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Short Communication

Rare loss-of-function variants reveal threshold and multifactorial inheritance of dextrocardia

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Congenital heart disease (CHD) is the most common type of congenital malformation. Recent studies using massively parallel sequencing (MPS) have shown that some familial forms of CHD, previously considered to be Mendelian due to single-gene mutations, may actually be oligogenic or polygenic inheritance [1]. Transcription factors, signaling molecules, and structural proteins, are all involved in cardiac development [2]. Although the genetic architecture of CHD is not fully understood, more than half of the CHD-causing genes identified through whole-exome sequencing (WES) have been linked to ciliopathies in mouse models [3], indicating that CHD may be a new type of ciliopathy [4]. Clinically, ciliary dysfunction has been observed in over 40% of CHD patients with heterotaxy [5]. In addition, damaging variants in ciliary genes have been identified in human transposition of the great arteries (TGA) patients [6], one of the most severe types of CHD, supporting the idea that CHD is a type of ciliopathy.

Dextrocardia, a condition where the heart is located on the right side of the thoracic cavity, occurs in approximately 0.83 of 10,000 pregnancies [7] and is often associated with complex CHDs. Complex CHDs refers to a group of CHDs that are more severe and require more complex surgical procedures to correct compared to other types of CHDs. The recent "omnigenic" model suggests that many complex diseases are directly affected by a relatively small number of core genes and are largely driven by peripheral genes with no direct role in the disease [8]. A study on human neural tube defects (NTDs) using whole genome sequencing (WGS) demonstrated that complex birth diseases such as NTDs can be measured by the accumulation of singleton loss-of-function variants (SLoFVs) or the threshold of SLoFVs [9]. While the omnigenic threshold model has been validated for NTDs [9], it remains unclear whether this model also applies to dextrocardia.

In this study, we recruited 194 children with dextrocardia from different provinces at the Shanghai Children's Medical Center. All study protocols were reviewed and approved by the medical ethics committee of the Shanghai Children's Medical Center (approval No. SCMC-201015), conducted in accordance with the *Helsinki Declaration*, and written consent was obtained from the parents of the subjects and/or the subjects themselves before study enrollment. These dextrocardia patients had a mean age of 19.1 ± 37.9 months and exhibited various CHD phenotype, including 95 (49.0%) with situs solitus (isolated dextrocardia), 47 (24.2%) with situs inversus, and 52 (26.8%) with situs ambiguous (Table S1 online). More than half of the dextrocardia patients had associated ventricular septal defect (VSD, 58.8%), atrial septal defect (ASD, 52.1%), and pulmonary stenosis (PS, 54.6%). Additionally, almost half of all patients had double outlet right ventricle (DORV,44.8%). In summary, all dextrocardia patients in this study exhibited diverse CHD phenotypes.

The developmental origins of dextrocardia can be traced back to the establishment of the left-right axis during gastrulation, which is influenced by the movement of cilia in the embryonic node [10]. Therefore, we initially evaluated rare loss-of-function (LoF) variants of 428 ciliary genes (187 established and 241 candidate

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ciliopathy-associated genes [11]) in our Chinese dextrocardia cohort using WES (100× coverage) (Fig. S1 online). The selected LoF variants were further compared with 1000 Genomes Project (1KGP) and ExAC databases. Our analysis revealed that 44.3% (86/194) of human dextrocardia patients carried rare LoF variants ($MAF_{1KGP} < 0.001$ and $MAF_{ExAC} < 0.001$) in 78 genes, including both established and candidate ciliopathy-associated genes (Table S2 online). We then compared the burden of rare LoF variants in 194 Han Chinese individuals with dextrocardia to 208 normal Han Chinese controls from 1KGP (higher coverage in coding region) in 428 ciliary genes. However, no single established and candidate ciliopathy-associated gene showed a significant ($P < 0.05$) excess of rare LoF variants in the dextrocardia cohort (Table S2 online). Therefore, we performed a burden test using a binomial test to investigate rare LoF variants of ciliary gene sets. The results demonstrated that there was no significant increase in rare LoF variants within both the established ciliopathy-associated gene set (enrichment = 1.3, binomial test, $P = 0.18$) and the candidate ciliopathy-associated gene set (enrichment = 1.3, binomial test, $P = 0.18$) (Fig. 1a, andTable S3 online). We subsequently detected the combined effect of rare LoF variants in all 428 ciliary genes, revealing a marginally significant 1.3-fold (binomial test, $P = 0.05$) enrichment of rare LoF variants (Table S3 online). Furthermore, when we compared the rare damaging alleles of ciliary genes in TGA replication and controls of obese (OBE) children without cardiac defects using data from published research [6], we observed no statistically significant excess of rare damaging alleles in the ciliary gene set at a significance level of $P < 0.01$ in the TGA replication group (Fig. 1b and Table S4 online). These genetic patterns of ciliopathy-associated genes highlight their pathogenic roles in dextrocardia. These results implicate that dextrocardia is likely influenced by genetic interactions rather than a single mutation. However, further investigations with larger sample sizes are necessary to fully understand the potential pathogenic role of ciliary genes in patients with dextrocardia.

Since defects in genes expressed during heart development are likely to be closely associated with CHD due to their critical role in providing essential developmental cues, we investigated 387 genes with elevated expression in heart tissues, including 162 heart-specific (“tissue/group-enriched”) genes and 225 “tissue-enhanced genes” (classification criteria can be found in the Methods section online). We observed a significant excess of rare LoF variants in the 387 elevated genes in the heart of the dextrocardia cohort compared to controls (enrichment = 1.4, binomial test, $P = 0.03$) (Table S5 online). However, there was no significant excess of rare LoF variants in the 225 heart tissue-enhanced genes between the dextrocardia and control group (binomial test, $P = 0.74$) (Fig. 1c, and Table S5 online). Interestingly, when we removed the tissue-enhanced genes, the excess became more pronounced (enrichment = 1.9, binomial test, $P = 6.0 \times 10^{-3}$) (Fig. 1c, and Table S5 online). Furthermore, we found a significant excess in the 162 heart-specific genes compared to the 225 tissue enhanced genes among the dextrocardia patients (Chi-squared test, $P = 9.0 \times 10^{-3}$) (Table S6 online). Our findings also indicated that none of the ciliary genes identified with rare LoF variants in the dextrocardia cohort were classified as tissue/group-enriched genes. Based on the involvement of transforming growth factor (TGF)-beta signaling in the transmission and interpretation of mechanical signals generated by ciliated cells in the node, which is crucial for the proper development and positioning of organs on the left and right sides of the body [12], we hypothesized that mutations in TGF-beta signaling components may contribute to the development of dextrocardia. Although a significant excess of rare LoF variants were identified in TGF-beta signaling pathway (Fig. S2 online), the statistical significance did not reach the Bonferroni-corrected threshold after adjustment. Further research is required to understand their mediated mechanisms in dextrocardia.

Although the occurrence of dextrocardia with complex CHD is associated with both the asymmetrical development of the left-right axis and the development of the heart itself, no significant excess of rare LoF variants identified in the ciliary gene set, heart-specific gene set or TGF-beta signaling gene set (Table S7 online) after applying the Bonferroni correction. The study’s findings suggest that additional genes are involved in the causation of dextrocardia through a combination of inherited rare LoF variants and an omnigenic threshold model. The number of SLoFVs has been suggested to measure the risk of NTDs [9], as it is similar among different ethnic control population [13]. We then compared the number of SLoFVs between dextrocardia patients and controls. The results showed a significant difference in the number of SLoFVs between dextrocardia cases and controls, with dextrocardia samples having a median of 13 SLoFVs per person compared to the controls’ median of 6 SLoFVs (two-sided Wilcoxon test, $P = 6.4 \times 10^{-49}$) (Fig. 1d). The robustness of this findings was tested by comparing the number of SLoFVs between systemic lupus erythematosus (SLE) cases (100× coverage) and controls from 1KGP, where SLE cases carried more SLoFVs than controls (7 vs. 6, two-sided Wilcoxon test, $P = 0.002$) but did not reach significance level based on the Bonferroni-corrected threshold based on correction for testing ~ 20,389 genes ($P < 2.5 \times 10^{-6}$) (Fig. 1d). In contrast, comparison between TGA cases, a subtype of CHD with left–right asymmetry disorders, and common controls demonstrated that TGA cases (55× coverage) carried significantly more SLoFVs than controls (13 vs. 6, two-sided Wilcoxon test, $P = 3.6 \times 10^{-57}$; Fig. 1e) or SLE cases (13 vs. 7, two-sided Wilcoxon test, $P = 1.4 \times 10^{-24}$). These results demonstrate that the number of SLoFVs can be utilized to compare the risk between dextrocardia cases and matched controls, as well as the risk between TGA and controls. Furthermore, the odds ratios (ORs) for dextrocardia were calculated with different numbers of SLoFVs, revealing a threshold number of SLoFVs for dextrocardia risk. When the number of SLoFVs reached 8, the OR for dextrocardia was greater than 1 (OR = 6.9 (1.2–21.2)) (Fig. 1f). As the number of SLoFVs increases, the risk for dextrocardia increases exponentially, with an OR of 22.8 (11.4–44.7) for 12, OR = 59.6 (25.3–151.2) for 14, and OR = 357.8 (56.9–13,429.0) for 16 SLoFVs. We additionally demonstrated that the Chinese NTD cohort (primarily anencephaly) [9], exhibited a higher burden of SLoFVs compared to dextrocardia, which is a less severe structural birth defect (Fig. S3 online). However, due to the limited sample sizes available for analysis, the observed difference did not reach a Bonferroni-corrected threshold.

Based on the omnigenic theory [8], which suggests that core genes play a more significant role in determining phenotype than peripherally connected genes, we hypothesized that LoF mutations in core genes, such as heart-specific genes, are more likely to cause heart defect compared to tissue-enhanced genes that have only peripheral effects on heart defect. To test this hypothesis, we employed zebrafish models to knock down representative genes with LoF variants belonging to core and peripheral genes. Among the six candidate genes selected, myosin heavy chain 6 (*MYH6*) and myosin binding protein C3 (*MYBPC3*) were tissue-enriched genes that were highly expressed in the heart muscle, while myosin heavy chain 7 (*MYH7*) and cardiomyopathy associated 5 (*CMYA5*) were group-enriched genes expressed in the heart muscle (Fig. 2a). These highly expressed genes that are heart-specific (tissue- and group-enriched genes) represent the core genes, while the heart-enhanced genes desmin (*DES*) and solute carrier family 25 member 3 (*SLC25A3*), which are widely expressed, belong to peripheral genes. We found that rare LoF variants identified in these candidate genes were very rare or absent in the genomAD database and were classified as pathogenic (Table S8 online)

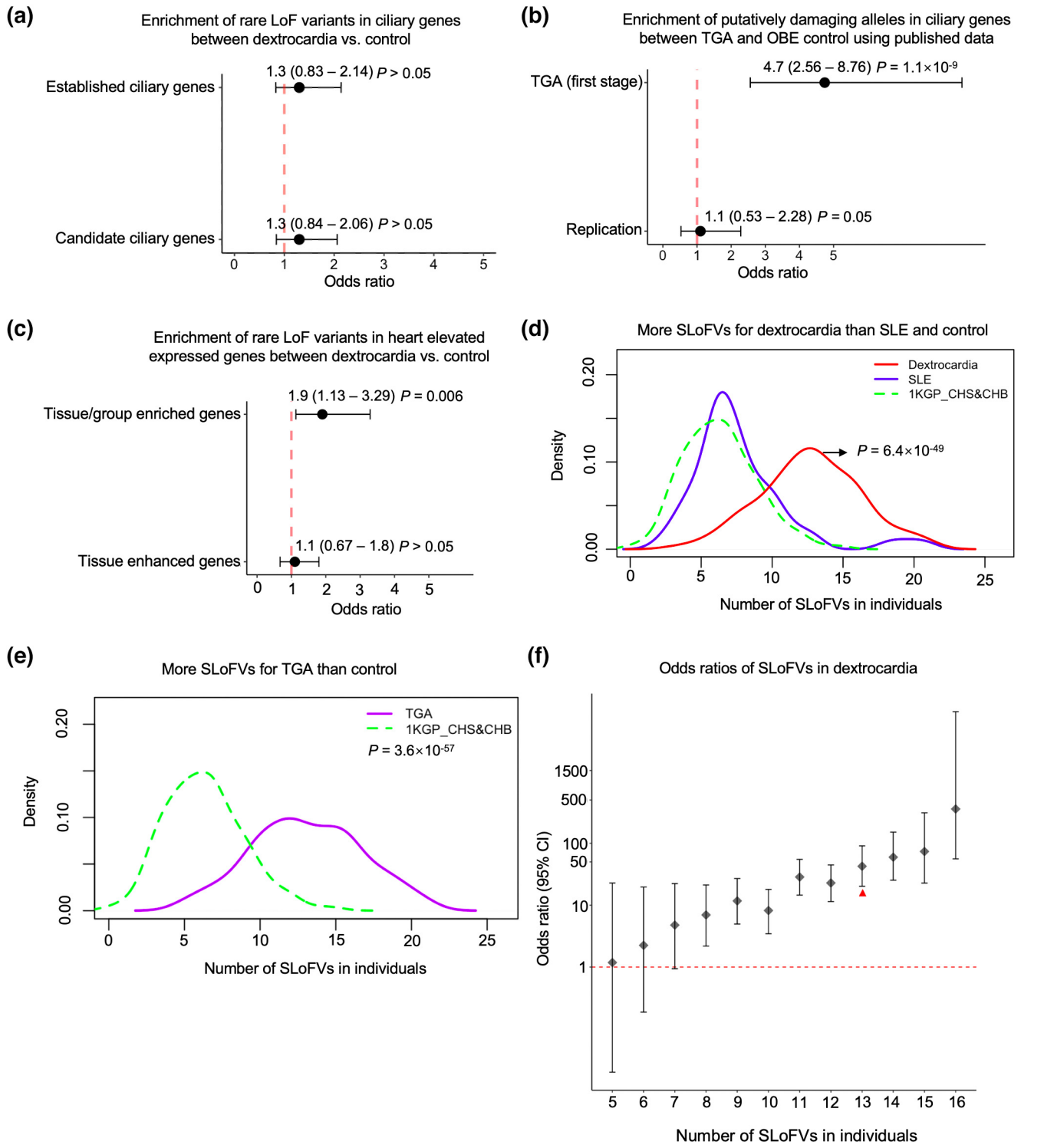

Fig. 1. Rare loss-of-function (LoF) variant burden analyses and omnigenic threshold model in human dextrocardia. (a) Enrichment of rare LoF variants in ciliary genes among dextrocardia cohort and controls. (b) Enrichment of putatively damaging alleles in ciliary genes between human transposition of the great arteries (TGA) patients and OBE (obese children without cardiac defects) controls based on published data. (c) Enrichment of rare LoF variants in elevated expressed genes in heart tissues between dextrocardia cohort and controls. (d) Significantly more singleton LoF variants (SLoFVs) were found in dextrocardia or TGA cohort as compared to Chinese controls from 1000 Genomes Project (1KGP) controls and systemic lupus erythematosus (SLE) cohort. (e) Significantly more SLoFVs were found in the TGA cohort as compared to Chinese controls. (f) Odds ratios of SLoFVs in dextrocardia. The red arrowhead represents the median number of SLoFVs in dextrocardia.

according to the ACMG/AMP guidelines. Using microinjection of morpholinos (MOs) for each of the enriched or enhanced genes into 12 cell staged zebrafish, we observed that knockdown of heart-enriched core genes led to more severe cardiac abnormalities, such as arrested looping and pericardial effusion, with a statistically higher ratio compared to knockdown of all-elevated peripheral genes, such as *DES* and *CMYA5*. Specifically, the abnormal ratios in *myh6*-MO, *mybpc3*-MO, *myh7*-MO, *cmya5*-MO, *desma*-MO, and *slc25a3b*-MO zebrafish models were 84.4%, 56.5%, 54.7%, 34.1%, 31.6%, and 8.8%, respectively (Fig. 2b, c). The number of

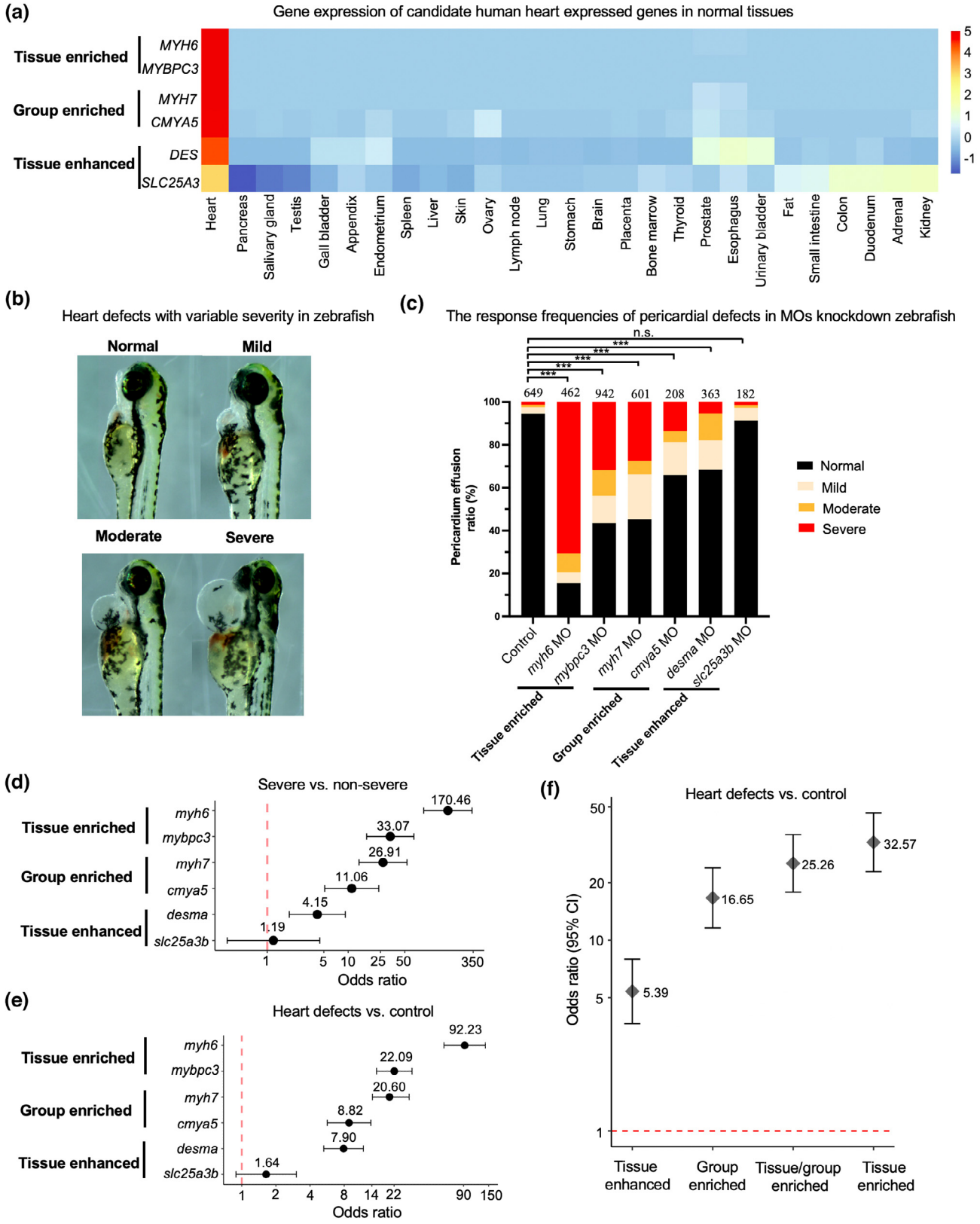

Fig. 2. The damaging core genes in dextrocardia produce a significantly higher rate of heart abnormality in morpholinos (MOs) knock down zebrafish. (a) Two human heart tissue enriched genes (*MYH6* and *MYBPC3*), two group enriched genes (*MYH7* and *CMYA5*) and two tissue enhanced genes (*DES* and *SLC25A3*) are differentially expressed in different tissues. (b) Typical three days postfertilization zebrafish morphology of four categories according to heart defects with variable severity: normal heart; mild defect: heart shows mild looping arrested, pericardium effusion; moderate defect: heart shows moderate looping arrested, pericardium effusion; severe defect: malformed embryo with small, string-like heart, severe pericardium effusion. (c) Distribution of the four categories in each group after we injected *myh6*-MO (n = 462), *mybpc3*-MO (n = 942), *myh7*-MO (n = 601), *cmya5*-MO (n = 208), *desma*-MO (n = 363), *slc25a3b*-MO (n = 182) and controls (STD CTR, n = 649) into zebrafish embryos, respectively. Association of *myh6*-MO, *mybpc3*-MO, *myh7*-MO, *cmya5*-MO, *desma*-MO, and *slc25a3b*-MO with (d) severe heart defects compared to non-severe and (e) heart defects compared to controls in zebrafish. (f) Combined association of tissue enhanced genes (*desma*-MO and *slc25a3b*-MO), group enriched genes (*myh7*-MO and *cmya5*-MO), tissue and group enriched genes, and tissue enriched genes (*myh6*-MO and *mybpc3*-MO) with heart defects compared to controls in zebrafish.

severe heart defects increased significantly in *myh6*-MO (OR = 170.5), *mybpc3*-MO (OR = 33.1), *myh7*-MO (OR = 26.9), *cmya5*-MO (OR = 11.1), and *desma*-MO (OR = 4.2) zebrafish as compared to the controls ($P < 0.001$) (Fig. 2d). Furthermore, the OR for *myh6*-MO (OR = 92.2), *mybpc3*-MO (OR = 22.1), *myh7*-MO (OR = 20.6), *cmya5*-MO (OR = 8.8), and *desma*-MO (OR = 7.9) zebrafish were significantly associated with heart defects as compared to the controls ($P < 0.001$), whereas *slc25a3b*-MO (OR = 1.6) did not reach the significant level ($P > 0.05$) (Fig. 2e). The OR for heart-specific genes was 25.3, $P = 4.5 \times 10^{-130}$ (Fig. 2f). It is worth noting that, although we have observed that knockdown of heart-specific genes is more likely to result in cardiac malformations under identical experimental conditions, off-target effects may also be present and have a certain degree of impact on phenotype variation, even though it is unlikely to significantly affect the overall statistical results.

In conclusion, our study has demonstrated that the genetic influence on dextrocardia is partly due to ciliopathy-associated genes, and additional genetic mutations also contribute to its genetic etiology. Additionally, the omnigenic threshold model offers a plausible explanation for the genetic cause of dextrocardia. We highlight the significance of genes that are specifically expressed in heart cells, which could indicate a greater risk for dextrocardia. These findings provide a much clearer understanding of the genetic architecture of dextrocardia and could offer guidance for the diagnosis and therapeutic management of the condition.

Conflict of interest

The authors declare that they have no conflict of interest.

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Author contributions

Hongyan Wang, Zhongzhong Chen, and Yunqian Gao designed and led the project. Zhongzhong Chen, Nan Li, and Jianguo Zhang performed genetics and bioinformatics analysis. Yunqian Gao, Lei Lu, Pei Liu, Rui Peng, and Lingling Liu conducted the experiments. Hongyan Wang, Qihua Fu, Haifa Hong, Jianguo Zhang, and Hefeng Huang supervised the study and commented on the project. Finally, all authors reviewed and approved the manuscript.

Appendix A. Supplementary materials

Supplementary materials to this short communication can be found online at https://doi.org/10.1016/j.scib.2023.08.005.

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