *ALK2* variant that was identified in a patient with trisomy 21 and a primum-type ASD (ASD type I). In addition to the *ALK2* variant, two other variations were detected in another type I BMP receptor gene, *ALK3*, and in the epidermal growth factor receptor family, *ErbB3*. Using both *in vitro* and *in vivo* assays, we show that the *ALK2* variant has reduced BMP-inductive capacity and a mild dominant-interfering effect on BMP signaling. This effect on BMP signaling from the *ALK2* variation in

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conjunction with the additional variations identified in this patient is likely to contribute to the manifestation of a CHD in this individual.

MATERIALS AND METHODS

This study was prospectively reviewed and approved by the Local Research Ethics Committee of the University Medical Center Utrecht and the Academic Medical Center, Amsterdam, The Netherlands.

Genetic material and clinical evaluation

The patient described in this study was identified in a large-scale candidate gene sequencing screen as previously described.¹⁴ In total, nine candidate genes were sequenced in this patient (*ALK2*, *ALK3*, *EGFR*, *GATA4*, *HAS2*, *UGDH*, *ERBB2*, *ERBB3* and *CRELD1*). Patient material was acquired from the CONCOR (CONgenital CORvitalia) database.¹⁵ DNA material was obtained from the patient and first-degree relatives by buccal swabs. Family members were clinically evaluated and individuals testing positive for the *ALK2*, *ERBB3* and *ALK3* variant were examined by echocardiography. 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, that comprises 80% of individuals of European ancestry (according to the Central Bureau of Statistics, The Netherlands, for 2008).

Detection of variants

PCR primers (sequence available upon request) for amplification were designed using an in-house developed management system (<http://limstill.niob.knaw.nl>), which was also used for the semi-automated identification and classification of mutations.¹⁶ Selected genes were analyzed by dideoxy sequencing of PCR-amplified exonic fragments.

Cell lines and transfections

Bovine aortic endothelial cells¹⁷ were used for luciferase assays and cos7 cells were used for construct expression. All transfections were performed using Lipofectamine (Invitrogen, Carlsbad, CA, USA) according to manufacturer's instructions.

Constructs, luciferase assay and kinase activity assays

Expression constructs encoding full-length human *ALK2* were cloned into the pcDNA3 vector as previously described,¹⁸ and mutant variants were generated by site-directed mutagenesis using a QuikChange kit (Stratagene, La Jolla, CA, USA). Inserts were fully sequenced and recloned into the original parental vector or the pCS2+ vector (for mRNA synthesis) to ensure against additional mutations. The luciferase assay was performed as previously described.¹⁹ This included an injection with vector only or in combination with a short

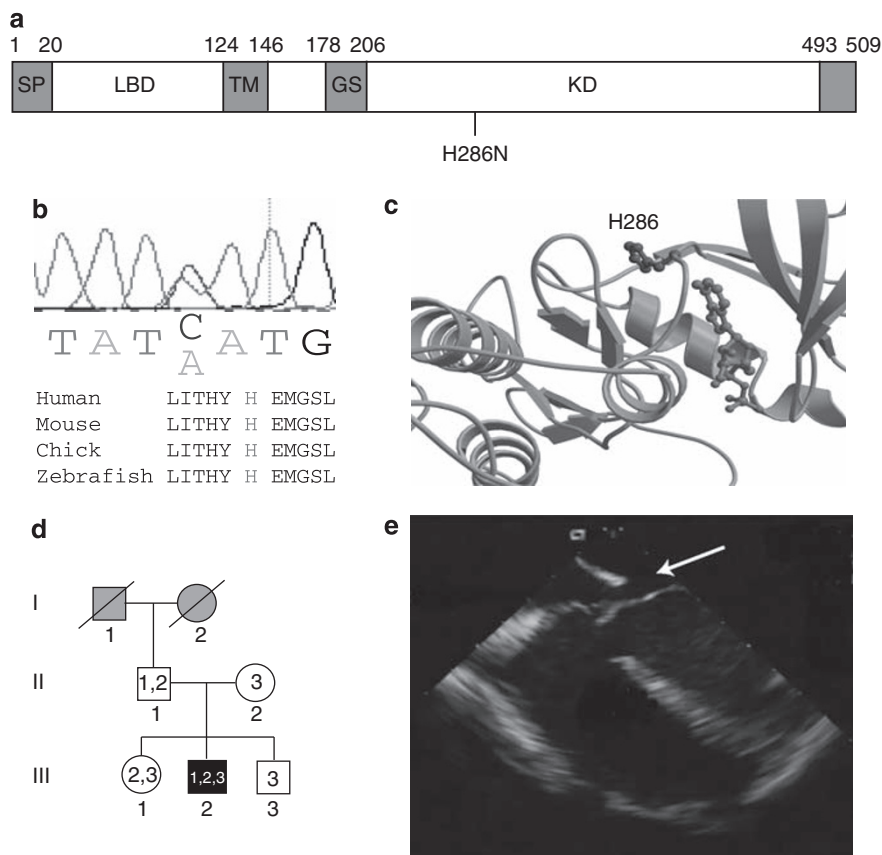


Figure 1 Identification of *ALK2* variant in *DS*. (a) Schematic representation of the *ALK2* protein and structural domains: signal peptide (SP), ligand-binding domain (LBD), transmembrane domain (TM), GS domain (GS) and kinase domain (KD). Arrows indicate approximate locations of p.His286Asp in *ALK2*. (b) Sequencing chromatogram with alignment below (aligned by ClustalW) showing the *ALK2* p.His286Asp variant in genomic DNA from the proband with *DS* and a primum-type atrial septal defect. The protein alignment shows strong conservation between species of the *ALK2* p.His286Asp residue. (c) TGF β RI (pdb entry 1PY5) as a model for *ALK2*, showing H268 in red, ADP in blue and the β -sheet in yellow. (d) Family pedigree of proband (III:2). Only the father, without *DS*, was in possession of the *ALK2* variant and did not have a congenital heart defect by echocardiography. Carriers of variants are depicted with a number: 1 (*ALK2* p.His286Asp), 2 (*ALK3* p.Glu415Lys) or 3 (*ErbB3* p.Thr1169Ile), and individuals with or without CHDs are shaded black or white, respectively. Gray coloration indicates the genotype or phenotype was unable to be determined. Deceased individuals are annotated by a diagonal strike-through. (e) Echocardiogram of proband carrying the p.His286Asp variant (III:2) before surgery, demonstrating a large primum-type atrial septal defect (arrow) and overriding AV valve.

hairpin RNA, the latter to knockdown endogenous bovine ALK2 but not vector-based human ALK2, as described previously.¹⁴

Zebrafish lines, mRNA synthesis and injections

Wild-type fish were kept under standard conditions as previously described.²⁰ pCS2+ ALK2 constructs were linearized with *NotI* enzyme, and capped mRNA was prepared with the Message Machine kit (Ambion, Austin, TX, USA). mRNA was diluted to appropriate concentration in nuclease-free water and 1 nl (see ref. 20) per embryo injected at the 1–2-cell stage. Embryos were imaged live and mounted in methylcellulose.

Statistical analysis

Results are expressed as mean \pm SEM. Statistical significance was determined by one-way analysis of variance, followed by a Bonferroni *t*-test using the program SigmaStat version 2.03 (Jandel Scientific, San Rafael, CA, USA).

RESULTS

We recently performed a screen for the detection of cSNPs in patients with CHDs arising from improper endocardial cushion formation.¹⁴ During this screen we identified a patient with DS in possession of compound cSNPs in *ALK2* that resulted in a His286Asp substitution (*ALK2* p.His286Asp) (Figure 1b); the *ALK3* cSNP caused a Glu414Lys substitution (*ALK3* p.Glu414Lys), while the cSNP in *ERBB3* resulted in a Thr1169Ile substitution (*ERBB3* p.Thr1169Ile) (Supplementary Figure 1). These cSNPs were analyzed with the prediction tools PolyPhen^{21,22} and SIFT²³ (Table 1). Only *ALK2* p.His286Asp was predicted to damage protein function in both models and was not detected in 350 controls of the Dutch population. The coding regions of a total of nine genes were sequenced in this individual, including *CRELD1*, and no other cSNPs were identified.

This patient with DS was diagnosed with a primum-type atrial septal defect with a cleft mitral valve and the coronary sinus draining into the left atrium (Figure 1e). Sequencing of DNA from peripheral blood lymphocytes confirmed the three cSNPs in this individual (Figure 1d). To determine heritability and segregation of the cSNPs, we screened all first-degree relatives of the proband for the presence of each substitution (Figure 1d). The father of the proband was found to possess both the *ALK2* p.His286Asp and the *ALK3* p.Glu414Lys variants, whereas the mother was a carrier of the *ERBB3* p.Thr1169Ile variant. Of the proband's two siblings, only the sister carried the *ALK3* p.Glu414Lys and the *ERBB3* p.Thr1169Ile variants, whereas the brother was negative for the three cSNPs. None of these relatives were found to have a CHD by cardiac echocardiography.

We utilized our previous experience in functionally characterizing ALK2 variants to investigate the ALK2 p.His286Asp variant. Structural modeling of the cytoplasmic domain of the ALK2 receptor demonstrated that H286 resides within the ATP binding pocket of the kinase domain, indicating that a missense substitution at this residue may impair receptor activity (Figure 1a and c). To test this hypothesis, ALK2 p.His286Asp was examined for its capacity to induce BMP-specific transcriptional reporter activity.¹⁹ In this reporter assay, the ALK2 p.His286Asp variant showed significantly lower luciferase

activity with and without BMP6 induction compared with wt ALK2 ($P < 0.05$) (Figure 2a). Next, endogenous ALK2 expression was knocked down by an RNAi construct as previously described.¹⁴ Transfection with the ALK2 p.His286Asp variant demonstrated significantly lower levels of luciferase activity in the uninduced state ($P < 0.05$), but a difference from wt ALK2 was not observed upon stimulation with BMP6 (Figure 2b). These results indicate that the ALK2 p.His286Asp receptor is capable of propagating a signal when stimulated with a BMP ligand, but this activity is impaired when the ALK2 p.His286Asp variant is in the presence of the wt ALK2 form of the receptor.

To investigate the effect of the ALK2 p.His286Asp variant on receptor function *in vivo*, synthetic wt ALK2 and ALK2 p.His286Asp mRNAs were injected into zebrafish embryos at the single-cell stage. Injection of the ALK2 p.His286Asp variant resulted in mild dorsalization of the zebrafish (C1 in 17 versus 3% of injection with wt ALK2; $P < 0.05$),²⁴ which demonstrates a mild dominant-interfering effect of the ALK2 p.His286Asp variant on BMP signaling in the gastrulating

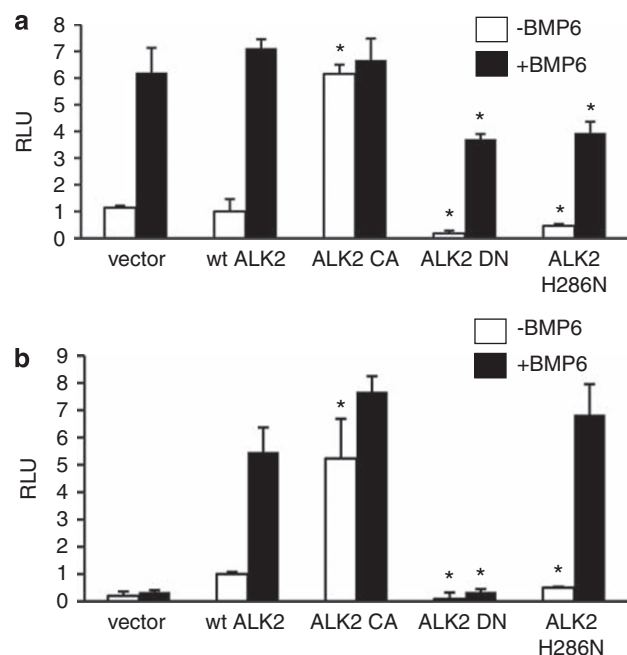


Figure 2 Representative luciferase assay on bovine aortic endothelial (BAE) cells transiently transfected with a BMP-responsive element fused to luciferase, renilla (to control the transfection) and ALK2 constructs. ALK2 activity was measured as relative luciferase units (RLU), without (white) and with (black) induction by BMP6 protein. (a) Significantly higher RLU was observed for ALK2 constitutively active (CA) without induction, whereas significantly lower RLU was observed for ALK2 dominant negative (DN) with and without BMP6 induction. For the p.His286Asp variant, both without and with induction with BMP6 significantly lower RLU was observed. (b) Luciferase assay on BAE cells as described above, including transfection of the bovine ALK2 RNAi construct to knockdown endogenous bovine ALK2 but not vector-based human ALK2. A similar pattern for ALK2 CA, ALK2 DN and wt ALK2 was observed as with injection of vector only. However, transfection with the p.His286Asp variant demonstrated significantly lower RLU without BMP6 induction, whereas induction with BMP6 did not demonstrate a significant difference between wt ALK2 and the p.His286Asp variant. RLU were measured as luciferase activity/renilla activity and expressed relative to unstimulated wt ALK2 activity. RLU are depicted as mean \pm SEM, where $n=5$ (3 replicates per experiment). Statistical significance, compared with wt ALK2, was determined by *t*-test, where $*P < 0.05$.

Table 1 Predictions of ALK3 and ErbB3 variant using PolyPhen and SIFT

	PolyPhen	SIFT
ALK3 p.Glu415Lys	Benign variant	Affect protein function
ErbB3 p.Thr1169Ile	Benign variant	Alter protein function (low confidence)

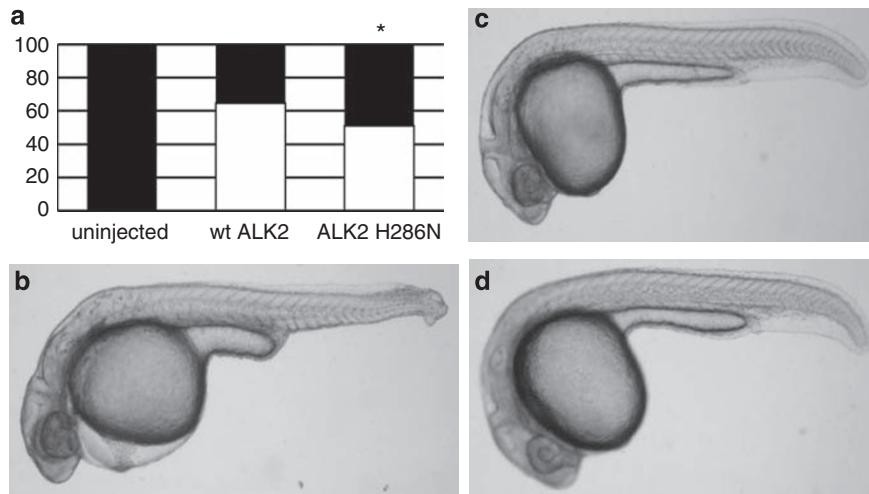


Figure 3 Rescue experiments in *laf* mutants. Rescue experiments in the zebrafish *alk2/8* mutant, *lost-a-fin* (*laf*). (a) Injection of wt ALK2 or p.His286Asp RNA at the single-cell stage in *laf/Alk2* mutants. Wt ALK2 RNA rescues phenotype in 65% of mutant embryos, while injection of p.His286Asp rescues the phenotype in 51% of mutant embryos. This is significantly less effective compared with wt RNA injection. Statistical significance was determined by Student's *t*-test, where $*P < 0.05$. (b) *laf* embryo identifiable by its C1 dorsalization phenotype, with cardiac edema and absent ventral fin. (c) Rescued *laf* zebrafish sibling after injection with ALK2 p.His286Asp demonstrating wt phenotype. (d) wt zebrafish.

embryo (Figure 3a). As a further measure of the reduction of ALK2 signaling for the ALK2 p.His286Asp variant, rescue experiments were performed in the zebrafish ALK2/8 mutant, *lost-a-fin* (*laf*) which is identifiable for its C1 dorsalization phenotype (Figure 3c). Although both wt ALK2 and variant ALK2 mRNA were able to rescue the C1 dorsalization phenotype in *laf/alk2* embryos, the ALK2 p.His286Asp did so less effectively than wt (51 versus 65%, respectively; $P < 0.05$; Figure 3a).

DISCUSSION

We recently reported a dominant-negative ALK2 allele in a patient with a CHD.¹⁴ In the current study, we report on a patient with DS and a primum-type atrial septal defect who harbors three cSNPs in genes associated with endocardial cushion development. The cSNPs result in missense substitutions in the type I BMP receptor genes, ALK2 and ALK3, resulting in the genetic variants p.His286Asp and p.Glu414Lys, respectively, and in the epidermal growth factor receptor family gene, *ERBB3*, resulting in the p.Thr1169Ile variant.

We show that, although subtle, there is a statistically significant difference in BMP signaling activity in ALK2 p.His286Asp compared with the wt ALK2 allele by both *in vitro* and *in vivo* analysis. Additionally, a genetic variant in ALK3 was identified. How these compound substitutions in two distinct type I BMP receptors affect overall BMP signaling was not tested here, but could affect BMP signaling to a greater extent when present in a compound manner, because ALK2 and ALK3 can form a heterodimeric complex.²⁵ Furthermore, it is difficult to predict what adverse effects may arise when occurring in concert with the non-conservative *ERBB3* p.Thr1169Ile substitution. What can be determined from the family data is that none of these variants are sufficient to cause a CHD when present in isolation in the absence of trisomy 21.

The three variants were detected in the parents of the probands, demonstrating that these variants were not acquired *de novo*. The father of the proband carries the ALK2 p.His286Asp and ALK3 p.Glu414Lys variants, and the sister of the proband harbors the ALK3 p.Glu414Lys and the *ERBB3* p.Thr1169Ile variants. Neither of these individuals presented with a CHD, demonstrating that the possession of either of these two combinations of cSNPs is either

not sufficient to cause the heart defect or the variants are not completely penetrant.

Our data are consistent with the notion that ALK2 is a susceptibility gene for primum-type atrial septal defects, in combination with variants at other loci or in combination with trisomy 21. This case is also suggestive that additional predisposing genetic factors exist for heart defects in DS patients, thus underlining the polygenic nature of these defects. These genetic factors can be identified by large-scale candidate gene screens.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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Supplementary Information accompanies the paper on European Journal of Human Genetics website (<http://www.nature.com/ejhg>)