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Assessment of Heart Laterality Defects in Zebrafish to Study Variants of Uncertain Significance in Primary Ciliary Dyskinesia

Abstract

Primary ciliary dyskinesia (PCD) is an autosomal recessive orphan disease (OMIM#244400) characterized by motile ciliary dysfunction. These hairlike organelles are responsible for the mucociliary clearance of the lungs, and varying degrees of infections in the upper respiratory tract—including the inner ear, nasal passage, and lungs—are common in affected children present with PCD. Moreover, ciliary function is critical for embryonic development, and defects in cilia can lead to situs anomalies, which are sometimes associated with congenital heart disease. To date, more than 50 genes have been implicated in the etiology of PCD, each affecting different parts of the motile ciliary apparatus. Testing via multi- or single-gene panels is recommended for confirmation of diagnosis, which enables timely treatment initiation and familial risk counselling. Unfortunately, for a significant proportion of children with clinical features consistent with PCD, a molecular diagnosis cannot be established. For many of these children, genetic testing returns a variant of uncertain significance (VUS) in a known PCD gene. Hence, there is a pressing need to develop strategies to validate unresolved PCD variants for their pathogenicity. The overall aim of this project is to resolve VUSs in children with suspected but genetically unconfirmed PCD by use of zebrafish (*Danio rerio*). As a key PCD gene in humans, *dnaaf1* has a *dnaaf1* homolog in zebrafish, making this model highly relevant for studying human PCD phenotypes. By employing gene knock-down technology (antisense morpholino oligonucleotides; MO), we established and characterized PCD-specific developmental readouts for the zebrafish gene *dnaaf1*, which affect different aspects of motile ciliary ultrastructure. We observed an increase in ventral body curvature and hydrocephalus in embryos with *dnaaf1*-MO, with 38% of *dnaaf1*-MO knockdown embryos showing heart-laterality defects. These phenotypic outcomes not only provide a concrete framework for assessing PCD-related developmental defects in zebrafish but also offer a platform for validating VUSs in human PCD genes. By performing co-injection experiments with patient-derived VUSs and examining the resulting phenotypic alterations, we can directly link specific genetic variants to observable PCD-like traits, offering a robust methodology for determining the pathogenicity of previously uncharacterized variants. This approach aims to enhance the accuracy of genetic diagnoses in PCD and provide new insights into its molecular mechanisms.

Introduction

Motile ciliary dysfunction defines the orphan disease (OMIM#244400) primary ciliary dyskinesia (PCD), which follows an autosomal recessive inheritance pattern. PCD is a rare, heterogeneous disease associated with more than 50 monogenic causes resulting from a loss of function in the motile cilia apparatus[1]. Cilia are complex structures encoded by many genes in various cell types and play a significant physiological role in humans. Motile cilia are responsible for the mucociliary clearance in the lungs and is vital in embryonic development. During development, cilia directionally beat to guide the flow of embryonic fluid, which establishes proper left/right axis body asymmetry and organ development. Structural or functional abnormalities in the cilia are formed by variants in known PCD genes. This can lead to two forms of *situs inversus*, conditions in which the normal asymmetry of internal organs is reversed or disrupted. The first, *situs inversus totalis*, involves major organs, including the heart and liver, being mirrored to the opposite side of the body. The second, *situs inversus totalis*, is characterized by irregular or inconsistent organ positioning, resulting in complex and often unpredictable arrangements[2]. These ciliary defects not only disrupt the typical left-right body asymmetry but also impair mucociliary clearance, which can lead to chronic respiratory issues and abnormal embryonic development. As a result, ciliary defect individuals often present with recurrent respiratory infections, such as bronchitis and congenital heart disease. More specifically, about 50% of patients with *situs inversus*—whether *totalis* or *ambiguous*—are also affected by these associated complications, including bronchitis and heart defects[3] (Figure 1). Understanding variants in PCD genes can help elucidate the genetic basis of these conditions and potentially inform diagnostic and therapeutic strategies.

PCD is a childhood onset disease with an estimated prevalence of 1:7,500 to 1:20,000 in liveborn babies[1]. However, this may be an underestimate due to variable clinical presentation and a current absence of readily available diagnostic markers for PCD. A diagnosis of PCD can be obtained clinically by demonstration of structurally abnormal cilia via transmission electron microscopy (TEM) and/or low nitric oxide (NO) inhalation measurements[4]. However, TEM is costly, and not all patients exhibit observable cilia abnormalities. Additionally, nasal nitric oxide (NO) tests are limited to children above the age of 5 years[5]. Therefore, molecular testing via multi-gene panels is the recommended clinical mainstay for early and accurate confirmation of a PCD diagnosis. Identification of the underlying molecular defect enables early supportive treatment initiation, accurate familial and reproductive risk counseling, as well as access to novel personalized treatments. Unfortunately, for a significant number of children, the molecular diagno-

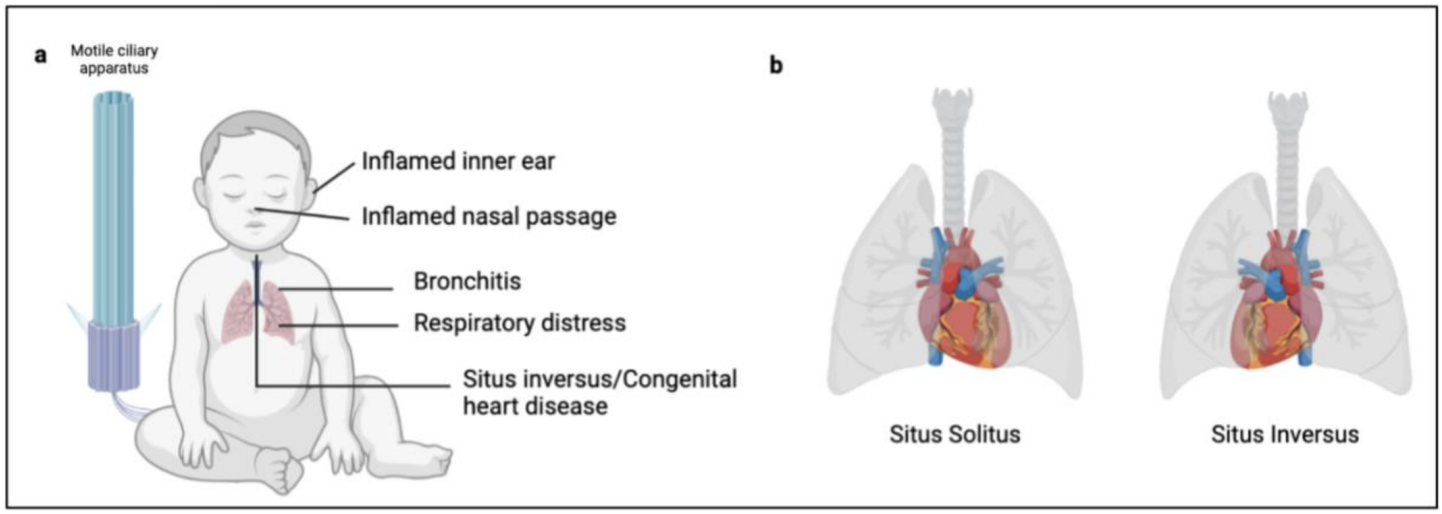

Figure 1. Clinical manifestations of primary ciliary dyskinesia (PCD) and its impact on organ laterality. **(a)** Common symptoms of PCD, including inflammation of the inner ear and nasal passages, bronchitis, respiratory distress, and *situs inversus* with or without congenital heart disease, are shown alongside a schematic of a motile ciliary apparatus. **(b)** Comparison of normal organ positioning (*situs solitus*) versus mirror-image organ arrangement (*situs inversus*), a hallmark phenotype observed in approximately 50% of PCD patients. Created in BioRender. Nurcombe, Z. (2025) https://BioRender.com/1czt0of

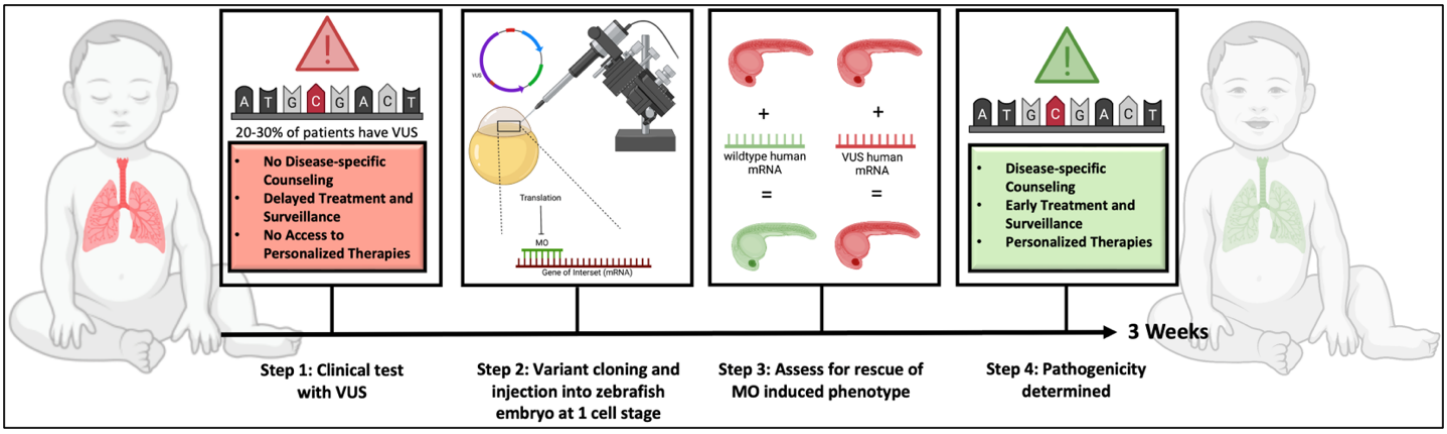

Figure 2. Workflow for assessing the pathogenicity of variants of uncertain significance (VUS) in primary ciliary dyskinesia (PCD) using a zebrafish model. Step 1: Clinical testing identifies patients with VUS, leading to challenges in treatment and counseling. Step 2: The VUS is cloned and injected into zebrafish embryos at the one-cell stage. Step 3: Phenotypic rescue is assessed by co-injecting wild-type and VUS mRNA. Step 4: Pathogenicity is determined, enabling disease-specific counseling, early treatment, and access to personalized therapies within approximately three weeks. Created in BioRender. Nurcombe, Z. (2025) https://BioRender.com/19xd4am

sis remains inconclusive due to a variant of uncertain significance (VUS) in PCD genes. Variants of uncertain significance are point mutations or deletions with unknown impact on gene and protein function, leading to undetermined pathogenicity. We propose zebrafish as a tool to rapidly validate VUS in PCD genes (Figure 2).

This project focuses on the study of the human ciliary PCD gene *Dynein Axonemal Assembly Factor 1* (*dnaaf1*). *dnaaf1* encodes a protein involved in the preassembly and transport of dynein arms, which are essential for the proper function of motile cilia. Dynein arms are motor protein complexes responsible for the coordinated beating of cilia, enabling functions such as mucociliary clearance in the respiratory tract and left-right body asymmetry establishment during embryonic development. Mutations in *dnaaf1* are associated with PCD, leading to defects in dynein arm assembly and loss of ciliary motility. Clinically, variants in *dnaaf1* lead to problems affecting the auditory (hearing loss), cardiovascular (dextrocardia), respiratory (sinusitis, bronchitis, bronchiectasis), and genitourinary (infertility in both male and female at birth) systems in patients. Most notably, 50% of patients with variants in *dnaaf1* present with *situs inversus* where the normal left-right asymmetry of internal organs is reversed[6,7].

The goal of this project is to confirm suspected PCD in patients with an inconclusive molecular diagnosis due to VUS in PCD-related genes by using zebrafish (*Danio rerio*) models. To achieve this, we developed an antisense mRNA probe for *cmlc2*, a gene essential for zebrafish heart development and a well-established marker for cardiac laterality. Since *dnaaf1* is crucial for dynein arm assembly in motile cilia, its disruption can impair ciliary function, leading to defects in left-right patterning during embryonic development. By using the *cmlc2* probe to visualize heart position in zebrafish embryos, we can assess laterality defects, which serve as an indicator of disrupted ciliary function. This approach allows us to determine whether specific *dnaaf1* variants impact motile cilia-driven processes, thereby linking genetic variants to observable phenotypes. Ultimately, this strategy provides functional validation of VUS in *dnaaf1* and other PCD genes, improving diagnostic accuracy for patients with unresolved genetic findings.

Zebrafish serve as an excellent model for establishing PCD phenotypes due to their high genetic homology (~70%) with humans, including the conservation of key genes such as *dnaaf1*, which is critical for ciliary function and motile cilia-driven processes[8]. One of the major advantages of zebrafish as a model organism is their rapid development and transparency during early

larval stages, allowing for real-time visualization of internal structures under light microscopy. Importantly, zebrafish develop a beating heart as early as 24 hours post-fertilization (hpf), providing a fast and accessible readout for assessing laterality defects associated with impaired ciliary function. Since ciliary dysfunction in PCD disrupts left-right asymmetry during embryonic development, the early formation of the zebrafish heart provides a rapid model for assessing whether defective cilia affect organ positioning—a hallmark phenotype of PCD. This rapid timeline enables efficient screening of genetic variants and accelerates functional validation of VUS in PCD-related genes. Overall, observing heart laterality in zebrafish is particularly valuable for investigating developmental pathways associated with PCD and translating these insights to human disease[9].

The *cmlc2* gene was used to determine heart laterality defects in zebrafish by serving as a molecular marker for cardiac tissue, enabling precise visualization of heart positioning. *cmlc2* is specifically expressed in the developing heart, making it an ideal target for assessing left-right patterning abnormalities associated with PCD-related ciliary dysfunction. To analyze heart laterality, we designed an antisense mRNA probe targeting *cmlc2* and used whole mount in situ hybridization (WISH) to stain and visualize the zebrafish heart. This allowed us to determine whether the heart was positioned normally (*situs solitus*, SS), abnormally mirrored (*situs inversus*, SI), located at the midline (midline heart, M), or duplicated (bilateral heart, B), all of which are key phenotypes linked to defective cilia function.

Morpholino knockdown technology is a widely used tool in zebrafish research to study gene function during development. Morpholinos are synthetic antisense oligonucleotides designed to bind complementary sequences in target mRNA, thereby blocking either translation or proper splicing. Unlike traditional RNA interference (RNAi), which relies on cellular machinery to degrade mRNA, morpholinos work by blocking ribosome access, preventing protein synthesis without triggering mRNA degradation. This makes them particularly useful for gene knockdown in early developmental stages.

By injecting morpholinos into zebrafish embryos, we were able to knock down known PCD-related genes and establish their corresponding phenotypes. This approach has already been successfully used to study *dnaaf1*, *dnaaf3*, and *dnah1* (Figure 1), revealing hallmark PCD phenotypes such as perturbed otoliths, hydrocephalus, body curvature defects, ciliary abnormalities in the eye, pronephric cysts, and *situs inversus*[9]. Notably, heart laterality defects—such as SS, SI, M, and B—have been well documented in *dnaaf1* and *dnaaf3* knockdown models[10,11]. Additionally, body curvature defects have been associated with *dnaaf1* loss, further confirming their role in cilia-driven developmental processes. Both heart laterality and body curvature abnormalities stem from defective ciliary function. Given the significance of these phenotypes associated with *dnaaf1* loss, we aimed to evaluate the pathogenicity of variants in *dnaaf1* through body curvature and hydrocephaly phenotypes. These two phenotypes are characteristic of PCD in zebrafish and therefore are a confident readout for these experiments.

To assess heart laterality defects in zebrafish models of PCD, researchers commonly probe for *cmlc2* (cardiac myosin light chain 2), a gene specifically expressed in the developing heart. *cmlc2* serves as a molecular marker for cardiac tissue, allowing visualization of heart positioning relative to the embryonic midline. By examining *cmlc2* expression patterns, we can determine whether the heart is properly positioned (SS) or exhibits abnormal laterality, such as leftward displacement (SI) or ambiguous positioning, all key phenotypes indicative of ciliary dysfunction.

Objective

We developed a probe to enable characterization of a PCD-specific phenotype in zebrafish. And we assessed heart laterality defects associated with mutations in the PCD gene, *dnaaf1*, to help confirm suspected PCD in patients with VUS.

Materials and Methods

Microinjections

Morpholinos (200 ng) (*Ensembl*) are injected to knock down gene expression in zebrafish and create knockdowns of targeted genes for up to 5 days post-fertilization (dpf)[12,13]. The *dnaaf1*-MO (Table 1a) inhibits expression of *dnaaf1* in zebrafish through a translation-blocking mechanism. Zebrafish embryos were injected (5 ng) at the 1-2 cell stage with 28.5 ˚C incubation, with either a non-targeting MO (Table 1b) as a negative control (250 uM) or a *dnaaf1*-MO (250 µmM), generating *dnaaf1* morphant embryos to establish the PCD-specific phenotypes in zebrafish. Embryos were fixed at 24 hpf in 4% paraformaldehyde (PFA).

a	*dnaaf1*-MO	5'-ATTTTTGTCTTCATTTCGCAGTGAT-3'
b	scrambled MO	5'-CCTCTTACCTCAGTTACAATTTATA-3'
c	PCR primers for *cmlc2* probe synthesis	f: 5' – GACCAACAGCAAAGCAGACA – 3' r: 5' – TAATACGACTCACTATAGGG GGGTCATTAGCAGCCTCTTG – 3'

Table 1. Sequences for PCR primers and MOs. a) *dnaaf1*-MO sequence. b) scrambled control MO sequences. b) PCR primers for *clmc2* probe synthesis. T7 RNA polymerase binding site in red.

Probe Synthesis

Designing an antisense mRNA probe for the *cmlc2* gene allows visualization of the developing zebrafish heart to assess heart laterality, a component of the PCD phenotype. The *cmlc2* gene contains a 77% similarity to the human homolog *MYL2*[14,15]. This zebrafish ortholog is a small transcript of roughly 1000 bp (NCBI Reference Sequence: NM_131329.3) and is expressed in the developing zebrafish heart at 24 hpf. To target a region of the *cmcl2* transcript (under 500 bp), forward and reverse primers for PCR were designed against the zebrafish *cmlc2* coding sequence. The reverse primer includes the TAATACGACTCACTATAGGG sequence, which serves as the T7 RNA polymerase binding site (Table 1c). PCR was carried out using 48 hpf zebrafish cDNA as the template, with Platinum SuperFi II polymerase according to manufacturer guidelines. This process ensured the incorporation of the T7 site at the 5' end of the *cmlc2* amplicon, enabling *in vitro* transcription. This T7 sequence allowed T7 RNA polymerase to synthesize antisense mRNA, which was used as the probe for detecting *cmlc2* expression. The alkaline phosphatase conjugated anti-digoxigenin (α-DIG) antibody reacts with the NBT/BCIP, which produces the purple signal of the probe in the zebrafish heart. The NBT/BCIP solution consisted of 4.5 mM nitro-blue tetrazolium chloride (NBT), 3.75 mM 5-bromo-4-chloro-3-indolyl phosphate (BCIP), 100 mM Tris buffer (pH 9.5), 50 mM magnesium chloride ($MgCl_2$), and 0.1% Tween-20. The DIG-labeled antisense mRNA probe hybridized to its complementary target in the zebrafish heart and was detected by an alkaline phosphatase (AP)-conjugated anti-digoxigenin (α-DIG) antibody. The colorimetric reaction occurs when NBT/BCIP, added in the staining buffer, serves as a substrate for AP, leading to the formation of an insoluble purple precipitate at the probe's location. This occurs because the antisense mRNA is labeled with digoxigenin (DIG)-UTPs during

in vitro transcription, allowing the α-DIG antibody to specifically bind to the probe and facilitate the colorimetric reaction.

Whole Mount mRNA In Situ Hybridization (WISH)

Standard WISH in zebrafish against the *cmlc2* transcript was carried out to visualize the heart, allowing us to assess laterality defects[16]. We fixed the embryos at 24 hpf in 4% PFA overnight at 4 ˚C. Fixed embryos were then dehydrated in 100% methanol and stored at –20 ˚C. Embryos were rehydrated through a series of methanol-phosphate buffered saline (PBS) washes (75/25, 50/50, 25/75, 0/100). The *cmlc2* probe (1:100 stock probe in hybridization buffer – formamide 50%, 5xSSC, Tween 20 0.1%, heparin 50ug/ml, torula RNA 500ug/ml, pH 6.0) is placed on the embryos and then are incubated at 70 ˚C overnight. Next, the embryos underwent two 30-minute washes in 2xSSC (saline sodium citrate buffer) at 70 ˚C and two 30-minute washes in 0.2xSSC at room temperature (RT). We then blocked the embryos with blocking buffer (PBT containing 2% sheep serum and 2 mg/ml bovine serum albumin (BSA)) for 1 hour at RT, followed by a 2-hour incubation with the anti-digoxigenin (α-DIG) antibody (diluted 1:1000 in blocking solution). After antibody removal, we incubated the embryos in PBT (PBS with 1% Tween 20) overnight at 4 ˚C. The embryos were washed twice in staining buffer (0.1 M Tris HCl pH 9.5, 0.1 M NaCl) for 15 minutes to eliminated residual PBS/PBT. Subsequently, they are diluted by adding NBT/BCIP (nitro blue tetrazolium/5-bromo-4-chloro-3-indolyl-phosphate) stock solution to the staining buffer (100 µm in 5 ml) and then added to embryos. Embryos were kept in the dark for roughly 30 minutes allowing time for the signal to develop. Once the signal had developed, we post-fixed the embryos in 4% PFA for 20 minutes at RT. The embryos then underwent a final wash and were stored in PBS at 4 ˚C for subsequent processing. We followed the Thisse and Thisse protocol[16].

Imaging

For assessment of body curvature and hydrocephalus, we live-imaged the embryos laterally at 48 hpf using basic light microscopy (LeicaMZ7.5 microscope with Opti Vision 4K Series CMOS microscope camera). For heart laterality, the embryos were fixed at 24 hpf, stained, and then individually mounted in 3% methylcellulose and imaged cephalically (Figure 2a) using basic light microscopy.

Quantification

To avoid bias during the assessment of heart position, the conditions were blinded during the quantification process. In zebrafish, heart position is classified in terms of its relative location to the eyes. During development, the heart initially forms at the midline and then loops to its designated side by approximately 48 hpf (ref. 17) (Figure 3a). The heart positions are classified as follows: If the heart is located from the lateral wall to the medial edge of the left eye, it is classified as *situs solitus* (SS). If the heart is located under the lateral wall to the medial edge underneath the right eye, it is classified as *situs inversus* (SI). Located between the two medial edges of the eyes is a midline heart (M) and if there are two hearts present it is classified as a bilateral heart (B). The embryos were appropriately quantified under the four conditions (SS, SI, M, B) and then subjected to further data analysis to assess the relevant phenotypic outcomes.

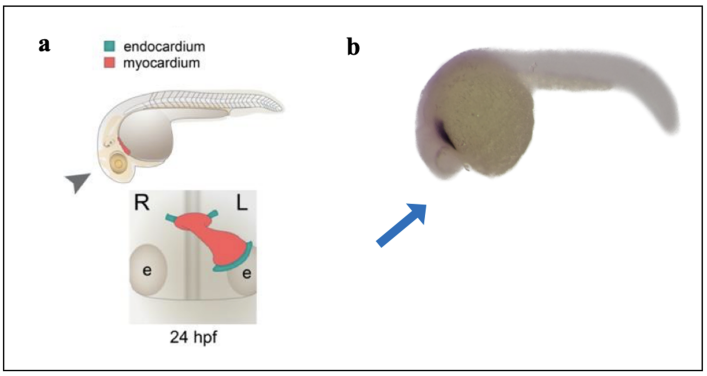

Figure 3. Imaging and quantification of zebrafish embryos at 24 hpf. a) Orientation of embryo for cephalic imaging via light microscopy of embryos fixed at 24 hpf to properly observe heart laterality b) Normal heart development position in zebrafish embryos at 24 hpf. Heart anchored medially with looping located directly under the left eye[17].

Figure 4. Body curvature phenotype in *dnaaf1*-MO knockdown embryos. a) Lateral view of *dnaaf1*-MO knockdown embryos at 48 hpf via live imaging exhibiting increased body curvature and hydrocephalus (red arrow).

Results

Body Curvature

To assess the role of *dnaaf1* in early zebrafish development, we performed morpholino (MO) knockdown and analyzed embryonic phenotypes at 48 hours post-fertilization (hpf). Compared to scrambled MO controls, *dnaaf1*-MO knockdown embryos exhibited pronounced ventral body curvature and increased hydrocephalus, suggesting defects in body axis formation and ciliogenesis. These phenotypic abnormalities are consistent with disrupted motile cilia function, which is required for proper left-right patterning and cerebrospinal fluid circulation. As shown in Figure 4a, the scrambled MO control embryo (top panel) displayed a normal elongated body axis and typical brain morphology. In contrast, the *dnaaf1*-MO knockdown embryo (bottom panel) exhibited a curved body axis and an enlarged brain ventricle indicative of hydrocephalus (red arrow). Quantification revealed that *dnaaf1*-MO embryos (n=17) presented with body curvature defects, supporting the role of *dnaaf1* in maintaining normal body symmetry. Statistical analysis was performed using a Chi-square test ensuring an appropriate comparison of categorical data. Replicates consisted of individual embryos from at least three independent experiments to confirm reproducibility.

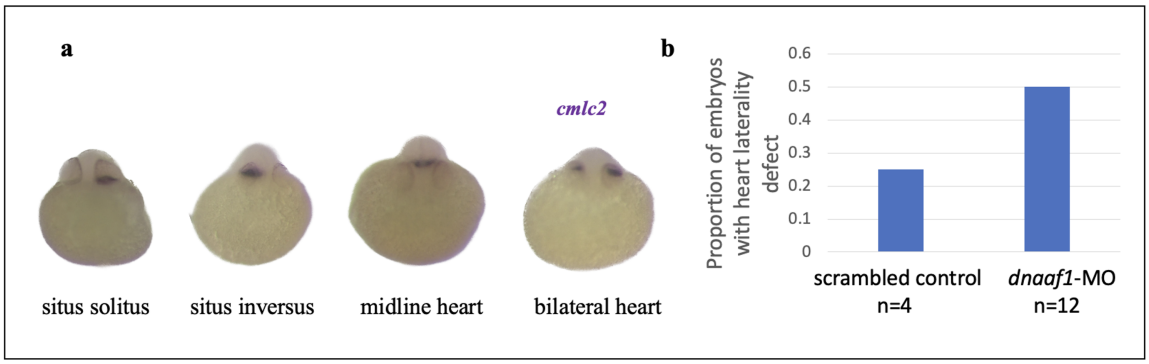

Figure 5. Laterality defect phenotypes and proportions in MO knockdown embryos. a) Cephalically imaged *dnaaf1*-MO knockdown embryos showing heart laterality phenotypes: *situs solitus* (normal), *situs inversus*, midline heart, and bilateral heart. b) Proportion of *dnaaf1*-MO knockdown embryos demonstrating heart laterality defects (0.14 versus 0.38; P=0.027).

Heart Laterality Defects

To evaluate heart laterality defects in *dnaaf1*-MO knockdown embryos, we used WISH with a *cmlc2* antisense probe to visualize *cmlc2* mRNA expression in cardiac tissue. *cmlc2* is a well-established marker of the zebrafish heart, allowing for precise localization and assessment of cardiac positioning in developing embryos. By comparing the heart's positioning in *dnaaf1*-MO embryos versus scrambled MO controls, we were able to identify defects in left-right asymmetry, a hallmark of PCD-related ciliopathy.

As shown in Figure 5a, we observed four distinct heart laterality phenotypes: *situs solitus* (normal), *situs inversus*, midline heart, and bilateral heart. Compared to controls, *dnaaf1*-MO knockdown embryos exhibited a significantly higher incidence of heart laterality defects, with a noticeable increase in *situs inversus*, midline heart, and bilateral heart phenotypes. Quantification of heart laterality defects (Figure 5b) revealed that 38% of *dnaaf1*-MO embryos (n=17/45) displayed abnormal heart positioning compared to only 14% of scrambled MO controls (n=7/50), with a statistically significant difference (P=0.027, Chi-square test). This analysis was conducted using biological replicates from at least three independent experiments, ensuring statistical robustness. Additionally, each n value represents individual embryos, with percentages calculated per replicate rather than as a single data point. These findings support the role of *dnaaf1* in proper left-right patterning.

Discussion

By morpholino mediated gene knockdown, we observed PCD phenotypes in 48 hpf zebrafish embryos. The first PCD phenotype observed is an increase in ventral body curvature in *dnaaf1*-MO knockdowns (Figure 3a). This is consistent with the primary PCD phenotype of ventral body curvature in zebrafish for the PCD gene *Lrcc50*, observed by Van Rooijen et al. (2009)[10]. These authors developed a line of zebrafish with a mutation in the *Lrcc50* gene. Their mutant embryos showed a pronounced ventral body curve, and cilia of Kupffer vesicles, which are required to break bilateral symmetry in zebrafish, showed no motility. Mutant pronephric tubules showed cysts and reduced brush border with short microvilli. Electron microscopy of pronephric mutant cilia revealed ultrastructural irregularities, and some axonemes completely lacked all dynein arms or had misplaced inner dynein arms. Van Rooijen et al. (2008) concluded that *Lrcc50* is critical for ciliary architecture, proper left-right asymmetry, organ positioning, and body axis curvature. Improper cilia function leads to malformation of the curvature of the embryos, making it a good marker to characterize PCD. Their methodology is congruent with ours in using zebrafish KOs as a model of disease. However, their experiments were conducted with 24 hpf embryos, different from our 48 hpf time point. The difference in developmental stages is an important consideration. At 24 hpf, zebrafish are still in the early stages of body plan establishment, and defects in body axis curvature may be more subtle or in an earlier phase of manifestation. In contrast, by 48 hpf, the curvature defects may be more pronounced and fully realized, which could explain the more obvious phenotypic observations in our study.

The other observed PCD phenotype from our study is an increase in heart laterality defects in *dnaaf1*-MO knockdown embryos at 48 hpf. The *dnaaf1*-MO embryos demonstrated *situs solitus* (SS), *situs inversus* (SI), midline heart (M), or bilateral heart (B) (Figure 4a). These heart laterality defects are a hallmark of ciliary dysfunction, and they are commonly associated with PCD in both zebrafish models and humans. The findings of our study align with the work of Van Rooijen et al. (2009)[10], who also reported similar heart laterality defects in their own PCD morphants zebrafish. Van Rooijen et al. (2009)[10] observed all heart laterality phenotypes in *dnaaf1* morphants except the bilateral heart phenotype. The cause of the bilateral heart phenotype is unknown and requires further investigation, however we still considered it as a laterality defect for quantification of this experiment.

In humans, mutations in *dnaaf1*, a gene involved in the assembly of dynein arms in motile cilia, lead to a variety of PCD-related defects, including heart laterality defects. Approximately 50% of individuals with *dnaaf1* mutations exhibit heart laterality defects, including *situs inversus* or situs *ambiguous* (partial *situs inversus*), in which the heart is either reversed or positioned incorrectly due to defects in left-right patterning[18]. As expected, we observed a statistically significant (P=0.027) proportion of heart laterality defects (Figure 4b), confirming its specificity for PCD. This result confirms the specificity of heart laterality defects as a reliable marker for studying PCD and further validates the zebrafish model for investigating the molecular mechanisms of this disease.

While MO knockdown remains a widely used approach for gene silencing in zebrafish, we must acknowledge its potential for off-target effects, which have been extensively documented in the field. To mitigate this, we ensured specificity by using a scrambled MO control and analyzing phenotypic consistency across multiple independent experiments. However, future studies using CRISPR-Cas9 or mutant lines would be valuable in further validating the role of *dnaaf1* in early zebrafish development.

Conclusion

The goal of this study was to develop a heart-specific probe and establish the heart laterality defect as a PCD-specific phenotype in zebrafish, par-

ticularly in the context of *dnaaf1* knockdown. We utilized MO-mediated gene knockdown technology to disrupt *dnaaf1* expression in zebrafish embryos, with the hypothesis that these embryos would exhibit an increased proportion of heart laterality defects, a hallmark of PCD. Through our experiments, we demonstrated that *dnaaf1*-MO knockdown embryos did indeed show an increased incidence of heart laterality defects, including *situs inversus*, midline heart, and bilateral heart phenotypes, providing strong evidence that *dnaaf1* is a key player in the establishment of left-right asymmetry and proper organ positioning in zebrafish. These findings confirm the utility of the heart laterality defect as a reliable and specific phenotype for PCD in zebrafish models.

Our findings contribute to the ongoing project aimed at establishing zebrafish as a robust *in vivo* model for the study of PCD. The overall aim is to establish zebrafish as a tool for rapid variant validation to confirm probable PCD diagnoses. By optimizing MO-knockdown protocols and the heart-specific probe we developed, our research lays the groundwork for future studies focused on the role of other PCD-associated genes, such as *dnaaf3* and *hydin*, where phenotypic characterization in zebrafish has yet to be established. These additional gene knockdowns will help broaden our understanding of the diverse phenotypic spectrum of PCD and contribute to the genetic and clinical characterization of this complex disease.

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