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Recent advances in causes, diagnosis, and therapeutics for congenital heart defects

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Recent advances in causes, diagnosis, and therapeutics for congenital heart defects

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Editorial: Recent advances in causes, diagnosis, and therapeutics for congenital heart defects

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Editorial on the Research Topic
Recent advances in causes, diagnosis, and therapeutics for congenital heart defects

Introduction

Congenital heart disease (CHD) is one of the most prevalent major birth defects, yet its causes remain largely unknown. Both genetic and environmental factors play a role. Animal and human induced pluripotent stem cell models have shown how these factors disrupt heart development (Liu et al., 2017; Xu et al., 2022), but the precise mechanisms in humans remain unclear.

Advanced genetic and genomic approaches have significantly improved CHD diagnosis and therapies, especially through prenatal genetic testing, enabling earlier and more accurate diagnosis and screening. As survival rates into adulthood improve, new research directions have emerged, including exploring the genetic basis of surgical outcomes and developing therapies to enhance the quality of life for CHD patients. The growing population of adults with CHD, who lacked access to modern genetic technologies during childhood, underscores the need for ongoing research and tailored medical care (Bhatt et al., 2015).

This Research Topic encompasses a total of 14 articles, including basic research studies, clinical case reports, and a mini review. The novel findings focus on both pediatric and adult CHD (ACHD), covering recent advances in the causes, diagnosis, and therapeutics of CHD. These studies collectively demonstrate that integrating genetic data with clinical

assessments provides crucial insights for developing precise diagnostic and therapeutic strategies.

New causes and biomarkers in heart development abnormalities

The identification of novel genetic markers and pathways is a pivotal advancement in CHD research. Several studies in this Research Topic revealed novel biomarkers and genetic factors in cardiac developmental abnormalities. Hu et al. have reported that the structural protein Sorbs2 promotes the development of the second heart field, with its deficiency resulting in atrial septal defects. Their continued research found increased cardiac macrophages in Sorbs2-deficient hearts, revealing a potential role for macrophages in responding to embryonic myocardial abnormalities. A significant breakthrough came from Xu et al., who identified Keratin 19 (Krt19) as a novel epicardial gene through cardiac single-cell mRNA sequencing analysis. This discovery provides valuable insights into epicardial contribution to embryonic and neonatal heart development, addressing a long-standing challenge in the field. Additionally, Li et al. identified novel markers associated with increased risk and pathogenesis of immune checkpoint inhibitor-associated myocarditis through serum autoantibody profiles, expanding our understanding of cardiac complications in immunotherapy.

Genetic diagnosis in human congenital heart disease

The application of advanced genetic and genomic technologies, such as microarray testing and next-generation sequencing (NGS), has revolutionized the diagnostic landscape of CHD. Mascho et al. found an association between left outflow tract obstruction and 5p deletion, with high mortality in the presence of additional copy number variants. Similarly, Yu et al. used microarray analysis to identify both *de novo* and inherited micro-CNVs at 16p13.11 in 21 Chinese patients with defective cardiac left-right patterning. Significant progress in understanding specific CHD subtypes emerged from several studies. Li et al. conducted whole exome analysis of 25 Patent foramen ovale (PFO) patients, identifying potential mutant genes like *LDLR, SDHC*, and *NKX2-5*, enhancing our understanding of PFO's genetic basis. Chen et al. explored the impact of a *KCNH2* missense variant on Long QT syndrome, revealing incomplete penetrance influenced by sex, potentially shedding light on the distinct penetrance behaviors and patterns of the *KCNH2* gene. In another study, Wang et al. studied complex arrhythmias in children with *RYR2* gene sequence variation, providing evidence for early detection and treatment. Additionally, Shen et al. reported a pathogenic MEIS2 sequence variation in a patient with multiple conditions, expanding the CHD symptom spectrum associated with *MEIS2* sequence variations.

Potential therapeutics and new challenges

New research and advances in genetic diagnostics have led to evidence-based therapies. This Research Topic highlighted several promising therapeutic developments and identified critical challenges in CHD management. Dyrka et al. showed the efficacy and safety of long-term recombinant growth hormone treatment on aortic dimensions in a girl with Loeys-Dietz Syndrome. Hiraya et al. identified implications for diagnostic strategies and therapeutic approaches by performing genetic testing and human leukocyte antigen analysis in patients with hypertrophic cardiomyopathy and connective tissue diseases.

Improved management of CHD has increased the life expectancy of adults with CHD. Many adults missed out on genetic evaluations during childhood due to the lack of modern genetic technologies. Oehlman et al. highlighted the need for guidelines to enhance access to genetic services and improve medical management by studying how ACHD cardiologists offer genetic services. Edwards et al. revealed significant gaps in genetics-related care, especially for those ACHD patients with congenital and neurodevelopmental comorbidities, through a retrospective chart review.

The identification of neurological deficits in adult CHD patients, as reviewed by Saric et al. , points to the necessity of integrating genomic data with clinical history for better therapeutic targeting. They also suggested that investigating the interactions among ciliary genetics, CHD, and neurodevelopment could improve therapeutic management.

Conclusion

In conclusion, this Research Topic advances our understanding of CHD by elucidating its genetic causes, improving diagnostics and therapeutics, and identifying gaps and challenges. These studies provide essential insights for greater diagnostic precision and new directions for enhancing CHD care and management. It also highlights multidisciplinary collaboration and leveraging of cutting-edge technologies will continue to drive innovations in CHD research, leading to better patient care and improved clinical outcomes.

Author contributions

XX: Writing–original draft, Writing–review and editing. CL: Writing–review and editing. LM: Writing–review and editing. LH: Writing–review and editing. DX: Writing–review and editing.

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Current approach to genetic testing and genetic evaluation referrals for adults with congenital heart disease

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Background: Congenital heart disease (CHD) is the most common congenital anomaly. Up to 33% have an identifiable genetic etiology. Improved medical and surgical management of CHD has translated into longer life expectancy and a rapidly growing population of adults living with CHD. The adult CHD (ACHD) population did not have access during childhood to the genetic technologies available today and therefore have not had a robust genetic evaluation that is currently recommended for infants with CHD. Given this potential benefit; the aims of this study were to determine how ACHD cardiologists offer genetics services to patients and identify the indications that influence decision-making for genetics care.

Methods: We performed a descriptive cross-sectional study of ACHD cardiologists. A study-developed questionnaire was distributed via emailed REDCap link. The recruitment email was sent to 104 potential respondents. The survey was open from 06/2022 to 01/2023.

Results: Thirty-five cardiologists participated in the study (response rate of 34%). Most cardiologists identified as white (77%) and male (66%). Cardiologists were more likely to refer patients to genetics (91%) than to order testing themselves (57%). Of the testing ordered, chromosomal testing (55%) was ordered more than gene sequencing (14%). Most cardiologists would refer a patient with a conotruncal lesion (interrupted aortic arch) over other indications for a genetics evaluation. There were more reported barriers to ordering genetic testing (66%) compared to referring to genetics for a genetics evaluation (23%). Cardiologists were more confident recognizing features suggestive of a genetic syndrome than ordering the correct test (p = 0.001). Regarding associations between clinical factors and current practices, more years in practice trended towards less referrals and testing. Evaluating a greater number of patients (p = 0.11) and greater confidence recognizing syndromic features (p = 0.12) and ordering the correct test (p = 0.09) were all associated with ordering more testing.

Conclusion: Testing for microdeletion syndromes is being offered and completed in the ACHD population, however testing for single-gene disorders

associated with CHD is being under-utilized. Developing guidelines for genetic testing in adults with CHD could increase access to genetic services, impact medical management, reduce uncertainty regarding prognosis, and inform recurrence risk estimates.

KEYWORDS

cardiology, genetic testing, inclusion of genetic services, ACHD, clinical practice

1 Introduction

Congenital heart disease (CHD) has a birth incidence of 0.8%–1.0% making it the most common major congenital anomaly seen in newborns (Bracher et al., 2017; Ito et al., 2017).

Most heart defects are isolated and have an unknown etiology, but up to 33% of patients with CHD have an identifiable genetic cause (De Backer et al., 2020). Genetic etiologies for CHD can be divided into chromosomal anomalies and single gene variants. Chromosomal associated CHD includes trisomy 21 (Down syndrome), monosomy X (Turner Syndrome), and microdeletions at 22q11.2 (DiGeorge, velocardiofacial syndrome), 7q11.23 (Williams Syndrome) and 1p36. Single-gene variants can cause syndromic CHD (e.g., Noonan syndrome, Holt-Oram syndrome, Alagille syndrome) as well as isolated genetic CHD (e.g., variants in NOTCH1 and FLT4) (Pierpont et al., 2007; Nees and Chung, 2020). Patients with CHD benefit from evaluation for chromosomal abnormalities, single gene variations, congenital exposures, and multi-system associations such as VACTERL (a disorder including vertebral defects, anal atresia, cardiovascular anomalies, tracheoesophageal fistula, esophageal atresia, renal anomalies, and limb defects) (Bracher et al., 2017).

Historically, infants with syndromic CHD were diagnosed based on characteristic facial features and symptoms resembling cohorts of other patients with genetic syndromes (Rimoin and Hirschhorn, 2004). More recently, diagnostic practices have shifted to identifying chromosomal and gene variations through karyotype, fluorescent *in situ* hybridization (FISH), chromosomal microarray (CMA), and next-generation sequencing (NGS) techniques (genetic panels, whole exome sequencing (WES), and whole genome sequencing (WGS)) (Pierpont et al., 2007). The benefits and limitations of genetic testing for patients with CHD affect how individuals and families are counseled and can be critical to the medical diagnosis and management of CHD (De Backer et al., 2020).

Confirming a genetic cause for a heart defect can reduce uncertainty and worry about a prognosis, inform recurrence risk estimates, and can impact medical management (Bernier and Spaetgens, 2006). For example, genetic testing can lead to the identification of individuals who are at risk for comorbidities such as heart failure, arrhythmias, and neurodevelopmental disorders (NDD). Understanding the cause of a heart defect can also guide cardiac screening (electrocardiograms, echocardiograms, etc.), screening of other organ systems, involvement of necessary specialists (vascular surgery, endocrinology, etc.) and timely interventions, and in the future, targeted curative therapies (De Backer et al., 2020). When considering preconception decision-making, meeting with a genetics provider can help families understand etiology and family risks and may influence when and how individuals with a history of CHD have children (van Engelen et al., 2013).

Medical advances have led to improved diagnosis and management of adults with congenital heart disease (ACHD). Two-dimensional echocardiograms first became available in the 1970s and greatly improved the diagnosis of CHD (Kiess, 2016; Lapum et al., 2019). Improving medical and surgical management of CHD has translated into longer life expectancy; with approximately 90% of patients with CHD born after 1990 having survived to adulthood. There is now a rapidly growing population of adults living with CHD (Mazor Drey and Marelli, 2015). Many of these adults did not have access during childhood to genetic technologies available today and therefore have not had a robust genetic evaluation. We propose that this population would benefit from the same genetic testing and counseling that is currently considered for infants with CHD (Parrott and Ware, 2012; Stout et al., 2019).

Given the potential benefit of genetic evaluation of adults with CHD, we sought to determine how Adult Congenital Heart Disease (ACHD) cardiologists currently approach offering genetic testing and genetic evaluations to patients with ACHD. We also sought to evaluate the indications and practice structures that influence decision-making by ACHD cardiologists when it comes to the provision of genetics care. We hypothesized that adults with CHD are not receiving indicated genetics services and that there is a need for concise and clear guidelines regarding genetic evaluation and testing in the ACHD population.

2 Materials and methods

2.1 Study population

This descriptive cross-sectional study surveyed cardiologists who self-reported that they provide care to adults with congenital heart disease (CHD). Approval from the Institutional Review Board at Cincinnati Children's Hospital Medical Center (CCHMC) was obtained (2022-0324). The survey email list was obtained from the Adult Congenital Heart Association. Cardiologists that self-reported being a currently practicing board-certified cardiologist were eligible to participate in the study. Physicians in other specialties and non-patient-facing cardiologists were excluded from the study.

2.2 Survey distribution

An invitation for participation in the survey was emailed to ACHD cardiologists using REDCap and included a personal link and URL to the questionnaire. Initially, the survey was emailed to the first 20 emails alphabetically to ensure cardiologists were able to access and answer the questionnaire. Three completed surveys were submitted from the first group before the email was sent to the

remainder of the email list. The recruitment email was sent to 113 total ACHD cardiologists. Eight emails could not be delivered and we received one request asking to be removed from the email list. This left 104 potential respondents. The survey was open from June 2022 to January 2023. Three reminder emails were sent to individuals on the email list who had not completed the survey.

2.3 Questionnaire development

A questionnaire was compiled for the purpose of the study and is available in the supplementary materials. The questionnaire is not validated. The questions were based on an article by Boynton and Greenhalgh (2004) on selecting, designing, and developing a questionnaire as well as questions used by other specialties for similar studies (Prochniak et al., 2012). The questions were organized into 7 categories in the following order: screening questions, demographics, access to genetics professionals, confidence with genetic knowledge and testing, using genetic testing, making genetics referrals, and perspectives on use of genetics in practice. The questionnaire was administered through REDCap hosted at CCHMC and skip-logic was utilized to avoid irrelevance and redundancy of questions (Harris et al., 2009 and 2019). The questionnaire was pre-tested by two ACHD cardiologists at CCHMC for question content and clarity.

2.4 Data analysis

The data from participation in the questionnaire was exported from REDCap. Descriptive statistics were used to characterize the study population. Frequency (percentage) was reported for all categorical variables. Median and inter-quartile range (IQR) was reported for continuous variable (knowledge and confidence scores). Fisher's exact tests were used to examine the associations between testing/referral practices and clinical factors (cardiologist's level of training and clinical experience). Knowledge score was calculated using three case-based knowledge questions. Cardiologists scored one point per question they answered correctly. The third question asked to select more than one patient and each option was assigned a quarter of a point. The knowledge score ranged from 0 to 3. For the confidence questions, numerical values were assigned to Likert answer scores ("not confident at all" = 1; "slightly confident" = 2; "Neutral" = 3; "fairly confident" = 4; "completely confident" = 5). The confidence score ranged from 1 to 5. Wilcoxon rank-sum tests were used to test association between knowledge/confidence in genetic testing and testing/referral practices. Given the exploratory nature of this study and our limited sample size, a p-value threshold of $p < 0.2$ was applied for significance. All the analyses were performed in R software, version 4.2.0 (GNU Project, Free Software Foundation, https://www.r-project.org).

3 Results

Of the 104 ACHD cardiologists who received an invitation to participate in the study, 35 cardiologists participated and met the inclusion criteria of the study (response rate of 34%). Two cardiologists did not finish the survey but their partial responses were included in data analysis.

3.1 Demographics

Most cardiologists identified as white (77%) and male (66%). All cardiologists had a Doctor of Medicine (M.D.) degree and four cardiologists (11%) had more than one advanced degree. Most cardiologists completed a fellowship in ACHD (89%), currently work at an academic medical center (89%), and practice in the United States (66%). Table 1 contains a complete list of cardiologist demographics.

3.2 Utilization of genetics professionals and testing services

3.2.1 Clinic structure

Regarding patient load and utilization of genetics services, most (74%) cardiologists indicated that they are involved in more than 400 appointments for ACHD per year. A subset of cardiologists (60%) also indicated that they are involved with pediatric CHD appointments. Of the thirty-one (89%) respondents that reported having a genetics professional at their institution, only ten (32%) indicated that genetics providers were embedded in their ACHD clinic. Cardiologists reported having less barriers to referring to genetics (77%) than to ordering genetic testing (34%). Of the reported barriers to referring, two were selected by more than one cardiologist: difficulties with the logistics of referring patients and connecting them with a genetics professional and long wait times for a genetic evaluation. The greatest barriers to ordering genetic testing according to cardiologists included no changes to management based on testing (29%), not knowing what test to order (17%), high cost to patients for genetic testing (11%), and not being able to contact a genetics professional (11%). For a complete description of reported clinic structures refer to Table 2.

3.2.2 Reported practices in the last year

The practices of cardiologists when referring and providing genetic testing in the last year are summarized in Table 3. In general, cardiologists were more likely to refer patients to a genetics professional than to order testing themselves. Nearly all cardiologists (91%) indicated that they referred one or more patients to a genetics professional in the last year and twenty cardiologists (57%) endorsed having ordered genetic testing in the last year. Of the cardiologists that did not order a genetic test in the last year, most (79%) indicated that they could easily refer to a genetics provider. Figure 1 compares the frequency of referring and ordering practices during the last year, as reported by cardiologists.

When ordering genetic testing, most cardiologists ordered only chromosomal testing (55%) and within that subset FISH was ordered most often (93%). Of the cardiologists that ordered gene sequencing (5 cardiologists, 14%), most (60%) ordered a gene panel.

3.3 Risk assessment

Cardiologists were asked if they would refer four potential testing candidates to a genetics professional (Table 4). Of the four cases, the greatest number of cardiologists would refer a 30 year-old male with interrupted aortic arch, type B (n = 26, 74%).

TABLE 1 Cardiologist demographics and training.

	N (n = 35)	%
Gender		
Male	23	66
Female	11	31
Self-identify—*free text*	0	0
No response	1	3
Ethnicity—*Could select more than one option*		
White	27	77
Black or African American	0	0
Hispanic or Latino	3	9
Asian	1	3
Other	3	9
No response	2	6
Degree—*Could select more than one option*		
Medical Doctor (MD)	35	100
Doctor of Osteopathy (DO)	0	0
Master of Public Health (MPH)	1	3
Master of Medical Science (MMSc/MMS)	2	6
Other—*free text*		
Master of Science in Clinical Investigation	1	3
ACHD Fellowship		
Yes	31	89
No	4	11
Years since training		
0–5 years	9	26
6–10 years	10	29
11–15 years	9	26
16–20 years	2	6
>20 years	4	11
No response	1	3
Work at an academic medical center		
Yes	31	89
No	2	6
No response	2	6
Country where workplace is located		
United States	23	66
Puerto Rico	1	3
Canada	3	9
Other (One each from Australia, Chile, Saudi Arabia, South Africa, Switzerland)	5	14
No response	3	9

Cardiologists were also asked to indicate how likely they are to refer or offer testing for specific indications (Table 5). Over 90% of cardiologists indicated that they would refer if they were suspicious of a genetic syndrome (97%), if the patient requested a referral to genetics (97%), and if the patient had a finding of NDD in addition to their CHD (93%). This was very similar to the response for the indications that would be offered a genetic test. Regarding isolated/simple CHD, only 4% of cardiologists would refer to a genetics provider and only 6% would be likely to offer genetic testing.

3.4 Confidence with recognizing indications for and ordering genetic testing

Cardiologists were asked their confidence level in recognizing features of a genetic condition based on patient presentations and they were then asked how confident they felt ordering the correct genetic test for their patients. Figure 2 depicts a comparison of the reported confidences between recognizing features of a genetic condition and ordering the correct genetic test. The greatest number of cardiologists endorsed being fairly

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TABLE 2 Clinic structure and barriers.

	N (n = 35)	%
How many ACHD appointments are you involved in per year?		
>400	26	74
301–400	5	14
201–300	1	3
101–200	2	6
1–100	1	3
Do you also evaluate pediatric CHD patients?		
Yes	14	40
No	21	60
How many pediatric CHD appointments are you involved in per year?	(n = 14)	
>400	2	14
301–400	0	0
201–300	2	14
101–200	4	29
1–100	6	43
What is the availability of genetics providers at your primary institution?		
At the same institution	31	89
At a different local institution	3	9
No genetics providers available	0	0
Other—*free text*		
Pediatric genetics cannot evaluate adults, only one adult geneticist	1	3
Are there geneticists or genetic counselors embedded in your clinic?	(n = 31)	
Yes	10	32
No	21	68
How are genetics appointments conducted?		
In person only	5	14
Telehealth only	0	0
Both in person and telehealth	24	69
I do not know	5	14
No response	1	3
Barriers to referring patients to genetics providers		
No barriers	27	77
Barriers—*Could select more than one option*	5	14
There is not a geneticist or genetic counselor in my clinic	1	3
Meeting with genetics would be logistically difficult for patients	2	6
Other—*free text*		
Very long wait to see a genetics provider	2	6
Scheduling is logistically difficult	2	6
Limited availability of genetics professionals	1	3
No response	3	9
Barriers to ordering testing for patients with ACHD		
No barriers	12	34
Barriers—*Could select more than one option*	22	63
I could easily refer to genetics professionals to order testing for me	11	31
I did not know how to order genetic testing	3	9
I did not know the best genetic test to order	6	17
I did not feel confident interpreting genetic testing	3	9
I was not able to contact a geneticist or genetic counselor	4	11
My patients were not interested in genetic testing	1	3

(Continued on following page)

TABLE 2 (*Continued*) Clinic structure and barriers.

	N (n = 35)	%
I was concerned about the patient's risk of genetic discrimination	2	6
Ordering genetic testing would not have changed management	10	29
Other—*free text*		
Concerns about the cost of genetic testing	4	11
Would not change management for post-menopausal patients	1	3
No response	1	3

TABLE 3 Referral and testing practices.

	N	%
Referred one or more patients in the last year	(n = 35)	
Yes	33	94
No	1	3
No response	1	3
Ordered testing in the last year	(n = 35)	
Yes	20	57
No	14	40
No response	1	3
Type of testing ordered in the last year	(n = 20)	
Chromosome testing only	11	55
Gene sequencing only	2	10
Both chromosome testing and gene sequencing	3	15
I do not know the type of testing ordered	4	20
Type of chromosome testing ordered—*can select more than one*	(n = 14)	
FISH	13	93
Microarray	6	43
Karyotype	4	29
Unknown	1	7
Type of gene sequencing ordered—*can select more than one*	(n = 5)	
Single gene	1	20
Gene panel	3	60
Whole exome	2	40

confident that they could recognize a genetic condition based on patient presentation (n = 12, 35%) and no cardiologists indicated that they were not confident at all. Regarding ordering the correct test, no cardiologists endorsed being completely confident and able to assist others with ordering the correct test for ACHD and the greatest number of cardiologists indicated that they were not confident at all and would need someone else to tell them what test to order (n = 13, 39%).

3.5 Influential factors for placing referrals and ordering testing

The associations between cardiologist demographics, experience, barriers, genetics knowledge/confidence, and the practices reported by cardiologists are summarized in Table 6. Only knowledge scores had a significant association with referral and testing practices but there were trends in how other factors affected ordering and testing.

Regarding years since training, cardiologists who have been out of training longer trended toward referring less patients to genetics providers ($p = 0.51$). Of the senior ACHD cardiologists (those with more than 16 years since training) that participated, 80% reported that they refer less than 5% of the patients they see in a year. Comparatively, less than half (44%) of junior ACHD cardiologists (those with 1–5 years since training) refer 1%–5% of their patients, the majority refer more than 5% of their patients to genetics services. There also seems to be a potential association between practicing outside of the United States and testing a smaller proportion of patients (17% vs. 46%, $p = 0.33$).

Cardiologists who ordered testing on more than 5% of their patients indicated that they see more than 400 patients for ACHD per year. There also appears to be a trend toward referring more patients when cardiologists see more ACHD patients (42% vs. 22%, $p = 0.43$). Having reported barriers to offering genetic testing is associated with ordering less genetic tests (13% vs. 50%, $p = 0.16$). Evaluating pediatric patients and having a genetics professional embedded in clinic did not have an effect on referral and testing practices.

Higher scores on knowledge questions were associated with referring a *smaller* proportion of patients to genetics providers (2.0 vs. 1.5, $p = 0.002$) and ordering *less* testing (2.0 vs. 1.3, $p = 0.009$). In comparison, greater confidence in recognizing features of a genetic condition and in ordering the correct genetic test led to cardiologists ordering more genetic testing (4.0 vs. 3.0, $p = 0.12$; 3.0 vs. 2.0, $p = 0.09$).

4 Discussion

Understanding how cardiologists decide when to provide ACHD patients with genetic knowledge can inform management guideline development for evaluating, testing, and counseling adults with CHD. In this study we investigated cardiologists' perspectives and practices for referring patients to genetics providers and ordering genetic testing themselves. We also explored factors that influence their decision-making.

The opportunity for cardiologists to specialize in ACHD as a subspecialty is relatively new; the American Board of Medical Specialties recognized ACHD as a separate subspecialty of cardiology in 2011 (Madan and Kim, 2015). According to the Accreditation Council for Graduate Medical Education (ACGME) website, there are currently 27 programs in the United States that offer a fellowship in ACHD. Given the small number of cardiologists specializing in ACHD, recruiting a large sample size of cardiologists for the study was difficult.

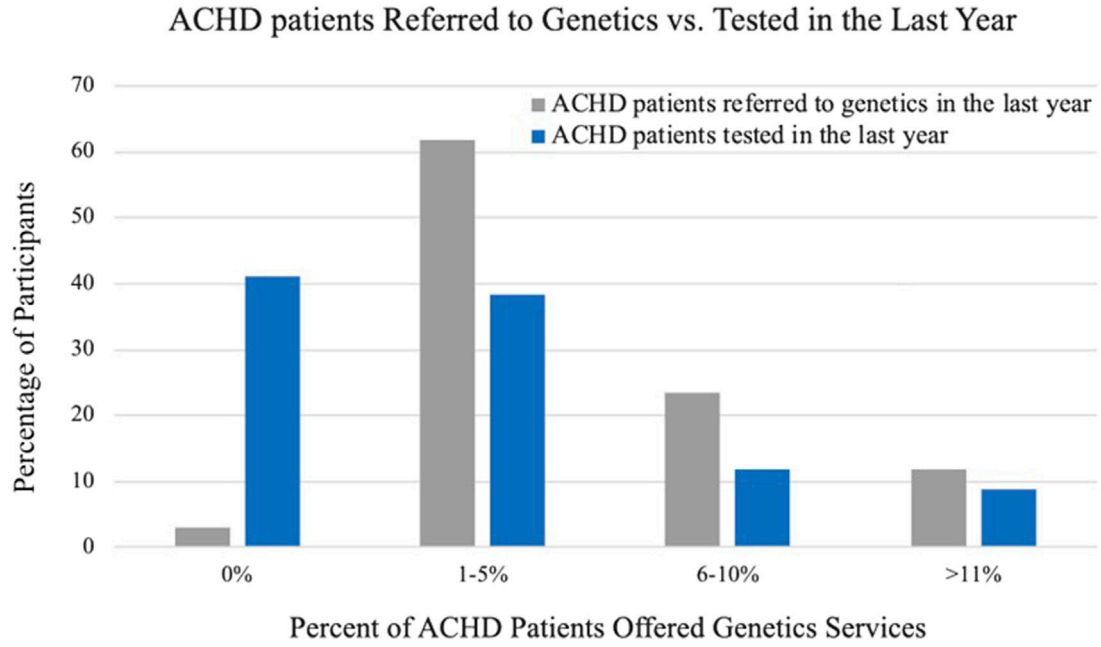

FIGURE 1
Referring and testing practices in the last year as reported by cardiologists.

TABLE 4 Referring practices based on patient presentation.

Potential genetic test candidate[a]	Cardiologists that would refer (%) n = 35
30yo male with interrupted aortic arch, type B	74
16yo male with a VSD and developmental delay	66
21yo female with heterotaxy and complex CHD	43
41yo female with transposition and family history of an ASD	40

ASD, Atrial Septal Defect VSD, Ventricular Septal Defect.
[a]Genetics professionals would recommend that all these patients be evaluated for a genetic cause.

TABLE 5 Likelihood of cardiologists referring or offering genetic testing.

Potential indications	Refer to genetics		Offer genetic testing	
	Mean rating[a]	% Of cardiologists likely to refer[b]	Mean rating[a]	% Of cardiologists likely to offer[b]
Suspicion of a syndrome	90	97	92	95%
Patient request	89	97	N/A	N/A
Presence of NDD	77	93	73	94%
Family history of CHD	72	84	69	71%
Extracardiac Anomaly	67	73	69	89%
Complex CHD	51	46	55	50%
Isolated/simple CHD	10	4	14	6%

NDD, Neurodevelopmental Disorders CHD, Congenital Heart Disease.
[a]0 = Very unlikely, 50 = Neutral, 100 = Very likely.
[b]Respondents answering 51–100.

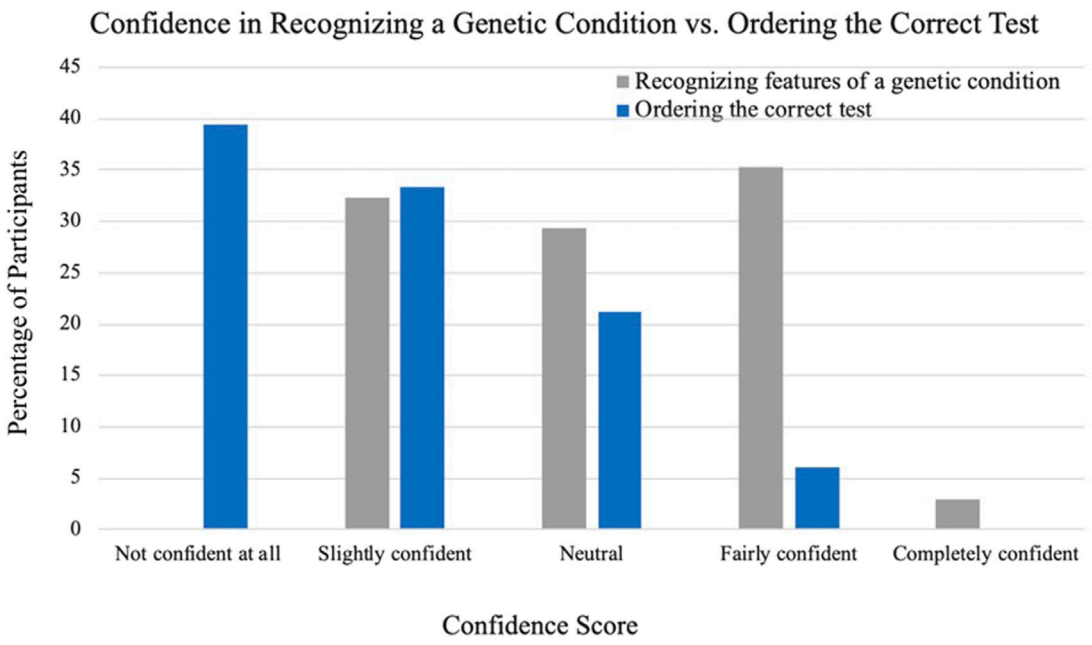

FIGURE 2
Cardiologist-reported confidence in recognizing a genetic condition compared to ordering the correct genetic test.

4.1 Utilization of genetics professionals and testing services

4.1.1 Clinic structure

4.1.1.1 Access to genetics services

ACHD cardiologists have a unique opportunity to connect patients with genetics services and to help them understand the possible causes of their CHD. Identifying an underlying genetic etiology can affect management, guide recurrence risk discussions, and empower patients to make informed decisions about their CHD health. A third of cardiologists in the study indicated that a genetics provider was embedded in their clinic. However, providers reported logistical challenges when placing a referral or ordering a genetic test and long wait times for genetics appointments once a referral was made.

This is indicative of a need for improved access to genetics providers evaluating ACHD patients. Potential interventions to improve access to genetics services include using electronic medical record (EMR) systems to generate referral orders and facilitate the referral process, increasing telehealth and telephone genetics appointments, and pre-visit education to increase the efficiency of genetics appointments (Bednar et al., 2022). Implementing guidelines for genetic testing in adults with CHD that are readily available to ACHD cardiologists could also streamline the process of ordering testing and referring to genetics services. In this scenario, the best test would be ordered by the cardiologist and then counseling and any additional non-cardiac management could be coordinated by the genetics providers at a follow-up visit.

4.1.1.2 Affordability of genetic testing

Multiple cardiologists reported concerns about the cost of genetic testing. While affording genetic testing continues to be a challenge, the cost of testing has decreased over the last decade and resources for patients with financial need has increased (Young and Argáez, 2019). Many genetic testing laboratories also offer family variant testing to relatives at no cost, or for a lower cost than broad testing. It is possible that with more defined guidelines and criteria for genetic testing in the ACHD population, coverage of genetic testing would improve.

When ordering testing, cardiologists should discuss opportunities for financial support with their patients and assist patients in making an informed decision. If a patient endorses that cost of testing is prohibitive, it is recommended that the patient be referred to genetics to determine additional options available for the specific patient. Expertise regarding billing practices for genetic testing is one of many advantages to having geneticists or geneticist counselors involved in the provision of genetic services.

4.1.1.3 Utility of a genetic diagnosis

Many cardiologists also indicated that genetic testing would not change the clinical management of their patients. One cardiologist specifically identifying post-menopausal women as a group for whom they would not offer testing, presumably because the genetic information would not inform recurrence risk in their children. Genetic evaluation offers more to patients than just management changes or recurrence risk. Limiting decision-making to direct clinical utility, overlooks the value of providing knowledge, counseling, and family testing to patients.

4.1.2 Reported practices

Nearly all cardiologists reported they had referred at least one patient for genetic services in the last year but only about half ordered a genetic test. The type of testing that was ordered by cardiologists was most frequently chromosomal testing for

TABLE 6 Association between clinical factors and testing and referral practices.

Influential factor	Percentage of patients referred			Percentage of patients tested		
	>5%	1%–5%	*p*-value	>5%	1%–5%	*p*-value
	N (%)	N (%)		N (%)	N (%)	
Years since training			0.51			0.45
1–5 years	5 (56%)	4 (44%)		3 (50%)	3 (50%)	
6–10 years	4 (40%)	6 (60%)		3 (43%)	4 (57%)	
11–15 years	2 (25%)	6 (75%)		0 (0%)	4 (100%)	
>16 years	1 (20%)	4 (80%)		1 (33%)	2 (67%)	
Current workplace			0.67			0.33
In the United States	9 (41%)	13 (59%)		6 (46%)	7 (54%)	
Outside the United States	2 (25%)	6 (75%)		1 (17%)	5 (83%)	
Number of ACHD appointments			0.43			0.11
1–400	2 (22%)	7 (78%)		0 (0%)	5 (100%)	
>400	10 (42%)	14 (58%)		7 (47%)	8 (53%)	
Also care for pediatric CHD			0.72			1
Yes	6 (43%)	8 (57%)		3 (38%)	5 (63%)	
No	6 (32%)	13 (68%)		4 (33%)	8 (67%)	
Genetics provider in clinic			0.69			0.6
Yes	4 (44%)	5 (56%)		3 (50%)	3 (50%)	
No	7 (33%)	14 (67%)		3 (27%)	8 (73%)	
Barriers to accessing genetics			1			0.16
Yes	2 (40%)	3 (60%)		1 (13%)	7 (88%)	
No	10 (37%)	17 (63%)		6 (50%)	6 (50%)	
	Median (IQR)	Median (IQR)	*p*-value	Median (IQR)	Median (IQR)	*p*-value
Knowledge Score	1.5 (1.3–1.6)	2.0 (2.0–3.0)	0.002	1.3 (0.9–1.5)	2.0 (2.0–2.25)	0.009
Confidence recognizing features	3.0 (2.0–4.0)	3.0 (2.0–4.0)	0.83	4.0 (3.0–4.0)	3.0 (2.0–4.0)	0.12
Confidence ordering correct test	2.0 (1.75–3.0)	2.0 (1.0–2.0)	0.25	3.0 (2.5–3.0)	2.0 (1.0–2.0)	0.09

aneuploidies and microdeletions; only five cardiologists ordered gene sequencing. Single nucleotide variants (SNVs), detected only by gene sequencing, account for about 10% of all CHD (De Backer et al., 2020). This is especially important for adults with CHD, as isolated cardiovascular defects are frequently caused by SNVs (Parrott and Ware, 2012). We would expect that for adults with syndromic CHD, the presence of extracardiac findings would have already developed and led to a genetic evaluation. Therefore, testing ACHD patients for SNVs could be more important than ordering chromosomal testing. Based on the small number of cardiologists that endorsed ordering gene sequencing, single-gene disorders associated with CHD are being under-evaluated and under-diagnosed.

The reported practices are indicative of a need for a more standardized approach to providing genetics care for adults with CHD. Providing ACHD patients with genetics services can influence patient management and decision-making, as supported by a recent study analyzing the perception of adults with CHD regarding genetic testing, preconception counseling, and family risk assessments. Participants in the study disclosed that they purposefully postponed having children until after they met with a genetics provider with some changing their plans about having a child after their genetic consultation. Most attended their genetics appointments to learn about the recurrence risk in their children and evaluate the cause of their CHD (van Engelen et al., 2013). Another important finding of the study was that most participants were referred by their cardiologist, further supporting that ACHD cardiologists have a unique opportunity to connect patients with geneticists and genetic counselors. In our study, 97% of cardiologists endorsed referring ACHD patients to genetics providers and helping their patients access genetics services.

Our understanding of the genetics of CHD is continuously evolving. For example, The Pediatric Cardiac Genomics Consortium (PCGC) researches the genetic etiologies of CHD. A recent publication from PCGC systematically evaluated CHD candidate genes and created a list of genes likely to be associated with CHD. The goal of the study was to improve the utility and yield of clinical genetic testing. Of the initial 558 candidate genes, a total of 99 genes were classified as having strong or definitive clinical validity for CHD. Furthermore, 18 of the 99 genes were associated with isolated CHD and 81 with syndromic CHD. Once genes were classified as having strong clinical validity, test results were disclosed to participants and followed up with a survey. Individuals who participated in the survey reported that understanding the cause of their CHD was important for life planning, managing future pregnancies, and improving knowledge about their diagnoses. One participant stated that having a *de novo* genetic variant was "settling" for her parents

who feared they had caused her CHD (Griffin et al., 2023). The PCGC article further supports the importance of determining the underlying genetic cause of CHD for patients and their families. The reclassification of candidate CHD genes to "clinically valid" will also affect gene sequencing testing options and interpretation of results. Updated knowledge on the genetic etiologies of CHD may also warrant re-evaluation for additional genetic testing or re-interpretation of previous results such as periodic exome reanalysis.

4.2 Risk assessment

A genetics evaluation should be considered for any child or adult with a congenital anomaly, including CHD, given the benefits of a genetics evaluation. Additionally, many genetic conditions are characterized by incomplete penetrance and variable expressivity, making them more difficult to detect just by family history alone. Certain findings such as family history, additional congenital anomalies, and certain types of CHD may increase the yield of genetic testing. In this study, cardiologists were provided four clinical cases and asked if they would refer them to a genetics provider. All four clinical cases would benefit from a genetics evaluation, so our hypothesis was that the patients would all be referred with similar frequencies close to 100%. The results of the survey indicated that this was not the case. According to our survey, the hypothetical patient with an interrupted aortic arch would be referred by the greatest number of cardiologists. The three other cases would be referred in decreasing frequency for developmental delay, heterotaxy and complex CHD, and transposition with a positive family history. This response may be indicative of limited training on the genetic etiologies of CHD and the emphasis that is placed on referring and testing for conotruncal lesions and 22q deletion syndrome (Valente and Landzberg, 2018). The emphasis on 22q deletion syndrome may also explain why most cardiologists (n = 13/20) ordered FISH testing. Regarding ACHD training on genetic etiologies, the ACGME has two core competencies related to knowledge about the genetics of CHD and 2% of the ACHD board certification exam covers "genetic syndromes and associations." These competencies and questions do not include specific information about diagnosing and counseling patients on the genetics of their CHD (acgme.org, abim.org).

Cardiologists also indicated that they were likely to refer and offer testing for CHD with findings other than simple/isolated CHD. Given the low rates of testing reported by cardiologists, possible reasons may include that cardiologists are 1) mostly managing patients with simple CHD or 2) referring to genetics providers when additional findings are observed, instead of ordering testing themselves. Referring patients with complex CHD or extracardiac findings to genetics professionals can provide additional education and counseling opportunities; however, if there are barriers to accessing these services, patients may not receive the recommended or requested care and go undiagnosed.

4.3 Confidence with genetics

Cardiologists who participated in the survey reported being more confident in recognizing features of a genetic condition than in ordering a correct genetic test. The reported referral and ordering practices reflect the difference in confidence and may explain why almost all cardiologists referred at least one patient but a much smaller subset ordered testing for patients. Most cardiologists selected that they were not confident at all and would require guidance for ordering genetic testing. Based on this result, cardiologists who do not have a genetics professional in their clinic could potentially benefit from consulting with a genetics provider to seek instruction on what testing is most appropriate, when referring to genetics is not feasible. The low confidence scores may also be indicative of a gap in ACHD training and lack of published guidance for genetic testing in adults with CHD. Therefore, we recommend an algorithm for testing in ACHD patients be developed that mirrors testing strategies used in pediatric CHD patients. Fellowship training for ACHD cardiologists and continuing medical education (CME) should also include additional education on the process of ordering testing and diagnosing genetic conditions in patients with ACHD.

4.4 Influential factors when deciding to refer or test

The power of our study was limited by the number of ACHD cardiologists we could invite to participate in our study, as well as our final sample size. Although we could not draw definitive conclusions, we were able to observe some trends in the associations between referral and testing practices and clinical factors. One trend we observed was that physicians who have practiced longer referred less patients and ordered less testing. Given how young the specialty of ACHD is and how quickly the field of genetics has grown, it can be expected that cardiologists who trained before the development of ACHD as a subspecialty are less likely to offer genetics services to their patients. Implementing additional education and training for ACHD cardiologists through continuing medical education (CME) could benefit physicians who trained prior to current genetic testing methodologies. It is also possible that junior cardiologist see more new patients than senior cardiologists who may follow more established patients with prior genetic testing that would not need additional testing or genetics services.

Cardiologists outside the United States indicated ordering less testing and having more barriers to testing. We investigated the availability of genetic testing outside the United States and found that as of February 2023, GeneTests[1] listed 284 genetic testing labs in the United States and 228 non-US laboratories, mostly in Canada and Europe. There are nearly as many labs in the United States as outside the United States so the availability of testing outside the United States may be limited and dependent on the country. Therefore, the difference in testing practices indicated by cardiologists practicing outside the United States could be influenced by the availability of genetic testing labs and the types

1 GeneTests is federally funded and includes an international directory of laboratories offering clinical and research genetic testing; it is maintained by the University of Washington, Seattle, Washington

of testing offered. Testing practices outside of the United States may also be dependent on the culture of the region and the resources available to patients within a hospital system itself.

We observed that a higher number of ACHD appointments per year were associated with higher rates of genetics referrals and testing; but evaluating pediatric patients in addition to adult patients did not influence referral or testing practices. It is possible that cardiology clinics with high volumes of patients are associated with hospitals that also have high patient volumes, or with academic medical centers. In these situations, there may be more genetics providers available to consult and discuss testing. We initially anticipated that managing pediatric patients in addition to adult CHD patients would lead to higher testing and referral practices since cardiologists who cared for both populations would be familiar with pediatric CHD guidelines for making genetic referrals and providing genetic testing.

Interestingly, cardiologists who scored higher on the case-based knowledge questions in the questionnaire indicated that they referred a smaller proportion of patients and ordered less testing than their counterparts who scored lower on those questions. This association should be evaluated with caution given the small sample size of the study but it could mean that cardiologists who understand the type of testing that is indicated for specific cases are not referring or ordering as much as those who do not. It is possible that these cardiologists have resources in place to help with ordering testing and therefore they order less testing independently.

4.5 Conclusion

Adults with CHD are a growing population who have not had the same access to genetic services during childhood as is currently offered to children with CHD. Current genetic testing technologies and recommendations did not exist when they were children. Therefore, adults with CHD should be provided the same comprehensive access to genetic evaluation and counseling that is currently offered to infants and children. There is a need for additional guidance to increase genetics assessments in adults with CHD and other indicators of genetic risk (e.g., a non-cardiac congenital diagnosis, NDD, or family history of CHD).

The genetic etiologies of conotruncal defects are emphasized in fellowship training of cardiologists and this study supports that ACHD providers are more likely to refer and offer testing to patients with conotruncal anomalies, especially to evaluate for 22q11.2 microdeletion syndrome. For adults with CHD, testing for SNVs via gene sequencing may be of greater importance given that isolated cardiac defects are more likely to be caused by a single-gene variant and less likely to have been evaluated during childhood. Interestingly, cardiologists in this study reported ordering less gene-sequencing than chromosomal testing for their patients. Broader genetic testing for single-gene variants can also be beneficial in conditions such as Noonan Syndrome or RASopathies, where more than one gene is associated with overlapping clinical features. In these cases, adults may have a clinical diagnosis of a condition but determining what gene is affected could guide targeted testing for other family members. There is a need for improved access to genetics services and implementation of interventions such as using electronic medical record (EMR) systems to facilitate the referral process. Additional pre-visit education could increase efficiency of genetics appointments and help improve access for adults with CHD.

4.6 Limitations

Limitations of this study include a small sample size overall (n = 35) and a small sample of cardiologists who ordered testing in the last year (n = 20). Additionally, a response rate of 34% raises concern for potential selection bias and the generalizability of the trends identified in the sample. In the study, all the cardiologists were medical doctors (M.D.s), and most were white men working at academic medical centers. For this reason, our study may not be generalizable to all cardiologists who evaluate patients for ACHD. There is limited information on the demographics of ACHD cardiologists, but according to a Professional Life Survey (PLS) of U.S. cardiologists conducted in 2015 by the American College of Cardiology (ACC), 64% of cardiologists were white and 58% were men (Thomas et al., 2021). By surveying cardiologists outside of the United States we tried to increase the diversity of the study and capture differences in referral and testing practices but our questionnaire did not include questions specific to cardiologists practicing outside of the United States.

4.7 Future research

A similar provider survey could be used to evaluate how pediatric cardiologists offer genetics services to children with CHD, identify the indications that influence decision-making for genetics care, and determine if published recommendations for genetics evaluations in children with CHD are being utilized by providers. Further investigation on how ACHD fellows are trained on the genetic etiologies of CHD, especially single-gene disorders, and possible educational tools that could fill in the knowledge gap is warranted based on the findings of this study. A study focused on ACHD provider knowledge and confidence recognizing a genetic syndrome and ordering testing would provide a more robust evaluation than what we were able to achieve in this study.

Data availability statement

The original contributions presented in the study are included in the article/supplementary material, further inquiries can be directed to the corresponding author.

Ethics statement

The studies involving humans were approved by the Cincinnati Children's Institutional Review Board (IRB). The studies were conducted in accordance with the local legislation and institutional requirements. The ethics committee/institutional review board waived the requirement of written informed consent for participation from the participants or the participants' legal guardians/next of kin because all of the research was conducted in a commonly accepted setting (electronic questionnaire), potential participants were provided

an explanation of the purpose of the study and informed that completion of the questionnaire would serve as their consent to participate in the research project, and the study procedures involved no increase in the level of risk or discomfort to participants.

Author contributions

LO: Conceptualization, Data curation, Formal Analysis, Investigation, Methodology, Software, Visualization, Writing–original draft, Writing–review and editing. AO: Conceptualization, Investigation, Methodology, Visualization, Writing–review and editing. KW: Conceptualization, Methodology, Writing–review and editing. NB: Conceptualization, Methodology, Writing–review and editing. CB: Conceptualization, Methodology, Writing–review and editing. EM: Conceptualization, Methodology, Writing–review and editing. HH: Data curation, Formal Analysis, Software, Validation, Writing–review and editing. Amy R AS: Conceptualization, Data curation, Methodology, Project administration, Writing–review and editing.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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A whole-exome sequencing study of patent foramen ovale: investigating genetic variants and their association with cardiovascular disorders

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Background: Patent foramen ovale (PFO) has a genetic predisposition and is closely associated with cryptogenic stroke (CS), migraine, decompression sickness, and hypoxemia. Identifying PFO-related mutant genes through whole-exome sequencing (WES) can help in the early recognition of cardiovascular genetic risk factors, guide timely clinical intervention, and reduce the occurrence of cardiovascular events.

Methods: We analyzed mutant genes from ClinVar and OMIM databases. WES was performed on 25 PFO patients from Zhejiang Provincial Hospital of Chinese Medicine. Pathogenicity of variants was evaluated using American College of Medical Genetics and Genomics (ACMG) and Association for Molecular Pathology. (AMP) guidelines.

Results: In ClinVar (4 Feb 2023), 113 coding gene mutations were found, including 83 associated with PFO. From OMIM (18 Apr 2023), 184 gene mutations were analyzed, with 110 mutant coding genes. WES identified pathogenic mutations in two of 25 PFO patients (8%). LDLR, SDHC, and NKX2-5 genes were linked to PFO and primarily involved in myocardial tissue function. NKX2-5 may play a crucial role in PFO development, interacting with NOTCH1, GATA4, MYH6, SCN5A signaling pathways regulating cardiomyocyte characteristics.

Conclusion: We identified pathogenic mutations in LDLR, SDHC, and NKX2-5 genes, implying their role in PFO development. Functional enrichment analysis revealed NKX2-5's interaction with signaling pathways regulating cardiomyocyte function. These findings enhance our understanding of PFO's genetic basis, suggesting potential therapeutic targets for future research.

KEYWORDS

patent foramen ovale, whole exome sequencing, pathogenic mutations, functional enrichment analysis, nkx2-5

1 Introduction

Patent foramen ovale (PFO) is a common congenital heart disorder that affects approximately 25% of adults worldwide and demonstrating a familial aggregation (Mojadidi et al., 2019). It is characterized by a small hole in the diaphragm of the left and right atria, allowing blood to bypass the pulmonary circulation and flow directly from the right atrium to the left atrium (Homma et al., 2016). This physiological pathway is crucial during fetal development, but if it remains open after 3 years age, it is referred to as PFO. PFO has been associated with various diseases, including migraine, cryptogenic stroke (CS), and decompression sickness (Gillow and Lee, 2015; Lafère et al., 2017; Mojadidi et al., 2021b). CS, as a severe adverse outcome of patent foramen ovale (PFO), is influenced significantly by genetic factors (Bo et al., 2018; He et al., 2018). Research has shown that genetic variants associated with coagulation function, genetic predisposition to cardiac structural abnormalities, and gene variations related to arterial wall elasticity and stability may contribute to the occurrence of CS(Austin et al., 2002; Harland et al., 2002; Cinelli et al., 2019; Lin et al., 2022). These findings highlight the importance of genetic factors in the development of CS as a consequence of PFO. The diagnosis of PFO relies primarily on clinical manifestations and is further supported by complementary examinations, including transesophageal and transthoracic echocardiography (TEE,TTE), as well as transcranial Doppler (TCD) (Liu et al., 2021). There are mainly two treatment options for PFO patients. One option is to use antiplatelet and anticoagulant drugs (such as vitamin K antagonists, direct thrombin inhibitors, or Xa factor inhibitors) to treat PFO complicated stroke (Mas et al., 2017; Kasner et al., 2021). Another method is to insert a special plugging device at the PFO to prevent blood from flowing from the right side of the heart through the ovale foramen into the left atrium (Akobeng et al., 2018). Both treatment modalities have demonstrated efficacy in preventing recurrent ischemic events among patients with cryptogenic stroke and transient ischemic attack (TIA). It has been suggested that heart development, specifically the atrial septum, may be influenced by a combination of different genetic factors rather than a single gene variant (Paolucci et al., 2021). However, PFO patients exhibit variability in onset age, genotype heterogeneity (where one phenotype can be caused by multiple genes), incomplete penetrance, and unclear inheritance patterns. Therefore, molecular genetic testing may offer valuable insights for early diagnosis and subsequent treatment of PFO.

Previous studies have indicated an association between gene polymorphism and PFO patients with CS (Liu et al., 2022). The occurrence of cardiovascular events in PFO patients is closely related to their genetic cardiovascular risk factors. Whole-Exome Sequencing (WES) is an efficient genomic sequencing technique that focuses on analyzing the exonic regions of the genome responsible for encoding proteins. With WES, we aim to identify new genes and rare variations in known genes associated with PFO-related cardiovascular diseases, unravel the genetic complexity of these conditions, and facilitate risk assessment and personalized treatment based on an individual's genetic profile. This approach may propel the advancement of precision medicine in the field of cardiovascular diseases. The objective of this study is to employ WES technology to identify pathogenic genes, promoter genes, and signaling pathways linked to PFO patients. This will offer more precise guidance for the prevention, diagnosis, and treatment of PFO.

2 Materials and methods

2.1 Analysis of mutant genes in ClinVar and OMIM databases

To identify PFO-related mutate genes, we searched the terms "patent foramen ovale" in both the ClinVar (https://www.ncbi.nlm.nih.gov/clinvar/) and OMIM (https://omim.org) databases. In the ClinVar database (as of 4 February 2023), we obtained a range of genetic mutation types, including single-allele mutations, multi-copy mutations, non-coding gene deletions, coding gene deletions, and coding gene mutations. In the OMIM database (as of 18 April 2023), we identified gene deletions and gene mutations. We selected mutations in coding genes from both databases for further analysis.

Next, we compared the mutant genes from both databases to find overlapping genes. These overlapping genes were then imported into the String database (https://www.string-db.org) to construct a protein-protein interaction (PPI) network using Cytoscape software. After obtaining gene clusters, we conducted Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) functional enrichment analysis. GO enrichment analysis involves annotating the functions of genes or proteins into different GO terms to identify functional classes associated with diseases or other conditions of interest. KEGG enrichment analysis utilizes pathway annotation information to classify and enrich genes and proteins, providing insights into the pathways associated with specific diseases or biological processes.

2.2 Patients

Twenty-five patients with PFO from the cardiovascular department of Zhejiang Provincial Hospital of Chinese Medicine between May 2022 and January 2023 were enrolled for WES, including 20 females and 5 males (Table 1). Right-to-left shunt (RLS) classification criteria: 1) RLS 0, without shunts; 2) RLS 1, 1-9 microbubbles; 3) RLS 2, 10-30 microbubbles; 4) RLS 3, more than 30 microbubbles. All patients signed informed consent forms.

Approximately 5 mL of venous blood was collected into Ethylene diamine tetraacetic acid (EDTA) tube from each patient and stored in a −80°C refrigerator for future detection. The diagnostic criteria and evaluation of PFO patients were based on the Chinese Expert Consensus on Ultrasonic Diagnosis of Patent Foramen Ovale. Patients had to meet at least one of the following conditions: 1) Microbubble signals were observed in the left cardiac system within 3-5 cardiac cycles after the right cardiac system was filled with microbubble signals following active saline injection in contrast TTE; 2) Gaps and shunt between the primary and secondary septum were observed in TEE examination; 3) Microbubble signals in the middle cerebral artery were observed within 10 s after intravenous infusion of activated saline in contrast TCD ultrasonography. If the microbubble signal was not detected in

TABLE 1 Clinical characteristics of 25 PFO patients.

Characteristics	Simple	Complex	*p*-value
n	17	8	
PFO type, n (%)			<0.001
simple	17 (100.00%)	0 (0.00%)	
long tunnel (length≥8 mm)	0 (0.00%)	6 (75.00%)	
long Euclidean lobe or Hiarli network	0 (0.00%)	1 (12.50%)	
combined atrial septal prolapse tumor	0 (0.00%)	1 (12.50%)	
Years, mean ± sd	46.294 ± 15.459	46.125 ± 15.56	0.98
Migraine, n (%)			0.017
YES	15 (88.24%)	3 (37.50%)	
NO	2 (11.76%)	5 (62.50%)	
Dizzy, n (%)			0.359
NO	13 (76.47%)	4 (50.00%)	
YES	4 (23.53%)	4 (50.00%)	
Syncope, n (%)			1
NO	16 (94.12%)	7 (87.50%)	
YES	1 (5.88%)	1 (12.50%)	
Stroke, n (%)			0.231
NO	16 (94.12%)	6 (75.00%)	
YES	1 (5.88%)	2 (25.00%)	
Hypertension, n (%)			0.283
NO	15 (88.24%)	5 (62.50%)	
YES	2 (11.76%)	3 (37.50%)	
Hyperlipidemia, n (%)			0.059
NO	15 (88.24%)	4 (50.00%)	
YES	2 (11.76%)	4 (50.00%)	
Hyperglycemia, n (%)			1
NO	16 (94.12%)	8 (100.00%)	
YES	1 (5.88%)	0 (0%)	
TTE (Valsaval), n (%)			0.057
RLS I	5 (29.41%)	2 (25.00%)	
RLS II	7 (41.18%)	0 (0.00%)	
RLS III	5 (29.41%)	6 (75.00%)	

the resting state, it was detected when activated saline was administered intravenously again after the Valsalva manoeuvre.

2.3 WES

Deoxyribonucleic acid (DNA) was extracted from the venous blood of PFO patients using a DNA blood test kit (Idt xgen exome research panel v1.0). The integrity of the DNA was further assessed by 1% agar gel electrophoresis to determine the degree of DNA degradation and the presence of Ribonucleic acid (RNA) and protein contamination. DNA concentrations were quantified using qubit, and libraries with a minimum of 500 ng could be constructed normally. DNA libraries were prepared by randomly fragmenting DNA into 180-280bp fragments using a nucleic acid interrupter. After terminal repair and A-tail addition, the DNA libraries were ligated to both ends of the fragments. The libraries were then pooled and indexed, followed by hybridization with a

biotin-labeled probe in liquid phase. Magnetic beads with streptomycin were used to capture the exons on the gene, and the library was linearly amplified by Polymerase chain reaction (PCR). Once qualified, the library was further quantified using the Qubit instrument and the insert size of the library was determined using Agilent 2100. The effective concentration of the library (3 nM) was accurately quantified using the qPCR method. To ensure the quality of the library, PE150 sequencing was performed on the NovaSeq 6000 platform (Illumina Inc., United States) based on the effective concentration of the qualified library and data production requirements.

2.4 Bioinformatics analysis

2.4.1 Screening of pathogenic genes

Offline data analysis process V1.0 for high-throughput sequencing, Human Genome Reference UCSC hg19 February 2009. The application software used for mutation detection is proprietary software self-programmed by Dean Laboratories and BWA. Annotation of variation was made using the public database 1000 Genome (phase I), gnomAD database, variation database ClinVar, dbNSFP, and the proprietary databases LOVD and ZJU-DB. The naming convention for variations is based on Human Genome Variation Society (HGVS).

2.4.2 Variation analysis

The Rare Exome Variant Ensemble Learner (REVEL) (Ioannidis et al., 2016), ClinPred (Alirezaie et al., 2018), Sorting Intolerant FromTolerant (SIFT) (Kumar et al., 2009) and Polymorphism Phenotyping v2 (PolyPhen2) (Adzhubei et al., 2013) were used in combination to analyze the harmfulness of mutation sites. REVEL and ClinPred analyze the pathogenicity of mutation sites, while SIFT and PolyPhen-2 predict the pathogenicity of the encoded proteins. Based on the predictions from these software tools, and in conjunction with the patient's clinical information, the pathogenicity of the patient is classified according to the American Society for Medical Genetics and Genomics (ACMG) and the American Society for Molecular Pathology (AMP) guidelines (Richards et al., 2015).

REVEL is an integrated method for predicting missense mutations. It combines the prediction results of multiple software tools, including MutPred, FATHMM, VEST, PolyPhen, SIFT, PROVEAN, Mutation Assessor, Mutation Taster, LRT, GERP, SiPhy, phyloP, and phastCons. The method utilizes pathogenic and rare neutral nonsense mutations for training and employs the random forest algorithm, resulting in improved prediction efficacy for rare missense mutations. The predicted score for a single mutation range from 0 to 1, with a score >0.75 generally indicating harm. ClinPred is a prediction tool to identify Disease-Relevant Nonsynonymous Single-Nucleotide Variants. It predicts the harm of mutation by integrating two machine learning algorithms: random forest (cforest) and gradient boosting decision tree (xgboost). The predicted score range for ClinPred is from 0 to 1, with scores above 0.5 predicted as deleterious and scores below 0.5 predicted as harmless. The higher the score, the stronger the pathogenicity of the variation. SIFT predicts the impact of a missense replacement on protein function, thereby determining the harmfulness of the amino acid substitution. SIFT scores range from 0 to 1, with scores less than 0.05 indicating a deleterious effect, while scores greater than or equal to 0.05 suggest no significant harm. PolyPhen2, based on the HumDiv database, is commonly used for complex diseases. A score of 0.957 or higher is predicted to be harmful, a score between 0.453 and 0.956 is predicted to be potentially harmful, and a score of 0.452 or lower is predicted to be harmless.

2.4.3 Pathogenicity rating

The pathogenicity rating of the variation was determined by conducting a literature search and querying databases. Pathogenicity rating refers to the classification standards and guidelines set by the ACMG and AMP, which include categories such as pathogenic, likely pathogenic, benign, likely benign, and of uncertain significance (Richards et al., 2015).

3 Results

3.1 PFO-related mutant genes in ClinVar and OMIM databases

The ClinVar database (as of 4th February 2023) yielded 268 results when searching for "patent foramen ovale". An analysis was conducted on the mutated coding genes, resulting in the detection of 82 distinct gene mutations. The total number of gene mutations observed was 113, with NKX2-5 being the gene showing the highest mutation frequency (25/113) (Figures 1A, C). In the OMIM database (as of 18th April 2023), a search for "patent foramen ovale" generated 116 results. Among these, 190 results indicated gene mutations, including 6 cases of gene deletions and 184 cases of gene mutations. Analysis of the mutant genes revealed the presence of 110 different gene mutations, with FLNA (10/110) and NKX2-5 (6/110) being the genes displaying relatively higher mutation frequencies (Figures 1B, D).

3.2 GO and KEGG functional enrichment analysis

Venn performed an intersection of the mutant genes from both databases, resulting in the identification of 20 overlapping genes associated with PFO (Figure 2A). By analyzing the constructed protein-protein interaction (PPI) network, we discovered several genes potentially implicated in PFO formation. These genes include NKX2-5, TBX20, KAT6A, PTPN11, CACNA1C, KMT2D, PAGR1, SCAF4, MED12, MED13L, MYOD1, NOTCH1, COL11A1, and ADAMTS3 (Figure 2B).

Enrichment analysis of mutant genes in these two-database revealed that the effects of these genes primarily involve ventricular formation, myocardial tissue morphogenesis, and venous vessel development at the biological level. At the cellular component level, these genes are associated with MLL3/4 complex, histone mediator complex, methyltransferase complex, and more. Furthermore, at the molecular functional level, these genes are involved in RNA polymerase II specific DNA-binding transcription factor binding, DNA-binding transcription

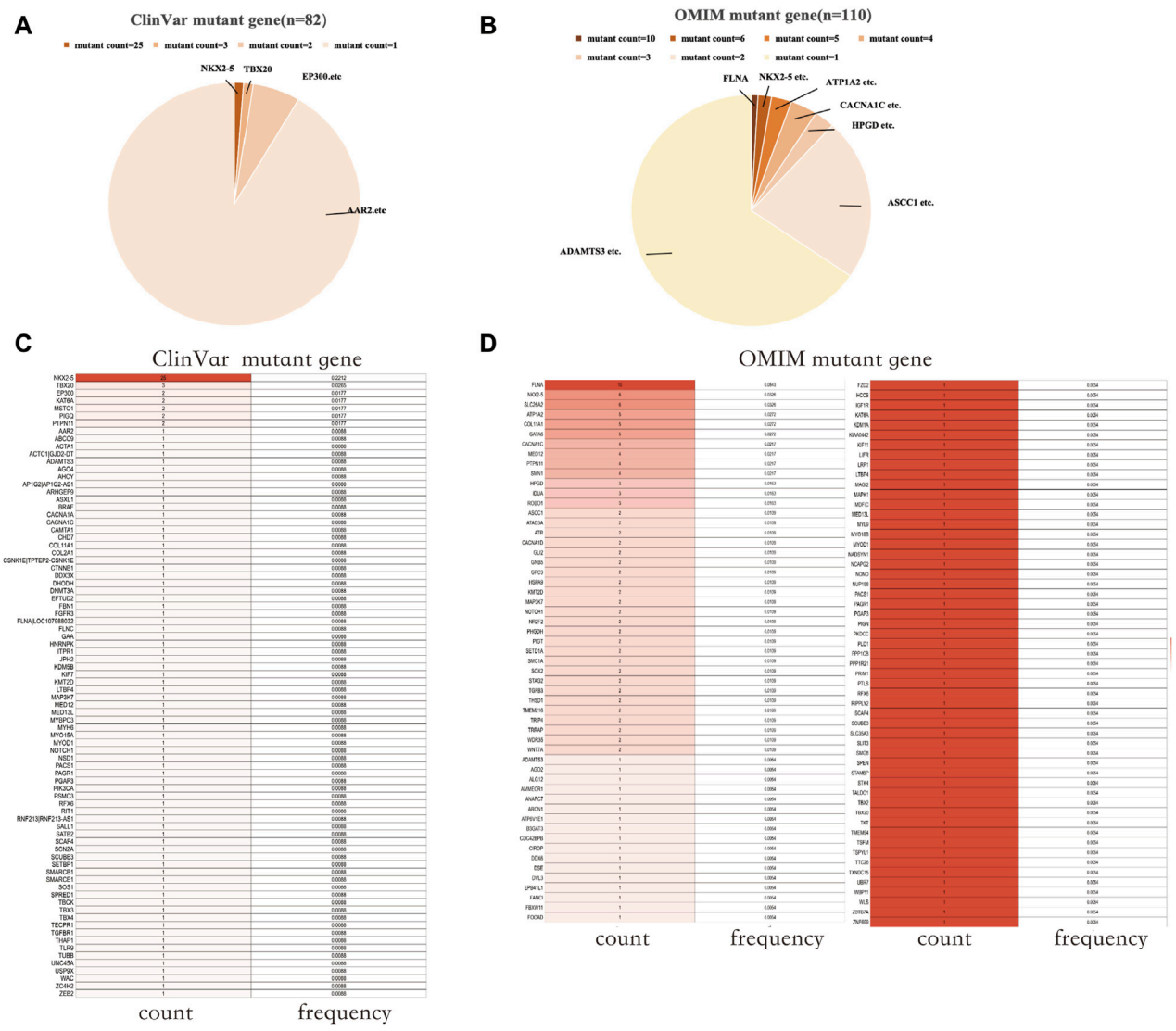

FIGURE 1
PFO-related mutant genes in public databases. **(A)** Mutant count of PFO-related mutant genes in the ClinVar database. **(B)** Mutant count of PFO-related mutant genes in the OMIM database. **(C)** The count and frequency of mutations in PFO-related mutant genes in the ClinVar database. **(D)** The count and frequency of mutations in PFO-related mutant genes in the OMIM database. PFO: Patent foramen ovale.

factor binding, transcriptional coregulatory activity, and others (Figure 2C).

3.3 Identification of gene pathogenicity through WES

Through WES, a total of 48 different mutant genes were detected in the blood samples of 25 patients with PFO, involving 59 mutation sites. The analysis of these gene variations yielded three categories: primary findings, secondary findings, and other potential variations. The primary findings were that the mutant genes were highly relevant to the patient's clinical information and can be reasonably explained, with sufficient evidence of pathogenicity. The secondary findings were that the mutant genes were highly relevant to the patient's clinical information and can be reasonably explained, although the evidence for pathogenicity was not robust, but the possibility of pathogenicity was not ruled out. Other potential variations were that the mutant genes were related or possibly related to the patient's clinical information but cannot be reasonably explained.

In the primary findings, two genes with two specific mutation sites were identified as potentially associated with patent foramen ovale. These genes are LDLR (c.947A>G) and SDHC (c.412dup) (Table 2). The LDLR gene is involved in cellular cholesterol metabolism and has implications in familial hypercholesterolemia, while the SDHC gene is linked to the tricarboxylic acid cycle within the mitochondria. The secondary findings revealed that 42 mutant genes (87.5%) were classified as variants of uncertain significance in terms of clinical relevance (Table 3). In addition to these variations, five other potential mutations (10.4%) were also identified (Table 4). Among the

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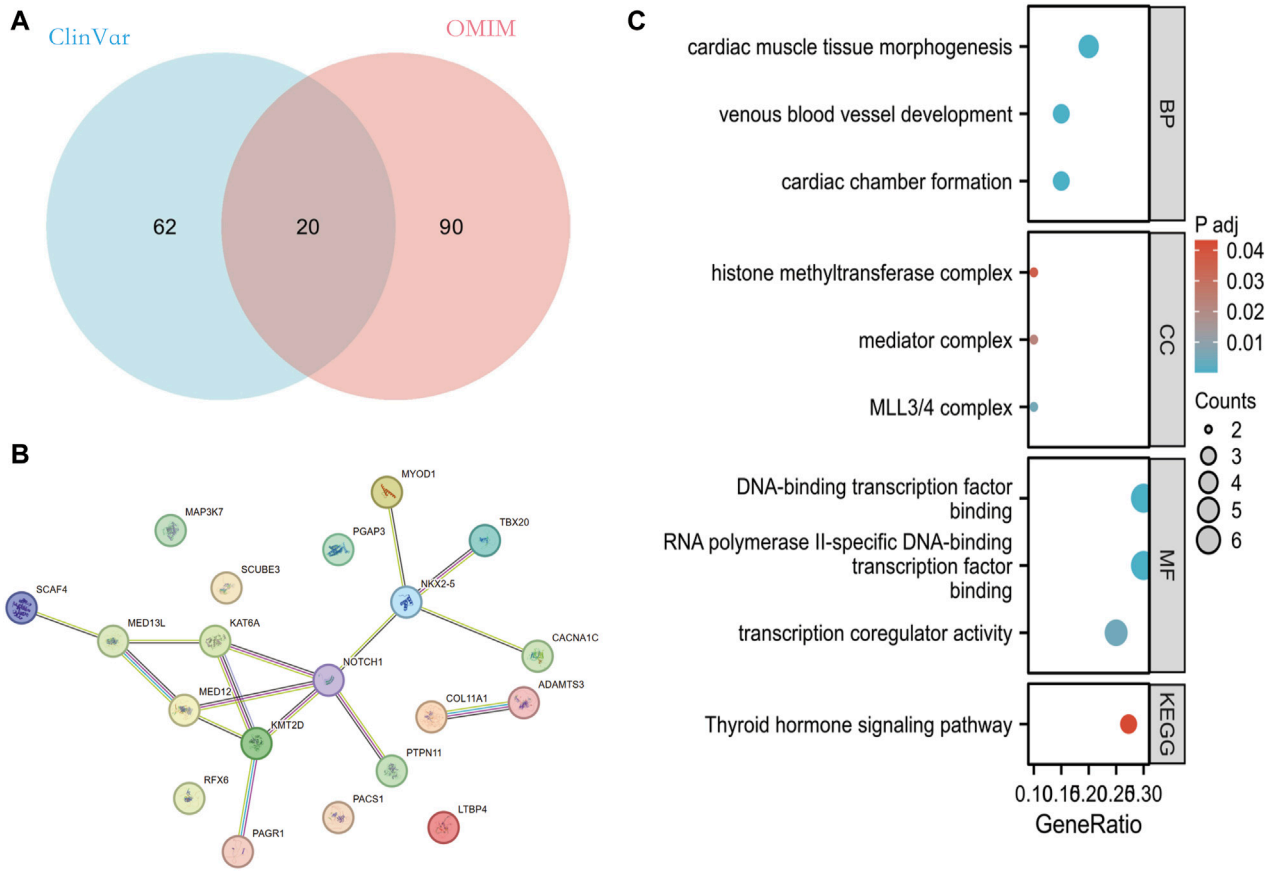

FIGURE 2
Analysis of mutant genes associated with PFO in ClinVar and OMIM database. **(A)** Overlapping mutant genes related to PFO in the ClinVar and OMIM databases. **(B)** PPI network of these 20 overlapping genes built through String database. **(C)** The GO and KEGG pathway enrichment analysis of these 20 genes. PFO: Patent foramen ovale; PPI: protein-protein interaction network; GO: Gene Ontology; KEGG: Kyoto Encyclopedia of Genes and Genomes.

TABLE 2 The primary findings related to PFO.

Gene	Number of people	Nucleotide change (amino acid change)	Zygotism	ACMG classification
LDLR	1	c.947A>G (p.Asn316Ser)	heterozygous	Likely pathogenic
SDHC	1	c.412dup (p.Asp138GlyfsTer69)	heterozygous	Likely pathogenic

The primary findings: mutant genes were highly relevant to the patient's clinical information and can be reasonably explained, with sufficient evidence of pathogenicity.

59 mutation sites we detected, we identified 12 novel mutation sites that are not recorded in the ClinVar database and have not been observed in the East Asian population. We utilized the REVEL and ClinPred software to score and predict the potential pathogenicity of these mutation sites. Following this, we employed SIFT and Polyphen2 to forecast their impact on protein function (Table 5).

3.4 Functional enrichment analysis of mutant genes in PFO patients

We constructed a protein-protein interaction (PPI) network (Figure 3A) using a String database for these 48 different genes. To further understand the interactions among these genes, we applied the MCC algorithm in Cytoscape software to select the top 10 genes. It is noteworthy that the genes NKX2-5, SCN5A, MYH6, and GATA4 are the most important genes associated with PFO disease (Figure 3B). Functional enrichment analysis was performed on these 10 genes, revealing their significant involvement in cardiac cell biology functions. In terms of molecular function, these genes were found to potentially influence various protein bindings, including calcium-binding proteins, glycoproteins, myosin, and dystrophin. Furthermore, the analysis suggested their potential involvement in several KEGG pathways, such as thyroid hormone signaling, hypertrophic cardiomyopathy, dilated cardiomyopathy, adrenergic signaling in cardiac cells, as well as their association with viral myocarditis (Figure 3C).

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TABLE 3 The secondary findings related to PFO.

Gene (n = 42)	Number of people	Transcript number	Nucleotide alteration
ABCC6	1	NM_001171.5	c.2633C>T
ABCG2	1	NM_004827.3	c.376C>T
ACE	1	NM_000789.4	c.945 + 6C>T
AKAP9	1	NM_005751.5	c.3532 + 7T>C
AKT2	1	NM_001626.6	c.441 + 16A>G
APOB	1	NM_000384.3	c.3404G>A
CACNA1A	1	NM_001127221.2	c.4391 + 19A>G
CELA2A	1	NM_033440.3	c.760C>T
CITED2	1	NM_006079.5	c.769A>G
CLCN2	1	NM_004366.6	c.694-11A>C
COL4A1	1	NM_001845.6	c.3506-13C>G
DMD	1	NM_004006.3	c.1704 + 16G>T
DSC2	1	NM_024422.6	c.2488A>C
ELN	1	NM_001278929.2	c.2101T>C
EPAS1	1	NM_001430.5	c.1444-5C>G
EPOR	1	NM_000121.4	c.52C>G
ESR1	1	NM_001122740.2	c.433G>A
EYA4	1	NM_00410.5	c.978C>G
FLNC	2	NM_001458.5	c.5791C>T
		NM_001458.5	c.6527G>A
FLT4	1	NM_182925.5	c.2406 + 16C>A
GATA4	1	NM_002052.5	c.49G>A
HCCS	1	NM_005333.5	c.560G>C
LAMA4	1	NM_002290.5	c.292T>C
LRP6	1	NM_002336.3	c.268G>A
MYBP3	1	NM_000256.3	c.1458-7C>T
MYH11	1	NM_002474.3	c.3616G>A
MYH6	1	NM_002471.4	c.5491G>A
NKX2-5	1	NM_004387.4	c.257T>C
NOTCH1	2	NM_017617.5	c.164C>T
		NM_17617.5	c.562C>G
NOTCH3	3	NM_000435.3	c.6604G>A
		NM_00435.3	c.3371A>G
		NM_00435.3	c.4039G>C
POBO4	1	NM_001301088.2	c.244 + 12G>A
PPARC	1	NM_015869.5	c.328C>T
PRDM16	2	NM_022114.4	c.37 + 15C>T
		NM_022114.4	c.686C>T

(Continued on following page)

TABLE 3 (*Continued*) The secondary findings related to PFO.

Gene (n = 42)	Number of people	Transcript number	Nucleotide alteration
PSEN2	1	NM_000447.3	c.893T>C
RNF213	3	NM_001256071.3	c.10424-3C>T
		NM_001256071.3	c.10424-3C>T
		NM_001256071.3	c.12805C>T
		NM_001256071.3	c.12942 + 15C>T
		NM_001256071.3	c.13186-18_13186-14dup
		NM_001256071.3	c.1978C>T
RYR2	1	NM_00103	c.4990G>A
SCN5A	1	NM_198056.3	c.1975C>T
SCNN1B	1	NM_000336.6	c.1074C>A
SDHD	1	NM_003002.4	c.205G>A
TNNI3K	1	NM_015978.3	c.1153T>C
VCL	1	NM_014000.3	c.2191A>G
WBP11	1	NM_016312.3	c.1889A>G

The secondary findings: mutant genes were highly relevant to the patient's clinical information and can be reasonably explained, although the evidence for pathogenicity was not robust, but the possibility of pathogenicity was not ruled out.

TABLE 4 Other potential variations related to PFO.

Gene	Number of people	Transcript number	Nucleotide change
G6PC1	1	NM_000151.4	c.361A>G
SERPINA1	1	NM_000295.5	c.187C>T
NOTCH1	1	NM_017617.5	c.1214C>T
FOXC1	1	NM_001453.3	c.1304C>G
EXPHX2	1	NM_001979.6	c.910 + 4G>A

Other potential variations: mutant genes may be related or potentially related to the clinical information of the clinical subjects, but they cannot be reasonably explained in the context of the subjects' clinical information.

4 Discussion

PFO, the most common congenital heart abnormality in adults, has been found to have a higher incidence in stroke patients compared to healthy individuals (Gonzalez and Testai, 2021). Nearly half of CS patients have PFO. Research by Del Sette M et al. suggests that PFO occurrence is characterized by family aggregation, indicating the presence of genetic abnormalities (Del Sette et al., 2004). The prevalence of PFO in siblings of young patients with ischemic stroke (IS) is three times greater than in siblings of patients without PFO (Limborska and Filippenkov, 2022). Hereditary hypercoagulability has also been proven to be an important risk factor for PFO-related CS (Harland et al., 2002). In our study, we performed WES to identify mutant genes in 25 PFO patients with clinical symptoms who visited our hospital. Patient outcomes among those with PFO exhibit significant variability. The risk of cardiovascular events in PFO patients is closely related to the anatomical characteristics of the PFO, their genetic background, and the presence of other cardiovascular risk factors (Mojadidi et al., 2021a). In our cohort of 25 PFO patients, 3 cases experienced a stroke, all at an age younger than 45 years. In one of these patients, a suspected pathogenic gene variant was detected (LDLR NM_00527.5 c.947A>G). This mutation results in the substitution of asparagine (Asn) with serine (Ser) at the 316th amino acid of the LDLR protein, which is associated with Familial Hypercholesterolemia (FH). FH leads to abnormally elevated levels of low-density lipoprotein cholesterol (LDL-C) in the blood, increasing the risk of cardiovascular diseases. Mary F Lopez found that in PFO-stroke patients, there was a slight increase in HDL (high-density lipoprotein) and a decrease in cholesterol levels following the closure of the PFO (Lopez et al., 2015). Detecting genetic mutations associated with PFO can help to gain a deeper understanding of the pathogenesis of PFO, the interaction between genes and environmental factors, determine whether patients have genetic risk, and develop personalized treatment plans for patients.

In the ClinVar and OMIM databases, 82 and 110 PFO-related genes were identified, with 20 genes belonging to overlapping

TABLE 5 Novel mutation sites not included in the ClinVar database and not detected in East Asian populations.

Gene	Transcript number	Nucleotide alteration	Amino acid change	REVEL	ClinPred	SIFT	PolyPhed-2
MYH11	NM_002474.3	c.3616G>A	p.Glu1206Lys	0.653	0.985	Harmless	Potentially harmful
NOTCH1	NM_17617.5	c.562C>G	p.Leu188Val	0.176	0.051	Harmless	Harmless
ABCC6	NM_001171.5	c.2633C>T	p.Ser878Phe	0.218	0.105	Harmless	Harmless
WBP11	NM_016312.3	c.1889A>G	p.Tyr630Cys	0.492	0.995	Harmful	Potentially harmful
HCCS	NM_005333.5	c.560G>C	p.Gly187Ala	0.957	/	Harmful	Harmful
ELN	NM_001278929.2	c.2101T>C	p.Phe701Leu	0.034	0.782	Harmful	Harmful
EPOR	NM_000121.4	c.52C>G	p.Leu18Val	0.342	0.782	Harmful	Harmful
NOTCH3	NM_000435.3	c.6604G>A	p.Val2202ILE	0.135	0.005	Harmless	Harmless
GATA4	NM_002052.5	c.49G>A	p.Ala17Thr	0.358	0.534	Harmless	Harmless
LAMA4	NM_002290.5	c.292T>C	p.Cys98Arg	0.948	1.000	Harmful	Harmful
NOTCH3	NM_00435.3	c.3371A>G	p.Asp1124Gly	0.688	0.938	Harmful	Harmful
NOTCH1	NM_017617.5	c.164C>T	p.Pro55Leu	0.030	0.060	Harmful	Harmless

REVEL, rating: >0.750, predicted to be harmful; ClinPred rating: >0.500, predicted to be harmful.

genes. Through functional enrichment of 20 overlapping genes, these genes mainly focusing on ventricular formation, myocardial tissue morphogenesis, and venous vessel development at the biological process (BP) level. At the cellular component (CC) level, these genes are related to MLL3/4 complex, histone mediator complex, methyltransferase complex, and others. At the molecular functional (MF) level, these genes are associated with RNA polymerase II specific DNA-binding transcription factor binding, DNA-binding transcription factor binding, transcriptional coregulatory activity, and more. Most of these mutant genes are closely related to the development of heart structure, and they may lead to PFO formation by affecting heart development.

We performed WES on 25 patients with clinical symptoms undergoing PFO closure and identified mutations in 48 related genes. Among these genes, LDLR and SDHC were considered as suspected pathogenic genes. The LDLR gene is primarily involved in cholesterol metabolism at the cellular level, while the SDHC gene is mainly involved in mitochondrial tricarboxylic acid circulation. Furthermore, the SDHC gene has also been linked to gastrointestinal stromal tumor and paraganglioma, although these variants are potential pathogenic factors. Additionally, other pathogenic variants, G6PC1 and SERPINA1 genes, were found. G6PC1 is a pathogenic variant associated with glycogen storage disease type 1a, and SERPINA1 is a suspected pathogenic variant associated with α1-antitrypsin deficiency. Moreover, enrichment analysis revealed that the genes in PFO patients were mainly related to the biological function of muscle tissue, particularly the myocardium. At the cellular level, these genes were associated with myofibril and contractile fiber cells. At the molecular function level, the genes were found to be involved in triosan binding, nitric oxide synthase binding, ankyloprotein binding, actin binding, calmodulin binding, and more. In terms of KEGG pathways, these genes were associated with thyroid hormone signaling pathways, hypertrophic cardiomyopathy, dilated cardiomyopathy, and viral myocarditis.

The analysis of mutation genes in databases and clinical data revealed that NKX2.5 has the highest mutation frequency and has been detected in ClinVar, OMIM, and clinical data. According to the PPI network, NKX2.5 has protein interactions with CACNA1C, MYOD1, TBX20, GATA4, MYH6, RYR2, SCN5A, and NOTCH-1. Cao Y's study in 2016 suggested that a variation in the single nucleotide site of NKX2-5 may be linked to the occurrence of Atrial Septal Defect (ASD) (Cao et al., 2016). NKX2-5 regulates the proliferation, migration, differentiation, and function of cardiomyocytes through signaling pathways involving GATA4, MYH6, and others (Välimäki et al., 2017; Wang et al., 2020; Andreasen et al., 2022). Furthermore, several studies have suggested that NOTCH-1 mutation genes are among the most prevalent causes of Congenital Heart Disease (CHD) (Samira et al., 2020; Sarah et al., 2022). Josef Finsterer et al. state that transcription factors NKX2-5 and NOTCH-1 signaling are involved in the pathogenesis of left ventricular hypertrabeculation (LVHT) (Finsterer and Stöllberger, 2020). These studies further confirm the oligogenic effect in the pathogenic process of PFO.

Identification of PFO-related genetic variations through WES allows for the early detection of high-risk PFO patients, enabling timely interventions to reduce potential complications, such as CS. Furthermore, understanding the specific genetic variations in patients who have already been diagnosed with PFO can aid in the formulation of personalized treatment plans. For instance, patients carrying variations in genes such as NKX2-5 or NOTCH-1 may require more intensive monitoring and targeted therapeutic strategies. Additionally, this information on genetic variations can guide patients in genetic counseling, helping them understand the hereditary risks and family history of the disease.

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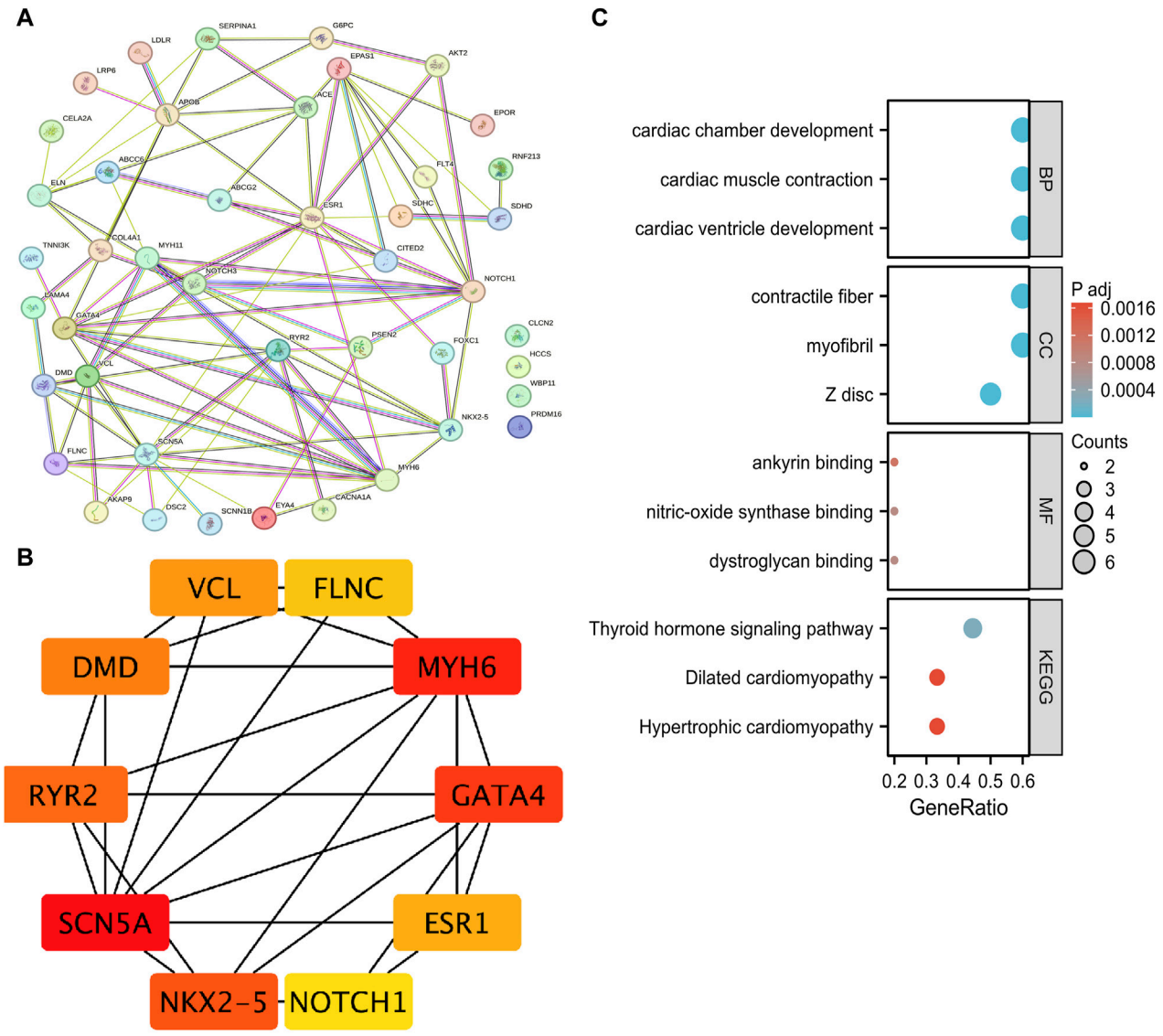

FIGURE 3
Mutation genes analysis of whole exome detection in 25 clinical PFO patients. **(A)** PPI network of mutation genes built through String database. **(B)** The top ten genes in PPI network obtained using Cytoscape software and MCC algorithm. **(C)** The GO and KEGG pathway enrichment analysis of these 10 genes. PFO: Patent foramen ovale; PPI: protein-protein interaction network; GO: Gene Ontology; KEGG: Kyoto Encyclopedia of Genes and Genomes.

Future research can further explore the impact of these genetic variations on treatment outcomes, thereby providing more precise personalized therapies for PFO patients.

Starting from the analysis of genes can provide a deeper understanding of the formation and pathogenesis of PFO, facilitating the identification of specific pathways for targeted interventions that may lead to effective prevention and non-invasive treatment strategies in the future. However, it is important to acknowledge the limitations of this study, such as the small sample size, which may introduce sampling errors and variations in the distribution of disease-causing genes among the sample subjects. Therefore, future research should consider increasing the sample size and employing direct sequence analysis or whole genome sequencing in combination with clinical diagnosis to achieve the highest detection rate of pathogenic genes.

5 Conclusion

In this study, we employed WES to identify potential mutant genes and gene mutation sites in patients with PFO. Subsequently, we analyzed PFO-related mutant genes using ClinVar and OMIM databases and found that NKX2-5 genes are involved in the pathogenesis of PFO through other mutation sites and signaling pathways. The findings of our study provide important insights for genetic counseling and gene therapy in PFO patients.

Data availability statement

The original contributions presented in the study are included in the article/supplementary material, further inquiries can be directed to the corresponding authors.

Ethics statement

The studies involving humans were approved by Ethics Committee of the First Affiliated Hospital of Zhejiang University of Traditional Chinese Medicine. The studies were conducted in accordance with the local legislation and institutional requirements. The participants provided their written informed consent to participate in this study.

Author contributions

XL: Writing–original draft. LX: Writing–original draft. JD: Data curation, Formal Analysis, Funding acquisition, Writing–review and editing. XZ: Data curation, Formal Analysis, Funding acquisition, Writing–review and editing. TC: Data curation, Funding acquisition, Methodology, Software, Writing–review and editing. WM: Funding acquisition, Writing–review and editing.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

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Keratin 19 (Krt19) is a novel marker gene for epicardial cells

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Epicardial cells regulate heart growth by secreting numerous growth factors and undergoing lineage specification into other cardiac lineages. However, the lack of specific marker genes for epicardial cells has hindered the understanding of this cell type in heart development. Through the analysis of a cardiac single cell mRNA sequencing dataset, we identified a novel epicardial gene named *Keratin 19* (*Krt19*). Further analysis of the expression patterns of *Krt19* and *Wt1*, a well-known epicardial gene, revealed their preferences in major cardiac cell types. Using lineage-tracing analysis, we analyzed *Krt19-CreER* labeled cells at multiple time windows and found that it labels epicardial cells at both embryonic and neonatal stages. Furthermore, we studied the function of epicardial cells using a diphtheria toxin A chain (DTA)-based cell ablation system. We discovered that *Krt19-CreER* labeled cells are essential for fetal heart development. Finally, we investigated the function of *Krt19-CreER* and *Wt1-CreER* labeled cells in neonatal mouse development. We observed that the *Krt19-CreER*; *Rosa-DTA* mice displayed a smaller size after tamoxifen treatment, suggesting the potential importance of *Krt19-CreER* labeled cells in neonatal mouse development. Additionally, we found that *Wt1-CreER*; *Rosa-DTA* mice died at early stages, likely due to defects in the kidney and spleen. In summary, we have identified *Krt19* as a new epicardial cell marker gene and further explored the function of epicardial cells using the *Krt19-CreER* and *Wt1-CreER*-mediated DTA ablation system.

KEYWORDS

KRT19, epicardial cell, DTA, ablation, lineage tracing, WT1

Introduction

Heart development is an essential embryonic process that involves multiple cell lineages and is tightly regulated at the cellular and molecular levels. If this process goes awry, it will lead to congenital heart diseases (CHDs), which account for a significant portion of stillbirths and is present in 1%–2% of all live births (Triedman and Newburger, 2016). The epicardium, a membranous layer covering the outside of the myocardium, not only acts as a pool of multi-potential progenitor cells contributing to the development of fibroblasts and smooth muscle cells but also acts as an important source of mitogenic signals to maintain the continued growth and differentiation of the heart (von et al., 2011; von Gise and Pu, 2012; Riley, 2012; Simoes and Riley, 2018). A systematic analysis of epicardial cell function is critical not only for understanding these normal heart development processes but is also required for understanding the molecular mechanisms of CHDs such as Left Ventricular Non-Compaction Cardiomyopathy (Zhang et al., 2013; Villa et al., 2016; Jang et al., 2022).

Epicardial cells are known to develop from the proepicardial organ (PEO) and highly express many genes, including *Wt1* (Cao et al., 2020). *Wt1* is a transcription

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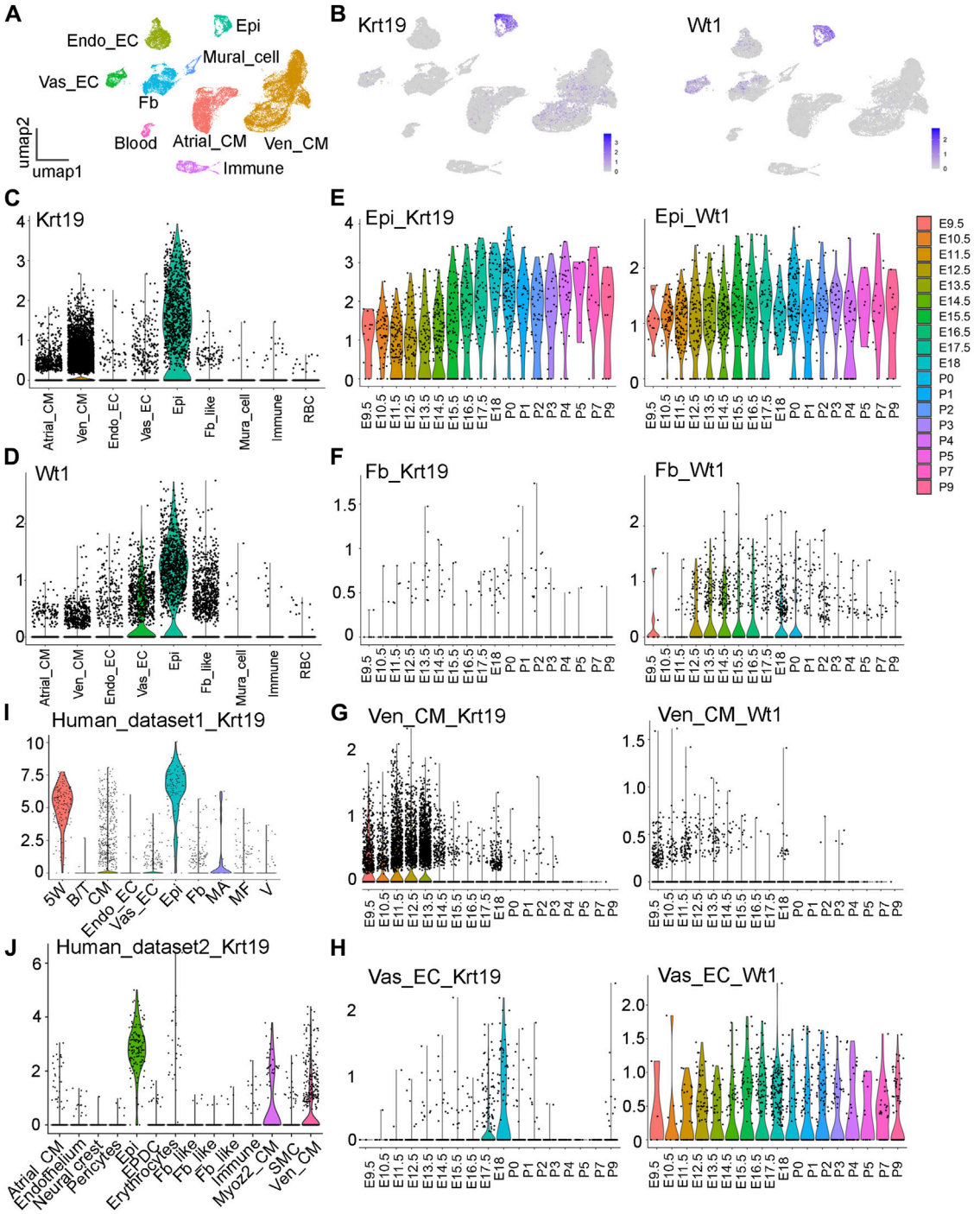

FIGURE 1
Analysis of the *Krt19* and *Wt1* expression patterns in scRNA-seq data. **(A)** UMAP plot of the CD1 scRNA-seq dataset with cell type annotation. **(B)** Feature plots of *Krt19* and *Wt1* expression in the scRNA-seq data of 18 stages of developing hearts. **(C, D)** Violin plots of *Krt19* and *Wt1* expression at different cardiac cell types. **(E–H)** Violin plots of *Krt19* and *Wt1* expression at different stages in each cell type. **(I, J)** Violin plots of *Krt19* expression at two human fetal heart scRNA-seq datasets.

factor, mutations of which in mice lead to death at early stages before E14.5[2]. The *Wt1-CreER* mouse line has been utilized to trace the lineage descendants of epicardial cells and is widely applied to manipulate gene expression in epicardial cells and epicardium-derived lineages (Simoes and Riley, 2018; Cao et al., 2020). While controversies persist regarding the lineage differentiation of Wt1-CreER labeled epicardial cells into endothelial cells and cardiomyocytes (Zhou et al., 2008; Rudat and Kispert, 2012; Zhou and Pu, 2012; Lupu et al., 2020), there has been a lack of systematic study on Wt1 expression across various cardiac cell types at the single-cell level.

To investigate the function of epicardial cells, we employed a conditional ablation system using *DTA*. *DTA*, a gene-encoded cell toxin, has been extensively utilized to selectively eliminate target cells in various tissues (Sturzu et al., 2015; Fenlon et al., 2020; Xu et al., 2021). In *Rosa26-DTA* mice, DTA expression is controlled by CRE recombinase-mediated gene recombination (Ivanova et al., 2005). Cardiac progenitor cells and cardiomyocytes have been ablated using this system to explore their function in embryonic heart development (Sturzu et al., 2015). The epicardium was selectively ablated in *Wt1-CreER*; *Rosa26-DTA* mice at E11-11.5 to explore its involvement in macrophage recruitment (Stevens et al., 2016). However, a comprehensive examination of epicardium function in myocardium development was not performed.

In this study, we have identified *Krt19* as a novel epicardial cell marker gene. *Krt19*, an intermediate filament protein belonging to the keratin family, functions in maintaining the structural integrity of epithelial cells. Mice homozygous for *Krt19* mutations remain viable and fertile. Notably, *Krt19-CreER* mice have been employed to lineage trace epithelial cells and mesothelial cells across multiple tissues (Means et al., 2008; Westcott et al., 2021). For example, in mouse adult adipose tissue, *Krt19*, but not *Wt1*, was found to be a highly specific marker for the mesothelium (Westcott et al., 2021). Through the combination of single cell RNA sequencing (scRNA-seq), and lineage tracing experiments, we demonstrated that *Krt19* is an epicardial cell gene with differential expression pattern from *Wt1*. We further utilized it together with *Rosa-DTA* mice to study epicardium function.

Results

Identification of *Krt19* as a novel epicardial cell marker gene

Through the analysis of an 18-staged cardiac single-cell RNA sequencing (scRNA-seq) dataset in CD1 mice, previously published by our team and deposited in GEO with the accession number GSE193346 (Feng et al., 2022), we identified a new epicardial cell gene named *Krt19* (Figures 1A,B). We found that *Krt19* is highly expressed in epicardial cells at all analyzed stages, ranging from embryonic (E) day 9.5 to postnatal (P) day 9 (Figure 1E). Additionally, *Krt19* is also expressed in atrial and ventricular cardiomyocyte (Atrial_CM, Ven_CM) at early developmental stages (mainly before E14.5) (Figures 1C,G). To better understand *Krt19*'s expression pattern, we compared it with the expression pattern of the well-known epicardial gene *Wt1*. We found that *Wt1* is highly expressed in epicardial cells at all stages (Figures 1B,E) and in atrial and ventricular cardiomyocytes at early stages. However, *Wt1* is also expressed in vascular endothelial cell (Vas_EC) and fibroblast (Fb) at all the analyzed stages, while *Krt19* is barely detected in these 2 cell types at most of the analyzed stages (Figures 1F,H). Moreover, we analyzed *Krt19* expression in two human fetal heart scRNA-seq datasets and found that it had high expression in epicardial cell and moderate expression in CM (Asp et al., 2019; Cui et al., 2019) (Figures 1I,J). In summary, we have identified a novel epicardial cell gene, *Krt19*, which is highly expressed in epicardial cells and exhibits a differential expression pattern compared to *Wt1*.

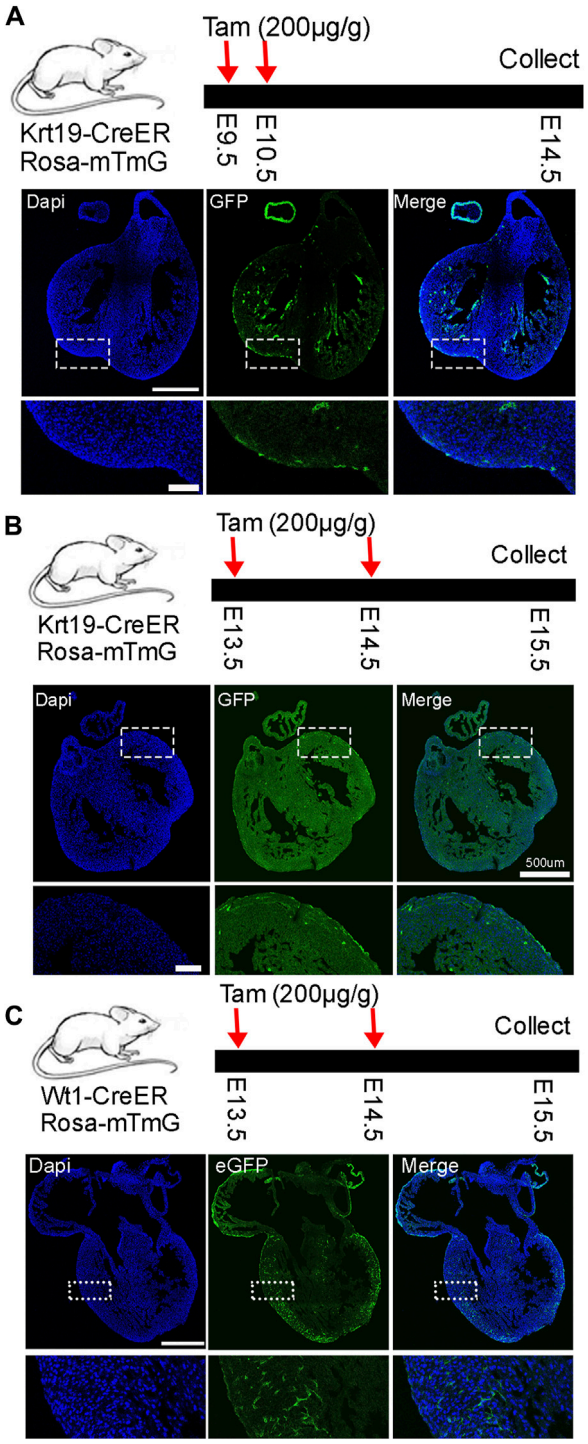

FIGURE 2
Lineage analysis of *Krt19-CreER* and *Wt1-CreER* labeled cells at early embryonic stages. **(A–C)** Analysis was conducted at early stages by treating the mice with tamoxifen and analyzing them at different stages. Scale bar is 500 μm for the whole heart and 100 μm for the enlarged images.

Lineage analysis of *Krt19-CreER* labeled cells

Next, to understand the lineage development of *Krt19* positive cells, we lineage traced them by breeding *Krt19-CreER*

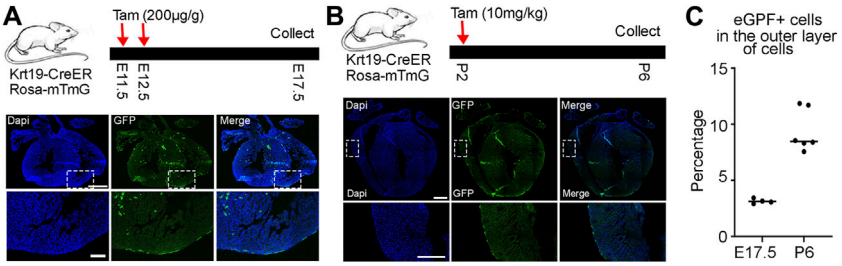

FIGURE 3
Lineage analysis of *Krt19-CreER* labeled cells at embryonic and neonatal stages. **(A)** Long term lineage analysis of the *Krt19-CreER* positive cells by treating the mice with tamoxifen at E11.5 and E12.5 and analyzing them at E17.5. **(B)** The mice were treated with tamoxifen at P2 and harvested at P6. **(C)** Quantification of the percentages of eGFP-positive cells in the outer layer of epicardial cells. Scale bars are 500 μm for the whole heart and 100 μm for the enlarged images.

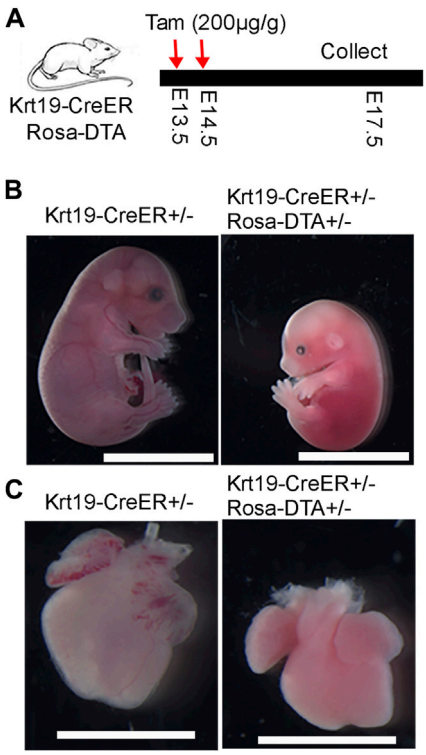

FIGURE 4
Phenotypic analysis of the embryo and heart defects after ablation of the *Krt19-CreER* labeled cells. **(A)** Diagram of the experimental workflow. **(B, C)** The ablated embryos and hearts were smaller than controls. Scale bar is 1 cm for the embryos and 250 μm for the hearts. Four control and three ablated embryos with similar phenotype were observed in the experiment.

mice with *Rosa26-mTmG* mice and administering tamoxifen at different time points. Initially, we treated the mice with tamoxifen at E9.5 and E10.5 and analyzed their hearts at E14.5 (Figure 2A). We observed strong eGFP signals on the outer surface of the heart, indicating efficient labeling of epicardial cells by the mice. Additionally, we observed eGFP-positive cells inside the chamber and septum, which could be derived from the labeled epicardial cells or non-epicardial cells expressing *Krt19*. We then moved the analysis to a later time window by treating the mice with tamoxifen at E13.5 and E14.5 and analyzing the hearts at E15.5 (Figure 2B). Again, we observed strong eGFP signals in epicardial cells, but also some eGFP-positive cells inside the chamber. In contrast, when we analyzed *Wt1-CreER*; *Rosa26-mTmG* mice at the same time period using the same dose of tamoxifen, we observed strong eGFP signals at the chamber surface and inside the chambers (Figure 2C), which could represent epicardial cells and vascular endothelial cells, respectively. These results are largely consistent with their expression pattern identified in the scRNA-seq analysis.

Subsequently, we conducted a long-term lineage tracing experiment by treating the mice with tamoxifen at E11.5 and E12.5 and analyzed their hearts at E17.5. We observed clear eGFP signals in epicardial cells and many eGFP-positive cells inside the chamber (Figure 3A). Finally, we treated the mice with tamoxifen at P2 and analyzed their hearts at P6 to understand their performance at the neonatal stage (Figure 3B). We found that all eGFP signals were on the outer surface, indicating that only epicardial cells were labeled by the *Krt19-CreER*; *Rosa-mTmG* mouse line during this time window. Finally, we quantified the percentages of eGFP-labeled cells within the outer layer of epicardial cells. We observed that about 3 percent of cells at the embryonic stage and 9 percent of cells at the neonatal stage were labeled (Figure 3C). However, please note that the labeling efficiency may vary under different doses of tamoxifen treatments.

In summary, the lineage tracing results suggest that the Krt19-CreER mouse line labels epicardial cells at the embryonic stage and displays specificity for epicardial cells at the neonatal stage. This indicates its potential utility in studying the function of epicardial cells and gene regulations within this cell type.

Functional analysis of *Krt19-CreER* labeled cells at embryonic stage

To investigate the function of epicardial cells, we crossed *Krt19-CreER* mice with *Rosa26-DTA* mice for ablation purposes. Tamoxifen was administered to pregnant dams at E13.5 and E14.5, and embryos were collected at E17.5 (Figure 4A). Strikingly, we observed that the ablated embryos, as well as their

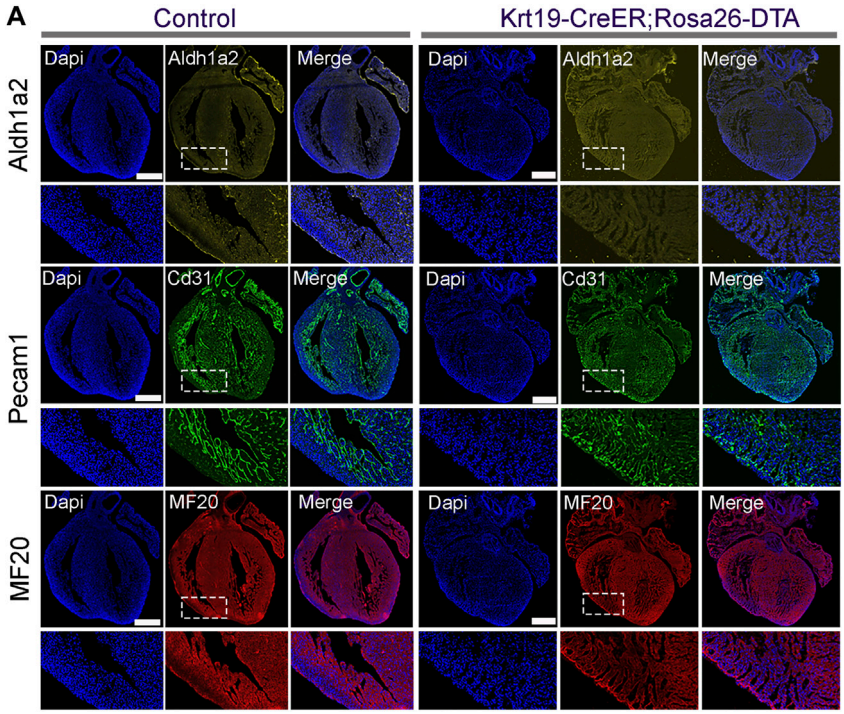

FIGURE 5
The cellular defects in the ablated embryonic hearts. **(A)** ALDH1A2 signal was largely absent on the outer surface of the ablated hearts. Hyper-trabeculae defects were observed in the ablated hearts based on the staining signal of PECAM1 and MF20. Scale bar is 500 μm in the whole hearts and 100 μm in the enlarged images.

hearts, were noticeably smaller compared to the controls (Figures 4B,C). These results suggest that *Krt19*-positive cells are essential for embryo and heart development.

To gain further insights into the molecular defects in the ablated hearts, we conducted immunofluorescence analysis. Firstly, we analyzed the expression of the epicardial cell marker gene *Aldh1a2*. We observed strong fluorescence signals on the outer surface of the chambers in control hearts; however, in the ablation hearts, we found that the signal was largely eliminated (Figure 5A). This result indicates efficient epicardial cell ablation in the *Krt19-CreER*; *Rosa-DTA* mice. Furthermore, we stained the hearts with antibodies against PECAM1 for the endothelial cell lineage and MF20 for the cardiomyocyte lineage. Interestingly, we found that the ablated hearts have an obviously hyper-trabeculated myocardium compared to the control hearts (Figure 5A). These results suggest that the *Krt19-CreER* labeled epicardium is important for embryonic heart development, likely by regulating the growth of compact myocardium.

The function of *Krt19-CreER* and *Wt1-CreER* labeled cells at neonatal stage

Lastly, we crossed *Krt19-CreER* mice with *Rosa-DTA* mice to investigate the function of the epicardium in neonatal mouse heart development. Newborn mice were treated with tamoxifen from P1 to P4, and their hearts were analyzed at P25. We observed that the ablated animal and its heart was noticeably smaller than its controls. Given that we have only successfully retrieved one ablated mouse after multiple breeding, these results can only imply that *Krt19*-positive epicardial cells are likely important for neonatal heart growth (Supplementary Figure S2). Next, we conducted similar experiments with *Wt1-CreER*; *Rosa-DTA* mice, treating them with tamoxifen at P1. However, we found that all the ablated mice died at P4. To better understand the causes of their death, we sacrificed them at P3 for detailed analysis (Figure 6). After inspecting multiple tissues, including the heart, liver, lung, kidney, and spleen from both control and ablated mice, we found that the ablated mice likely died due to kidney hemorrhage or spleen defects, which appeared wider and shorter than those in control mice (Figure 6). These results suggest that utilizing the *Wt1-CreER* mouse line to study epicardium function during neonatal heart development is challenging, while the *Krt19-CreER* mouse line emerges as a potential ideal candidate.

Discussion

In this study, we have identified a novel epicardial gene, *Krt19*, and found that it exhibits a differential expression pattern from *Wt1*. Furthermore, we conducted lineage tracing experiments to confirm the labeling of epicardial cells by the *Krt19-CreER* mouse line. Finally, we utilized an ablation system controlled by this mouse line to eliminate epicardial cells, thus confirming the importance of the epicardium in embryonic and neonatal heart development.

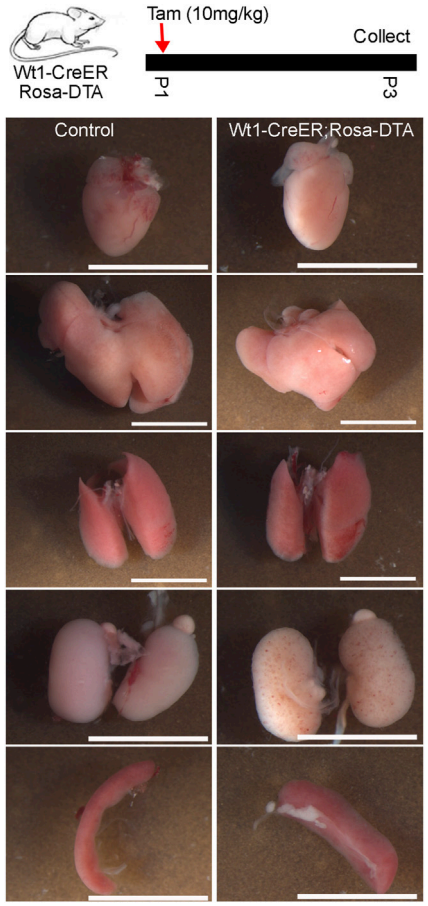

FIGURE 6
"Multi-organ analysis of control mice and mice subject to Wt1-CreER-mediated cell ablation at the neonatal stage. Tamoxifen was given at P1 and five tissues (heart, liver, lung, kidney, spleen) were analyzed at P3. The scale bar is 5 mm.

The epicardium is well known to serve not only as a reservoir of multipotential progenitor cells but also as a crucial source of mitogenic signals orchestrating heart development (Ruiz-Villalba and Perez-Pomares, 2012; Simoes and Riley, 2018; Cao et al., 2020). Anomalies in epicardial development and their signaling mechanisms in diverse mouse models manifest as defective cardiac development, mirroring human CHDs (Ruiz-Villalba and Perez-Pomares, 2012; Gittenberger-de Groot et al., 2016). Notably, the primary CHD arising from aberrant epicardium is left ventricular non-compaction cardiomyopathy, with additional implications for coronary vascular anomalies, valvulopathies, and conduction system anomalies (Zhang et al., 2013; Gittenberger-de Groot et al., 2016). Consequently, undertaking a systematic inquiry into the function of the epicardium becomes imperative, promising valuable insights into the mechanisms underlying CHDs.

Besides epicardial cells, *Krt19* is also expressed in epithelial cells and mesothelial cells in many other tissues, such as adipose and liver (Pepe-Mooney et al., 2019; Westcott et al., 2021). The lethal phenotype observed in *Krt19-CreER*; *Rosa-DTA* embryos could be caused by defects in other tissues. Regarding the defects observed in the hearts, it is possible that they were secondary to defects in other tissues, although the chance is low given the previous publications of a similar phenotype in epicardial gene mutants (von et al., 2011). This issue may also apply to other available epicardium related CreER mouse lines, given that they are also driven by genes expressed not only in epicardial cells but also in other cell types. Furthermore, the defects in the heart's compact myocardium could potentially be caused by the ablation of *Krt19*-positive CMs. To explore this possibility, we analyzed *Krt19* expressions in compact and trabecular CMs using 18 staged mouse scRNA-seq data. We found that *Krt19* is expressed in both types of CMs and appears to have slightly higher expression in compact CMs (Supplementary Figure S1). However, considering that *Krt19* was mainly expressed in ventricular CMs at stages before E14.5 (Figure 1G), while tamoxifen was administered at E13.5 and E14.5 in the embryonic ablation experiments in Figure 4, and it takes time for the *DTA* gene expression to respond to the tamoxifen treatment, we believe that the embryonic heart defects in *Krt19-CreER*-mediated DTA ablation were likely mainly caused by epicardial cell ablation.

Based on the lineage tracing results (Figure 3B), we have learned that the *Krt19*-CreER mouse line specifically labels epicardial cells at neonatal stage. In contrast, other epicardium labeling strains, including *Wt1-CreER* and *Tbx18-CreER*, have been shown to label epicardial cell and epicardial cell derived cells such as fibroblasts and smooth muscle cells (Cai et al., 2019; Deng et al., 2023). Additionally, the immediate lethal phenotype observed after *Wt1-CreER*-based *DTA* ablation at the neonatal stage also suggests the ablation of critical cell types in other tissues by this line. These results collectively suggest that the *Krt19-CreER* mouse strain may be an ideal model for studying epicardium function and regulation at the neonatal stage. However, Considering that we have only recovered one ablated mouse at P25 from six breedings, including 4 litters of neonatal mice that were not treated with tamoxifen at all (Supplementary Figure S2C), we are cautious about the use of this strain until we understand more about the cause of the low recovery rate. This could be attributed to factors such as the age of the female mice used in the breedings, or potential developmental defects associated with the double heterozygous mice.

Methods

Mouse strains

The animal experiments have been approved by the University of Pittsburgh Institutional Animal Care and Use Committee (IACUC). The transgenic mice, including *Krt19-CreERT2* (Strain #:026925) (Means et al., 2008), *Wt1-CreERT2* (Strain #:010912) (Zhou et al., 2008), *Rosa26-mTmG* (Strain #:007676) (Muzumdar et al., 2007), and *ROSA26-eGFP-DTA* (Strain #:032087) (Ivanova et al., 2005) were ordered from the Jackson Laboratory.

Tamoxifen treatment and mouse dissection

To induce Cre activity, the pregnant mice were given the default dosage of 200 μg of tamoxifen per gram of body weight (200 μg/g)

through oral gavage and the neonatal mice were given 10 μg/g of tamoxifen by direct injection into their stomach (Hortells et al., 2020). The pregnant and neonatal mice were euthanized using CO2 and decapitation-based methods, respectively. Following the standard procedure described previously, the mouse hearts were isolated and fixed at 4% paraformaldehyde for immunofluorescence staining.

Immunofluorescence staining

The staining was performed following a standard procedure. Briefly, mouse hearts were fixed in 4% PFA overnight, embedded in OCT, and sectioned at 10 μm. After a brief wash in PBS to remove the OCT, samples were blocked for 1 h in blocking buffer (10% goat serum, 1% BSA, 0.1% Tween 20) and then incubated with primary antibodies in the primary antibody buffer (1% BSA in PBST) at 4°C overnight. On the second day, the samples were stained at room temperature with fluorophore-conjugated secondary antibodies in blocking buffer for 1 h. Finally, the samples were stained with DAPI, mounted with fluoromount-g, and imaged with a confocal microscope. The antibodies used in the study include anti-CD31 (BD, #550274), anti-Aldh1a2 (Sigma, #HPA010022), and MF20 (DSHB, #MF20). The outer layer of cells in Figure 3C was counted based on the DAPI staining signal.

Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: https://www.ncbi.nlm.nih.gov/geo/, GSE193346, GSE106118, and https://ega-archive.org/, EGAS00001003996.

Ethics statement

The animal study was approved by University of Pittsburgh Institutional Animal Care and Use Committee (IACUC). The study was conducted in accordance with the local legislation and institutional requirements.

Author contributions

GL: Investigation, Supervision, Writing–original draft, Writing–review and editing. JX: Investigation, Methodology, Validation, Writing–review and editing. YD: Investigation, Writing–review and editing.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fgene.2024.1385867/full#supplementary-material

SUPPLEMENTARY FIGURE S1
Feature plots showing the expression of compact and trabecular CM genes in Ven_CMs. **(A)** The expression pattern of compact myocardium genes Mycn and Hey2 and trabecular myocardium genes Bmp10 and Slit2. **(B)** The expression pattern of Krt19.

SUPPLEMENTARY FIGURE S2
DTA mediated ablation of Krt19-CreER labeled cells at neonatal stage. **(A)** Diagram of the experimental design. **(B)** Smaller heart was observed after the ablation of Krt19-CreER labeled cells at neonatal stage. Only 1 neonatal heart was analyzed at this stage. **(C)** Summary of the mouse genotyping results from 6 breeding sessions. The scale bar is 5 mm.

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Clinical characteristics and follow-up of complex arrhythmias associated with *RYR2* gene mutations in children

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Objective: The aim of this study was to analyze the diagnosis, treatment, and follow-up of six cases of complex arrhythmias associated with *RYR2* gene mutations in children.

Method: A retrospective analysis was conducted on six children diagnosed with complex arrhythmias associated with *RYR2* gene mutations. The study included an analysis of the age of onset, initial symptoms, electrocardiographic characteristics, genetic results, treatment course, and follow-up outcomes.

Results: Among the six cases included in the study, there were four males and two females, with an average age of 3.5 ± 0.5 years. The average time from initial symptoms to diagnosis was 2.7 ± 1.3 years. The most common clinical manifestation was syncope, with exercise and emotions being the main triggers. All six children had *de novo* missense mutations in the *RYR2* gene identified through whole-exome sequencing. In Holter electrocardiogram, atrial arrhythmias and sinoatrial node dysfunction were commonly observed in younger children. Four patients underwent exercise stress testing, with two experiencing bidirectional ventricular premature contractions and two experiencing bidirectional ventricular tachycardia and polymorphic ventricular tachycardia. Initial treatment involved oral propranolol or metoprolol. If arrhythmias persisted, flecainide or propafenone was added as adjunctive therapy. Two patients received permanent cardiac pacemaker treatment (single chamber ventricular pacemaker, VVI). All patients survived, with three experiencing occasional syncope during treatment. The follow-up period ranged from 12 to 37 months, with an average follow-up time of 24.3 ± 3.7 months.

Conclusion: Complex arrhythmias associated with *RYR2* gene mutations in children can present with various clinical manifestations. Atrial arrhythmias combined with sinoatrial node dysfunction are commonly observed in younger children, and the combination of pharmacological therapy and cardiac pacemaker treatment yields favourable treatment outcomes.

KEYWORDS

children, RyR2 gene, complex arrhythmias, atrial arrhythmias, gene mutation

1 Introduction

The *RYR2* gene is a key pathogenic gene encoding a calcium ion release channel located in the sarcoplasmic reticulum of myocardial cells, which is an important component of myocardial excitation-contraction coupling (Pérez-Riera et al., 2018; Baltogiannis et al., 2019). Complex arrhythmia begins with a single abnormal complex that is progressing to grouped, sustained complexes associated with worsened symptoms and outcome. The clinical manifestations of complex arrhythmias related to *RYR2* gene mutations are varied, and electrocardiogram manifestations in children can vary, leading to potential misdiagnosis and missed diagnosis. In adult patients, most cases with *RYR2* gene mutations present as ventricular arrhythmias. But in children, it can develop various types of tachycardia (e.g., ectopic atrial tachycardia, atrial flutter) and sinus node dysfunction. This study aims to retrospectively analyze the clinical data and treatment follow-up of six children with complex arrhythmias associated with *RYR2* gene mutations, to provide evidence for early detection and rational treatment of this disease.

2 Patients and method

2.1 Patients

This was an observational retrospective, single-centre trial. Patients were enrolled between January 2017 and January 2023 at Hunan Children's Hospital, Changsha, China. The definitions of complex arrhythmias associated with the *RYR2* gene are as follows: 1) Development of multiple types of arrhythmias. 2) The patient has *RYR2* gene mutations. This study protocol was approved by the Ethics Committee of the hospital (HCHLL-2023-128). All patients' family members were aware that their clinical data might be used for a clinical study and signed written informed consent.

2.2 Method

Retrospective analysis of the clinical data of enrolled children was conducted, including medical history, physical examination, electrocardiogram, Holter ECG, cardiac ultrasound, and pathogenic gene analysis results. The exercise treadmill test (GET2100 exercise treadmill, United States) was performed with an improved Bruce regimen for submaximal exercise. The indications for termination of the exercise test were: 1) achieving the target heart rate [(220-age) x 85%]; 2) Malignant arrhythmias such as ventricular tachycardia appear; 3) Development of intolerable symptoms such as palpitations, dizziness, etc. Whole exome gene sequencing was performed by Guangzhou Jiajian Medical Laboratory.

The assessment of the pathogenic role of the genetic variants identified should be multiparametric. Judicious use of bioinformatic predictors, which are known for a high rate of false-positives, and *in vitro* studies where available, should be coupled with data derived from the families of the index case and population data. According to ACMG guidelines, we calculate the correlation using the following six scoring criteria: 1. Group frequency: total frequency of population variation. 2. Conservative analysis: the region where this mutation occurs is an important component of this protein, and the amino acid sequences of different species are highly conserved. 3. Algorithm prediction: Computer assisted analysis predicts a higher likelihood of this mutation affecting protein structure/function. 4. Public data. 5. Family analysis: parents carry *RYR2* gene mutation or not. 6. Clinical notes: Based on the clinical manifestations and family analysis of the examinee, the clinical correlation is strong. The score of case 1 is PM2-P + PP3-A1+PP3-B1+PS4-M + PP2+PM6. The score of case 2 is PM2-P + PP3-A1+PP3-B2+PM1+PP2+PM6. The score of case 3 is PM2-P + PP3-A1+PP3-B1+PM1+PP2+PM6. The score of case 4 is PM2-P + PP3-A1+PP3-S + PM1+PP2+PM6. The score of case 5 is PM2-P + PP3-A1+PP3-B1+PM5+PP2+PS2. The score of case 6 is PM2-P + PP3-A1+PP3-B1-M + PM5+PP2+PS2.

2.3 Treatment and follow-up procedure

All children were advised to limit their exercise and avoid emotional stimulation. They were started on β-receptor blocker therapy, initially with a dose of 0.5–1 mg/(kg·d) of propranolol, administered orally in three times, and gradually increased to a tolerable maximum dose of 2–4 mg/(kg·d). Alternatively, a starting dose of 0.5–1.0 mg/(kg·d) of metoprolol tartrate was administered orally in two times. If arrhythmias persisted despite the use of β-receptor blockers, oral flecainide (1 mg/kg, twice a day) or propafenone (5–7 mg/kg, three times a day) was added. If obvious sinus bradycardia occurred after medication treatment, implantation of a pacemaker was considered. Outpatient follow-up was conducted after discharge, with Holter ECG and echocardiography reviewed every 3–6 months to evaluate heart function and the presence of cardiovascular-related symptoms. Children over 5 years old were evaluated through exercise treadmill tests to clarify the heart rate limit for sinus tachycardia before arrhythmias occurred, with the aim of avoiding exceeding the threshold in daily life.

3 Results

3.1 Clinical characteristics

Among the six cases of complex arrhythmias associated with *RYR2* gene mutations, there were four boys and two girls. Three children were initially misdiagnosed as "epilepsy" and treated with antiepileptic drugs in other hospitals. Two children were misdiagnosed as "myocarditis and tachycardia cardiomyopathy", and one child was misdiagnosed as "supraventricular tachycardia". For three children who were misdiagnosed with epilepsy at the first diagnosis, their initial symptoms were syncope and convulsions. They were admitted to neurology department for the first time, and diagnosed with epilepsy according to their clinical symptoms and therefore they were misdiagnosed. Without epileptic wave in electroencephalogram (EEG), electrocardiogram (ECG) or exercise treadmill was performed to detect that they have complex arrhythmias. Finally, genetic test was performed to detect *RYR2* mutation and the diagnosis was corrected. For two children who were misdiagnosed with myocarditis and tachycardia cardiomyopathy at the first diagnosis. The onset of the disease was

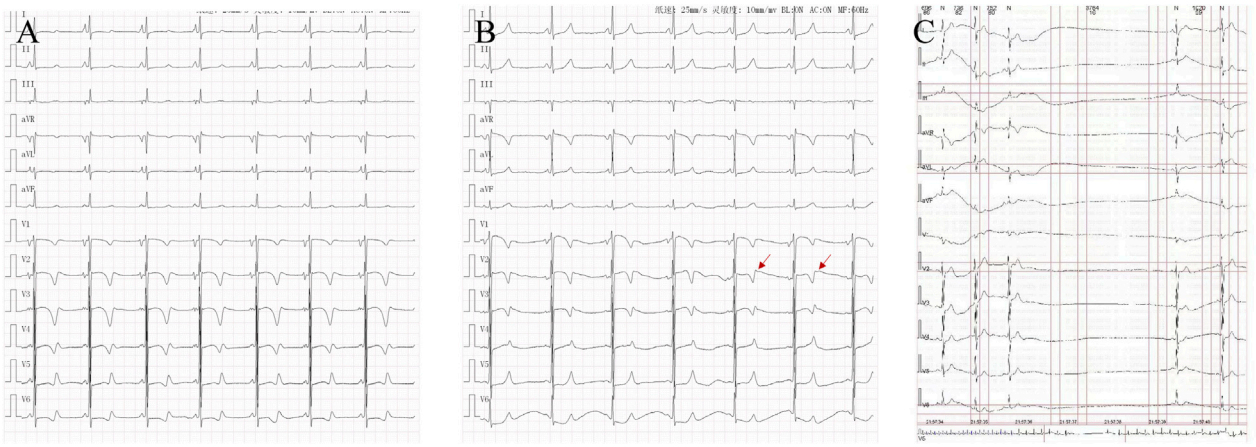

FIGURE 1
Characteristics of electrocardiogram in cases: **(A)**. Case 5: QT interval prolongation in electrocardiogram. **(B)**. Case 5: T-wave notches in lead V_2 in electrocardiogram. **(C)**. Case 1: Holter ECG electrocardiogram recorded sinus arrest for 3.764 s.

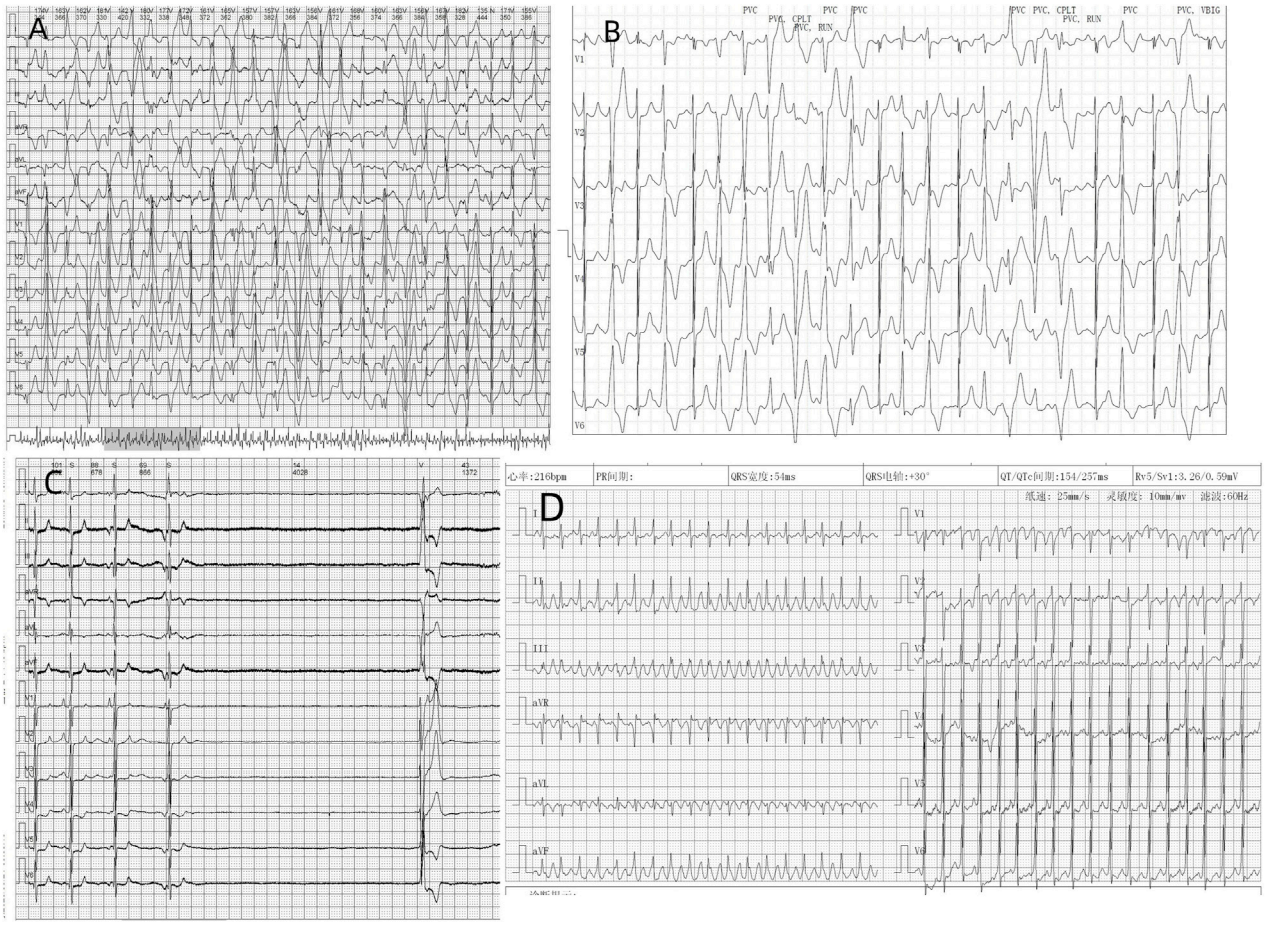

FIGURE 2
Different electrocardiogram manifestations in cases: **(A)**. Case 1 showed ventricular tachycardia recorded on dynamic electrocardiogram. **(B)**. Case 2: Ventricular premature and short paroxysmal ventricular tachycardia were recorded on the exercise treadmill. **(C)**. Case 3: Dynamic electrocardiogram recorded sinus arrest for 4.02 s. **(D)** Case 5: Routine electrocardiogram recording of atypical atrial flutter.

heart failure, with abnormal elevation of myocardial enzyme and cardiac troponin. And cardiac ultrasound showed cardiac enlargement. But later, they developed with complex arrhythmias such as atrial tachycardia and sinus node dysfunction. Finally, Genetic test was performed to confirm the diagnosis and found *RYR2* gene mutation. For two children who were misdiagnosed with supraventricular tachycardia at the first diagnosis. As the electrocardiogram diagnosis was supraventricular tachycardia, she developed with syncope and Holter ECG showed sinus arrest. So genetic testing was performed to confirm the diagnosis, revealing *RYR2* gene mutation. Three children developed syncope during exercise, one child during emotional arousal, and one child experienced transient loss of consciousness due to sinus bradycardia. The duration of syncope ranged from more than 10 s to a few minutes, with spontaneous recovery of consciousness. The age of initial symptoms ranged from 2.0 to 5.0 years, with an average age of 3.5 ± 0.5 years; the age at confirmed diagnosis ranged from 2.1 to 11 years, with an average age of 6.3 ± 1.4 years. The time from initial symptoms to diagnosis ranged from 0.1 to 8.4 years, with an average time of 2.7 ± 1.3 years.

3.2 Examinations

Two patients were admitted, and their routine surface electrocardiogram showed atrial tachycardia; one exhibited borderline tachycardia, one had occasional premature atrial contractions, and two patients had T-wave notches in lead V_2. Six patients showed abnormalities in the Holter ECG, including four cases of atrial tachycardia, two cases of atrial flutter, one case of atrial fibrillation, three cases of sinus bradycardia and cardiac arrest (RR interval 2–4.4 s), and two cases of QT interval prolongation (QTc 476–545 m). One case developed short burst multi-source ventricular tachycardia, and three cases developed ventricular premature beats. The patients' characteristics of electrocardiograms are shown in Figure 1 and Figure 2.

3.3 Genetic examination results

All six cases underwent whole exome gene sequencing and parental verification, revealing *de novo* missense mutations, with no family history of related genetic diseases in the family members. The mutation site c.12534C>A (p.N4178K) in case 2 has not been reported in clinical cases, but other pathogenic mutations p.N4178S and p.N4178Y at the same amino acid position have been reported in clinical cases. The mutation site c.536A>G (p.D179G) in case 4 has not been reported in clinical cases, but the pathogenic mutation p.G178A near this amino acid position has been reported in clinical cases. The mutation site c.41T>G (p.L14R) in case 6 has not been reported in the literature, but the region of variation is an important component of this protein. The mutation site c.12515T>A (p.F4172Y) in case 3 has not been reported in the literature. Cases 1 and 5 have been reported in clinical cases (Ohno et al., 2015; Kaneshiro et al., 2017; Kannankeril et al., 2017; Wilde et al., 2022). Based on the clinical manifestations and test results of the examinees, and according to the American College of Medical Genetics and Genomics (ACMG) mutation classification guidelines, the pathogenicity of the mutation sites in all six children is possible. The genetic examination results are shown in Table 1. The gene test had been performed in their parents and no *RYR2* gene mutations were found in their parents. Their family members were unwilling to undergo the gene test. And the exome gene sequencing and mapping results, especially for the mutation sites, are shown in this paper (Figure 3; Figure 4).

3.4 Treatment and follow-up

All six children were strictly restricted in exercise and received treatment with antiarrhythmic drugs. After oral administration of propranolol, two children did not develop syncope. Two patients still experienced intermittent palpitations and syncope after initial oral administration of metoprolol. However, after switching to propranolol, no palpitations or syncope occurred. One patient with atrial arrhythmias and frequent sinus arrest was treated with permanent epicardial pacing (VVI, VVI means a pacing mode: single chamber ventricular pacemaker), and after oral administration of propranolol and flecainide, the arrhythmias were significantly reduced. One patient developed significant sinus bradycardia after oral administration of metoprolol, and underwent permanent endocardial pacing (VVI, VVI means a pacing mode: single chamber ventricular pacemaker). One patient still experienced short paroxysmal ventricular tachycardia after taking propranolol orally, and propafenone was added orally. All cases survived, with a follow-up period of 12–37 months, with an average time of 24.3 ± 3.7 months. Please see Table 2 for details.

4 Discussion

Mutations in the *RYR2* gene are most commonly observed in patients with familial catecholaminergic polymorphic ventricular tachycardia (CPVT). Under catecholaminergic stimulation, these mutations can result in an excess Ca^{2+} load during diastole, leading to delayed afterdepolarization and subsequent arrhythmogenesis (Kim et al., 2020). In adult patients, most cases with *RYR2* gene mutations present as ventricular arrhythmias (Kawata et al., 2016). Faggioni et al. (Wilde et al., 2022) found that approximately 16% of patients with *RYR2* gene mutations develop various types of tachycardia (e.g., ectopic atrial tachycardia, atrial flutter) and sinus node dysfunction. The incidence rate of complex arrhythmias associated with *RYR2* gene mutations is low, and there are few cases reported globally. The early clinical manifestations are not typical, leading to insufficient understanding of the disease among pediatric clinicians and a propensity for missed diagnosis or misdiagnosis. In this series of cases, the age of initial symptoms ranged from 2.0 to 5.0 years, with all cases experiencing misdiagnosis during initial diagnosis. The time from initial symptoms to diagnosis ranged from 0.1 to 8.4 years (average time 2.7 ± 1.3 years). Older children are more likely to be misdiagnosed with epilepsy, while younger children are more likely to be misdiagnosed with paroxysmal supraventricular tachycardia, consistent with the reports of Al-Khatib et al. (Al-Khatib et al., 2018). In this series, four young children were found to develop various types of atrial arrhythmias (such as atrial fibrillation and

TABLE 1 The results of gene test.

Patient	Gene	Heterozygous/ homozygous	Nucleotide changes	Changes in amino acids	Source	Pathogenicity
1	RYR2	Heterozygous	c.14311G>A	p.V4771I	Old	Would be Pathogenicity
2	RYR2	Heterozygous	c.12534C>A	p.N4178K	New	Would be Pathogenicity
3	RYR2	Heterozygous	c.12515T>A	p.F4172Y	New	Would be Pathogenicity
4	RYR2	Heterozygous	c.536A>G	p.D179G	New	Would be Pathogenicity
5	RYR2	Heterozygous	c.11997G>A	p.M3999I	Old	Would be Pathogenicity
6	RYR2	Heterozygous	c.41T>G	p.L14R	New	Would be Pathogenicity

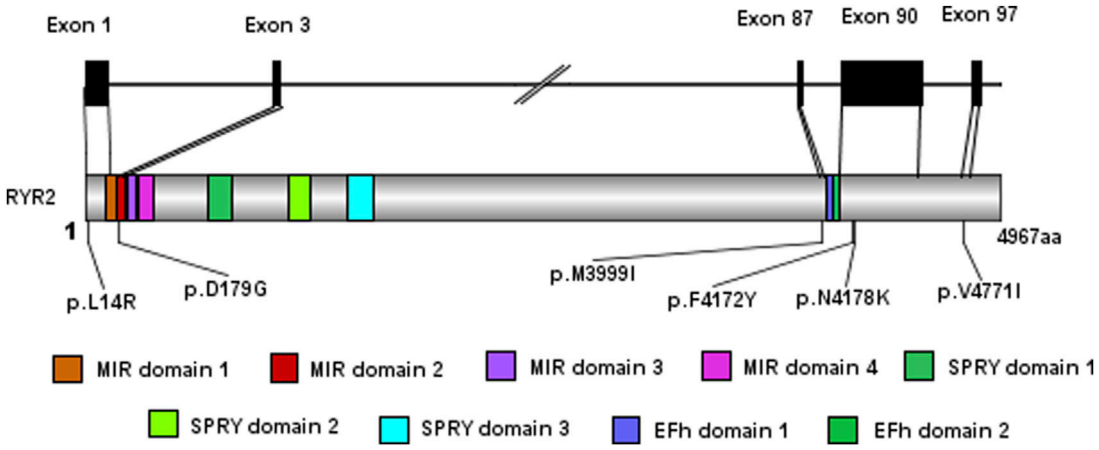

FIGURE 3
The model of exome gene sequencing and mapping results.

atrial flutter) in their initial Holter ECG, accompanied by obvious sinus bradycardia or even sinus arrest. The arrhythmias were variable, accompanied by heart failure and enlargement, making diagnosis challenging. This study also discovered that in young children with *RYR2* gene mutations, the likelihood of developing atrial tachycardia is higher than that of ventricular tachycardia. As age increases, the probability of developing ventricular tachycardia and the probability of syncope also increase. Sinus node dysfunction and chronotropic dysfunction are also characteristic changes in this group of children. Dysfunction of calcium channels and voltage-gated channels plays an important role in the occurrence of sick sinus syndrome and has a significant influence on the sinus rhythm (Faggioni et al., 2014).

RYR2 is a Ca^{2+} channel protein present in the sarcoplasmic reticulum of myocardial cells, playing a crucial role in regulating intracellular calcium ion flow and excitation-contraction coupling. Mutation in the *RYR2* gene can impair the function of the *RYR2* protein, leading to calcium leakage in the sarcoplasmic reticulum and causing fatal arrhythmias (Priori et al., 2013; Bongianino et al., 2017; Yamaguchi et al., 2022). To date, more than 350 mutations of *RYR2* have been reported, including splicing, deletion, insertion, and nonsense mutations, but most of them are missense mutations. It is challenging to clarify the association between mutation sites and pathogenicity, primarily due to the large size of the *RYR2* gene and the unclear relationship between numerous rare single nucleotide mutations and structural domains (Miyata et al., 2018). Currently, it has been found that 3.7% of the population carry benign *RYR2* gene mutations, making the interpretation of genetic results difficult. The expertise of professional genetic physicians is required to interpret the gene results (Steinberg et al., 2023). All cases in this study were newly diagnosed with missense mutations except case 1 and case 5, with mutation sites located in important regions of the *RYR2* domain. However, no family history of related genetic diseases was discovered in the family.

For the treatment of children with complex arrhythmias associated with *RYR2* mutations, attention should first be paid to lifestyle modifications, such as avoiding emotional excitement and competitive sports. β-receptor blockers are the first-line treatment drugs (Medeiros-Domingo et al., 2009). In this study, cases 2 and 6 still had symptoms even after taking metoprolol, but their

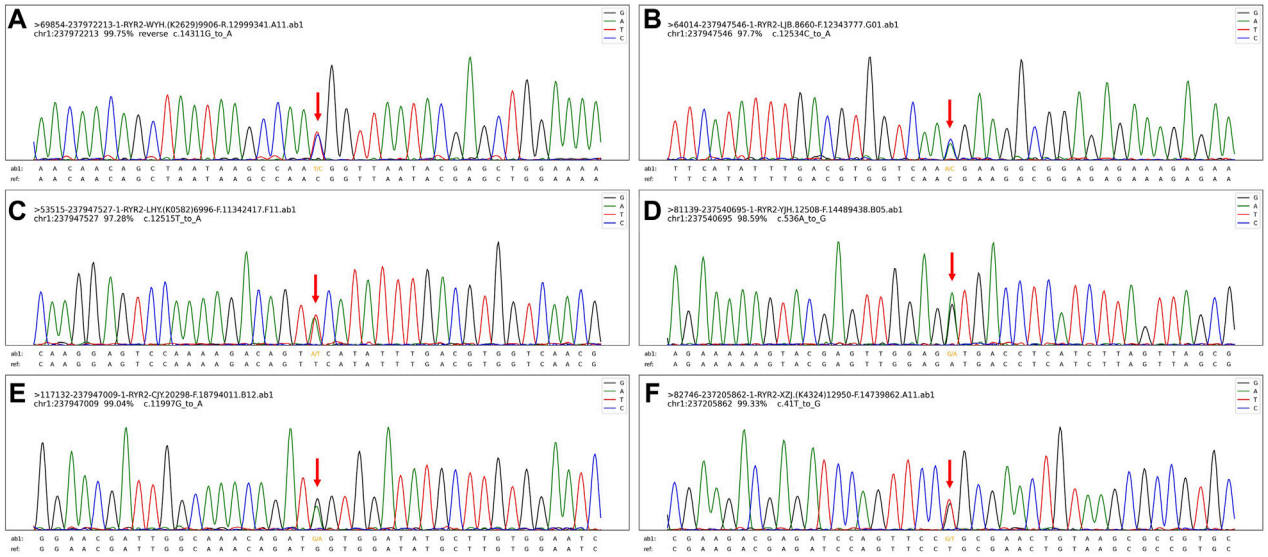

FIGURE 4
The exome gene sequencing and mapping results, especially for the mutation sites. **(A)**. The mutation site **(C)**.14311G>A (p.V4771I) in case 1. **(B)**. The mutation site **(C)**.12534C>A (p.N4178K) in case 2. **(C)**. The mutation site **(C)**.12515T>A (p.F4172Y) in case 3. **(D)**. The mutation site **(C)**.536A>G (p.D179G) in case 4. **(E)**. The mutation site **(C)**.11997G>A (p.M3999I) in case 5. **(F)**. The mutation site **(C)**.41T>G (p.L14R) in case 6.

TABLE 2 Clinical characteristics of the patients.

Patient	Gender	Syncope	Holter ECG electrocardiogram	Treatment [mg/(Kg.d)]	Follow-up time (month)	Follow-up result
1	Female	Yes	atrial tachycardia, sinus arrest, Ventricular Tachycardia	Propranolol (1.5) propafenone (5)	37	Syncope for 2 times
2	Male	Yes	atrial tachycardia, ventricular premature, sinus bradycardia	Metoprolol(1), Later changed to Propranolol(2), VVI permanent pacing	28	Intermittent palpitations
3	Male	No	atrial tachycardia, atrial fibrillation, atrial flutter, sinus arrest	Propranolol (1.5), flecainide (2), VVI permanent pacing	30	Syncope for 1 time
4	Male	Yes	ventricular premature	Propranolol (2)	20	No discomfort
5	Female	No	atrial tachycardia, atrial flutter, sinus bradycardia, QT prolongation	Propranolol (2)	12	No discomfort
6	Male	Yes	junctional tachycardia, ventricular premature	Metoprolol(1), Later changed to Propranolol(2)	19	Syncope for 1 time

condition improved significantly after switching to propranolol. Flecainide is an IC class antiarrhythmic drug that can inhibit delayed depolarization-mediated triggering activity by blocking sodium channels, as well as inhibit the release of sarcoplasmic reticulum calcium ions, reducing the occurrence of ventricular arrhythmias in these patients. In a randomized controlled study conducted in 2016 (Kannankeril et al., 2017), it was found that flecainide is more effective than β-receptor blockers in reducing the occurrence of ventricular tachycardia, although the number of patients in the study was small. In case 3, the occurrence of arrhythmia significantly decreased after the addition of flecainide.

For patients with sinus node dysfunction, increasing the rate of the sinus node can reduce the incidence of ventricular tachycardia and syncope. A study has found that using atropine to increase supraventricular heart rate can reduce the incidence of ventricular arrhythmias after exercise on a treadmill (Kannankeril et al., 2020). To avoid symptoms related to long R-R intervals occurring after β-blocker administration, patients can be administered β-receptor blockers under the protection of cardiac pacing therapy to reduce the occurrence of malignant arrhythmias (Writing Committee Members et al., 2021). In this study, cases 2 and 3 exhibited obvious symptoms related to long R-R intervals after receiving β-blockers. After receiving cardiac pacing treatment to increase the ventricular rate, no more malignant arrhythmias occurred, and good results were achieved.

We provide some potential advices on the diagnosis and treatment. 1. For children with syncope, while electroencephalogram were performed to exclude epilepsy, Holter ECG and exercise treadmill tests should be performed as early as possible. 2. When complex arrhythmias are detected by Holter ECG

and exercise treadmill tests, genetic testing should be performed. 3. Timely and sufficient use of non-selective β Receptor blockers can control arrhythmia, avoiding emotional excitement and competitive exercise can significantly improve the prognosis of such pediatric patients. 4. For patients with sinus node dysfunction and tachycardia, adequate β receptor blockers administered under the protection of cardiac pacing can reduce the occurrence of malignant arrhythmias.

5 Study limitation

This is a single-centre study, and more valuable findings are likely to be uncovered in a nationwide, multicentre, large-sample study.

6 Conclusion

Complex arrhythmias associated with *RYR2* gene mutations in children can present with various clinical manifestations. Atrial arrhythmias combined with sinoatrial node dysfunction are commonly observed in younger children, and the combination of pharmacological therapy and cardiac pacing treatment yields favourable treatment outcomes.

Data availability statement

The original contributions presented in the study are included in the article/Supplementary material, further inquiries can be directed to the corresponding author.

Ethics statement

The studies involving humans were approved by Ethics Committee of Hunan Children's Hospital. The studies were conducted in accordance with the local legislation and institutional requirements. Written informed consent for participation in this study was provided by the participants' legal guardians/next of kin. Written informed consent was obtained from the minor(s)' legal guardian/next of kin for the publication of any potentially identifiable images or data included in this article.

Author contributions

YW: Funding acquisition, Writing–original draft, Writing–review and editing. YY: Funding acquisition, Writing–original draft, Writing–review and editing. NX: Formal Analysis, Writing–review and editing. YX: Formal Analysis, Funding acquisition, Investigation, Writing–review and editing. CZ: Writing–review and editing. ZC: Supervision, Writing–review and editing.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Exploring the impact of a KCNH2 missense variant on Long QT syndrome: insights into a novel gender-selective, incomplete penetrance inheritance mode

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Background: Long QT syndrome (LQTS) is an inherited malignant arrhythmia syndrome that poses a risk of sudden death. Variants in the Potassium Voltage-Gated Channel Subfamily H Member 2 (*KCNH2*) gene are known to cause Long QT syndrome through an autosomal dominant inheritance pattern. However, as of now, there have been no reports of any *KCNH2* variant leading to Long QT syndrome exhibiting incomplete penetrance that is influenced by gender.

Methods: Whole-exome sequencing (WES) was conducted on the proband to identify pathogenic variants. Subsequently, Sanger sequencing was employed to validate the identified likely pathogenic variants in all family members.

Results: We analyzed a pedigree spanning three-generations afflicted by Long QT syndrome. WES revealed a novel *KCNH2* missense variant (p.Val630Gly, c.1889 T>G) as the causative factor for the family's phenotype. Within this family, all three male carriers of the *KCNH2* variant carriers exhibited the Long QT syndrome phenotype: one experienced sudden death during sleep, another received an implantable cardioverter defibrillator (ICD), and a younger man displayed a prolonged QTc interval without any instances of syncope or malignant arrhythmia to date. Interestingly, the middle-aged female carrier showed no Long QT Syndrome phenotype. However, her offspring, diagnosed with Turner syndrome (45, X) and also a carrier of this variant, experienced frequent syncope starting at 12 years old and was diagnosed with Long QT syndrome, leading to an ICD implantation when she was 15 years old. These observations suggest that the manifestation of Long QT syndrome associated with this KCNH2 variant exhibits incomplete penetrance influenced by gender within this family, indicating potential protective mechanisms against the syndrome in females affected by this variant.

Conclusion: Our investigation has led to the identification of a novel pathogenic *KCNH2* variant responsible for Long QT syndrome within a familial context characterized by gender-selective, incomplete penetrance. This discovery highlights a unique pathogenic inheritance pattern for the *KCNH2* gene associated with Long QT syndrome, and could potentially shed light on the

distinct penetrance behaviors and patterns of the *KCNH2* gene. This discovery broadens our exploration of the KCNH2 gene in cardiac arrhythmias, highlighting the intricate genetic dynamics behind Long QT syndrome.

KEYWORDS

kcnh2, Long QT syndrome, gender selective, turner syndrome, incomplete penetrance

Introduction

Long QT syndrome (LQTS) is an inherited arrhythmia syndrome, which characterized by a prolonged corrected QT interval on the Electrocardiogram (ECG) without structural heart disease and drug influence (Priori et al., 2013; Schwartz and Ackerman, 2013). LQTS is associated with malignant arrhythmias that can lead to syncope and sudden death (Schwartz, 2021). Most patients with LQTS have pathogenic variants in genes encoding ion channels or their regulating proteins (Wilde et al., 2022). The three most common subtypes of LQTS are distinguished based on characteristic features and genotype-phenotype relationships, which have been widely described (Tester et al., 2005; Kapplinger et al., 2009).

LQT2, accounting for approximately 30% of LQTS cases, exhibits high penetrance (Wilde et al., 2022). Loss-of-function variants in potassium voltage-gated channel subfamily H member 2 (*KCNH2*) gene are responsible for LQTS2 (Wilde et al., 2022). To date, a significant number of pathogenic or likely pathogenic *KCNH2* variants have been reported, with the majority showing no gender-specific differences in morbidity. However, select studies have reported that females may possess a heightened risk of developing morbidity and life-threatening arrhythmias associated with LQTS2 (Mazzanti et al., 2018; Ke et al., 2023).

Turner syndrome is a rare genetic disorder characterized by features such as female hypergonadotropic hypogonadism, dysplasia, infertility, endocrine and a spectrum of endocrine and metabolic syndromes (Gravholt et al., 2019). This condition arises from the complete or partial absence of one X-chromosome, leading to the pathogenesis of Turner syndrome (Barr and Bertram, 1949). Consequently, individuals with Turner syndrome may exhibit features typically associated with "maleness" (Steiner and Saenger, 2022). Additionally, there is an increased risk of cardiovascular diseases in these patients, including hypertension, coronary heart disease, heart failure, and aortic disease (Mortensen et al., 2018).

In this study, we identified a novel pathogenic *KCNH2* variant through whole exome sequencing in a three-generation pedigree. This variant causes LQTS with a novel-gender selective, incomplete penetrance inheritance pattern.

Methods

Participant recruitment

We identified a three-generation pedigree with multiple individuals suffering from LQTS. This family was recruited at Tongji Hospital in 2021, with the Institutional Review Board of Tongji Hospital granting approval for the study. Additionally, we recruited 500 healthy, unrelated Han Chinese individuals' as controls from those undergoing routine health examinations. Informed consent, including consent for portraits, was obtained from all participants. ECGs were collected from all family members. Echocardiography and 24-h Holter monitoring were specifically performed for the proband. Furthermore, program-controlled telemetry for the implantable cardioverter defibrillator (ICD) of the proband was conducted regularly. The QT intervals were measured using the Tangent method, and the Bazett correction formula was applied to calculate the rate-corrected QT (QTc) (Vink et al., 2018). The diagnosis of LQTS was determined based on the Schwartz scoring scale (Priori et al., 2015; Westphal et al., 2020).

Whole exome sequencing

Genomic DNA was extracted from the leukocytes of peripheral blood samples using the QIAamp DNA Mini Kit (Qiagen, Germany). Following extraction, the DNA underwent fragmentation through sonification. The fragmented DNA's tail ends were subsequently repaired, and adapters were ligated using the Agilent SureSelect Human All Exon V6 Kit (Agilent, Santa Clara, CA). These prepared libraries were then amplified and sequenced on the Illumina HiSeq platform (Illumina, California, America).

Bioinformatics analysis

The sequence reads were mapped to the GRCh37 human reference genome using the Burrows–Wheeler Aligner (BWA) (Li and Durbin, 2010). Coverage analysis was performed using BWA, while the Genome Analysis Toolkit (GATK) (McKenna et al., 2010) was used for marking duplicates, sorting BAM files, recalibrating base quality scores, and variant calling. Variants were then annotated using ANNOVAR and reported following the Human Genome Variation Society nomenclature guidelines (http://www.hgvs.org/mutnomen). Their evaluation was based on the American College of Medical Genetics (ACMG) standards (Richards et al., 2015). Given that LQTS is a rare inherited condition, variants displaying minor allele frequencies greater than 1% were excluded, as they were deemed unlikely to be deleterious. Population allele frequency data for variants were obtained from databases such as the 1000 Genomes Project (http://browser.1000genomes.org/), the Exome Aggregation Consortium (ExAC) (http://exac.broadinstitute.org/), the Exome Sequencing Project (ESP) (https://evs.gs.washington.edu/EVS/), and the Genome Aggregation Database (gnomAD) (https://gnomad.broadinstitute.org/). Variants also underwent filtering against our exome database of 500 healthy Han Chinese controls to further refine the analysis. The pathogenicity of all variants was assessed through databases like the Human Gene Mutation Database (HGMD) (http://www.hgmd.

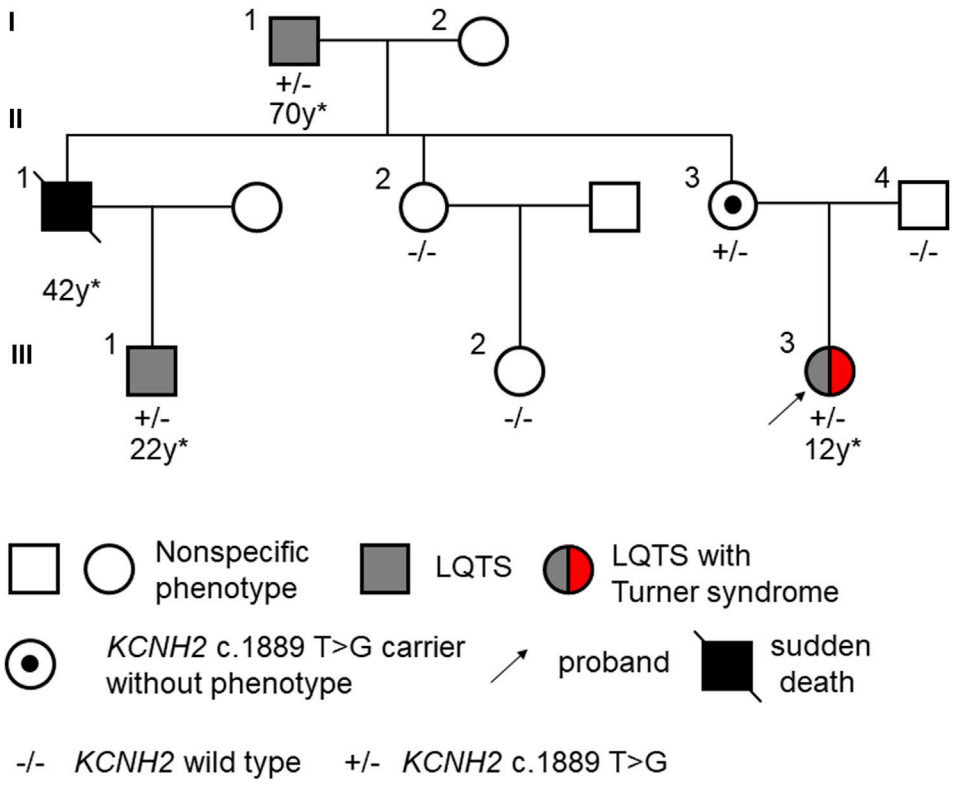

FIGURE 1
Pedigree of LQTS displaying gender-selective incomplete penetrance. The asterix (*) indicates the age at the first onset of a malignant event or diagnosis of LQTS.

cf.ac.uk/ac/index.php) and ClinVar database (http://www.ncbi.nlm.nih.gov/clinvar). Additionally, all missense variants were evaluated based on their effect on the function of the coding protein using SIFT, PolyPhen2 HDIV, PolyPhen2 HVAR, LRT, MutationTaster, MutationAssessor, FATHMM, PROVEAN, MetaSVM, MetaLR, VEST, M-CAP, CADD, GERP++, DANN, fathmm-MKL, Eigen, GenoCanyon, fitCons, PhyloP and SiPhy scores. (Chen et al., 2020). Protein structure of mutation was predicted using Pymol. Briefly, the pdb file (A0PJW5) of KCNH2 was downloaded from Alphafold Protein Structure Database (https://alphafold.ebi.ac.uk/) and mutagenesis as well as plot was generated with Pymol.

Sanger sequencing validation

Sanger sequencing was conducted using Applied Biosystems 3500xl capillary sequencer (Applied Biosystems, Foster City, USA) to validate all variants identified as likely pathogenic and associated with the phenotypes observed in this pedigree. Furthermore, each variant confirmed as likely pathogenic was subjected to Sanger sequencing in a group of 500 healthy controls for further validation.

Statistical analysis

Statistical analysis was performed using the SPSS software, version 26.0. Group differences were assessed with the Pearson chi-square test, and a p-value <0.05 was considered statistically significant.

Results

Case presentation

In our study, we identified a three-generation Chinese family presenting multiple cases of LQTS exhibiting gender-selective incomplete penetrance (Figure 1). The proband (III-3), a 15-year-old female with short stature and skeletal dysplasia, has a medical history of Turner syndrome and diabetes (Figures 2A,B). She has been on regular estrogen and metformin therapy. The proband reported symptoms of palpitations and amaurosis over 3 years, accompanied by four episodes of syncope—two occurring during the day with cold sweats and two at night, accompanied by urinary incontinence. Upon admission, a 12-lead electrocardiogram (ECG) revealed a prolonged QTc interval of 485 m for the proband. Further, a 24-h Holter monitor recorded a maximum QTc interval of 518 m, with alterations in the T wave observed across multiple leads (Figure 2C). Echocardiography examination showed no abnormalities, with a left ventricular dimension of 40 mm and an ejection fraction of 61%. Therefore, the diagnosis of this patient was considered as LQTS.

The proband's grandfather (I-1) began to experience syncope at the age of 70 and received an implantable cardioverter defibrillator

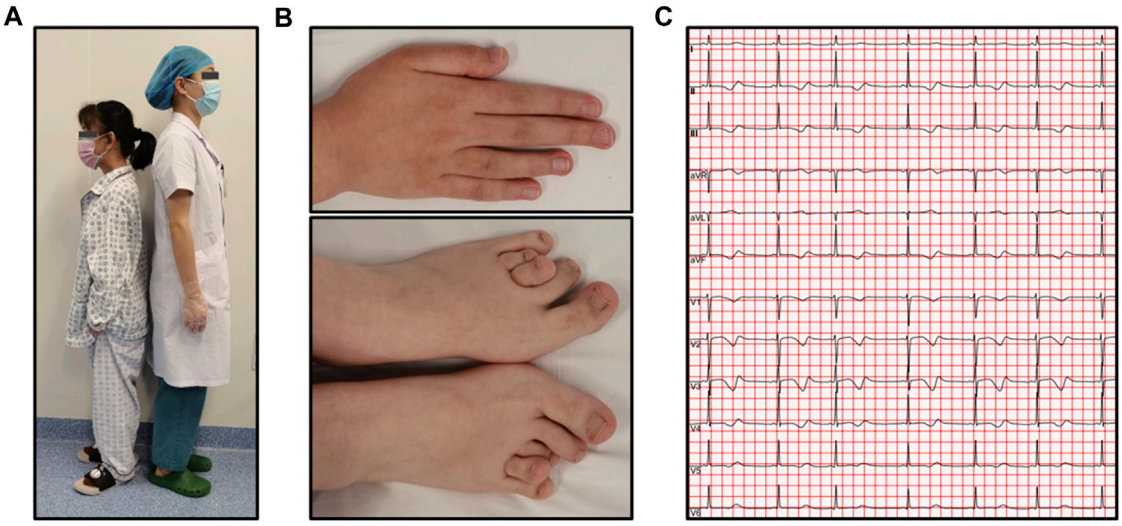

FIGURE 2
Phenotypes of the proband affected by Turner syndrome. **(A)** Short stature compared to a reference female model, whose height is 160 cm on the right side of the panel; **(B)** Skeletal dysplasia caused by Turner syndrome; **(C)** An ECG demonstrating a prolonged QTc interval with T-wave alterations in multiple leads.

TABLE 1 Clinical characteristics of all members of this pedigree.

Family member	Sex	Age (y)	QT/ QTc (ms)	Clinical symptoms	Schwartz-score	Genotype
I-1	M	70[a]	450/472	syncope, malignant arrhythmia, ICD implanted	6	*KCHN2* c.1889 T>G
I-2	F	70	342/417	no	1	WT
II-1	M	42[a]	NA	syncope, SCD	2	NA
II-2	F	47	338/409	no	1	WT
II-3	F	45	408/429	no	1	*KCHN2* c.1889 T>G
II-4	F	46	346/442	no	1	WT
III-1	M	22[a]	424/498	no (notched T waves)	5	*KCHN2* c.1889 T>G
III-2	F	19	388/407	no	1	WT
III-3	F	12[a]	452/485	syncope, malignant arrhythmia, ICD implanted	7	*KCHN2* c.1889 T>G
						Turner syndrome (45, X)

[a]Indicates the age at the first onset of malignant event or diagnosis of LQTS; F, female; ICD, implantable cardioverter defibrillator; M, male; NA, not available; SCD, sudden cardiac death; WT, wild type.

(ICD) 2 years later. The proband's uncle (II-1) suffered multiple syncope episodes starting at the age of 42, but did not undergo any specific treatment. Tragically, he passed away in his sleep that same year. Furthermore, this man's son (III-1), who had never shown symptoms of palpitation, amaurosis, or syncope, was discovered to have a prolonged QTc interval of 498 m with notched T waves at the age of 22 (Supplementary Figure S1). Meanwhile, the proband's grandmother (I-2), aunt (II-2), mother (II-3), father (II-4) and cousin sister (III-2) showed no specific symptoms, displaying normal QTc intervals in their 12-lead ECGs. No history of specific cardiology diseases was found in the paternal lineage of the proband. The clinical characteristics of the family members are listed in Table 1.

Genetic screening

Whole exome sequencing was conducted on the proband, revealing a chromosomal pattern of 45, X, which is consistent with the diagnosis of Turner syndrome. Moreover, a novel

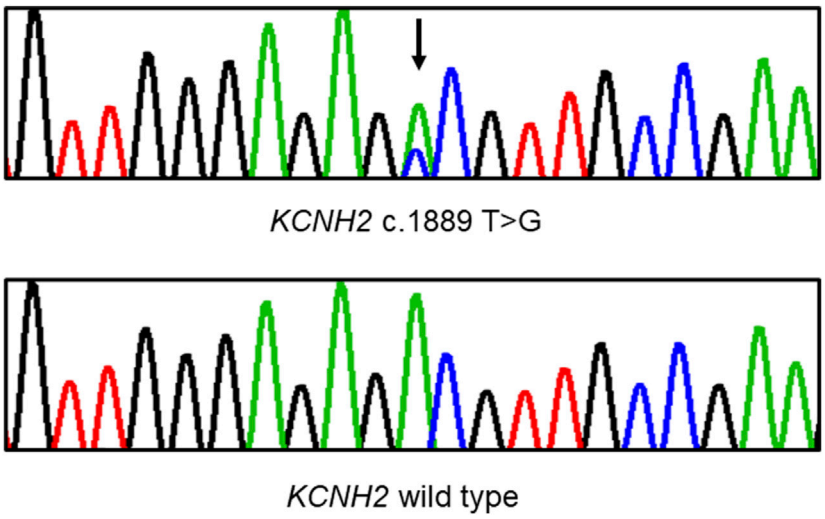

FIGURE 3
Sanger sequencing demonstrates a heterozygous missense variant in the *KCNH2* gene within the pedigree. A black arrow indicates the position of the mutation.

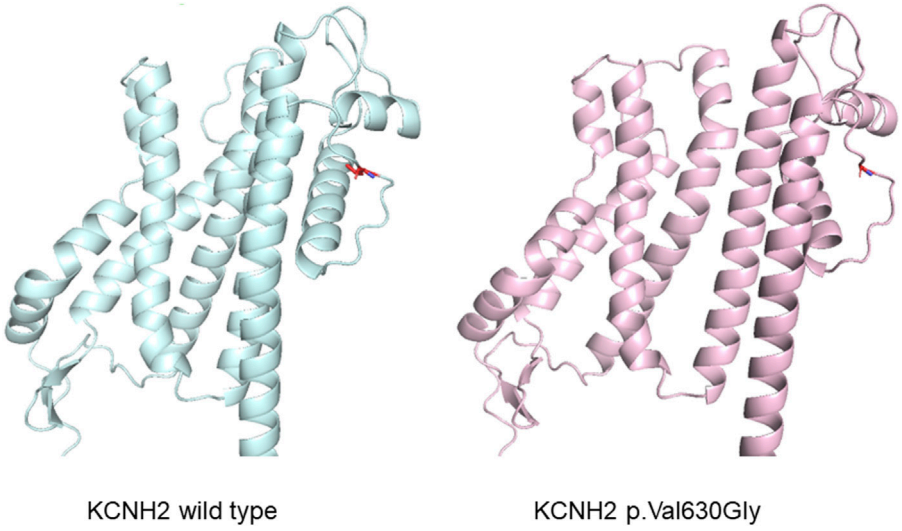

FIGURE 4
Protein structure prediction illustrating an alteration in the structure of the *KCNH2* protein caused by the variant.

missense variant in the *KCNH2* gene (p.Val630Gly, c.1889 T>G) was identified and deemed responsible for the LQTS phenotype observed in this family (Figure 3). This variant has not previously been reported in the 1000 Genomes Project, ExAC, gnomAD, ESP, HGMD, or ClinVar databases, nor was it found in the 500 healthy controls. The missense constraint metrics of *KCNH2* from gnomAD database (missense Z score = 2.48) is quite high, indicating that *KCNH2* is intolerance to missense variants. Additionally, this variant is predicted to be deleterious by *in silico* algorithm (Supplementary Table S1). Protein structure predictions suggest that this variant might alter the structure of KCNH2 protein (Figure 4). Sanger sequencing confirmed the presence of this variant in all relevant family members. The proband's grandfather (I-1), uncle (II-1), mother (II-3), and cousin brother (III-1) were identified as carriers of the *KCNH2* variant. In contrast, the genotype of the proband's grandmother (I-2), aunt (II-2), father (II-4) and cousin sister (III-2) were validated as wild type (Table 1; Figure 3). Additionally, whole exome sequencing performed on the proband's grandfather (I-1), mother (II-3), and cousin brother (III-1) revealed no other pathogenic variants in *KCHN2* or any other genes known to be associated with LQTS. The *KCNH2* gene is recognized as a known causative gene of LQTS. The identified variant co-segregated with the LQTS phenotype in the proband with Turner syndrome and all male family members, except for the proband's mother. Interestingly, no LQTS phenotypes were observed in the only 'confirmed' female carrier of this variant.

These findings suggest the existence of protective mechanisms that shields females from LQTS associated with this variant, indicating a gender-selective incomplete penetrance in this pedigree.

Treatment and follow-up

The ICD was implanted in the proband at the age of 15. Additionally, 10 mg propranolol has been taken for three times daily. In the seventh month after ICD implantation, 52 occurrences of ventricular flutter and 40 episodes of ventricular fibrillation were documented, resulting in five defibrillation events. She was admitted to hospital and diagnosed as electrolyte disturbance (hypokalemia) caused by diarrhea. She was treated with potassium chloride solution, suitable fluid supplement. The ventricular arrythmia attenuated and she recovered promptly. She kept on regular taking propranolol to control the ventricular arrythmia. After that, several occurrences of ventricular flutter, but no defibrillation events again in the first-year follow-up.

Discussion

This study discovered a novel missense variant in *KCNH2* gene (p.Val630Gly, c.1889T>G) through whole exome sequencing in a three-generation pedigree affected by LQTS. Among the family members carrying this variant, all male carriers and one proband with Turner syndrome exhibited LQTS phenotypes, while the female carrier displayed no abnormalities. Therefore, a novel gender-selective, incomplete penetrance inheritance mode was observed in this pedigree.

The *KCNH2* gene located on chromosome seven which encodes the α-subunit of the voltage-gated K^+ channel Kv11.1, which comprises six α-helical transmembrane segments (S1-S6). The segments from S1 to S4 constitute the voltage sensor domain, and S5 to S6 form the pore domain (Sanguinetti et al., 1995; Trudeau et al., 1995; Wang and MacKinnon, 2017). This gene is responsible for the rapid delayed rectifier current I_{Kr} (Wilde et al., 2022). Loss-of-function variants in *KCNH2* can decrease the amplitude of I_{Kr}, leading to a prolonged ventricular action potential duration (Ono et al., 2020). Previous studies have shown that more than 90% of variants in *KCNH2* disrupt the intracellular transport or trafficking of the Kv11.1 channel to the cell surface membrane (Ono et al., 2020). In our study, referencing the SMART database (http://smart.embl-heidelberg.de/), the identified *KCNH2* variant (p.Val630Gly, c. 1889 T>G) is associated with a transmembrane region in S5-S6, potentially affecting the spatial structure and functionality of the pore domain.

Previous studies have highlighted gender differences in the manifestation of LQTS. Notably, a comprehensive follow-up study including 1,710 LQTS patients indicated that females are at a higher risk of experiencing life-threatening arrhythmic events (Mazzanti et al., 2018). Additionally, a case report outlined a pedigree with a putative *KCNH2* mutation that exclusively affected females (Ke et al., 2023). Our research similarly identified a family with LQTS carrying a *KCNH2* variant, which exhibited gender-selective incomplete penetrance. Contrary to expectations, female carriers of the *KCNH2* variant did not exhibit LQTS phenotypes; instead, male carriers and those with Turner syndrome (45, X) demonstrated typical LQTS phenotypes. Previous studies suggested that this gender-selective pattern might be influenced by androgen levels, considering the differential impact of sex hormones on potassium currents (Grouthier et al., 2021; Ke et al., 2023). However, our findings challenge this theory, as the Turner syndrome patient, despite undergoing regular estrogen therapy for years, began experiencing arrhythmias at a younger age compared to all male patients in the study. This observation leads us to speculate that the presence of complete female sex chromosomes (XX) could offer protective mechanisms against the development of LQTS in carriers of this variant. Supporting this, published data indicate that male LQTS2 patients with mutations in the pore region are at a higher risk of cardiac event than those with non-pore mutations (Platonov et al., 2018). This observation may partially explain the gender-selective incomplete penetrance inheritance pattern observed in this family's pedigree.

A previous study demonstrated that most LQTS2 patients start showing symptoms around puberty (Wilde et al., 2022). In this family we studied, male patients began experiencing arrhythmias at an older age. However, we observed a trend where the onset of malignant arrhythmias or sudden cardiac death occurred progressively earlier with each generation. Despite this, the proband (III-3), who has Turner syndrome, started suffering from malignant arrhythmias at a significantly younger age than other family members, displaying symptoms more frequently. Prior studies have shown that Turner syndrome patients are at a higher risk of cardiovascular diseases, including hypertension, congenital heart disease, coronary heart disease, and aortic disease (De Groote et al., 2017; Klaskova et al., 2017; Mortensen et al., 2018). This suggests that Turner syndrome may cause latent cardiac structural changes, potentially hastening the onset of LQTS in the proband or affected individuals.

Limitation

This study identified a LQTS pedigree exhibiting a gender-selective incomplete penetrance inheritance pattern. However, a limitation of our study is the absence of a second female carrier of the variant within this pedigree. Future research should include functional studies on this variant to deepen our understanding.

Conclusion

This study identified a novel missense variant in the *KCNH2* gene, linked to LQTS in a Chinese Han family/pedigree, characterized by gender-selective incomplete penetrance. This suggests the existence of protective mechanisms that may shield female carriers from the onset of LQTS. The discovery of this variant introduces a novel pathogenic inheritance mode for *KCNH2* associated LQTS, offering valuable insights into the gene's penetrance mechanisms. This contribution enriches our understanding of the genetic factors influencing LQTS and highlights the importance of considering gender in genetic studies of inherited arrhythmias.

Data availability statement

The data presented in the study are deposited in the following depository: https://pan.baidu.com/s/1-SEpn19b49lPxVfkZr5uyw acess code: 5dpt

Ethics statement

The studies involving humans were approved by the Institutional Review Board of Tongji Hospital. The studies were conducted in accordance with the local legislation and institutional requirements. Written informed consent for participation in this study was provided by the participants' legal guardians/next of kin. Written informed consent was obtained from the individual(s), and minor(s)' legal guardian/next of kin, for the publication of any potentially identifiable images or data included in this article.

Author contributions

PC: Funding acquisition, Investigation, Resources, Data curation, Formal Analysis, Methodology, Project administration, Software, Writing–original draft. ZZ: Data curation, Investigation, Methodology, Software, Writing–review and editing. HW: Investigation, Writing–review and editing, Project administration. LW: Investigation, Writing–review and editing, Conceptualization, Funding acquisition, Resources, Supervision.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fgene.2024.1409459/full#supplementary-material

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Genetic testing and human leukocyte antigen in patients with hypertrophic cardiomyopathy and connective tissue diseases

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Hypertrophic cardiomyopathy (HCM) is caused by myocardial hypertrophy, often due to mutations in cardiac sarcomere protein genes such as beta-myosin heavy chain (MYH7) and myosin-binding protein C (MYBPC3). However, a significant proportion of HCM cases lack identified genetic mutations, and genotype-phenotype correlations remain unclear. Concurrently, potential associations between HCM and human leukocyte antigen (HLA) types, as well as connective tissue diseases, have been proposed. In this single-center study, we aimed to investigate the genetic and HLA profiles of patients with obstructive hypertrophic cardiomyopathy (HOCM) and connective tissue diseases, particularly focusing on the prevalence of genetic variants and HLA types. We conducted a detailed analysis of five patients with HOCM and connective tissue diseases and sarcoidosis, identifying rare variants in causative genes for HCM in two cases and observing specific HLA types that were relatively common. Notably, 15% of all HOCM cases presented with connective tissue diseases, mainly rheumatoid arthritis. These findings underscore the complexity of HCM etiology and suggest potential implications for both diagnostic strategies and therapeutic approaches in patients with concomitant inflammatory conditions.

KEYWORDS

hypertrophic cardiomyopathy, genetic mutations, human leukocyte antigen, rheumatoid arthritis, connective tissue diseases

1 Introduction

Genetic testing is central to understanding the pathogenesis of hypertrophic cardiomyopathy (HCM), with more than 2000 mutations identified in over 11 sarcomere genes (Authors/Task Force members et al., 2014; Kitaoka et al., 2021). Mutations in the genes encoding the beta-myosin heavy chain (MYH7) and myosin-binding protein C (MYBPC3) account for the majority mutations. However, the detection rate of known pathogenic mutations in HCM patients without a family history or in older patients is only 30%–40%, highlighting significant gaps in our understanding (Bonaventura et al., 2021). Furthermore, genotype-phenotype correlations remain inconsistent, underscoring the need for further investigation (Marian and Braunwald, 2017; Maron, 2018). There is emerging evidence suggesting an association between obstructive hypertrophic cardiomyopathy (HOCM) and human leukocyte antigen (HLA), indicating potential links between immune responses and HCM. HLA genes encode

major histocompatibility complex (MHC) proteins that play a crucial role in immune responses and have been implicated in various diseases, including autoimmune and chronic inflammatory conditions (Choi et al., 2021). There are many types of HLA antigens, of which HLA-A, HLA-B, and HLA-DRB1 are highly polymorphic (Tokunaga, 2014). The involvement of HLA-DR4 and HLA-DR1 in connective tissue diseases, particularly rheumatoid arthritis (RA), has been previously reported (Roudier, 2000). Several studies have demonstrated the involvement of HLA-DR4 in patients with HOCM combined with RA and mixed connective tissue diseases (Nakamura et al., 2008; Dawood et al., 2018). Despite these insights, there is limited research exploring the involvement of HLA and concomitant inflammatory conditions in HCM. Previous studies have hinted at the presence of "inflammatory hypertrophic cardiomyopathy", suggesting a novel avenue for investigation. However, the precise role of HLA and its implications in HCM remain largely unexplored. Therefore, this study aims to address this gap in the literature by investigating the role of HLA and performing genetic testing in patients with HCM and connective tissue diseases.

2 Materials and methods

On 14 February 2024, we investigated the prevalence of connective tissue diseases in 61 patients with HOCM who had outpatient appointments at the University of Tsukuba Hospital, Tsukuba, Japan. HOCM was defined as follows: i) maximum left ventricular wall thickness ≥15 mm; ii) cardiac magnetic resonance imaging (MRI) or endomyocardial biopsy ruling out secondary cardiomyopathy; and iii) left ventricular pressure gradient ≥30 mmHg at rest or during physiological provocation (Kitaoka et al., 2021). A rheumatologist made a definitive diagnosis of connective tissue diseases.

2.1 HLA analysis

Venous blood (5 mL) was collected in tubes containing ethylenediaminetetraacetic acid (EDTA) and used either fresh or frozen. The samples were sent to an external institution (LSI Medience Co., Tokyo, Japan) for human leukocyte antigen (HLA) genotyping. The genotyping was conducted using a reverse sequence-specific oligonucleotide probe protocol with a Luminex 100 × MAP flow cytometry dual-laser system, following the polymerase chain reaction (PCR)-Luminex method (Itoh et al., 2005; Azuma, 2021). Sets of polystyrene color-coded microbeads (Multi-Analyte Microsphere Carboxylated; Luminex, Austin, TX, USA), approximately 5.5 μm in diameter, were labeled with oligonucleotide annealing probes as part of the xMAP technology. Each set of beads was color coded by the manufacturer using a specific ratio of two different fluorescent dyes (red and infrared) embedded in the beads. Up to 100 different fluorescently labeled microbeads were coded and identified using the Luminex 100 flow cytometer by adjusting the concentration of each fluorochrome. One of the dual lasers on the Luminex 100 excited the internal dyes within the beads, identifying the exact code number of the fluorescent microbeads by determining the preset ratio of the internalized dyes. Target DNA was PCR-amplified using 5′-biotin-labeled primers highly specific to certain sequences of HLA genes. After denaturation at 95°C, amplified DNA hybridized to complementary DNA probes coupled to microbeads. The hybridized PCR product on the oligobeads was labeled with streptavidin-phycoerythrin. The Luminex apparatus was used to identify the fluorescence intensity of phycoerythrin on each coded oligobead hybridized with the biotin-labeled PCR product. Genosearch typing software assisted in determining the HLA genotype (alleles) of the sample DNA. HLA-DRB1*04 was serologically identified as HLA-DR4, and HLA-DRB1*01 was identified as HLA-DR1.

2.2 Genetic testing

The genetic analysis was reviewed and approved by the University of Tsukuba Clinical Research Ethics Review Committee (approval number: R02-300). After obtaining written informed consent from the patients, next-generation sequencing was conducted using the Ion Proton System (Thermo Scientific, Waltham, Massachusetts, USA) with the Ion AmpliSeq™ Cardiovascular Research Panel and the Ion AmpliSeq™ Library Kit 2.0. Primary processing of reads utilized Ion Proton Software (Thermo Fisher Scientific). Subsequent steps involved alignment with the human reference genome (GRCh38–hg19), base calling, trimming, and filtering of poor signal reads using the Ion Torrent Software Suite (ISS) version 5.4.0. The VCF file was uploaded and annotated using the wANNOVAR software. Variants classified as pathogenic or likely pathogenic for HCM according to ClinVar were considered causative genes and were confirmed by direct sequencing.

2.3 Endomyocardial biopsy

An endomyocardial biopsy was performed to exclude secondary cardiomyopathy. Myocardial specimens were collected from the right ventricular septum via the internal jugular venous approach using a 7-Fr bioptome (Cordis; Johnson and Johnson Co., New Brunswick, NJ, USA). At least three specimens were procured, with one undergoing electron microscopic evaluation and the others subjected to light microscopic examination. Biopsy samples for light microscopy analysis were transferred from the bioptome to a fixative (10% neutral buffered formalin), embedded in paraffin, and sectioned into 3-μm-thick sections. These sections were sequentially stained with hematoxylin and eosin, Masson's trichrome, and Congo red (Yamamoto et al., 2023).

3 Results

Among the 61 patients with HOCM, 9 (15%) had connective tissue disease (5 with RA, 1 with systemic lupus erythematosus, 1 with scleroderma, 1 with Takayasu arteritis, and 1 with sarcoidosis). HLA analysis and genetic testing were conducted in five of these patients (Table 1). Only two of the five patients with a family history had rare variants in HCM causative genes (case 1 and 2). In HLA analysis, case 2 had HLA-DR4, case 4 and 5 had HLA-DR1, case 1, 3 and 5 had HLA-A26, case 2, 4 and 5 had HLA-B7, and case 2, 3 and 5 had HLA-DR9, respectively.

Two cases (case 1 and 2) are described in detail.

TABLE 1 List of patients with obstructive hypertrophic cardiomyopathy and chronic inflammatory diseases.

Case no.	Age, sex	Family history of HCM	Maximum LV wall thickness (mm)	LV-PG (mmHg)	Types of chronic inflammatory diseases (disease duration)	Medication	CRP (mg/dL)	Pathological findings	HLA types	Genetic testing
1	44, F	Mother Grandmother	19	126	RA (10 years)	MTX ETN	1.19	NA	A*11, A*26 B*15, B*67 DRB1*14, DRB1*15	MYH7(NM_000257.4): c.1870T>A: p.Tyr624Asn
2	59, F	Mother	19	122	RA (3 years)	MTX	0.26	Hypertrophy and disarray of cardiomyocytes, interstitial fibrosis	A*24, -B*07, B*40 DRB1*04, DRB1*09	MYL2(NM_000432.4): c.173A>G: p.Arg58Gln
3	61, F	None	22	62	RA (29 years)	PSL MTX TAC	0.09	NA	A*26, A*31 B*40, B*48 DRB1*09, DRB1*14	Under analysis
4	69, F	None	15	72	RA (9 years) PM (1 year)	PSL	2.10	Nuclei size differences in cardiomyocytes, interstitial fibrosis	A*02, A*11 B*07, B*67 DRB1*01, DRB1*14	No pathogenic variants
5	68, F	None	17	69	Sarcoidosis	PSL	0.85	Interstitial fibrosis	A*24, A*26 B*07, B*15 DRB1*01, DRB1*09	No pathogenic variants

CRP = C-reactive protein; ETN = etanercept; F = female; HCM = hypertrophic cardiomyopathy; HLA = human leukocyte antigen; LV = left ventricular; MTX = methotrexate; MYH7 = ; NA = not available; PG = pressure gradient; PM = polymyositis; PSL = prednisolone; RA = rheumatoid arthritis; TAC = tacrolimus.

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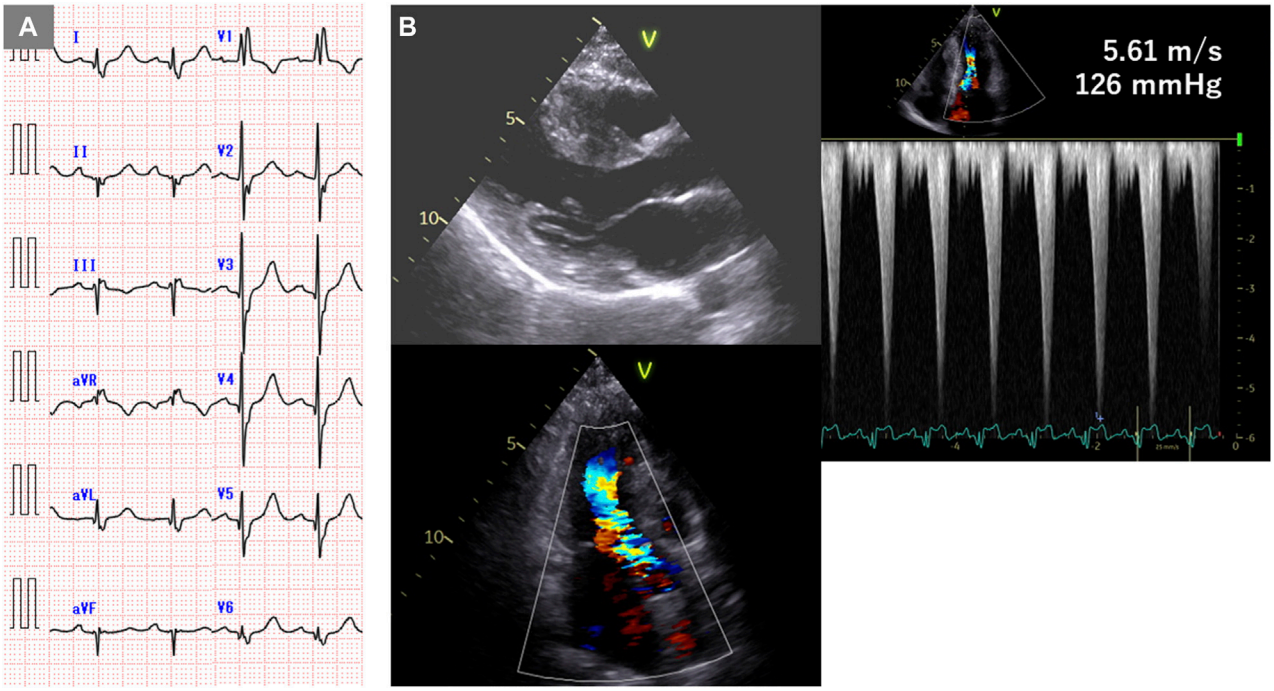

FIGURE 1
Electrocardiogram and echocardiogram of case 1. **(A)** Electrocardiogram showing complete right bundle branch block and left anterior hemiblock. **(B)** Echocardiogram showing mid-septal wall thickness of 19 mm and resting pressure gradient of 126 mmHg.

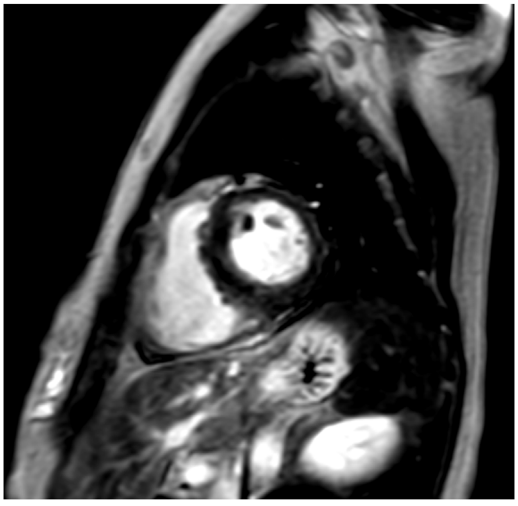

FIGURE 2
Cardiac magnetic resonance imaging (MRI) of case 1. Cardiac MRI showing no late gadolinium enhancement (LGE).

3.1 Case 1

The patient, a 44-year-old woman, had a familial history of sudden death from HCM (Ohsuzu et al., 1997). Electrocardiography revealed a complete right bundle branch block and left anterior hemiblock (Figure 1A). The mid-septum wall thickness was 19 mm, with a resting pressure gradient of 126 mmHg from the mid-ventricle to the outflow tract (Figure 1B). Diagnosed with RA at 34 years, the patient was treated with methotrexate and etanercept. Serum CRP levels were elevated at 1.19 mg/dL. Cardiac magnetic resonance imaging (MRI) showed no late gadolinium enhancement (LGE) (Figure 2), and no endomyocardial biopsy was performed. HLA-DRB1*14 and HLA-DRB1*15 were identified. Previous genetic testing revealed a missense variant (c.1870T>A, p. Y624N) in the MYH7 gene (NM_000257.4). This variant has been reported in an HCM cohort, and is not present in population databases. Therefore, this is predicted to be a pathogenic variant causative for HCM, but has been reported as VUS in ClinVar.

3.2 Case 2

The patient, a 59-year-old woman with a family history of cardiac hypertrophy, exhibited a grade I atrioventricular block, left axis deviation, and ST-T abnormality on electrocardiography (Figure 3A). The mid-septum wall thickness measured 19 mm, with a resting pressure gradient of 122 mmHg observed in the left ventricular outflow tract (Figure 3B). Diagnosed with RA at 56 years old, the patient underwent methotrexate treatment, while her serum CRP level was slightly elevated at 0.26 mg/dL. Cardiac MRI revealed LGE in the septum of the right ventricular junction (Figure 4). Pathological examination revealed hypertrophy, myocardial cell disarray, and interstitial fibrosis (Figure 5), with no observed inflammatory cell infiltration. HLA-DRB1 types were determined as HLA-DRB1*04 and HLA-DRB1*09, while genetic testing revealed a missense variant (c.173G>A, p. R58Q) of the myosin light chain 2 (MYL2) gene (NM_000432.4), which has been classified as pathogenic for HCM.

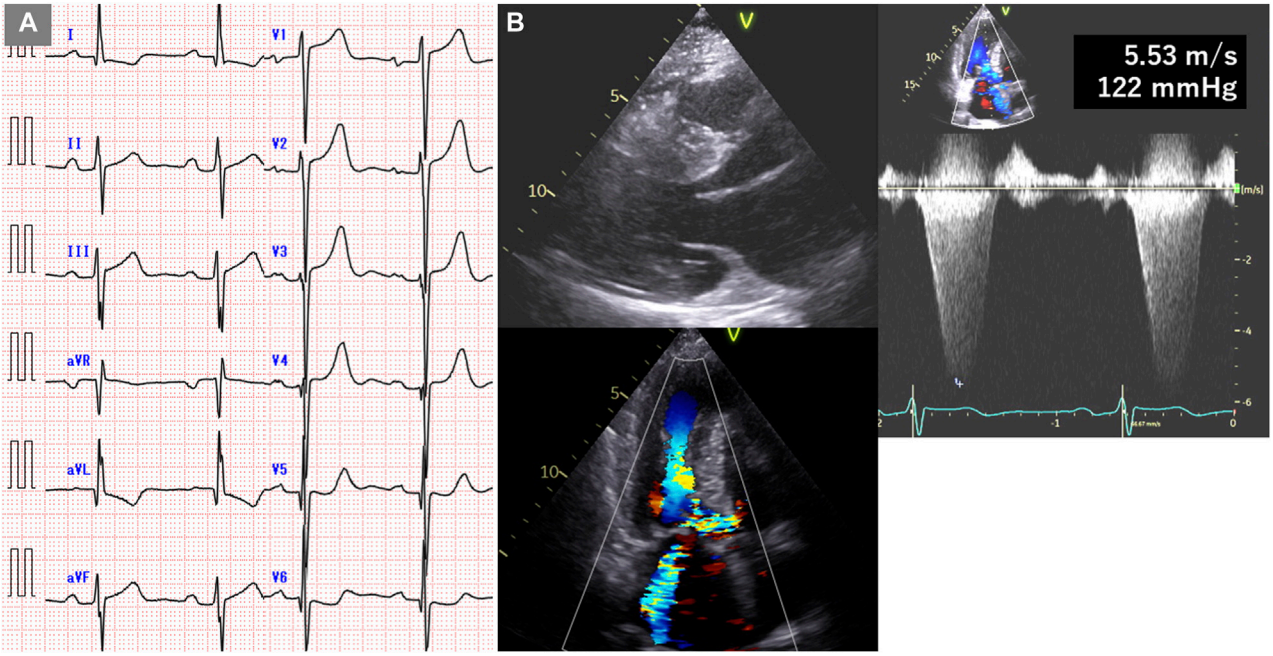

FIGURE 3
Electrocardiogram and echocardiogram of case 2. **(A)** Electrocardiogram showing grade I atrioventricular block, left axis deviation, and ST-T abnormality. **(B)** Echocardiogram showing mid-septal wall thickness of 19 mm and resting pressure gradient of 122 mmHg.

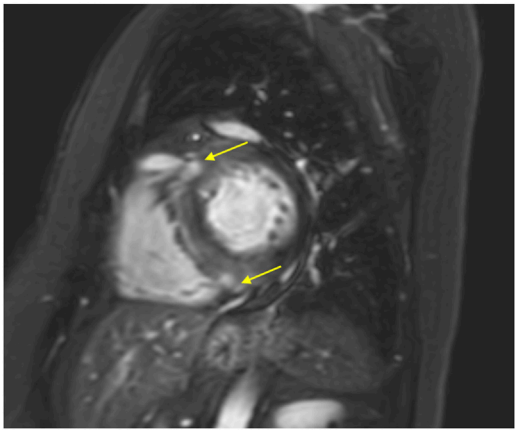

FIGURE 4
Cardiac MRI of case 2. Cardiac magnetic MRI showing LGE in the septum at the right ventricular junction (yellow arrows).

4 Discussion

A previous study reported the prevalence of RA in Japan to be 0.6%–1.0% (Yamanaka et al., 2014). In this study, out of 61 patients with HOCM, 9 (15%) had connective tissue disease, and five (8%) of them had RA. These results suggest a close relationship between HOCM and chronic inflammation. HLA is directly involved in antigen presentation, self- and non-self-identification. The HLA-class I molecules, HLA-A, HLA-B, and HLA-C, are expressed on the cell membranes of all nucleated cells and platelets. They identify non-self-endogenous peptides and stimulate $CD8^+$ killer T cells. HLA class II molecules, HLA-DR, HLA-DQ, and HLA-DP, are expressed on the membranes of antigen-presenting cells such as monocytes, macrophages, dendritic cells, and B cells, and identify exogenous peptides that activate $CD4^+$ helper T cells (Ogawa, 2016a).

Several studies have reported the presence of HLA-DR4 in patients with HOCM and connective tissue diseases. The involvement of HLA-DR4 and HLA-DR1 in RA has been previously reported. In this study, one of the five patients had HLA-DR4 (case 2), and two had HLA-DR1 (cases four and 5). HLA-DRB1, a major risk allele for RA, stimulates $CD4^+$ helper T cells, which enhances the production of interleukin-17 and interferon-γ, leading to chronic inflammation (Eid et al., 2009). No significant inflammatory cell infiltration was observed in the myocardial biopsy samples analyzed in this study. This is probably because myocardial hypertrophy is induced by chemokines and cytokines due to systemic inflammation rather than by inflammatory cell infiltration into the myocardium itself. In RA, transforming growth factor β (TGF-β) is secreted from fibroblast-like synoviocyte during a series of immune and inflammatory processes (Mclnnes and Schett, 2011). Because TGF-β stimulates cardiac microvascular endothelial cells and is involved in their conversion from endothelial to mesenchymal cells and migration into the myocardium, TGF-β signaling is a crucial mechanism for increases fibrosis in HCM. Treatment of HCM mice with TGF-β-neutralizing antibodies markedly reduces the expression of periostin, which promotes differentiation into fibroblasts and improves cardiac hypertrophy (Teekakirikul et al., 2010). Moreover, JAK/STAT and MAPK pathways, which are activated in RAs and are important for disease progression, also play a major role in the progression of cardiac hypertrophy. The close

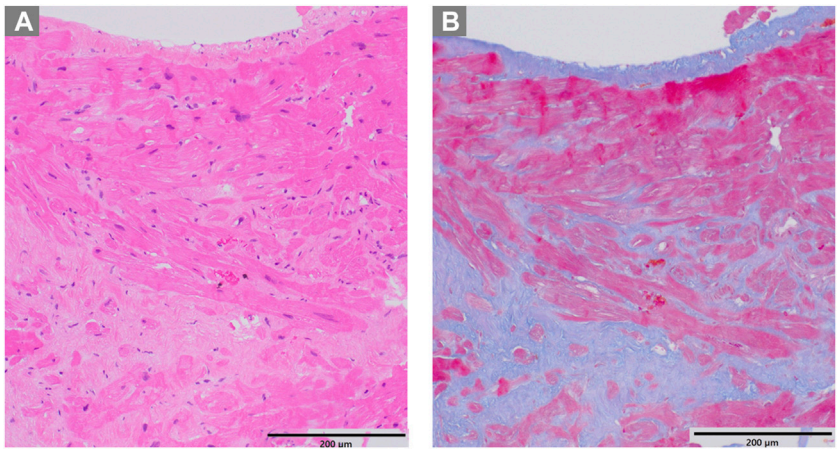

FIGURE 5
Pathological examination of case 2. **(A)** Hematoxylin-eosin staining revealing hypertrophy and disarray of myocardial cells **(B)** Masson trichrome staining showing interstitial fibrosis.

involvement of common pathways in disease progression may be one of the mechanisms by which HCM occurs in patients with RA.

In this study, we found that three of the five patients had HLA-A26, HLA-B7, HLA-DR9, and/or HLA-DR14, suggesting that HOCM may be associated with some HLA types, although the number of cases was small. Further investigation is required to confirm these findings.

Patients in this study received treatment for connective tissue diseases using prednisolone or methotrexate. While steroids are associated with the development of cardiovascular disease, anti-rheumatic drugs like methotrexate, tumor necrosis factor (TNF) inhibitors, and IL-6 inhibitors have been reported to mitigate this risk (Crowson et al., 2013; Giachi et al., 2022). Controlling inflammation may aid in suppressing myocardial hypertrophy and fibrosis in patients with HCM complicated by connective tissue diseases. In addition, T cells and TGF-β could potentially emerge as future therapeutic targets. However, anti-inflammatory treatment mechanisms are intricate, necessitating consideration of cytokine and chemokine interaction networks, rather than individual pro-inflammatory factors.

This study had several limitations. Firstly, it was a small, single-center study, and detailed genetic testing is ongoing; therefore, it remains unclear whether known genetic mutations are implicated in patients with HCM and connective tissue diseases. Secondly, due to racial and regional variations in HLA phenotypes, the results of this study cannot be generalized worldwide. Thirdly, the highly polymorphic nature of the HLA gene necessitates clarification of the odds ratio to establish its association with HCM (Ogawa, 2016b). Furthermore, a detailed nucleotide sequence investigation of HLA was not conducted, precluding determination of whether specific HLA alleles or antigens contribute to HCM or other connective tissue diseases. Lastly, the deposition of amyloid A protein in the myocardium due to chronic inflammation was not investigated. While our study provides valuable insights into the association between HCM and concomitant inflammatory diseases and proposes the existence of a clinical condition called "inflammatory HCM", it is important to acknowledge certain limitations that may affect the interpretation and generalizability of our findings. Further discussion regarding the potential impact of these limitations on the reliability and applicability of our results could provide with a clearer understanding of the study scope and implications.

To address the limitations of our study and further elucidate the relationship between HCM and HLA, future research endeavors could focus on conducting larger, multicenter studies involving diverse racial and regional populations. In addition, detailed investigations into the deposition of amyloid A protein in the myocardium and comprehensive nucleotide sequence analyses of HLA genes could provide valuable insights into the underlying mechanisms linking chronic inflammation to HCM. If the association between HCM and connective tissue diseases is confirmed, it is expected to advance not only diagnostic and preventive medicine but also the development of novel immune system-targeted treatments.

Data availability statement

The datasets presented in this article are not readily available because they contain personal genetic information and have not been approved in our ethical review for inclusion in publicly available databases. Requests to access the datasets should be directed to the correspondence (d.hiraya@md.tsukuba.ac.jp).

Ethics statement

The studies involving humans were approved by the institutional review board of the University of Tsukuba. The studies were conducted in accordance with the local legislation

and institutional requirements. The participants provided their written informed consent to participate in this study.

Author contributions

DH: Conceptualization, Data curation, Funding acquisition, Investigation, Methodology, Writing–original draft. NM: Data curation, Investigation, Writing–review and editing. MI: Conceptualization, Validation, Writing–review and editing. DX: Supervision, Validation, Writing–review and editing. TI: Supervision, Validation, Writing–review and editing.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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The role of primary cilia in congenital heart defect-associated neurological impairments

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Congenital heart disease (CHD) has, despite significant improvements in patient survival, increasingly become associated with neurological deficits during infancy that persist into adulthood. These impairments afflict a wide range of behavioral domains including executive function, motor learning and coordination, social interaction, and language acquisition, reflecting alterations in multiple brain areas. In the past few decades, it has become clear that CHD is highly genetically heterogeneous, with large chromosomal aneuploidies and copy number variants (CNVs) as well as single nucleotide polymorphisms (SNPs) being implicated in CHD pathogenesis. Intriguingly, many of the identified loss-of-function genetic variants occur in genes important for primary cilia integrity and function, hinting at a key role for primary cilia in CHD. Here we review the current evidence for CHD primary cilia associated genetic variants, their independent functions during cardiac and brain development and their influence on behavior. We also highlight the role of environmental exposures in CHD, including stressors such as surgical factors and anesthesia, and how they might interact with ciliary genetic predispositions to determine the final neurodevelopmental outcome. The multifactorial nature of CHD and neurological impairments linked with it will, on one hand, likely necessitate therapeutic targeting of molecular pathways and neurobehavioral deficits shared by disparate forms of CHD. On the other hand, strategies for better CHD patient stratification based on genomic data, gestational and surgical history, and CHD complexity would allow for more precise therapeutic targeting of comorbid neurological deficits.

KEYWORDS

congenital heart disease, genetic variants, cilia, brain, neurodevelopment

1 CHD-associated neurological and behavioral impairments

Children with CHD face the highest risk of infant mortality due to birth defects (Gilboa et al., 2010). Surgical technique advances have dramatically improved survival rates, however assessments of long-term outcomes in these patients have revealed a propensity for neurodevelopmental and neurobehavioral deficits. Cognitive delay, motor skill deficits, higher rates of autism spectrum disorder (ASD) and attention deficit hyperactivity disorder (ADHD) diagnoses as well as language difficulties represent the most frequently encountered neurological sequelae (Bellinger et al., 2003; Bellinger et al., 2009;

Marelli et al., 2016; Morton et al., 2017). Longitudinal studies have found correlations between lower arterial oxygen saturation levels and worse motor skills in CHD patients, according to diagnostic criteria of the Bayley Scales of Infant Development III (Hoffman et al., 2016). In addition, subclinical perioperative seizures detected via electroencephalography (EEG) in a subset of CHD patients was predictive of executive function deficits, reduced sociability, and restrictive behaviors (Gaynor et al., 2013). Many of the impairments persist into late childhood and adolescence, manifesting as a lower intelligence quotient (IQ), worse scholastic performance, attenuated visuo-spatial and visuo-motor skills, and a greater frequency of behavioral issues (Bellinger et al., 2009). Neuroanatomically, the most consistent findings using magnetic resonance imaging (MRI) and diffusion tensor imaging (DTI) are white matter injury and immaturity (Gaynor, 2004; Beca et al., 2013), suggesting that CHD patients have impaired structural and functional connectivity. These clinical studies underscore the variety, yet specificity of detrimental neurobehavioral outcomes found among CHD patients, which likely involve multiple brain regions and circuits that are particularly vulnerable to CHD-linked risk factors.

2 Genetic risk factors

Evidence for a major role of genetic variants in CHD has mostly come from large-scale genomic and exome sequencing efforts, such as those performed by research groups of the Pediatric Cardiac Genomics Consortium (PCGC). These studies have focused on dissecting the genetic contribution to CHD cases presenting with or without neurodevelopmental deficits using parent offspring trios (Homsy et al., 2015). Many of the identified gene hits in the neurodevelopmental group were *de novo* loss of function variants which were associated with known genetic syndromes, with some genes also being linked to isolated CHDs.

2.1 Key genetic variants

Among the genes with damaging (premature truncation, frame shift or splice site mutations) *de novo* variants identified as significantly associated with CHD, key ontological categories include transcriptional regulation, morphogenesis, cilia formation, chromatin regulation and the connectome (Homsy et al., 2015; Ji et al., 2020). These variants were particularly enriched among CHD individuals with accompanying neurodevelopmental deficits in contrast to those without extra-cardiac anomalies. Many of these genes were also demonstrated to have high levels of expression in both the heart and brain. Independent CHD association studies have found multiple genomic hotspots for copy number variants (CNVs) with known pleiotropic and dosage-sensitive effects on cardiac and brain development, whose contribution is estimated at 10%–15% of CHD cases (Ehrlich and Prakash, 2022; Landis et al., 2023). Single nucleotide polymorphisms (SNPs) conferring elevated risk of CHD have also been detected in individual genomic loci (Wang F. et al., 2016). Alongside single gene alterations chromosomal aneuploidies such as trisomy 21/Down syndrome are well known for frequently manifesting CHD (Dimopoulos et al., 2023). Collectively these studies illustrate the existence of extensive genetic heterogeneity among CHD patients with comorbid neurological impairments, some of which is shared with equally multifactorial neurodevelopmental disorders.

2.2 Preponderance of cilia-related damaging variants in CHD

One of the most striking findings of the large-scale sequencing and forward genetic screening studies in CHD has been the extensive genetic contribution of cilia-associated variants. Loss of function mutations in genes such as *Foxj1*, *Cep110*, *Jbts17* and *Fuz* and their association with ciliary defects point to a central role for cilia signaling in CHD pathogenesis (Zaidi et al., 2013; Li et al., 2015). The uncovered variants cluster into categories related to ciliogenesis, endocytic function and cilia-transduced intracellular signaling (Figure 1). These findings suggest that disruption of multiple processes upstream and downstream of normal cilia function enhances the likelihood of CHD-linked cardiac malformations. In addition, since many of the same genes are required for appropriate neural development and brain maturation, it is conceivable that the same damaging mutations affect both the developing heart and brain in CHD patients with neurological impairments. For instance, loss of function of the *Foxj1* gene, encoding a transcription factor, was demonstrated to detrimentally impact murine postnatal ependymal cell differentiation in the neurogenic niche of the lateral ventricles (Jacquet et al., 2009) as well as postnatal olfactory bulb neurogenesis (Jacquet et al., 2011). In addition, *Foxj1* targeted mutations elicit reductions in length and numbers of motile cilia in mice and zebrafish, while patient heterozygous *FOXJ1* mutations cause ciliopathies associated with situs inversus and isolated CHD (Padua et al., 2023). Ciliopathies such as Joubert and Meckel syndromes are frequently associated with neurological defects (Valente et al., 2014) including hydrocephalus, corpus callosum hypoplasia, ataxia and intellectual disability. Since cilia can be both motile and immotile/primary, the functional consequences on cardiac and neural development might differ substantially, depending on the nature and origin of the ciliary insult.

3 Role for primary cilia

Primary cilia are organelles consisting of microtubular filaments which form predominantly singular protrusions from the cell membrane (Goetz and Anderson, 2010). The initial putative links between CHD and ciliopathy came from observations of randomized left-right patterning of the developing heart due to loss of motile cilia and the resultant randomized morphogen flow in the extraembryonic fluid of the embryonic murine node organizer (Nonaka et al., 1998; Brennan et al., 2002). Ciliary mutations causing primary ciliary dyskinesia (PCD) result in heart laterality defects, including transposition of the great arteries (TGA) and double outlet right ventricle (DORV) (Desgrange et al., 2018). Further evidence for a ciliary role in CHD pathogenesis came from a large-scale forward mutagenesis screen carried out in murine fetuses using ethylnitrosourea (ENU) (Li et al., 2015). Interestingly, there was a clear observation of segregation between motile and immotile/primary cilia genes according to the presence of heart laterality defects, with a roughly equal split between motile and primary cilia genes within the laterality deficit group. Motile cilia function has been suggested to impact the developing

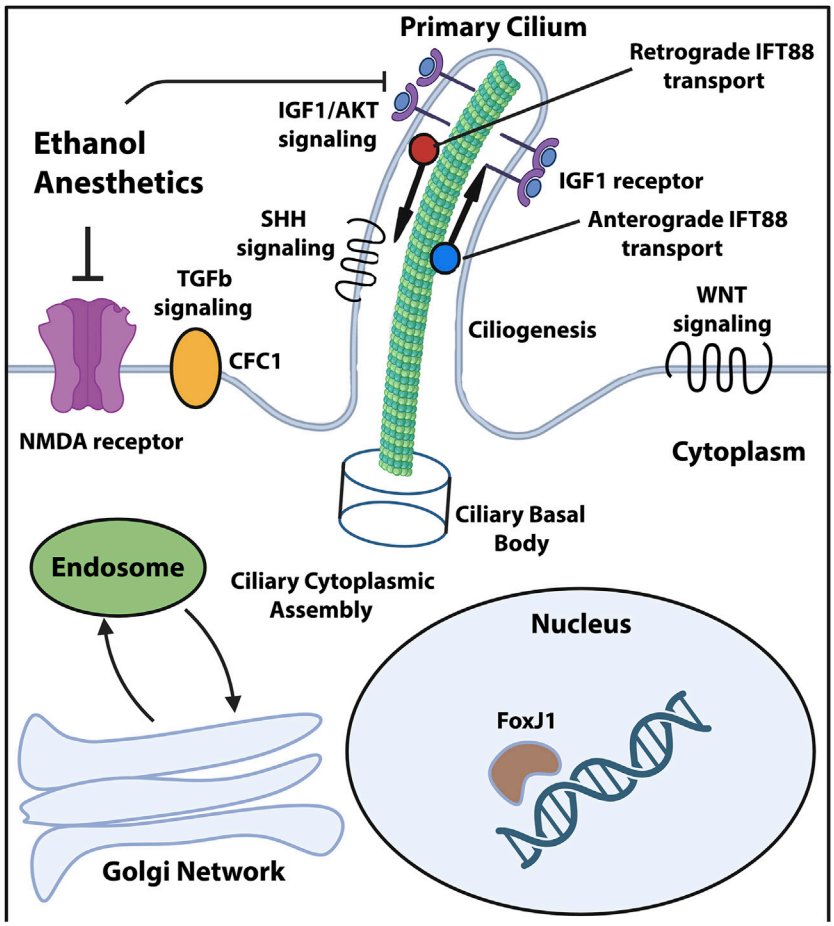

FIGURE 1
Ciliary compartments and pathways targeted by the known CHD mutations and environmental stressors. Diagram showing primary cilium and its compartments along with signaling pathways known to be affected by damaging genomic variants and environmental stressors associated with CHD and neurological impairments. Created in Biorender and adapted from Valente et al. (2014).

brain by altering cerebrospinal fluid flow patterns and volume, leading to brain dysplasia (Panigrahy et al., 2016). Primary cilia have also been identified as key regulators of neural development, being required for developmental phases spanning early neurogenesis, appropriate expansion of different precursor cell classes and neuronal maturation (Youn and Han, 2018; Park et al., 2019).

3.1 Neurodevelopmental functions of primary cilia

Ciliary patterning of the embryonic forebrain is an important determinant of early dorsoventral and rostrocaudal division formation, as demonstrated by the marked telencephalic disorganization in hypomorphic intraflagellar transport 88 (*Ift88*) mutant mice (Willaredt et al., 2008). The *Ift88* mutants lack key microtubular components of the intraflagellar transport (IFT) system which serves to transport protein cargo bidirectionally along the cilium axoneme. Loss of primary cilia in neural progenitors of the early mouse embryo (earlier than embryonic day 9) results in increased immature progenitor proliferation (Wilson et al., 2012), which is largely attributable to dampened Gli3 signaling, itself a negative regulator of the Sonic hedgehog (SHH) pathway (Matissek and Elsawa, 2020). Later, mid-gestational, loss of primary cilia does not result in overt deficits in neocortical development (Tong et al., 2014), suggesting that their developmental function is critical specifically during early forebrain patterning. In contrast to murine corticogenesis, the human fetal neocortex has significantly higher basal levels of hedgehog signaling, rendering its expansion potentially more sensitive to ciliary disruptions (Wang L. et al., 2016). Individuals with hypoplastic left heart syndrome (HLHS) are indeed known to have a greater risk of microcephaly, cortical mantle immaturity and agenesis of the corpus callosum (Glauser et al., 1990), while also harboring ciliome-related loss of function variants (Yagi et al., 2018). On the other hand, conditional ablation of primary cilia in murine hippocampal and cerebellar precursors results in significant reductions in adult hippocampal neural stem cells (Breunig et al., 2008) and cerebellar hypoplasia (Spassky et al., 2008) respectively. These findings argue that a subset of the ciliary mutations recovered from CHD screens regulate key stages of early neurodevelopment of different brain regions and therefore might be responsible for more severe neurological outcomes in CHD patients.

3.2 Ciliary neuroprotection

Emerging pre-clinical evidence points to a neuroprotective function for primary cilia from environmental exposures. In a forebrain-specific model of ciliopathy acute perinatal exposure to ethanol led to neuronal caspase activation and subsequent dendritic degeneration of deep layer pyramidal neurons (Ishii et al., 2021). Intriguingly, the phenotype was solely observed when ethanol exposure was combined with *Ift88* inactivation-induced primary cilia loss, indicative of a gene-environment interaction. This effect was dependent on caspase 3-mediated cytoskeletal remodeling and was reversible through pharmacological activation of the insulin-like growth factor 1 receptor-protein kinase B (IGF1R-AKT) pathway, which is known for its growth-promoting and anti-apoptotic cellular functions (Hemmings and Restuccia, 2012). Curiously, this study did not find evidence for caspase-mediated apoptotic loss of neurons following ethanol, suggesting a non-apoptotic mechanism of action. Ethanol acts as both a facilitator of γ-amino butyric acid (GABAergic) and inhibitor of N-methyl-D-aspartate (NMDA) neurotransmission (Nagy, 2008), a property which classifies it as a CNS depressant and which is shared with most general anesthetics (Petrenko et al., 2014).

3.3 Ciliary dysfunction and CHD perioperative anesthesia

A significant fraction of CHD neonates (estimated at 25%) require heart surgery within their first year of life, necessitating the use of general anesthesia (Moller et al., 1994). Despite being essential for suppressing consciousness and pain management during surgeries, inhalable and injectable anesthetics have been scrutinized due to a plethora of pre-clinical evidence pointing to their potential for developmental neurotoxicity (Jevtovic-Todorovic et al., 2003; Yon et al., 2005). A recent clinical trial did not find significantly altered neurobehavioral outcomes in 5-year-old children undergoing a short 1-h course of general anesthesia (McCann et al., 2019), however it did not account for genetic status of the participants. This is of particular concern given that pre-existing genetic susceptibility in CHD infants, such as ciliary gene variants, might adversely interact with general anesthetic exposures as previously described (Saric et al., 2022). More in depth pre-clinical modeling as well as careful clinical studies which account for multiple relevant variables are needed to dissect the risk and mechanisms of genetic status and anesthesia interactions.

4 Diagnostic and therapeutic outlooks

The multitude of factors which can influence risk of CHD-relevant neurological impairments necessitates a combination of detailed diagnostic patient stratification and therapies targeted at each potential high-risk phase for neurological injury. Currently proposed CHD therapeutic strategies span both pre- and post-operative phases and include maternal oxygen supplementation, progesterone, tetrahydrobiopterin (BH4) as well as mesenchymal stromal cell (MSC) administration during surgery (Kobayashi et al., 2021). In addition to therapy, more precise diagnostic tools encompassing genomic data and developmental history are needed to identify CHD patients at high risk of comorbid neurological impairments, coupled with the appropriate, specific treatment regime. Given that primary cilia function constitutes a key node for damaging genetic variants, potential environmental stressors such as anesthesia and gene-environment interactions, future diagnostic tools will necessarily be more comprehensive to account for these factors. Ciliome-directed therapies will likely target ciliogenesis, signaling pathways such as Sonic hedgehog (SHH), Wingless (WNT), transforming growth factor β (TGFβ) and insulin growth factor (IGF), as well as ciliary microtubular transport (Figure 1).

5 Concluding remarks

Here we aimed to account for ciliary dysfunction as a potential key causative element in the adverse neurodevelopmental and behavioral sequelae commonly encountered in CHD patients. In conclusion, while many of the ciliary genetic and environmental risk factors associated with CHD can produce neurodevelopmental deficits in independent fashion, there is an increasing appreciation for their interactions which can act as strong phenotypic modifiers. Investigating how these interactions might induce or enhance neurological impairment will yield potential avenues for better therapeutic management.

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NS: Conceptualization, Writing–original draft, Writing–review and editing. NI: Conceptualization, Funding acquisition, Resources, Supervision, Writing–review and editing.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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De novo and inherited micro-CNV at 16p13.11 in 21 Chinese patients with defective cardiac left-right patterning

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Objective: Copy number changes at Chromosomal 16p13.11 have been implicated in a variety of human diseases including congenital cardiac abnormalities. The clinical correlation of copy number variants (CNVs) in this region with developmental abnormalities remains controversial as most of the patients inherit the duplication from an unaffected parent.

Methods: We performed CNV analysis on 164 patients with defective left-right (LR) patterning based on whole genome-exome sequencing (WG-ES) followed by multiplex ligation-dependent probe amplification (MLPA) validation. Most cases were accompanied with complex congenital heart disease (CHD).

Results: CNVs at 16p13.11 were identified in a total of 21 cases, accounting for 12.80% (21/164) evaluated cases. We observed a marked overrepresentation of chromosome 16p13.11 duplications in cases when compared with healthy controls according to literature reports (15/164, 9.14% versus 0.09% in controls). Notably, in two independent family trios, *de novo* 16p13.11 micro-duplications were identified in two patients with laterality defects and CHD. Moreover, 16p13.11 micro-duplication was segregated with the disease in a family trio containing 2 affected individuals. Notably, five coding genes, NOMO1, PKD1P3, NPIPA1, PDXDC1, and NTAN1, were potentially affected by micro-CNV at 16p13.11 in these patients.

Conclusion: Our study provides new family-trio based evidences to support 16p13.11 micro-duplications predispose individuals to defective cardiac left-right patterning and laterality disorder.

KEYWORDS

16p13.11, copy number variation, deletion, duplication, congenital heart disease, ciliopathy, laterality disorder, left-right patterning

Introduction

Abnormalities in the ultrastructure of cilia result in a rapidly expanding spectrum of clinical symptoms, termed ciliopathies, that mainly include nephronophthisis, Bardet–Biedl syndrome, primary ciliary dyskinesia (PCD), and retinal degeneration, laterality disorder (Fliegauf et al., 2007; Reiter and Leroux, 2017). Classified by

microtubular structure, two types of cilia are found: primary/non-motile cilia and regular motile cilia. Most regular motile cilia consist of a ring of nine peripheral microtubule doublets that surrounds a central pair of single microtubules (9 + 2 structure). In contrast to the 9 + 2 pattern of regular cilia, primary or sensory cilia are solitary, immotile organelles that have 9 + 0 microtubule configuration and are present on most cell types.

Proper left-right (LR) asymmetry is an essential aspect of embryonic development (Hamada et al., 2002). Embryonic cilia-driven fluid flow plays essential roles in LR organization in mammals (Hirokawa et al., 2006). Defects in LR organization cause a range of laterality disorders including situs inversus (SI) totalis (SIT) and heterotaxy (Htx) (Sutherland and Ware, 2009). SIT is a congenital condition in which organs in the chest and abdomen are arranged in a complete mirror-image reversal of the usual positions; the prevalence of SIT is estimated to range from 1/25,000 to 1/8,000 (Bartoloni et al., 2002). Up to 20% of SIT patients have Kartagener syndrome (KS), which is a triad of nasal polyps, bronchiectasis, and SIT, and a subgroup of PCD (Leigh et al., 2009). Approximately, 50% of patients with PCD present with SIT. Htx is a condition that involves the internal organs being abnormally arranged. However, unlike SIT, it does not result in a mirror-image reversal of organ positions. Over 80% of individuals with Htx present with complex CHDs including transposition of the great arteries (TGA) and double outlet right ventricle (DORV) (Fakhro et al., 2011). SIT patients have a lower risk of CHD (3%–9%) than Htx patients (Taketazu et al., 2006). However, the risk of CHD in SIT is still significantly higher than that in the normal condition, situs solitus (0.6%–0.8%) (Peeters and Devriendt, 2006). Till date, dozens of candidate genes including *DNAH5, DNAH9, TTC21B, NUP205, NEK3, NPHP3, CCDC40* and *NEK8* have been reported (Reiter and Leroux, 2017; Nöthe-Menchen et al., 2019; Chen et al., 2022; Zhang et al., 2020; Chen et al., 2019).

Copy number changes at chromosomal 16p13.11 have been recently implicated in pseudoxanthoma elasticum (van Soest et al., 1997), a variety of neuropsychiatric disorders (Johnstone et al., 2019), congenital anomalies of the kidney and urinary tract (CAKUT) (Verbitsky et al., 2019), megacystis-microcolon-intestinal-hypoperistalsis syndrome (MMIHS) (Kloth et al., 2019) and congenital cardiac abnormalities (Allach El Khattabi et al., 2018; Hamad et al., 2023). The population frequency of 16p13.11 duplication is estimated to be 0.09% in controls (Ingason et al., 2011; Kuang et al., 2011), 0.30% in schizophrenia (Ingason et al., 2011), 1.04% in aortic dissections (Kuang et al., 2011), and approximately 0.20% in CAKUT (Verbitsky et al., 2019). Although genotype-phenotype analysis does not reveal the impact of the size of duplicated segments on the severity of the phenotype, ciliary-related nuclear distribution E (NDE1) was proposed to be a response for the neuropsychiatric phenotype (Heinzen et al., 2010). In addition, modulators of Nodal, Nomo1 and Nomo3, were presented in this region (Warburton et al., 2014). These findings suggested that 16p13.11 alterations might be involved in the normal biological processes underlying ciliary function.

Methods

Study participants

Individuals with LR pattering defects were diagnosed by X-ray and color ultrasonic diagnosis at Pediatric Cardiovascular Center of the Children's Hospital affiliated to Fudan University. Typical symptoms of PCD, including neonatal rhinosinusitis, airway infections, and ottis media, were determined by standard clinical diagnostic criteria and nasal nitric oxide measurements as previously described (Chen et al., 2022). Other malformations in the cardiovascular system were diagnosed by cardiac ultrasound. For studies of affected individuals and their families, written informed consent was obtained from all participants prior to the start of the study. All procedures in the study were approved by the Medical Ethics Committee of Children' Hospital of Fudan University (2016-079) (Shanghai, China).

WE-GS

Genomic DNA isolated from the peripheral blood of patients was used to perform whole genome or exome sequencing on the SureSelect human all exon platform (v.6; Agilent Technologies, Santa Clara, CA, United States). Exome-enriched genomes were multiplexed using a flow cell for paired-end 2 × 150-bp read sequencing based on protocols established for the HiSeq X10 platform (Illumina, San Diego, CA, United States). The Genome Analysis Toolkit software package was used to detect single-nucleotide variants and indels. On average, exome coverage and depth were more than 95% (depth >20) and 80×, respectively.

CNV analysis

All sequencing data in this study were trimmed and filtered with Fastp. The filtered sequencing reads were aligned to a hg19 reference genome (human_g1k_v37.fasta) with Burrows-Wheeler Aligner (bwa 0.7.16,http://bio-bwa.Sourceforge.net/) and the duplicates were removed with samtools markdup (samtools 1.9). Then the Genome Analysis Toolkit (gatk 4.0.12.0) was employed for base quality score recalibration and indel realignment. The common genetic variants were recalibrated using GATK Resource Bundle (dbSNP, HapMap, g1k snps and indels). We applied the popular somatic copy number alteration caller, VarScan (VarScan v2.3.9), to the pre-processed sequencing data with the workflow recommended by developers. The called copy numbers with Varscan between two samples were smoothed and segmented using the R library and DNAcopy.

Multiplex-ligation dependent probe amplification (MLPA)

MLPA was used to validate the specific small chromosomal abnormalities at 16p13.11 identified from CNV analysis based on WE-GS data by following standard protocol. Three different probes

TABLE 1 Clinical characteristics of 21 patients (12 males, nine females) with 16p13.11 copy number changes. All 21 patients displayed defective cardiac LR patterning, mainly SIT and dextrocardia. The majority (17/21) of these individuals present with complications including congenital heart disease (10/21), PCD (3/21), kidney disease (2/21), congenital anal atresia (1/21) and congenital nystagmus (1/21). *De novo* copy number changes were identified in 33.3% (7/21) individuals.Three patients harbor the second rare CNV at 16p11.2. M, male; F, female; Dup, duplication; Del, deletion; n.a, not available; PCD, primary ciliary dyskinesia; SIT, situs inversus (SI) totalis; VSD, ventricular septal defect.

Patients	Age	Gender	Defective LR patterning	Complications	16p13.11		Second CNV
No. 1	4	M	Isolated dextrocardia	VSD and pulmonary artery sling	Dup	*De novo*	—
No. 2	4	M	—	Congenital nystagmus	Dup	*De novo*	—
No. 3	26	M	SIT	—	Dup	Inherited	—
No. 4	51	F	SIT	—	Dup	Inherited	—
No. 5	6	M	SIT	Nephronophthisis	Dup	*De novo*	16p11.2 deletion
	3	F	—	Neonatal cholestasis, chronic renal disease	—	n.a	—
No. 6	5	F	Dextrocardia	Recurrent respiratory infection, pulmonary agenesis	Dup	*De novo*	—
No. 7	5	M	Dextrocardia	Atrial septal defect, Persistent Left Superior Vena Cava	Dup	Inherited	—
No. 8	7	M	Isolated dextrocardia	Persistent Left Superior Vena Cava	Dup	Inherited	—
No. 9	2	M	SIT	Bicuspid aortic valve	Dup	n.a	—
No.10	2	M	Dextrocardia	VSD, Biliary atresia, Ectopic kidney	Dup	n.a	—
No. 11	1	F	Dextrocardia	VSD, Double outlet right ventricle	Dup	inherited	—
No. 12	6	F	Dextrocardia	Complete transposition of great arteries, Asplenia	Del	*De novo*	—
No. 13	12	F	SIT	Patent foramen ovale, VSD	Del	*De novo*	—
No. 14	6	M	SIT	—	Del	Inherited	—
No. 15	5	F	Dextrocardia	Double outlet right ventricle, Patent ductus arteriosus	Del	*De novo*	—
No. 16	6	M	SIT	PCD	Del	Inherited	16p11.2 deletion
No. 17	4	F	SIT	—	Dup	Inherited	—
No. 18	8	M	SIT	Congenital anal atresia	Del	Inherited	—
No. 19	9	F	SIT	PCD	Dup	n.a	—
No. 20	3	M	SIT	VSD, Atrial septal defect	Dup	Inherited	16p11.2 deletion
No. 21	5	F	Dextrocardia	Transposition of great arteries, VSD	Dup	n.a	—

were designed for each targeted gene (*GAPDH* and *PDXDC1*). The sequences of probes were provided in Supplementary Table S1. GAPDH served as internal reference. Copy number status was determined based on the DQ (dosage quotient) values (0.80<DQ < 1.20, Normal; DQ = 0.00, homozygous deletion; 0.40<DQ < 0.65, heterozygous deletion; 1.3<DQ < 1.65, heterozygous duplication; 1.75<DQ < 2.15, heterozygous triplication/homozygous.

Statistical analysis

Here we compared the prevalence of duplication at 16p13.11 in our studied cohort with the prevalence in a control population and thoracic aortic aneurysm and dissection (TAAD) cohort from two previous studies. We calculated statistical significance using the Chi-square test ($\chi 2$). A *p*-value that is less than or equal to the 0.05 significance level signifies statistical significance.

Results

In this study, we initially recruited three unrelated family trios with laterality defects and CHD (Table 1; Figure 1A). In a monozygotic twin (Family-1), a 4-year-old boy (F1-II-1) displayed isolated dextrocardia accompanied by an absent right lung, an atrial septal defect, and a pulmonary artery sling (Figure 1B). The identical twin brother (F1-II-2) exhibited congenital nystagmus and already undergo ophthalmic surgery, without other developmental malformations. Through trio-based whole genome sequencing (WGS) analysis on four family members, we did not identify recessive mutations in well-known ciliary genes that could meet our filter strategy. Subsequent genome-wide CNV analysis then identified *de novo* 16p13.11 micro-duplication (chr16: 14.98–15.13, 140 kb) in both affected siblings (Figure 1C). Five coding genes, NOMO1 (NODAL modulator 1) (chr16:14927578-14990014), PKD1P3 (polycystin 1, transient receptor potential channel interacting pseudogene 3) (chr16:15011391-15029565), NPIPA1 (nuclear pore complex interacting protein family

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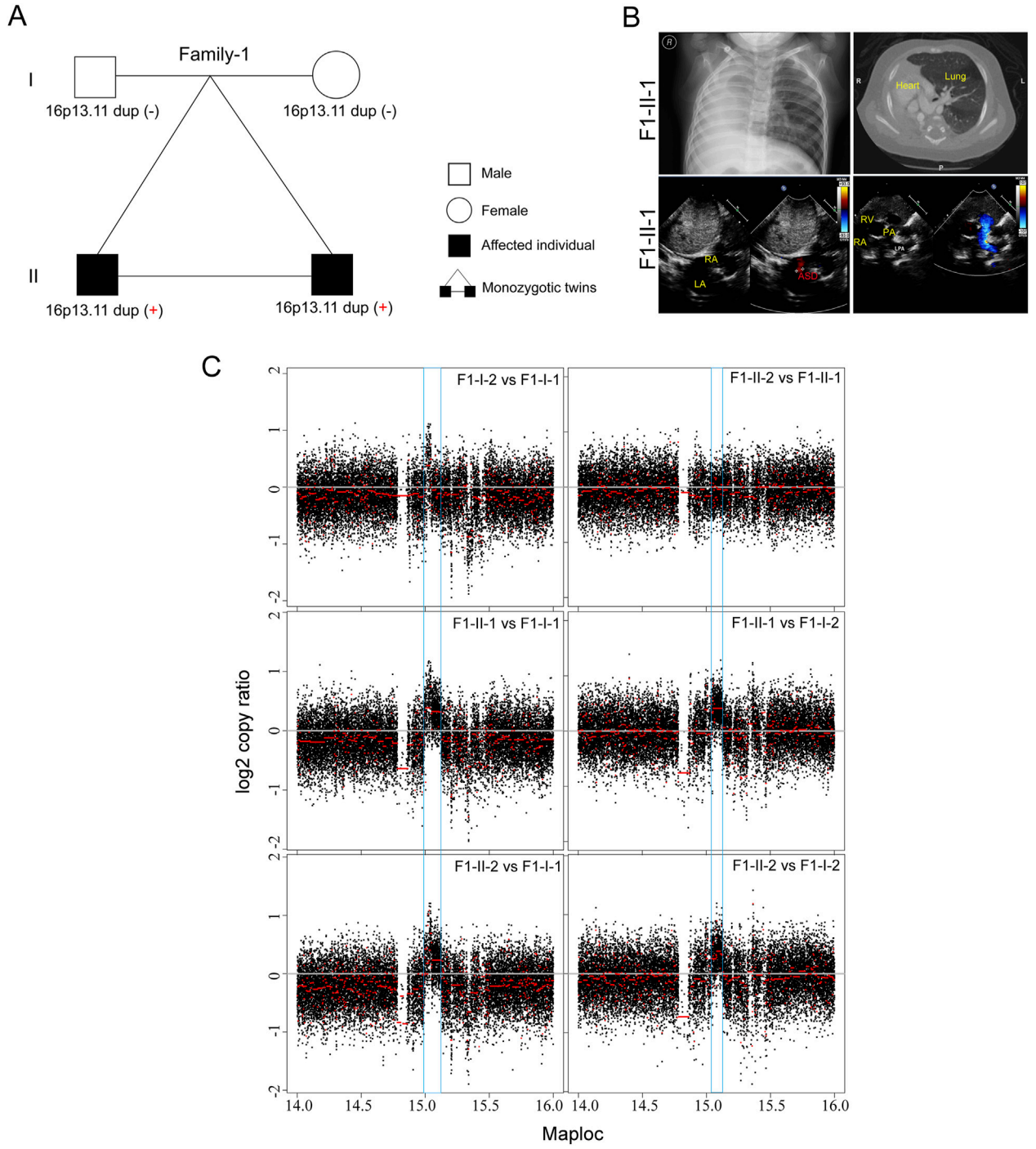

FIGURE 1
De novo 16p13.11 duplication in a monozygotic twin **(A)** Pedigrees of Family-1 (F1) indicating the affected individuals and the distribution of 16p13.11 duplication. **(B)** Chest X-ray and CT-scan show isolated dextrocardia in the proband (F1-II-1) (upper), and color ultrasound scans reveal an atrial septal defect (ASD), and a pulmonary artery sling in the patient (bottom). RA, right atrium; LA, left atrium; RV, left ventricle; LPA, left pulmonary artery; PA, pulmonary artery. **(C)** CNV analysis based on WGS data identified *de novo* 16p13.11 micro-duplication (chr16: 14.98–15.13, 150 kb, GRCh37.p13) in both sibilings when compared to the parents, independently.

member A1) (chr16: 15.03–15.05 Mb), PDXDC1 (pyridoxal dependent decarboxylase domain containing 1) (chr16: 15.07–15.12 Mb), NTAN1 (N-terminal asparagine amidase) (chr16:15131714-15149931) were potentially affected by the duplicated region.

In the second family (Family-2) trio containing 2 affected individuals with SIT and 2 unaffected individuals (Figures 2A, B). The proband (F2-II-1) was a 26-year-old male without detectable complications during his life history to the present (chief complaint but sperm morphological examination is not

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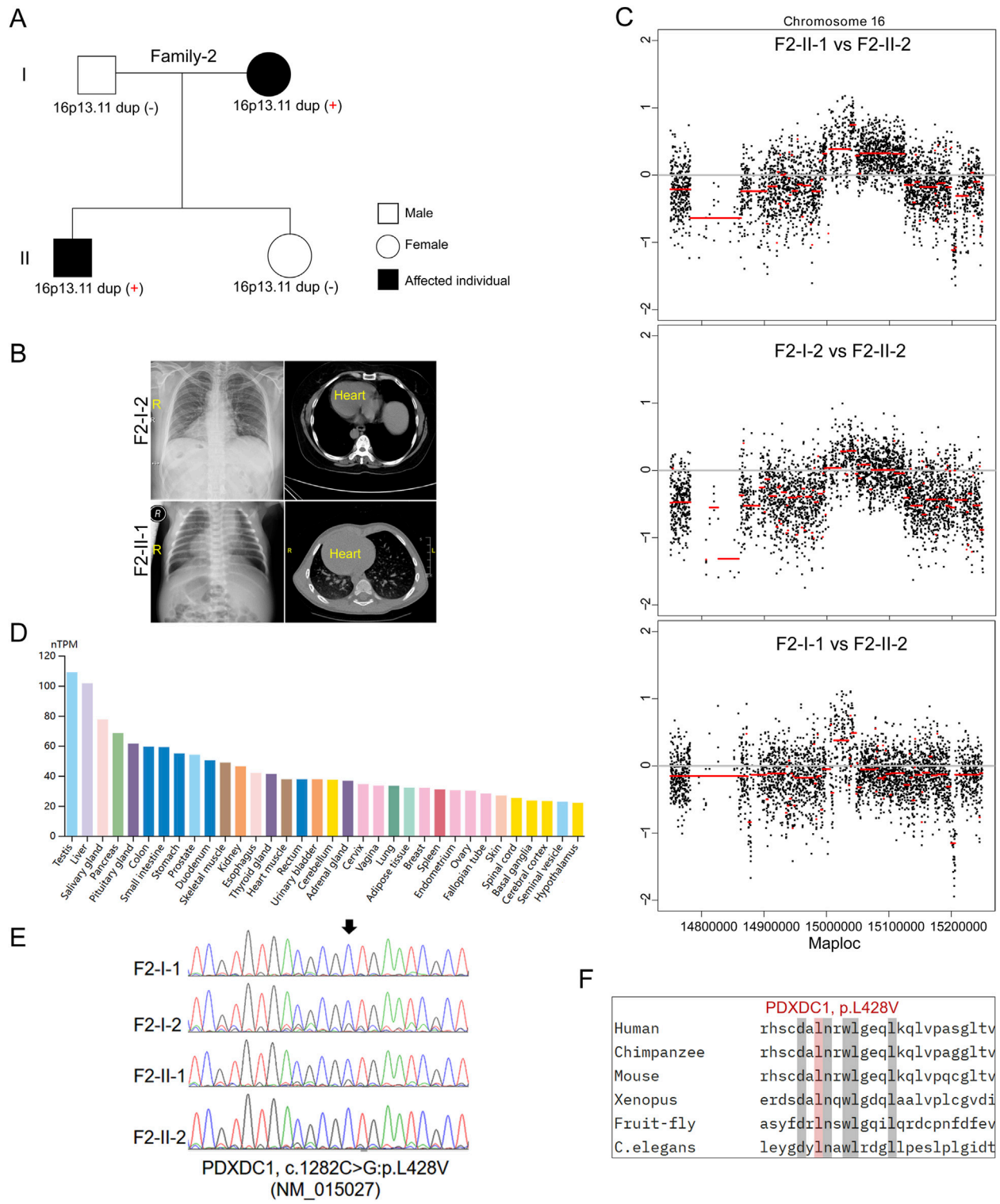

FIGURE 2
Inherited 16p13.11 duplication segregated with the disease in Family-2. **(A)** Pedigrees of Family-2 (F2) indicating the affected individuals and the distribution of 16p13.11 duplication. **(B)** Representative images of chest X-ray and CT scan shows the mirror-image arrangement of the abdominal organs in both patients (F2-I-2 and F2-II-1), R, right. **(C)** CNV analysis based on WGS data identified 16p13.11 micro-duplication (chr16: 14.98–15.13, GRCh37.p13) in both patients when compared to the unaffected individual, respectively. **(D)** Human Protein Atlas database showing PDXDC1 is widely expressed in various human tissues and has highest levels in testis. **(E)** Sanger sequencing on PDXDC1 variant (p.L428V) in the patients and unaffected members. **(F)** Sequencing alignment of missense variants p.L428V in different species as indicated. **(C)** elegans, *caenorhabditis elegans*.

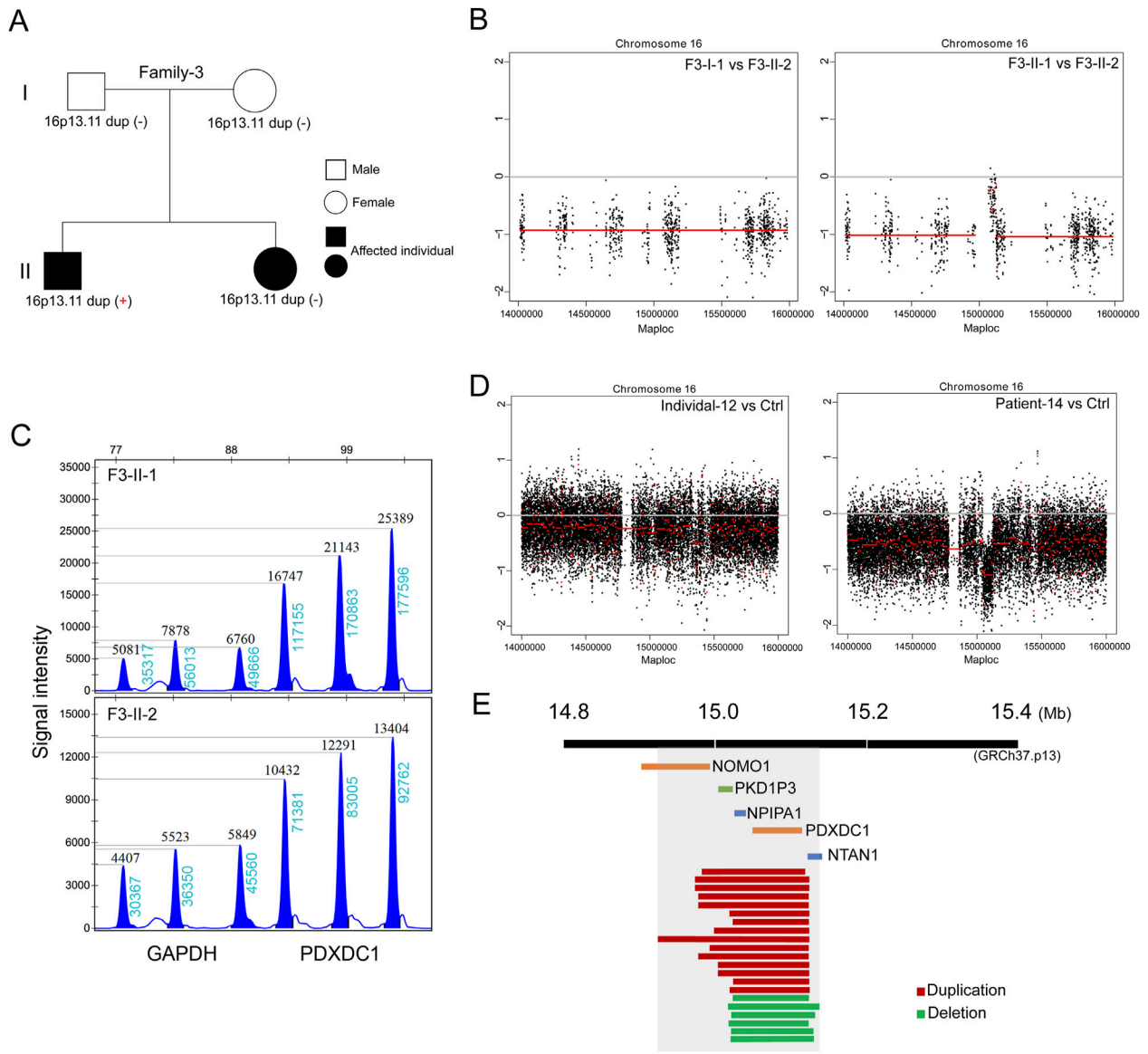

FIGURE 3
De novo 16p13.11 duplication potentially modulates the cardiac phenotype. **(A)** Pedigrees of Family-2 (F3) indicating the affected individuals and the distribution of 16p13.11 duplication in two siblings with different ciliopathies. **(B)** CNV analysis based on WES data identified micro-duplication at 16p13.11 in the brother (F3-II-1) but not in the sister (F3-II-2). **(C)** MLPA confirmation of the gain of copy in *PDXDC1*, located in the 16p13.11 region, that identified from CNV analysis in the F3-II-1 when compared with F3-II-2. Three independent probes were designed for each targeted gene as indicated. GAPDH served as the internal reference. Dosage quotient (DQ) values = 1.501 (1.3<DQ < 1.65, heterozygous duplication) **(D)** Genome-wide CNV analysis also identified 16p13.11 deletion in the patient with defective LR patterning when compared with health Ctrl (right panel). Left panel served as a internal Ctrl. **(E)** The diagram of 16p13.11 duplications (red) and deletions (green) in 21 patients. The scale is in megabases. The region that is spanned by all CNVs containing five coding genes, *NOMO1, PKD1P3, NPIPA1, PDXDC1 and NTAN1,* as indicated.

included). His mother (F2-I-2) is a 51-year-old woman and her typical PCD phenotypes including bronchitis, sinusitis, and otitis media, whom were excluded by physical examination as previously (Chen et al., 2022). Echocardiography did not reveal other detectable abnormalities in heart structure or motion in both individuals. It is uncommon that SIT occurs in an autosomal dominant pattern in a non-consanguineous marriage family. However, we did not identify potential causative recessive and sex-linked non-synonymous variants in the known candidate genes by trio-based WGS and WES. Subsequent CNV analysis found that micro-duplication at 16p13.11 was identified in both patients (F2-II-1 and F2-I-2) (Figure 2C), but not in two unaffected members, suggesting that 16p13.11 duplication might segregated with the disease in this family. Moreover, WGS analysis identified a heterozygous missense mutation in PDXDC1 (c.1282C>G, p.L428V, NM_015027) in both affected individuals. PDXDC1 is widely expressed in various human tissues and has highest mRNA level in testis (Figure 2D), which is a common feature of many cilia related genes. Sanger sequencing further validated the base substitution from C to G at c.1282 of PDXDC1 in the patients (Figure 2E). The

CADD_Phred and GERP++_RS score of PDXDC1 L428V is 11.72 and 2.90, respectively. Although sequence alignment indicated PDXDC1 L428V affected highly conserved residues across including *Xenopus* tropicalis and *Caenorhabditis elegans* (Figure 2F), allele frequency of PDXDC1 p.L428V in GnomAD (v2.1.1)_exome_all (MAF = 0.0353) and gnomAD_exome_EAS (MAF = 0.0417) is too high for a disease-causing factor. Therefore, we classify L428V as a VUS (Variant of Uncertain Significance) leaning towards likely benign if applying the ACMG (American College of Medical Genetics and Genomics) guidelines.

In the third family (Family-3) trio containing two affected siblings (Figure 3A). The proband is a 6-year-old boy (F3-II-1) diagnosed with nephronophthisis-related ciliopathies and SIT. The patient's 3-year-old sister (F3-II-2) exhibited severe neonatal cholestasis and chronic renal disease, but had no defects in heart development. Subsequent trio-based WES did not identify the well-known causative genes in both siblings, according to literature reports (Reiter and Leroux, 2017). Genome-wide CNV analysis then identified *de novo* 16p13.11 duplication in the proband but not in his sister (Figures 3B, C). This finding further support that 16p13.11 duplication might act as a modifier of phenotypic heterogeneity on cardiac LR patterning.

Based on our above-mentioned findings, we expand the clinical sample size and performed CNV analysis on patients with defective cardiac LR patterning based on WG-ES data to verify the main findings above. A total of 164 cases (median age: 3.25 years; range: 35 days-51 years) confirmed to have laterality disorders were recruited between January 2013 and July 2023. Associated conditions were diagnosed in 139 cases (84.75%). The most commonly associated conditions were congenital heart defects (n = 116, 70.73%) followed by primary ciliary dyskinesia (n = 15, 9.15%) and renal disorders (n = 6, 3.66%). We identified copy number changes at 16p13.11 in a total of 21 patients (Table 1; Supplementary Figure S1), 15 cases with 16p13.11 micro-duplication (15/164, 9.14%) and 6 cases with 16p13.11 micro-deletion (6/164, 3.65%) (Figure 3D). We found that the frequency of 16p13.11 duplication in laterality disorders (9.14%) was markedly higher than that in controls (0.09%) and other relative disease as aforementioned (Verbitsky et al., 2019; Ingason et al., 2011; Kuang et al., 2011), ranging from 0.3% to 1.04%. The common region (chr16: 15.02–15.13, 110 kb, GRCh37.p13) that is spanned by all CNVs in 21 patients containing two intact coding genes (Figure 3E), NPIPA1 and PDXDC1 as aforementioned. Furthermore, three coding genes NOMO1, PKD1P3 and NTAN1 were also potentially, at least in part, affected by these micro-CNVs at 16p13.11.

Discussion

Here we report a significant overrepresentation of chromosome 16p13.11 duplications in patients with defects in cardiac LR patterning (14.42% versus 0.09% in controls as previously) (Ingason et al., 2011; Kuang et al., 2011), indicating greater enrichment of this duplication in laterality disorders. Cardiac malformations were present in approximately 20% of patients carrying 16p13.11 duplications, indicating a significant risk of cardiovascular disease (Allach El Khattabi et al., 2018; Nagamani et al., 2011). In 2023, a multicentric analysis of 206 patients with 16p13.11 microduplication. Echocardiograms were performed in 50.5% (104/206) of the total patient cohort and a congenital cardiac anomaly was identified in 15% (16/104) of those patients (Hamad et al., 2023). Duplication of the distal 16p13.11 recurrent region has been associated with variable clinical phenotypes including developmental delay, intellectual disability, learning difficulties, behavioral abnormalities, and variable dysmorphic features (Verbitsky et al., 2019). Although primary cilia play the important roles in congenital heart defect-associated neurological impairments, none of 21 patients in our study exhibited these neuropsychiatric disorders to date. We understand that neurological phenotypes in these CHD cases might be ignored due to the relative poor quality of medical conditions in grass roots. Ciliary-related NDE1 was consistently included in the 16p13.11 deletions in 23 previously evaluated patients with epilepsy syndromes (Ingason et al., 2011) and eight patients with TAAD (Chen et al., 2022). However, we did not detect either gained or lost copies of NDE1 in our 21 patients, which raises the possibility of other pathogenic genes responding in non-neuropsychiatric phenotypes.

Five coding genes, NOMO1, PKD1P3, NPIPA1, PDXDC1 and NTAN1, were potentially affected by 16p13.11 alterations in these patients. NOMO1 was identified as part of a protein complex that participates in the Nodal signaling pathway during vertebrate development. NODAL flow plays essential role in the generation of left-right asymmetry. Overexpression of NOMO1 imposes a sheet morphology on the endoplasmic reticulum (Amaya et al., 2021). Nomo1-deficient zebrafish exhibit multiple neuropsychiatric behaviors such as hyperactive locomotor activity, social deficits, and repetitive stereotypic behaviors (Zhang et al., 2024). NPIPA1 could co-localize with cilia-related NUP62 (Johnson et al., 2001; Takao et al., 2017). Many components of nuclear pore complex (NPC), especially inner ring nucleoporins NUP93, NUP205 and NUP188, as well as NUP62 were involved in the ciliary function and LR determination (Chen et al., 2019; Chen et al., 2023; Del Viso et al., 2016; Kee et al., 2012; Marquez et al., 2021). PDXDC1 gene was frequently deleted in hearing loss patients and modulates prepulse inhibition of acoustic startle in the mouse (Feldcamp et al., 2017; Haraksingh et al., 2014). Interestingly, many PCD patients exhibited conductive hearing loss (Kreicher et al., 2018). Although PDXDC1 is not associated with any human disease in OMIM, this gene has highest mRNA level in testis and is conserved in different species. Interestingly, many ciliary genes including NPHP4, CCDC40, DNAH9 and RSPH6A have restricted expression toward testis, suggesting PDXDC1 might be involved in ciliary function. NTAN1 acts as a tertiary destabilizing enzyme that deamidates N-terminal L-Asn residues on proteins to produce N-terminal L-Asp. L-Asp substrates are subsequently conjugated to L-Arg, which is recognized by specific E3 ubiquitin ligases and targeted to the proteasome. The Ntan1 gene is expressed in perineural glia and neurons of adult *Drosophila* (Castañeda-Sampedro et al., 2022). Altered activity, social behavior, and spatial memory was detected in Ntan1 (−/−) mice (Kwon et al., 2000).

Previous study reported that 9.7% of patients carrying 16p13.11 microduplication were found to have a second genetic/

chromosomal diagnosis, especially where there were additional phenotypic features (Hamad et al., 2023). In this study, we found that three patients with 16p13.11 duplication were likely to harbor the second rare CNV at 16p11.2. For example, the patient in family-3 (F3-II-1) harbors a second rare CNV in 16p11.2 (Supplementary Figure S2). A recent study found that the rare 16p11.2 deletion could push the genetic background closer to the threshold for severe manifestation and therefore require a lesser contribution from other hits. Thus, accurate genetic diagnosis requires complete evaluation of the genetic background even after a candidate disease-associated variant is identified (Pizzo et al., 2019). This model might explain the phenotypic heterogeneity of disease-associated variants in different siblings.

One limitation of our study is that we do not evaluate the frequency of 16p13.11 micro-duplication in the ethnic matched controls, although the frequency of the 16p13.11 duplication was reported to be identical between several previous studies. Our main concern is the marked phenotypic heterogeneity of 16p13.11 CNVs that were associated with a variety of developmental diseases in human. In this study, most of the patients inherit the duplication from an unaffected parent, with maternal and paternal inheritance evident. These findings further support the hypothesis of incomplete penetrance and that imprinting does not seem to take effect in manifestations, as the micro-duplication can be inherited from either parent (Hamad et al., 2023; Nagamani et al., 2011). Investigating the roles of five affected candidate genes on ciliary function and cardiac LR patterning would be a potential avenue for further understanding of these observations. Despite being a preliminary evaluation, our study indicated that micro-CNV at 16p13.11 predispose individuals to defective establishment of cardiac LR patterning.

Data availability statement

The original contributions presented in the study are included in the article/Supplementary Material, further inquiries can be directed to the corresponding author.

Ethics statement

The studies involving humans were approved by Medical Ethics Committee of Children' Hospital of Fudan University. The studies were conducted in accordance with the local legislation and institutional requirements. The participants provided their written informed consent to participate in this study. Written informed consent was obtained from the individual(s) for the publication of any potentially identifiable images or data included in this article.

Author contributions

KY: Data curation, Writing–original draft, Investigation. WC: Writing–original draft, Investigation, Data curation. YC: Validation, Writing–original draft. LS: Writing–original draft, Validation, Formal Analysis. BW: Writing–original draft, Validation. YZ: Writing–original draft, Validation, Supervision. XZ: Writing–review and editing, Funding acquisition, Project administration, Writing–original draft.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fgene.2024.1458953/full#supplementary-material

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Autoantibody profiling of patients with immune checkpoint inhibitor-associated myocarditis: a pilot study

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Background: Immune checkpoint inhibitor (ICI)-associated myocarditis is a rare, but potentially fatal, immune-related adverse event. Hence, identifying biomarkers is critical for selecting and managing patients receiving ICI treatment. Serum autoantibodies (AAbs) in patients with ICI myocarditis may serve as potential biomarkers for predicting, diagnosing, and prognosing ICI myocarditis. We conducted a pilot study using a human proteome microarray with approximately 17,000 unique full-length human proteins to investigate AAbs associated with ICI myocarditis.

Methods and results: AAb profiling was performed using sera collected from three patients with ICI myocarditis before the start of ICI treatment and immediately after myocarditis onset. All patients received anti-programmed death-1 antibody monotherapy. At baseline, 116, 296, and 154 autoantigens reacted positively to immunoglobulin G (IgG) in the serum samples from Cases 1, 2, and 3, respectively. Among these proteins, the recombination signal-binding protein for the immunoglobulin kappa J region (RBPJ) was recognized by all three samples, and 32 autoantigens were recognized by any two of the three samples. At the onset of ICI myocarditis, compared to baseline, 48, 114, and 5 autoantigens reacted more strongly with IgG in the serum samples from Cases 1, 2, and 3, respectively. Among these, antibodies against eukaryotic translation initiation factor 4E binding protein 3 (EIF4EBP3) were the most upregulated, with a 38-fold increase. Gene ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment analyses highlighted that B-cell receptor signaling, leukocyte transendothelial migration, and thymus development were among the most affected pathways. Enrichment analyses using DisGeNET revealed that proteins reacting to AAbs detected in patients with ICI myocarditis are associated with several diseases, including dilated cardiomyopathy and muscle weakness.

Conclusions: This pilot study provides the first integrated analysis of serum AAb profiling in patients with ICI myocarditis and identifies novel candidate markers associated with an increased risk of developing ICI myocarditis and its pathogenesis. However, our results require further independent validation in clinical trials involving a larger number of patients.

KEYWORDS

cardio-oncology, onco-cardiology, immune-related adverse event, irAE, proteome, proteomics, autoimmunity

1 Introduction

Immune checkpoint inhibitors (ICIs) are a novel class of immunotherapeutic drugs that improve the treatment of a broad range of cancers. These drugs are increasingly being used for a large number of solid and hematological malignancies in the early stages, and several clinical trials are underway to expand their indications (1). However, the benefits of ICIs are often mitigated by the development of immune-related adverse events (irAEs) (2–4). In particular, myocarditis is recognized as a life-threatening complication with a mortality rate of up to 50% (5–7), but little is known about its immunological mechanisms and potential biomarkers.

In patients with classical myocarditis or inflammatory cardiomyopathy, autoantibodies (AAbs) against a wide range of self-antigens are detected. These AAbs can be specific or nonspecific to heart tissue, and several have been reported to be directly related to the pathophysiology (reviewed in (8, 9)). Immunoadsorption therapy has been performed to remove circulating AAbs in patients with inflammatory cardiomyopathy, resulting in an improvement in cardiac function and decreased myocardial inflammation in some small studies with a limited number of patients (10–12). However, reports on AAbs in patients with ICI myocarditis are limited. A recent study reported that the presence of anti-acetylcholine receptor (AChR) antibodies was more prevalent in patients with ICI myocarditis than in ICI-treated control patients. Among patients with ICI myocarditis, anti-AChR antibodies were associated with a higher incidence of cardiomyotoxic events (a composite of life-threatening arrhythmias, severe heart failure, severe respiratory muscle failure, or cardiomyotoxic death) (13). In addition to these findings, no comprehensive investigation on the presence of AAbs in patients with ICI myocarditis has been performed to date.

If AAbs associated with the development of ICI myocarditis are identified, screening could be useful for the diagnosis and prediction of ICI myocarditis. Moreover, treating ICI myocarditis with immunoabsorption or plasma exchange could be a valuable addition to the current standard corticosteroid therapy (14). Furthermore, AAb profiling may help further elucidate the pathogenesis of ICI myocarditis. Therefore, we conducted a pilot study to characterize AAb profiles in cancer patients with ICI myocarditis.

2 Material and methods

2.1 Clinical data and sample collection

This study included six serum samples from three patients with ICI myocarditis. Patients were enrolled in a prospective biospecimen collection protocol approved by the Ethics Committee of the University of Tsukuba Hospital (H30-221). Written informed consent was obtained from all patients. Clinical, radiographic, and laboratory data were collected from electronic medical records. Blood samples were collected before ICI treatment and at the onset of ICI myocarditis (before steroid therapy). The collected serum samples were aliquoted into smaller volumes and stored at −80°C until further use.

2.2 Serum autoantibody profiling

Comprehensive profiling of serum AAbs was conducted using HuProt™ Human Proteome Microarray v3.1 (CDI Laboratories, Inc, Baltimore, MD, USA), which contained about 17,000 unique proteins. One array per sample was used for the serum profiling. All serum samples were probed on the arrays at a 1:1000 dilution, as optimized by CDI labs, and incubated overnight at 4°C to enhance potential interactions. After probing, the arrays were washed according to the manufacturer's protocol and probed with anti-human immunoglobulin G (IgG) antibodies optimized by the CDI laboratories for signal detection.

Non-specific hits that directly bound to the secondary antibody were eliminated from the analysis. The CDI software was used to quantify the specificity of each sample for specific proteins on the array based on Z-scores. The Z-score is the average Z-score of the duplicate spots of a given protein (each protein is printed in duplicate on a HuProt™ array). Z score was calculated as: $Z = [F_{protein} - F_{average}]/F_{SD}$ where $F_{protein}$ is the fluorescence signal intensity of a specific protein, $F_{average}$ is the mean fluorescence signal intensity of all protein spots on the array, and F_{SD} denotes the standard deviation of all protein spots on the array. Positive hits were defined as Z-scores > 3.0. AAbs that increased after the onset of ICI myocarditis were identified by following

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TABLE 1 Patient information.

Case	Age	Gender	Malignancy	Number of ICI cycles	Time to myocarditis onset from ICI start (days)	irAEs	Treatment	In-hospital outcome
1	73	M	RCC	2	29	Myocarditis, Myositis, MG-like syndrome	High-dose corticosteroids	Alive
2	57	F	NSCLC	1	24	Myocarditis	High-dose corticosteroids	Dead
3	80	M	Bladder cancer	1	29	Myocarditis, Myositis	High-dose corticosteroids, intravenous immunoglobulin	Alive

ICI, immune checkpoint inhibitor; irAE, immune-related adverse event; MG, myasthenia gravis; NSCLC, non-small-cell lung cancer; RCC, renal cell carcinoma.

criteria: (1) statistical differences between baseline and after developing ICI myocarditis ($P < 0.05$), assessed using the Mann-Whitney U test; and (2) fold change (FC) ≥ 2.0.

Gene Ontology (GO), Kyoto Encyclopedia of Genes and Genomes (KEGG) functional, and DisGeNET disease enrichment analyses, based on Metascape software (https://metascape.org/, version: v3.520240101) were used to explore their biological significance and characterize diseases associated with AAbs, as performed previously (15, 16). We combined the increased autoantibodies from the three patients, according to the above description ($P < 0.05$ and fold change (FC) ≥ 2.0) in one list. Then, the list was mapped to the coding genes. Finally, the list of coding genes of the increased autoantibodies was performed GO enrichment analysis and KEGG pathway enrichment analysis via Metascape, respectively. GO or KEGG pathway terms with a P-value < 0.01, a minimum count of three, and an enrichment factor > 1.5 (the enrichment factor is the ratio between the observed counts and the counts expected by chance) were collected and grouped into clusters based on their membership similarities. Top 20 identified GO enrichment terms and KEGG pathway were presented.

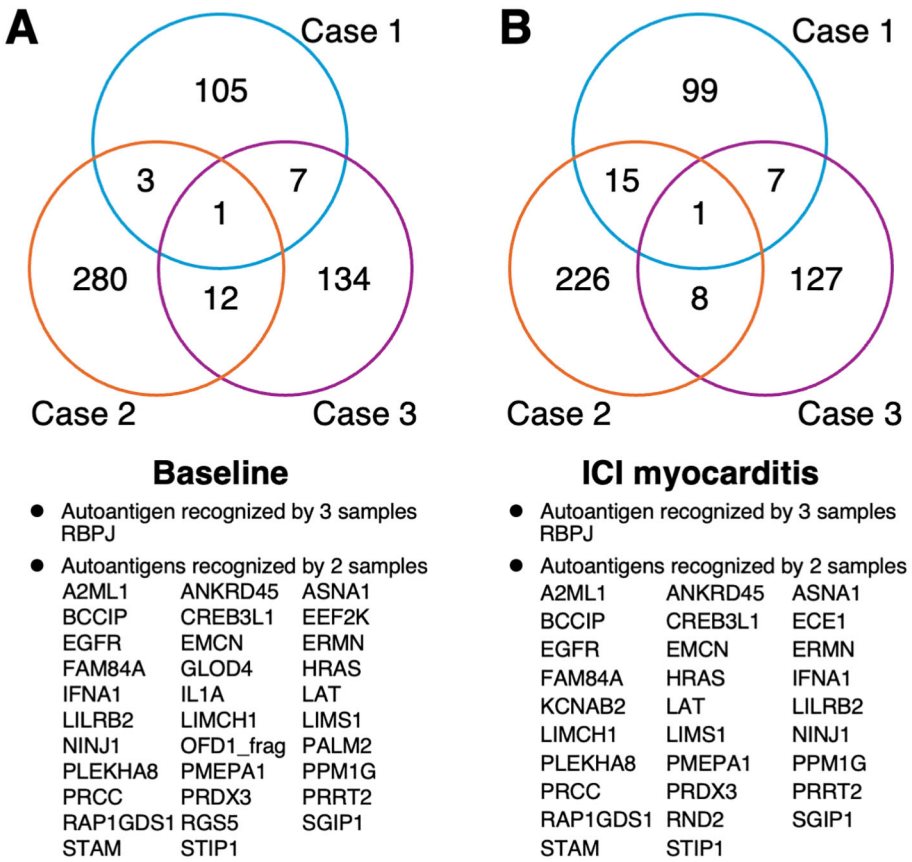

FIGURE 1
Venn diagram of the number of the immune-reactive autoantigens identified by human proteome microarray. **(A)** Before ICI treatment and **(B)** at the onset of ICI myocarditis **(B)**. ICI, immune checkpoint inhibitor.

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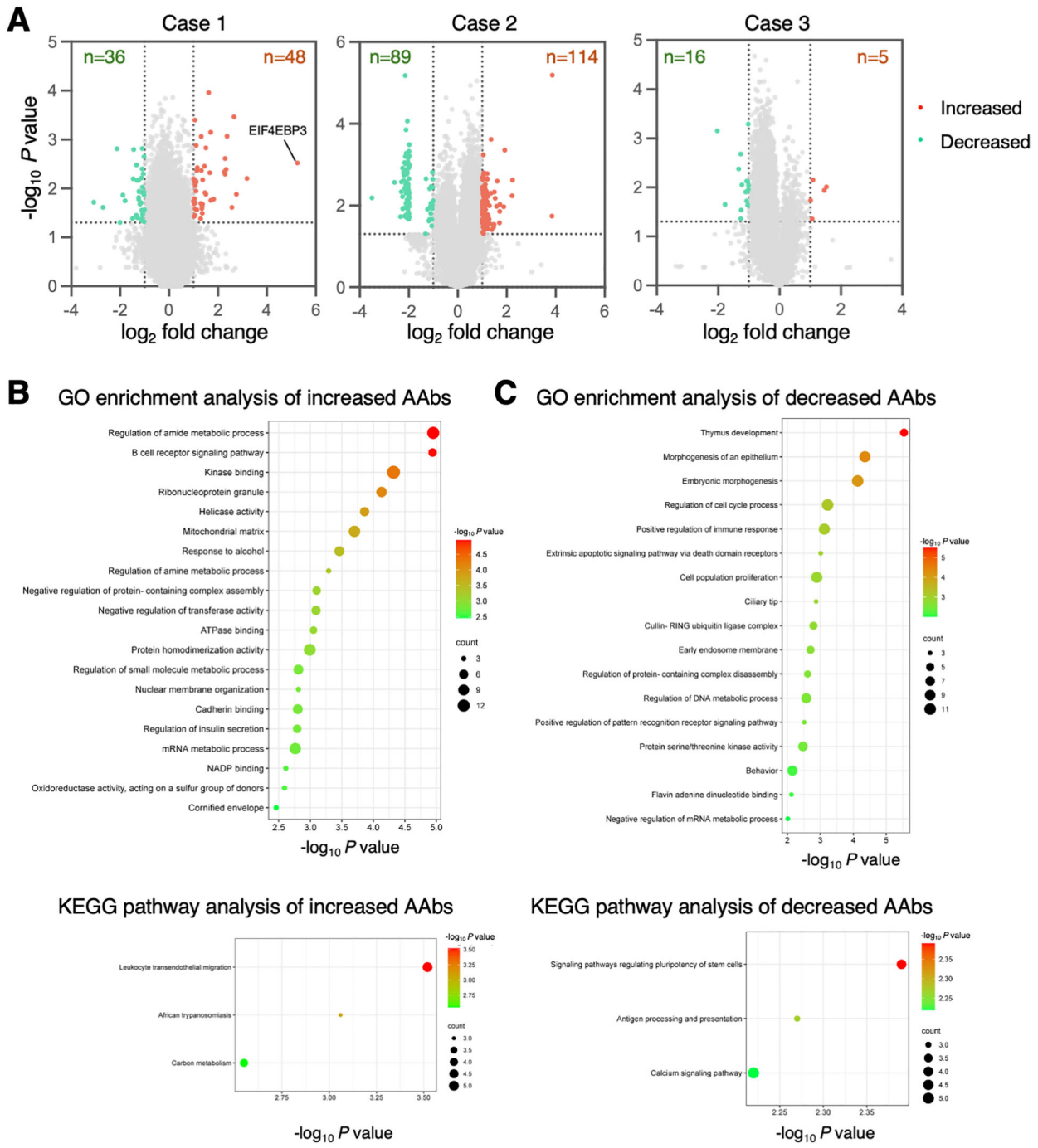

FIGURE 2
Differentially expressed autoantibodies in ICI myocarditis. **(A)** The volcano plots show the up- (red) or down-regulated (green) proteins at the onset of ICI myocarditis compared to baseline. **(B, C)** The top 20 GO enrichment terms and KEGG pathway categories involving upregulated **(B)** and downregulated IgG **(C)** antibodies. Negative $\log_{10}$-transformed *P*-value of significantly enriched GO terms and KEGG pathways ($P < 0.01$) are shown.

3 Results

Table 1 summarizes the clinical characteristics of the patients with ICI myocarditis included in this study. All patients received anti-programmed death-1 antibody monotherapy and developed myocarditis after 1-2 cycles of treatment. All patients were diagnosed with definite myocarditis based on definitions suggested by Bonaca et al. (17) and had tissue pathology suggestive of myocarditis on the endocardial biopsy. Two of the three patients had other concomitant myotoxicities (myositis with or without myasthenia gravis-like syndrome). All patients were treated with high-dose steroids, and one patient received additional intravenous immunoglobulin. Two patients were discharged alive, and one died of severe heart failure.

To identify AAb signatures in ICI myocarditis, we performed AAb screening using a human proteome microarray containing

approximately 17,000 unique full-length human proteins. We used sera collected before the initiation of ICI treatment (baseline) and immediately after the development of ICI myocarditis (before steroid treatment). At baseline, 116, 296, and 154 autoantigens reacted positively to IgG in the serum samples from Cases 1, 2, and 3, respectively (Supplementary Table 1). Among these proteins, one autoantigen (recombination signal binding protein for immunoglobulin kappa J region [RBPJ]) was recognized by all three samples, and 32 autoantigens were recognized by at least two of the three samples (Figure 1A). At the onset of ICI myocarditis, 122, 250, and 143 autoantigens reacted positively to IgG in the serum samples from Cases 1, 2, and 3, respectively (Supplementary Table 1). Among these proteins, RBPJ was recognized by all three samples. Additionally, 29 autoantigens were recognized by any two of the three samples (Figure 1B).

Next, we identified the specific and differentially expressed antibodies at the onset of ICI myocarditis. Antibodies with a change in antigen-antibody reactivity signal intensity of more than 2.0-fold or less than 0.5-fold ($P < 0.05$) between baseline and the onset of myocarditis were considered AAbs with different expression levels. The differentially recognized autoantigens are visualized in volcano plots (Figure 2A) and described in Supplementary Table 2. At the onset of ICI myocarditis, compared to baseline, 48, 114, and 5 autoantigens reacted more strongly with IgG in serum samples from Cases 1, 2, and 3, respectively, and 36, 89, and 16 autoantigens reacted less strongly, respectively. Among these, antibodies against EIF4EBP3 (eukaryotic translation initiation factor 4E [eIF4E] binding protein 3) were the most upregulated, showing a 38-fold increase (Figure 2A).

To better understand the biological relevance of the identified Abs, GO enrichment analysis was performed on their corresponding proteins. The results revealed that Abs increased during the development of ICI myocarditis and were profoundly involved in the regulation of amide metabolic processes and B-cell receptor signaling pathways (Figure 2B). In the KEGG pathway enrichment analysis, leukocyte transendothelial migration pathway was remarkably enriched (Figure 2B). In the AAbs that decreased during the development of ICI myocarditis, the most significantly enriched GO term was related to thymus development function (Figure 2C), and KEGG pathway enrichment analysis showed that the decreased AAbs were mainly involved in the signaling pathways regulating the pluripotency of stem cells and antigen processing and presentation (Figure 2C).

Finally, we performed a DisGeNET disease enrichment analysis to explore the diseases in which these AAbs were involved (Figure 3). Enrichment diseases include T lymphocyte deficiency, muscle weakness, and dilated or familial idiopathic cardiomyopathy.

4 Discussion

This pilot study represents the first integrated analysis of serum AAb profiles in patients with ICI myocarditis, shedding light on potential novel candidate markers associated with the heightened risk of developing this and its pathogenesis.

Although RBPJ wasn't involved in the pathways identified through GO or KEGG analysis, AAbs against RBPJ were detected in the sera of all patients with ICI myocarditis. RBPJ is a transcription factor that plays a crucial role in the Notch signaling pathway (18). The Notch signaling pathway mediated by RBPJ is involved in various cellular processes, including cell fate determination, differentiation, proliferation, and apoptosis, and plays critical roles in embryonic development, tissue homeostasis, and disease processes, such as cancer and heart diseases (19). There are few reports on AAbs targeting RBPJ, and their clinical significance remains unclear. Nickenig et al. reported an interesting finding that only 31% of healthy patients and 70.6% of patients with dilated cardiomyopathy (DCM) carry AAbs against RBPJ (20). They speculated that, because

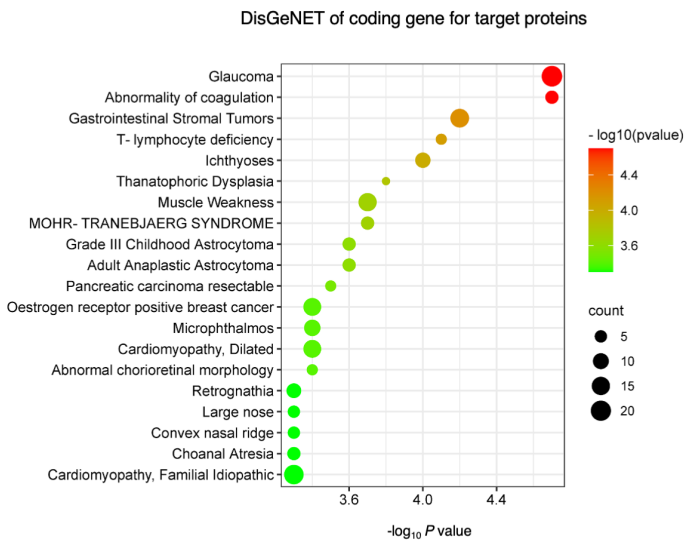

FIGURE 3
DisGeNET disease enrichment analysis. The top 20 diseases enriched in DisGeNETs were associated with upregulated and downregulated IgG antibodies. Negative log10-transformed P-value for significantly enriched diseases (P < 0.01) are shown.

RBPJ is involved in cellular immortalization and exerts anti-apoptotic effects, increased anti-RBPJ AAbs may inhibit this growth-regulating feature of RBPJ in patients with DCM. Further research is needed to determine whether anti-RBPJ AAbs can be used as biomarkers for ICI myocarditis and whether they are involved in the pathogenesis of ICI myocarditis.

The AAbs that increased most remarkably during the development of ICI myocarditis were anti-EIF4EBP3 AAbs in Case 1. EIF4EBP3 is an eIF4E-binding protein that inhibits translation initiation by competing with eukaryotic translation initiation factor 4G for a common binding site on eIF4E (21–23). In our study, EIF4EBP3 was involved in the "regulation of amide metabolic process" term in the GO analysis results of coding genes for target proteins recognized by increased AAbs. Little is known about the role of EIF4EBP3 under normal and abnormal conditions, and its role of EIF4EBP3 in the heart is completely unknown.

Recent studies have shown that the presence of anti-AChR AAbs is more prevalent in patients with ICI myocarditis than in ICI-treated controls (11-36% vs 4%) (13, 24). Among the patients with ICI myocarditis, those with anti-AChR antibodies have a poorer prognosis than those without (13). However, we did not detect any anti-AChR antibodies in our study.

Enrichment analyses using GO and KEGG highlighted that B-cell receptor signaling, leukocyte transendothelial migration, and thymus development were among the most affected pathways. Enrichment analyses using DisGeNET revealed that proteins reacting with AAbs detected in patients with ICI myocarditis were associated with several diseases, including DCM and muscle weakness. This pilot study suggests that profiling serum AAbs could enhance our understanding of the pathogenesis of ICI myocarditis and facilitate the development of effective therapies, a more comprehensive search for AAbs in more cases is needed in the future.

Although our study offers new insights into the properties and changes in the AAb repertoire associated with ICI myocarditis, it has some limitations. The small sample size included in this study limits the generalizability of the present findings. In addition, we did not perform AAb profiling in ICI-treated controls (i.e., without myocarditis). Consequently, it is crucial to validate the identified Abs in larger, more diverse, and independent cohorts with appropriate controls to elucidate their roles in ICI myocarditis.

5 Conclusion

Human proteomic microarrays offer a powerful platform for the discovery of novel antibodies in patients with ICI myocarditis. This pilot study provides the first integrated analysis of serum AAb profiling in patients with ICI myocarditis and identifies novel candidate markers associated with an increased risk of developing ICI myocarditis and its pathogenesis. However, our results require further independent validation in clinical trials involving a larger number of patients. Further identification and characterization of AAbs are likely to hold significant implications for diagnostic and biomarker discovery, immune profiling, and the development of effective treatments.

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Ethics statement

The studies involving humans were approved by Ethics Committee of the University of Tsukuba Hospital. The studies were conducted in accordance with the local legislation and institutional requirements. The participants provided their written informed consent to participate in this study.

Author contributions

SL: Data curation, Formal analysis, Investigation, Writing – original draft. DX: Project administration, Supervision, Visualization, Writing – review & editing. NM: Methodology, Project administration, Supervision, Writing – review & editing. ZY: Investigation, Methodology, Writing – review & editing. TI: Supervision, Writing – review & editing. KT: Conceptualization, Funding acquisition, Investigation, Writing – review & editing.

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Conflict of interest

KT received honoraria lecture fees from Bristol Myers Squibb, Pfizer, Ono Pharmaceutical, Chugai, and AstraZeneca.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2024.1423622/full#supplementary-material

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Genetic investigation and diagnosis in adults with congenital heart disease with or without structural or neurodevelopmental comorbidity: a retrospective chart review

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Introduction: Genetic evaluation is indicated for individuals with congenital heart disease (CHD), especially if extracardiac anomalies are also present. Timely recognition of genetic diagnoses can facilitate medical management and as well as provide assessment of reproductive risk. At least 20% of the pediatric population with CHD has a syndrome or genetic diagnosis. Further, at least 30% have extracardiac congenital malformations and/or neurodevelopmental differences (NDD), and this is known to increase the likelihood of a genetic/syndromic diagnosis. However, little is known regarding whether these statistics also apply to the current population of adults living with CHD, many of whom were born prior to currently available genetic testing.

Methods: The primary aim of this study was to determine the prevalence of documented genetic and syndromic diagnoses in a cohort of adults with CHD followed by a dedicated adult CHD (ACHD) clinic. The secondary aims were to describe genetic testing and genetic referral patterns in this population and identify the presence of extracardiac comorbidities which are known to be indicative of an underlying genetic diagnosis in the pediatric CHD population. To answer these questions, we performed a retrospective chart review on a sample of adults with CHD (excluding those with isolated bicuspid aortic valve) seen at Cincinnati Children's Hospital in the ACHD clinic between 2010–2021.

Results: Among 233 adult CHD patients, 36 (14%) had a documented genetic or syndromic diagnosis but only 29 (13.7%) had received genetic testing, while 27 (11.6%) had received genetic referrals. Furthermore, of 170 patients without any

Abbreviations: ACHD, Adult (or adults with) congenital heart disease; ADHD, Attention deficit hyperactivity disorder; CCHMC, Cincinnati Children's Hospital Medical Center; CHD, Congenital heart disease; EMR, Electronic medical record; NDD, Neurodevelopmental disorders; WES, Whole exome sequencing; WGS, Whole genome sequencing

documented genetics related care (defined as genetic testing, genetic referrals, or genetic diagnosis), 35 (20%) had at least one congenital and/or neurodevelopmental comorbidity. Factors associated with individuals having received genetics related care included younger age (<40), male sex, and presence of extracardiac comorbidities.

Discussion: Our results indicate important gaps in genetics-related care for adults living with CHD. The subset of our cohort with congenital and/or neurodevelopmental comorbidities who received no genetic-related care, represent a population of adults with CHD who may have unrecognized genetic diagnoses.

KEYWORDS

adult congenital heart disease, extracardiac comorbidity, genetic testing, neurodevelopmental comorbidity, cardiology

1 Introduction

Congenital heart disease (CHD) is one of the most prevalent birth defects, occurring in approximately 1 out of every 100 live births (Mitchell et al., 1971; Van Der Linde et al., 2011; Marino et al., 2012). Medical advances have decreased mortality rates in individuals with CHD. As many as 90% of children with CHD now reach adulthood (Khairy et al., 2010; Ntiloudi et al., 2016; Billotte et al., 2021), and adults living with CHD outnumber children with CHD (Pierpont et al., 2018).

The pediatric CHD patient population has been well-studied with respect to extracardiac comorbidities and neurodevelopmental disorders and have increased rates of both. Approximately 20%–30% of the pediatric CHD population have congenital extracardiac abnormalities (Massin et al., 2007; Ferencz et al., 1989; Helm and Ware, 2024). It has been shown that multisystem involvement increases the likelihood of genetic diagnoses in children with CHD (Massin et al., 2007; Bracher et al., 2017; Cohen et al., 2013; Shikany et al., 2020) and in general, patients with genetic syndromes are more likely to have extracardiac comorbidities compared with non-syndromic patients (Bracher et al., 2017; Khanna et al., 2019). However, not all patients presenting with CHD and coexisting extracardiac diagnoses have a specific, identifiable genetic syndrome, and individuals with a syndromic diagnosis may present with apparently isolated CHD and no other comorbidities. Therefore, isolated CHD and apparent lack of syndromic diagnosis does not exclude the possibility of genetic etiology for CHD (Massin et al., 2007; Bracher et al., 2017; Hoang et al., 2018).

The testing currently available and offered to infants with CHD was developed within the past one to 2 decades. With development of chromosomal microarray and next-generation sequencing (NGS) genetic testing technology in the early 2000s (Rauch et al., 2004; Slatko et al., 2018), diagnostic capabilities of genetic testing have greatly improved as has recognition of the utility of genetic testing for individuals with CHD. Clinical genetic evaluation and broad genetic testing that is now readily accessible (genome and exome sequencing) were essentially unavailable for adult patients at the time of their cardiac diagnosis (Lalani, 2020; Zaidi and Brueckner, 2017). We hypothesized that the prevalence of documented (recognized) genetic diagnoses in an ACHD population would be lower than pediatric populations and that the population of adults living with CHD would have relatively low rates of genetic testing and referral compared with what is typically provided to individuals born in more recent years. Identifying and defining gaps in genetics-related care for the ACHD population are important steps in improving referral and diagnosis rates and ultimately improving patient care. Therefore, we performed a retrospective study to identify the prevalence of three elements of genetic-related care (syndromic/genetic diagnoses, genetic referral, genetic testing) in an adult CHD population, and identify patient traits and characteristics, including congenital and neurocognitive comorbidities, that are associated with having received genetic-related care and/or are indicators of a population that would benefit from genetics care.

2 Materials and methods

2.1 Selection and description of participants

A retrospective chart review was performed with approval from the Cincinnati Children's Hospital Medical Center (CCHMC) Institutional Review Board for adults with CHD who received care at between 1/1/2010 and 10/31/2021. We queried the electronic medical record (EMR) to identify adult patients (≥18 years at time of query) who had a CCHMC cardiology visit of any of the 11 EMR visit types used in the ACHD Clinic (see Supplementary Table S1). Visit types in the EMR are digital templates designated by 3- to 5-digit codes that are designed for specific clinic use. In the ACHD clinics, this includes templates for new patients, follow-up visits, and Fontan clinic-specific scenarios. A total of 2,275 unique patients resulted from the EMR query. We determined that a minimum of 108 patients should be included for appropriate statistical power. This was calculated based on our primary hypothesis that the proportion of genetic diagnoses in adults with CHD differs from that in pediatric patients with CHD. Previous studies have suggested that the proportion in pediatric patients is 20%. In our adult cohort, the proportion is expected to be 10%. A total of 108 subjects will allow us to detect the difference with 80% power when the type I error rate is set at 0.05. Using the Microsoft Excel (Version 2201) randomizing tool, we randomly sampled the queried list of patients to select approximately equal number by age group (<40 years of age

TABLE 1 Characteristics of adults with CHD. N = 233 except where data was missing from chart and in "Genetic diagnosis," where 4 patients were excluded due to syndromic diagnoses not traditionally associated with CHD. Excluded patients were counted in "Genetic diagnosis by type" under "Other."

Characteristics of adults with CHD.	N	%
Age (y) (n = 233)		
18–40	132	43.4
40+	101	56.7
Sex (n = 233)		
Male	95	40.8
Female	138	59.2
Race(n = 230)		
White	200	87.0
Black	23	10.0
Other	4	1.7
Mixed race (2 or more)	3	1.3
Cardiac lesion (n = 233)		
Tetralogy of Fallot or DORV or pulmonary atresia	46	19.7
Left-sided obstructive lesions	35	15.0
AV septal defect	19	8.2
Valvar pulmonary stenosis	17	7.3
D-TGA or physiologically corrected TGA (systemic right ventricle) or s/p arterial switch (systemic left ventricle)	16	6.9
Ebstein/Uhl anomaly	8	3.4
Heterotaxy spectrum	4	1.7
Simple shunt lesions	60	25.6
Miscellaneous/other	16	6.9
Acquired comorbidities (n = 233)		
None	126	54.3
At least one	106	45.7
Congenital + ND comorbidities (n = 228)		
Only congenital comorbidity	45	19.5
Only ND comorbidity	13	5.6
Both congenital and ND comorbidity	21	9.1
None	152	65.8
Family history of CHD (n = 233)		
No	202	86.7
Yes	31	13.3
History of any CHD-related genetic testing (n = 211)		
No	182	86.3
Yes	29	13.7
History of genetic referral (n = 233)		
No	206	88.4
Yes	27	11.6
Genetic diagnosis (n = 233)		
No	197	84.5
Yes	32	13.7
Yes, likely unrelated to CHD	4	1.7
Genetic diagnosis by type (n = 36)		
Down	17	47.2
22q11 (DiGeorge, CATCH 22, VCF)	7	19.4
Turner	2	5.6
Williams	2	5.6
CHARGE	2	5.6
Other	5	13.9
Not specified	1	2.8

(Continued in next column)

TABLE 1 (*Continued*) Characteristics of adults with CHD. N = 233 except where data was missing from chart and in "Genetic diagnosis," where 4 patients were excluded due to syndromic diagnoses not traditionally associated with CHD. Excluded patients were counted in "Genetic diagnosis by type" under "Other."

and ≥40 years of age) and sex. We selected 325 cases for further review.

2.2 Inclusion/exclusion criteria

For 325 randomly selected cases, we verified patient age at most recent ACHD clinic visit, the presence of a personal CHD diagnosis, and adherence to all other inclusion and exclusion criteria. Patients were excluded if they were less than 18 years old at time of most recent ACHD clinic visit (8 patients); if they did not have a primary diagnosis of CHD (31 patients); or if they were not seen in the ACHD clinic (29 patients). Patients with isolated bicuspid aortic valve and/or thoracic aortic aneurysm and patients with aortic dilation were also excluded (24 patients). Of note, patients with Noonan syndrome and Marfan/Loeys Dietz syndromes are seen in separate dedicated clinics at CCHMC and therefore were excluded by our search criteria. Of the 325 initially selected cases, 92 were excluded. The final cohort consisted of 233 patients (demographics in Table 1).

2.3 Chart review methods

Data collected from the EMR were recorded in a REDCap database (see Supplementary Material). We collected information about established genetic diagnoses, history of genetic testing, and history of genetic referrals. We also collected detailed data on cardiac

history and lesion type, and extracardiac comorbidities (including acquired conditions and congenital/structural anomalies).

Patients were considered to have a genetic or syndromic diagnosis if they had a pathogenic/likely pathogenic genetic test result, or a clinical diagnosis of a syndrome (e.g., Down syndrome) documented in their medical record, even if confirmatory genetic testing was not conducted or if that record was missing. A patient was defined as having had genetic testing if records of genetic testing were available or if genetic testing was specifically referenced in clinical notes, even if the original report was missing. We recorded genetic referrals when the patient had documentation of a clinical genetic evaluation or if a genetics referral request had been placed in the EMR, even if this had not yet been completed.

CHD diagnoses were classified by dominant CHD type (i.e., the most severe diagnosis, in terms of impact on clinical status). If dominant CHD type was not clearly documented in the patient's chart, the chart was reviewed by an ACHD clinic cardiologist (AO) to clarify the dominant CHD type.

We collected information about each patient's neurodevelopmental status, acquired medical comorbidities (e.g., gastrointestinal reflux disease, hypertension), and congenital conditions (e.g., craniofacial dysmorphism, cleft palate). We assessed neurodevelopmental status based on documentation of neurocognitive disorders (e.g., intellectual disability) and highest level of school completed. We distinguished neurodevelopmental comorbidities from neurological comorbidities (e.g., stroke, seizures, migraines) and psychiatric comorbidities (e.g., depression, anxiety, schizophrenia). Neurodevelopmental comorbidities included attention deficit hyperactivity disorder (ADHD), cognitive impairment/developmental delay, and autism spectrum disorder.

We documented whether each patient had any biological children, any reported family history of CHD in first, second, and/or third-degree relative(s), and if affected relatives had extracardiac or neurodevelopmental comorbidities. Family history variables were collapsed into 'family history' or 'no family history' for analysis.

2.4 Statistical analyses

Prior to analysis, the quality and distribution of the data were examined. Demographics and clinical characteristics of the cohort were described using frequencies (proportions). To compare the genetic diagnosis rate of our cohort to that of previously reported pediatric cohorts, we conducted a one-sample proportion test. The associations of genetic diagnoses, referral, and testing with demographics and clinical characteristics were tested using Fisher's exact tests. All analyses were performed using SAS 9.4 (company, Cary, NC). A p-value ≤ 0.05 was used to indicate the statistical significance.

3 Results

Demographics and clinical characteristics of the cohort are summarized in Table 1. The majority (86.7%) of patients were White and approximately 59% were female. The cohort was roughly evenly split between those younger and older than 40 years at time of chart review. The two most prevalent types of cardiac lesions were left-sided obstructive lesions (19.7%) and tetralogy of Fallot (15%).

Genetic or syndromic diagnoses were documented in 36 patients (36/233, 15%). However, we discovered four patients with syndromic diagnoses that are not typically associated with CHD, including Long QT syndrome, Charcot-Marie-Tooth syndrome, and hypermobile Ehlers Danlos syndrome. Therefore, these were excluded from the group with genetic syndromes and not included in the nonsyndromic group. Down syndrome made up 47% of the syndromic diagnoses (17/36), followed by 22q11.2 microdeletion syndrome (7/36, 19%).

Twenty-nine patients (29/211, 13.7%) had documented genetic testing. Of note, the 36 patients with genetic/syndromic diagnoses only encompassed 10 of the 29 with documented pathogenic findings on genetic testing, indicating that the majority of the 36 had clinical diagnoses. Of those with genetic testing, the majority (21/29, 72.4%) had only one test conducted, with FISH being the most common single test performed. Twelve patients had FISH testing which confirmed diagnosis of 22q11.2 microdeletion syndrome in 5. Six patients had microarray, all of which had normal results. Eight patients received single gene and/or multigene panel testing, which produced a diagnostic result in a single patient. Four patients had single gene or multigene panel testing in tandem with at least one other genetic test. No patients had exome or genome sequencing (Supplementary Table S2).

Twenty-seven patients (27/233, 11.6%) received a referral to a geneticist and/or genetic counselor, with a consult completed in 19/27 (70%). The remainder of referrals were pending appointments (n = 5) or missing data (n = 2) about referral status. Of the 27 with referrals, 11 had a genetic/syndromic diagnosis.

We tested the associations of demographics and clinical characteristics with the likelihood of a genetic diagnosis, genetic referral, or genetic testing (Table 2). Younger patients (<40 years of age) were more likely to have genetic testing on record ($p = 0.048$) and more likely to have a documented genetic/syndromic diagnosis ($p = 0.003$). Younger patients were more likely to have received a genetics referral (15.1% vs 6.9%), though this difference did not reach statistical significance. Cardiac lesion type was associated with presence of a genetic diagnosis ($p < 0.001$). While there was not a significant association detected between lesion type and genetic testing, a higher percentage of patients with tetralogy of Fallot received genetic testing compared to other cardiac lesion groups (29.3% versus <25%).

Approximately 34% (79/228) of the cohort had at least one congenital and/or neurodevelopmental comorbidity (Figure 1). These patients were more likely to have genetic/syndromic diagnosis ($p < 0.001$), referral ($p < 0.001$), or testing ($p < 0.001$) if at least one congenital and/or neurodevelopmental comorbidity were present (Table 2). Increasing number of congenital and neurodevelopmental comorbidities is also associated with genetic referral ($p < 0.001$) (Table 3). By body system, those with a history of craniofacial ($p < 0.001$), skeletal ($p < 0.001$), or genitourinary and anorectal ($p = 0.048$) abnormalities were more likely to have genetic referrals on record. Of the neurodevelopmental comorbidities, ADHD and cognitive impairment were associated with genetic referral ($p < 0.05$). Of 170 patients who had no genetic testing, referral, or syndromic diagnosis, 35 (20%) had at least one congenital and/or neurodevelopmental comorbidity.

TABLE 2 Characteristics of adults with CHD by genetic referral, testing, and diagnosis. N is not equal across all categories due to missing data except in "Number of Relatives with CHD", which contains only the patients with a family history of CHD, and in "Genetic Diagnosis," where 4 patients were excluded due to syndromic diagnoses not traditionally associated with CHD.

Characteristics of adults with CHD by genetic referral, testing, and diagnosis status											
All traits	n	Genetic referral		p	n	Genetic testing		p	n	Genetic diagnosis		p
		Yes	No			Yes	No			Yes	No	
Age	233	N (%)	N (%)	0.06	211	N (%)	N (%)	0.048	229	N (%)	N (%)	0.003
18–40		20 (15.5)	112 (84.9)			21 (18.6)	92 (81.4)			26 (20)	104 (80)	
>40		7 (6.9)	94 (93.1)			8 (8.2)	90 (91.8)			6 (6.1)	93 (93.9)	
Sex	233			0.06	211			0.84	229			0.17
Male		16 (16.8)	79 (83.2)			13 (14.6)	76 (85.4)			9 (9.7)	84 (90.3)	
Female		11 (8)	127 (92)			16 (13.1)	106 (86.9)			23 (16.9)	113 (83.1)	
Race	230			0.38	209			0.84	226			
White		22 (11)	178 (89)			25 (13.9)	115 (86.1)			29 (14.8)	167 (85.2)	0.84
Black		5 (21.7)	18 (78.3)			4 (18.2)	18 (81.8)			2 (8.7)	21 (91.3)	
Other		0	7 (100)			0 (0)	7 (100			0 (0)	7 (100)	
Cardiac lesion	233			0.37	211			0.07	229			<0.001
ToF, DORV, PA		9 (19.6)	37 (80.4)			12 (29.3)	29 (70.7)			9 (19.6)	37 (80.4)	
Left-side obstructive		4 (11.4)	31 (88.6)			3 (9.1)	30 (90.9)			3 (8.6)	32 (91.4)	
AV septal		0	19 (100)			0	10 (100)			10 (52.6)	9 (47.4)	
Valvar PS		1 (5.9)	16 (94.2)			0	17 (100)			0	17 (100)	
D-TGA, phys. Corrected TGA, s/p arterial switch		3 (18.8)	13 (81.3)			1 (6.3)	15 (93.7)			0	16 (100	
SV Fontan or complex of SV unrepaired cyanotic		2 (16.7)	10 (83.3)			2 (16.7)	10 (83.3)			0	12 (100)	
Ebstein/Uhl		0	8 (100)			0	8 (100)			0	8 (100)	
Heterotaxy		1 (25)	3 (75)			1 (25)	3 (75)			0	4 (100)	
Simple shunt		6 (Helm and Ware, 2024)	54 (90)			7 (12.7)	48 (87.3)			7 (12.5)	49 (87.5)	
Miscellaneous/other		1 (6.3)	15 (93.8)			3 (20)	12 (80)					
Congenital/ND comorbidities	228			<0.001	208			<0.001	229			<0.001
Congenital		5 (11.1)	40 (88.9)			5 (13.9)	31 (86.1)			11 (24.2)	34 (75.6)	
ND		4 (30.8)	9 (69.2)			5 (41.7)	7 (58.3)			1 (7.7)	12 (92.3)	
Congenital + ND		8 (38.1)	13 (61.9)			10 (83.3)	2 (16.7)			16 (80)	4 (20)	
None		9 (5.9)	143 (94.1)			9 (6)	141 (94)			2 (1.3)	147 (98.7)	
Fam history CHD	233			0.22	211				30			0.58
Yes		6 (19.4)	21 (84)			7 (25)	21 (75)			5 (20.8)	19 (79.2)	
No		21 (10.4)	4 (66.7)			22 (12)	161 (88)			0	6 (100)	

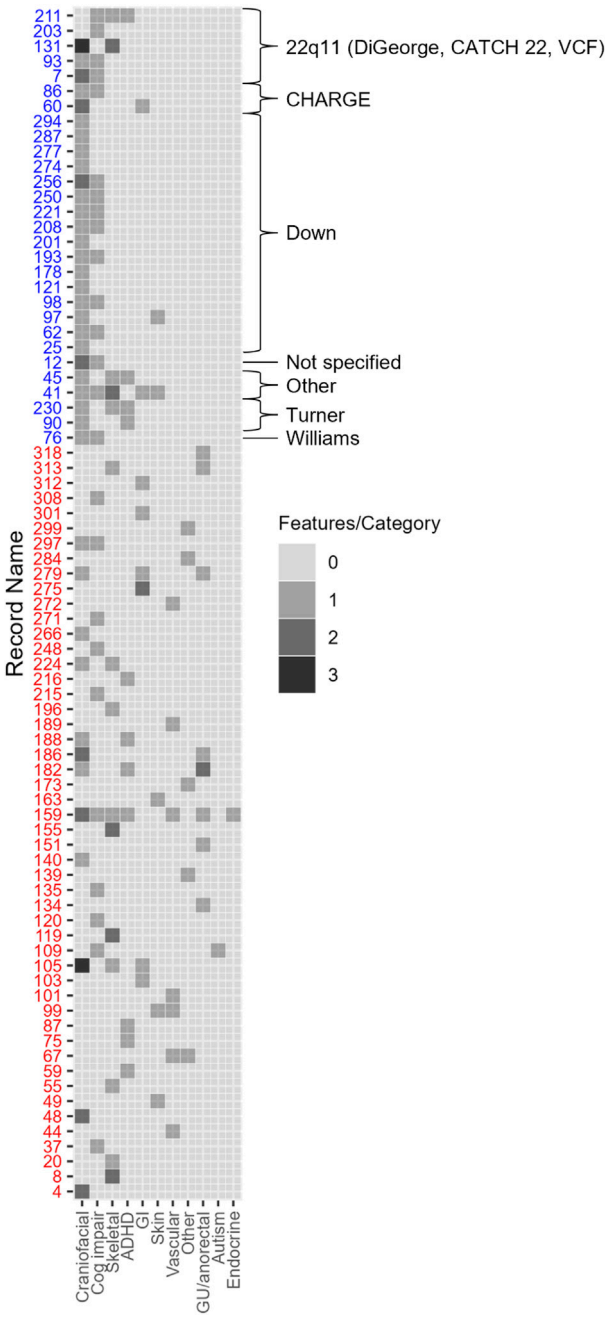

FIGURE 1
79 adults with CHD had extracardiac and/or neurodevelopmental comorbidities. Each row represents one patient with syndromic cases noted by blue text. Craniofacial comorbidities include craniofacial dysmorphism and anomalies of the brain, ear, nose, throat, palate, and eyes. Vascular comorbidities include anomalies of the lungs, lymphatic system, and arteriovenous malformations. GI and abdominal comorbidities include anomalies of the abdominal wall, kidney, spleen, pancreas, liver, and gall bladder. Number of comorbidities in each category was tabulated from each case from the data entry form (Supplementary Material) and boxes are shaded according to the number of manifestations present in each category shown on the *x*-axis.

Of the cohort, thirty-one patients (31/233, 13.3%) had a documented family history of CHD. Positive family history of CHD was not significantly associated with genetic testing, genetic referral, or genetic diagnosis, although a higher proportion of patients with a positive family history had genetic testing compared with those who had no family history (7/28, 25% versus 22/183, 12%). As expected, lesions with higher heritability (Oyen et al., 2009) had the highest incidence of positive family history (Table 4), including shunt lesions (25.8%), tetralogy of Fallot (16%), and left-sided obstructive lesions (16%) although this result did not reach statistical significance (p = 0.53). Five patients were reported to have a biological child with CHD which included 1 vascular ring, 1 ASD, 1 VSD and 1 unknown.

4 Discussion

There is widespread agreement that adults with CHD need multidisciplinary care in light of increased survival from childhood to adulthood and the spectrum of extracardiac and neurodevelopmental diagnoses in the CHD population (Bracher et al., 2017; Pierpont et al., 2007; Stout et al., 2018). Currently, there are no established guidelines governing referral of adult patients with extracardiac anomalies or neurocognitive diagnoses to outside specialties such as genetics (Cohen et al., 2013). Our study demonstrated that there are gaps in genetics-related care for adults with CHD at our institution. The overall prevalence of a genetic/syndromic diagnosis in our cohort was 14%, which is similar to what has been reported in incompletely sequenced/tested pediatric cohorts such as the initial description of the Pediatric Cardiac Genomics Consortium (PCGC) cohort which reported genetic/syndromic diagnosis in 11% of ~9,700 children (Hoang et al., 2018). Rates of trisomy 21 and deletion 22q11 syndrome, two genetic syndromes with well-established associations to CHD, were also similar between our adult CHD cohort and the 2018 PCGC cohort description (47% adult vs. 38% PCGC for Trisomy 21, and 19% adult vs. 24% PCGC for deletion 22q11). This, plus additional prior similarly described cohorts, suggests that the incidence of obvious genetic/syndromic diagnoses in CHD cohorts is around 10% (Massin et al., 2007; Zaidi and Brueckner, 2017; Egbe et al., 2014; Meberg et al., 2007).

In our study, factors associated with an adult having received genetics-related care (defined as genetic referral or testing) included younger age (18–40 years), male sex, and presence of extracardiac anomalies or neurodevelopmental diagnoses. The higher care rate in younger individuals likely reflects the increased availability of genetic testing and awareness of genetic contributions to CHD in more recent years. As expected, our data shows an association between cardiac lesion type and genetic diagnosis which we suspect was largely driven by the high rate of individuals with trisomy 21 and atrioventricular canal in our cohort. The types of genetic testing completed in our cohort reflect the technologies that were clinically available at the time, with karyotypes, microarray, and FISH being the most common. Microarray became available in our institution in 2008, only 16 years ago. Interestingly, documented family history of CHD was not found to have a statistically significant association with genetic referral or testing although a higher percentage of those with family history were referred to genetics compared to those without family history (19.4% versus 10.4%). Assessment of a larger cohort may provide further clarity. It is also worth noting that family history of

TABLE 3 Types and amounts of congenital and neurodevelopmental comorbities by referral status. N is not equal across all categories due to missing data.

Types and numbers of congenital and neurodevelopmental comorbidities by referral status							
		N	Genetic referral		No genetic referral		*p*-value
Congenital Comorbidities by Type			Number	%	Number	%	
	Craniofacial	231	28	71.2	11	28.1	$p < 0.001$
	Vascular systems	231	6	85.7	1	14.3	$p = 0.57$
	Endocrine	233	0	0	1	100.0	$p = 0.12$
	Gastrointestinal	231	6	75	2	25.0	$p = 0.22$
	Skeletal/Limb	233	7	46.7	8	53.3	$p < .001$
	Genitourinary/anorectal	231	5	62.5	3	37.5	$p = 0.048$
	Skin	233	4	66.7	2	33.3	$p = 0.14$
Number of Congenital Comorbidities		231	Number	%	Number	%	$p < .0001$
	0		13	7.9	152	92.1	
	1		4	8.5	43	91.5	
	2		3	27.3	8	72.3	
	3		3	60	2	40.0	
	4		2	100	0	0.0	
	5		1	100	0	0.0	
Neurodevelopmental Comorbidities by Type			Number	%	Number	%	
	ADHD	233	4	36.4	7	63.6	$p = 0.026$
	Cognitive impairment/developmental delay	233	10	40	15	60.0	$p < 0.001$
	Autism spectrum disorder	233	1	100	0	0.0	$p = 0.11$
Number of Neurodevelopmental Comorbidities		232	Number	%	Number	%	$p < 0.001$
	0		14	7.1	184	92.9	
	1		9	29	22	71.0	
	2		3	100	0	0.0	

CHD might not be fully assessed or documented by the managing cardiologist.

Our study, which found 79/233 (33%) of adults with CHD also had at least one extracardiac congenital abnormality and/or NDD, agrees with others that estimate 10%–50% of CHD patients have extracardiac comorbidities (Massin et al., 2007; Bracher et al., 2017; Hoang et al., 2018; Egbe et al., 2014). However, despite the general likelihood of our cohort to have received genetic-related care in the presence of congenital and/or neurodevelopmental comorbidities, there were many patients (35/233, 15%) with non-isolated CHD who have never received any genetic referral or testing. A genetic diagnosis is more likely to be identified in patients with CHD who have one or more extracardiac comorbidities and/or NDD (Bracher et al., 2017; Shikany et al., 2020). Therefore, these individuals may represent a population of adults with CHD who harbor unrecognized genetic diagnoses.

Our results suggest that adults with CHD may benefit from a more consistent and comprehensive genetic referral and testing strategy that utilizes available resources and newer technology and that aligns with current pediatric practices. This type of strategy should include analysis of both chromosomal and genetic sequence variants to minimize limitations of a less broad genetic testing approach. This study highlights the need to better utilize the available genetics expertise when evaluating genetic etiology of CHD. The utility of genetic testing for adults with CHD includes improved management and more accurate recurrence risk information. Even patients in our cohort who have received any historical genetic testing may be under-evaluated from a genetics standpoint in the current era. This is particularly true for the older ACHD population, who are less likely to receive any genetic-related care. Research has focused on the lack of knowledge or comprehension about cardiac lesion type, inheritance, pregnancy risks, and recurrence risks in adolescent patients, adult patients, and parents of patients, with emphasis that risk counseling has a positive impact on patient psychosocial functioning, medical management, and reproductive decision-making (Van Deyk et al., 2010; Van Engelen et al., 2011; Van Engelen et al., 2013; Blue et al., 2015; Shikany et al., 2019).

TABLE 4 Family history by cardiac lesion type.

Family history by cardiac lesion type					
	Family history		No family history		*p*-value
Cardiac lesion (n = 233)	Number	%	Number	%	0.53
Tetralogy of Fallot or DORV or pulmonary atresia	5	16.1	41	20.3	
Left-sided obstructive lesions	5	16.3	30	14.9	
AV septal defect	1	3.2	18	8.9	
Valvar pulmonary stenosis	2	6.5	15	7.4	
D-TGA or physiologically corrected TGA (systemic right ventricle) or s/p arterial switch (systemic left ventricle)	1	3.2	15	7.4	
Single ventricle (SV) Fontan-spectrum or Complex or SV unrepaired cyanotic (e.g., ToF/PA/MAPCA, mixing lesions s/p palliation)	2	6.4	10	5	
Ebstein/Uhl anomaly	3	9.7	5	2.5	
Heterotaxy spectrum	0	0	4	2	
Simple shunt lesions	8	25.8	52	25.8	
Miscellaneous/other	4	12.9	12	5.9	

4.1 Conclusions

Medical professionals providing care to adults with CHD should be aware that this population (particularly older adults, >40 years) is highly under evaluated and under-counseled with respect to genetics. All adults with CHD who have any non-cardiac congenital and/or neurodevelopmental comorbidities or family history of CHD should be referred for a formal genetics assessment. As our understanding of genetic etiologies for CHD continues to evolve, even adults with a history of limited genetic testing for CHD are advised to obtain updated genetic evaluation and to be offered more comprehensive genetic testing when appropriate.

4.2 Limitations

A retrospective chart review has expected limitations, such as survival bias, missing records, potential inconsistencies in documentation between providers, and misclassification bias, as well accrual of cases from a single tertiary care center. Additionally, cardiologists may not reliably observe or record all congenital and/or neurodevelopmental comorbidities, prenatal history, or family history, in comparison to common practice among geneticists and therefore this could introduce bias in our assessment of presence of extracardiac comorbidities. Genetic testing referral and testing rates may be under ascertained due to documentation inconsistency and/or noncompliance/lack of uptake of recommended evaluations. Statistical significance might not have been achieved in some calculations due to the study being underpowered for certain comparisons. Finally, it is important to acknowledge that due to the presence of specialty clinics within our institution for patients with Marfan and related syndromes, and for patients with Rasopathies, these patient populations are under-represented in our cohort.

4.3 Future research

With the current data, further investigation into the specific comorbidities and their genetic testing and/or evaluation could be conducted. Future studies should include prospective studies for adult patients with CHD, comparative studies of genetic-related care with patients from a cardiovascular genetics clinic, and further investigation of adult patient comorbidities correlated with results of comprehensive genetic testing and evaluation.

Data availability statement

The datasets presented in this article are not readily available because individual level patient clinical information could compromise anonymity. Requests to access the datasets should be directed to kathryn.weaver@cchmc.org.

Ethics statement

The studies involving humans were approved by CCHMC Institutional Review Board. The studies were conducted in accordance with the local legislation and institutional requirements. Written informed consent for participation was not required from the participants or the participants' legal guardians/next of kin in accordance with the national legislation and institutional requirements.

Author contributions

ME: Data curation, Formal Analysis, Investigation, Writing–original draft. XZ: Formal Analysis, Writing–review and editing. AO: Data curation, Methodology, Supervision,

Writing–review and editing. NB: Writing–review and editing, Data curation, Methodology. AS: Writing–review and editing, Conceptualization, Supervision. KW: Conceptualization, Supervision, Writing–original draft, Writing–review and editing.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fgene.2024.1412806/full#supplementary-material

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Case Report: An association of left ventricular outflow tract obstruction with 5p deletions

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Introduction: 5p deletion syndrome, also called Cri-du-chat syndrome 5p is a rare genetic syndrome with reports up to 36% of patients are associated with congenital heart defects. We investigated the association between left outflow tract obstruction and Cri-du-chat syndrome.

Methods: A retrospective review of the abnormal microarray cases with congenital heart defects in Children's Hospital of Pittsburgh and the Cytogenomics of Cardiovascular Malformations Consortium.

Results: A retrospective review at nine pediatric centers identified 4 patients with 5p deletions and left outflow tract obstruction (LVOTO). Three of these patients had additional copy number variants. We present data suggesting an association of LVOTO with 5p deletion with high mortality in the presence of additional copy number variants.

Conclusion: A rare combination of 5p deletion and left ventricular outflow obstruction was observed in the registry of copy number variants and congenital heart defects.

KEYWORDS

5p deletion, congenital heart defect, genetic disorder, left ventricular outflow tract obstruction, copy number variant

Abbreviations: ASD, atrial septal defect; BAV, bicuspid aortic valve; CCVM, Cytogenomics of Cardiovascular Malformations Consortium; CHD, congenital heart defect; CMA, chromosomal microarray; CNV, copy number variants; CoA, coarctation of the aorta; HLHS, hypoplastic left heart syndrome; LVOTO, left outflow tract obstruction; VSD, ventricular septal defect.

Introduction

5p deletion syndrome or 5p minus syndrome, also called Cri-du-chat syndrome, is a rare genetic syndrome that was first described with the distinctive, high-pitched, cat-like cry in 1963 by Lejeunne et al. (Lejeune et al., 1963). In Frech, Cri-du-chat translates to "cry of the cat." The most recognizable phenotypes are the characteristic shrill cry like the mewing of a cat, distinctive facial features, growth and developmental delay (Nguyen et al., 2015). However, there is a wide spectrum of features in the individuals with 5p deletion syndrome that may be attributed to the differences in their genotypes in whether terminal or interstitial 5p deletions occur at different breakpoints or the deleted genes in the 5p region (Nguyen et al., 2015). Cri-du-chat syndrome is a contiguous gene syndrome (Gersh et al., 1995). Studies by Overhauser et al. (1994) determined the deletion of 5p15.2 was correlated with facial dysmorphism and developmental delays and the deletion of 5p 15.3 was related to the characteristic cat-like cry (Gersh et al., 1995). Additional analysis by Zhang et al., in 2005 further localized the region of the cat-like cry to 5p 15.31, facial dysmorphism to 5p15.2 to 5p15.31, and speech delay to 5p15.32 to 5p 15.33 (5). Congenital heart defects (CHDs) are reported in approximately 15%–36% of 5p deletion syndrome, typically representing simple heart defects such as ventricular septal defect (VSD), atrial septal defect (ASD), patent ductus arteriosus (PDA), tetralogy of Fallot (TOF) or aortic stenosis (AS) (Hills et al., 2006; Mainardi et al., 2006; Nevado et al., 2021). Congenital heart defects are one of the most common causes of 5p deletion death (Nguyen et al., 2015). Left-sided lesions including left ventricular outflow tract obstruction (LVOTO) malformations have rarely been reported in 5p deletion syndrome. Nevado et al. (2021) observed up to 25% of additional copy number variants (CNVs) were noted in 5p deletion syndrome cohorts. Here we report two detailed case presentations of 5p deletion and LVOTO anomalies. Two more patients with 5p deletion in the presence of other CNVs were identified through a large multi-institution collaboration.

Methods

A retrospective review was performed on abnormal chromosomal microarray (CMA) cases (abnormal CNVs were detected either by SNP microarray or array CGH) with CHDs from UPMC Children's Hospital of Pittsburgh (CHP) and the Cytogenomics of Cardiovascular Malformations (CCVM) Consortium (Landis et al., 2023). The expression of related genes in the human heart tissues was obtained from the GTEx portal (https://gtexportal.org/home/singleCellOverviewPage). Enrichment of transcription factor targets was rendered using Metascape for genes within 5p deletions identified in a separate cohort of four complex CHD patients with LVOTO.

Case description

Patient 1 (009–0116)

A male was born at 36–5/7-week gestational age with prenatal concerns for aortic valve stenosis, aortic arch abnormality, and intrauterine growth restriction (IUGR). At birth, his weight was 2.078 kg (1.24 percentile), length was 44 cm (1.23 percentile), and head circumference was 31 cm (0.12 percentile). He demonstrated microcephaly, prominent supraorbital ridge, hypertelorism, downslanting palpebral fissures, prominent nasal root, and retrognathia. Right single palmar crease, tapered digits, and fifth digit clinodactyly were noted in his upper extremities. Bilateral metatarsus adductus and bilateral sandal gap between his first and second toes were noted in his lower extremities. An echocardiogram showed a dysplastic aortic valve with aortic stenosis (Figures 1B, C) and arch hypoplasia. The patient underwent aortic valvuloplasty at 2 days of life which was complicated by a posterior left ventricle (LV) pseudoaneurysm (Figure 1D). After the procedure there continued to be a significant gradient across the aortic valve and aortic arch with the concern of arch obstruction, therefore, he underwent arch reconstruction and aortic valvuloplasty at 10 days of life. He was noted to have a bicuspid aortic valve oriented in the anterior and posterior axis. There was a fusion of the commissures anteriorly. The aortic valve looked dysplastic and thickened. In addition, there was a raphe at the level of the junction between the right and left coronary cusps. His postoperative course was complicated by left vocal cord hypomobility and feeding difficulties. Subsequently, he was diagnosed with obstructive jaundice and was treated with biliary drainage (Figures 1E, F) when he was 2 months old. The drain was removed about a month later with no further issues. An echocardiogram at 5 months of age demonstrated the evolution of LV pseudoaneurysm. Given his congenital heart anomalies and multiple dysmorphic features, chromosomal microarray testing was ordered, which revealed a large deletion at 5p13.33-p13.2, specifically arr [GRCh37] 5p15.33p13.2 (22149_36232545) x1 encompassing 36.3 Mb and at least 320 genes (Figure 1A), including the critical region associated with Cri-du-chat syndrome which was identified in most studies as the region between 5p15.3-5p15.2 (Gersh et al., 1995; Overhauser et al., 1994; Church et al., 1995; Wu et al., 2005) and a 5.5 Mb deletion of 5p15.33-p15.32 (arr 5p15.33p15.32 (22178–5539182) x1 in a 3-generation family with atypical Cri-du-chat syndrome (Elmakky et al., 2014). This deletion also contains a 5p15 terminal (Cri-du-chat syndrome) region with sufficient evidence for haploinsufficiency (Zhang et al., 2005; Mainardi et al., 2006). Although he had multiple complications during his initial neonatal hospitalization and delayed milestones, he has been gaining weight and thriving on his growth curve (Figures 1G, H). He is 5 years old and is the only documented survivor in this case series, with the largest chromosomal deletion in the 5p region and no concurrent reported other CNVs. Based on ACMG guidelines this deletion is classified as pathogenetic CNV.

Patient 2 (009–0119)

A 37–4/7-week gestational age female was prenatally diagnosed with Cri-du-chat syndrome, had a 4.327 Mb deletion at 5p, specifically arr [GRCh37] 5p15.33 (22149_4349495) x1, and a 32.161 Mb duplication on 5q, specifically arr [GRCh37] 5q32q35.3 (148535314_180696806) x3 (Figure 1I). This deletion also contains 5p15 terminal (Cri- du-chat syndrome) region with sufficient evidence for haploinsufficiency (Zhang et al., 2005; Mainardi et al., 2006). The duplication contains 5q35 recurrent (Sotos syndrome) region (includes *NSD1*) with sufficient evidence

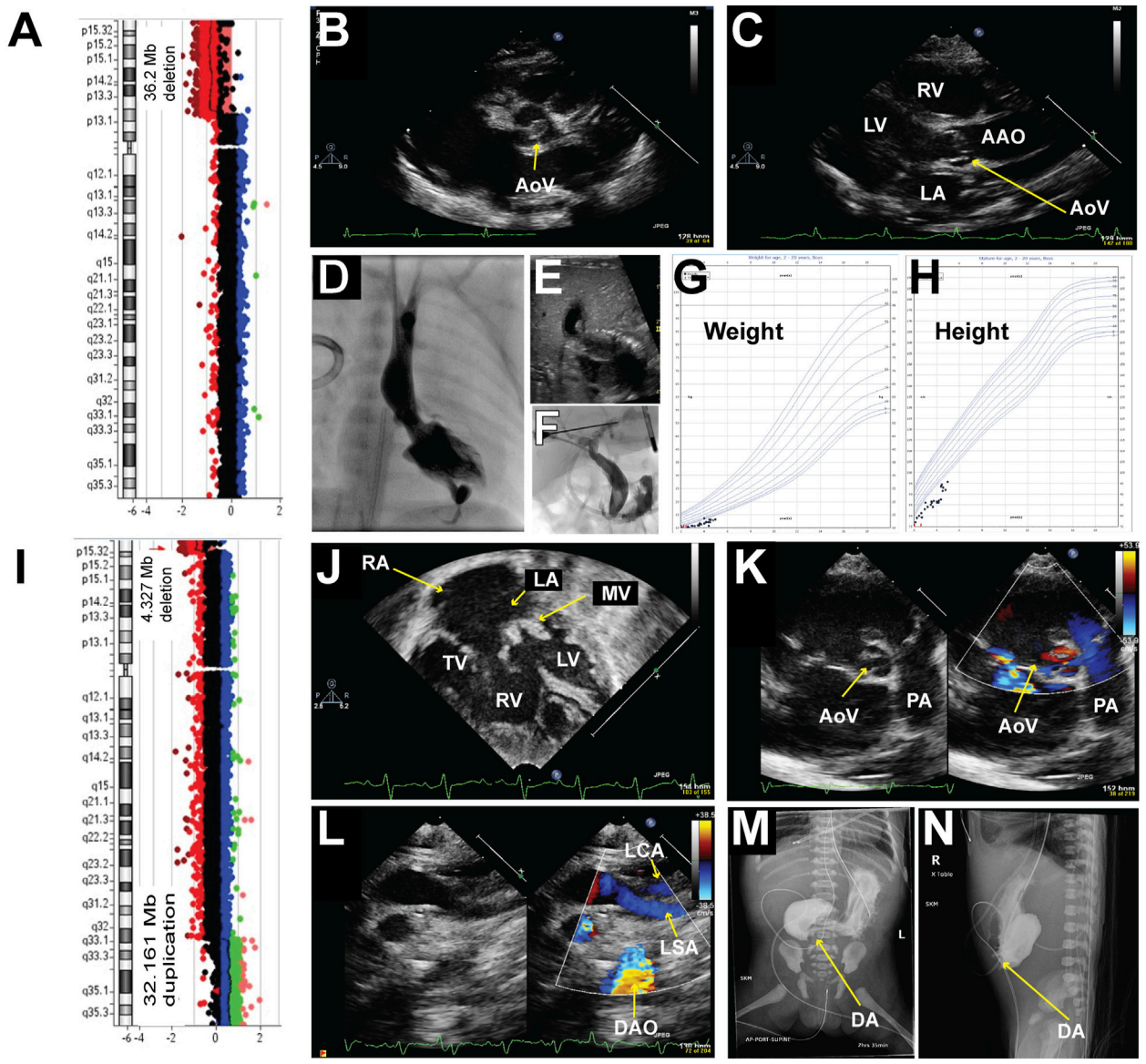

FIGURE 1
Chromosomal abnormalities and imaging from patients 1 and 2. Patient #1 **(A–H)** A Chromosomal microarray detected a 35.2 Mb deletion at 5p, specifically arr [GRCh37] 5p15.33p13.2 (22149_36232545) x1. **(B–C)**: Representative echocardiogram imaging demonstrating the patient's dysplastic aortic valve and post-stenotic dilatation of the ascending aorta. **(D)** Left ventriculogram demonstrating an apex-forming left ventricle with a hypoplastic aortic valve. The aortic valve annulus measured 4.46 mm in the anterior-posterior projection with mild post-stenotic dilation of the ascending aorta. A posterior pseudoaneurysm was noted with a small track extending apically through the myocardium. **(E)** Representative abdominal ultrasound image demonstrated dilation of the left intrahepatic biliary system as well as the intrahepatic common bile duct with gall sludge within the intrahepatic common bile duct. **(F)** An injection of contrast from a micro-puncture needle into a left hepatic duct filled a mildly dilated segmental biliary branch and communication with the dilated common hepatic and biliary ducts. **(G)** Growth chart of weight. **(H)** Growth chart of height. Patient #2 **(I–N) (I)** Chromosomal microarray detected a 4.327 Mb deletion at 5p, specifically arr [GRCh37] 5p15.33 (22149_4349495) x1, and a 32.161 Mb duplication at 5q, specifically arr [GRCh37] 5q32q35.3 (148535314_180696806) x3. **(J–L)**: Representative echocardiogram imaging demonstrating a hypoplastic left ventricle, dysplastic and thickened mitral valve, large secundum atrial septal defect (ASD), large ventricular septal defect (VSD), and a bicuspid aortic valve with thickened leaflets. Color flow Doppler in image **(L)** showed a lack of continuity between the left subclavian artery and the descending aorta consistent with interrupted aortic arch type **(A)**. **(M–N)**: Upper GI with small bowel follow-through demonstrating opacification of the stomach and blind-ending proximal duodenum is consistent with duodenal atresia. AAO = Ascending Aorta, DAO = Descending Aorta, LA = Left Atrium, LV = Left Ventricle, LSA = Left Subclavian Artery, MV = Mitral Valve, PA = Pulmonary Artery, RA = Right Atrium, RV = Right Ventricle, TV = Tricuspid Valve.

for triplosensitivity, which is associated with the Sotos phenotype (Rosenfeld et al., 2013). Prenatal ultrasound at around 20 weeks of gestational age demonstrated duodenal atresia and a decrease in LV function. She was born to a 26-year-old woman (gravida 7, para 1) with a history of six spontaneous abortions during prior pregnancies and a pericentric inversion- 46,XX,inv (Zhang et al., 2005) (p15.3q32). At birth, her weight was 1.665 kg (<0.01 percentile), length was 42.5 cm (<0.01 percentile), and head circumference was 30 cm (0.01 percentile). She was microcephalic, and her birth height and weight were below average. She was born without spontaneous

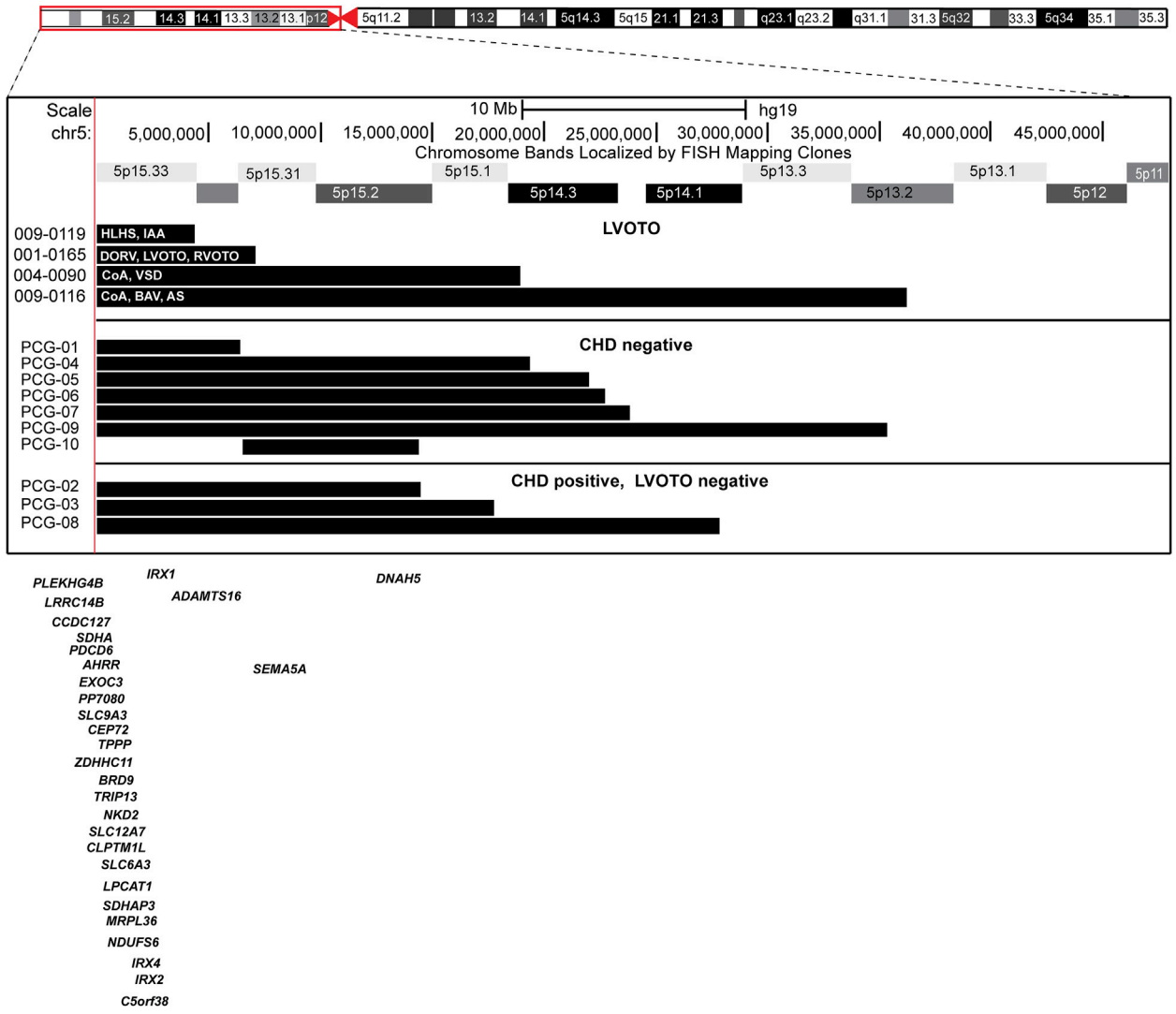

FIGURE 2
Ideogram of chromosome 5 with the expansion of the 5p region and location of the individual 5p deletions in each patient.

respiratory effort, necessitating an emergent intubation in the delivery room. Her initial heart rate at birth was less than 60 bpm without spontaneous movement. Her Apgar scores were 1 at 1 min, 3 at 5 min, and 3 at 10 min. Multiple facial dysmorphisms, including hypertelorism, low-set ears, and micrognathia, were noted after delivery. An echocardiogram was obtained due to severe metabolic acidosis and a murmur which demonstrated a hypoplastic LV, severe mitral valve dysplasia, a large VSD, a large ASD secundum (Figure 1J), a dysplastic aortic valve (Figure 1K) with an interrupted aortic arch type A (Figure 1L) and a large PDA. The upper gastrointestinal series confirmed the diagnosis of duodenal atresia (Figures 1M, N). She required multiple inotropic agents with a maximum Wernovsky inotrope score of 20 after birth. In conjunction with her comorbidities and ongoing critical illness, surgical palliation was not offered to her. She passed away on the day of life 6. Based on ACMG guidelines, deletion and duplication are classified as pathogenetic CNV(14). Also, this result suggests a parental balance inversion origin, genetic counseling is recommended. We found that the patient's mom carries a history of six spontaneous abortions during prior pregnancies and a pericentric inversion- 46, XX, inv (Zhang et al., 2005) (p15.3q32).

Additional patients

We identified five more patients with 5p deletion (Figure 2; Table 1; Supplementary Table S1) and LVOTO malformation from the CCVM Consortium (Hinton et al., 2015). This multi-site, cross-disciplinary collaboration has created a large database registry of patients with CHD who have had non-normal clinical CMA results. A total of 1,363 patients from nine pediatric centers across the United States were included in the study (Landis et al., 2023). There are 229 individuals with LVOTO in the CCVM Consortium. We excluded three patients who had small 5p deletion that is not consistent with the typical 5p deletion syndrome, with the size of deletion of 5p varying from 0.01 to 0.27 MB, which is less than the reported deletion size from 5 to 40 MB in Cri-du-chat syndrome and no genes in the deleted region that relate to heart development as well as not consistent with the typical 5p deletion syndrome (Simmons et al., 1995; Cerruti Mainardi, 2006) and these small

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TABLE 1 Patients characteristics.

Patient Number	ID	GA[e] (weeks)	Birth Body weight (kg)	Sex	Vital status	Cardiac Defect	Other diagnoses	Genetic Findings	Interval size (Mb[j])	5P minus syndrome
1	009-0116	36-5/7	2.078	M[i]	Alive	Hypoplastic aortic arch, AS[a]	IUGR[g], dysmorphic facial features, distal extremity abnormalities	arr[GRCh37] 5p15.33p13.2(22149-36232545x1)	5p deletion: 36.21	Yes
2	009-0119	37-4/7	1.665	F[d]	Decea sed age 6 days	HLHS[f], interrupted aortic arch, mitral valve dysplasia, large VSD[l], ASDII[b], PDA[k]	IUGR[g], duodenal atresia, subdural hemorrhages respiratory failure	arr[GRCh37] 5p15.33(22149-4349495)x1	5p deletion: 4.33	Yes
								arr[GRCh37] 5q32q35.3(148535314-180696806)x3	5q duplication: 32.16	
3	001-0165	Unknown	Unknown	M[i]	Unkno wn	Double outlet right ventricle (tetralogy of Fallot type); LVOTO[h], muscular VSD[l], ASDII[b], aortic root dilatation	8p partial trisomy, other extracardiac abnormalities unknown	arr[hg18] 5p15.33p15.31(66,648-7,175,604)x1	5p deletion: 7.11	Yes
								arr[hg18] 8p23.3p21.2(213-26,130,535)x3	8p duplication: 26.13	
4	004-0090	35	1.432	F[d]	Decea sed age 11 months	CoA[c], perimembranous VSD[l], ASDII[b], bilateral superior vena cava	Dysmorphism tethered cord, metabolic acidosis, pulmonary insufficiency, congenital hypotonia, abdominal wall hernia, IUGR[g]	arr[GRCh37] 5p15.33p14.3(22,149-18,927,458)x1	5p deletion: 18.91	Yes
								arr[GRCh37] 5p14.3p11(19,049,019-46,115,173)x3	5p duplication: 27.07	

[a]AS: Aortic stenosis; [b]ASDII: Atrial septal defect secundum; [c]CoA: coarctation of the aorta; [d]F: Female; [e]GA, gestational age; [f]HLHS: hypoplastic left heart syndrome; [g]IUGR, intrauterine growth restriction; [h]LVOTO, left ventricular outflow tract obstruction; [i]M: male; [j]Mb, megabases; [k]PDA: Patent ductus arteriosus; [l]VSD: Ventricular septal defect.

deletions were not consistent with pathological CNV according to ACMG guidelines (Rosenfeld et al., 2013). These 3 patients also have additional CNVs that are known to cause CHDs (Supplementary Table S1). The major birth defects of these additional 2 patients we included are summarized in Table 1. In total, there are four patients with a deletion in regions associated with Cri-du-chat syndrome (5p15.2-5p15.3, patients 1–4) (Zhang et al., 2005; Mainardi et al., 2006) in this report, of whom three patients had additional CNVs. Patient 2 (009–0119) had a 5q duplication in the region containing *HAND1* and *Nkx2-5* (Supplementary Table S2). Patient 3 (001–0165) had a 7.1 Mb deletion arr [hg19] 5p15.33p15.31966648_7175604) x1, and an 8p duplication in the region containing *MYOM2*. Interstitial duplication of 8p23 was noted to be associated with CHDs but 8p trisomy has not been reported to be associated with LVOTO (Gug et al., 2020). Patient 3 has a complex CHD with a double outlet right ventricle (DORV) and both left and right ventricular outflow tract obstruction. Patient 4 (004–0090) had an 18.9 Mb deletion arr [GRCh37] 5p15.33p14.3 (22149_18927458) x1 and arr [GRCh37]5p14.3p11 (19049019_46115173) x3, the 18.0 Mb duplication and a complex rearrangement of chromosome 5. Of these 4 patients, 2 died before 12 months of age and one did not have updated clinical information.

In summary, the 4 patients' 5p deletions are classified as pathogenetic CNV based on ACMG guidelines (Riggs et al., 2020) with an incidence of 1.3% in this cohort (Landis et al., 2023). We reported the association of LVOTO and 5p deletion and observed an association of LVOTO and 5p deletion with high mortality in the presence of additional copy number variants. This also indicates an expansion of the cardiac abnormalities' spectrum in the 5p deletion patients.

No common critical region for LVOTO defects in 5p deletion patients

To identify the specific region associated with LVOTO in patients with 5p deletion, we compared 10 patients with 5p deletion at CHP to the four with LVOTO. Of these 10, seven were without CHD and 3 had non-LVOTO CHD (Figure 2). There is not a common region associated with 5p deletion and LVOTO in this case series. This could be due to incomplete penetrance or the etiology of LVOTO may be concurrent copy abnormalities other than 5p deletion.

Discussion

Hills et al. reviewed a database of 98,000 congenital heart disease patients and identified twenty-one with Cri-du-chat syndrome

(Hills et al., 2006). When characterized by the most hemodynamically significant lesion, twenty-one patients either had a VSD, PDA, Tetralogy of Fallot, or right ventricular outflow tract obstruction (Hills et al., 2006). The patients described in our case series all had septal defects, but uniquely all had more hemodynamically significant congenital anomalies causing LVOTO. 5p deletion syndrome was reported to be associated with CHDs in 18%–36% of patients including AS (7). When characterized by the most hemodynamically significant lesion, these patients either had a VSD, PDA, or TOF (7). The patients described in our case series had LVOTO malformation, three patients (patients 2–4) have additional CNVs. This could be due to incomplete penetrance. In addition, two of four patients pass away before 12 months of age with complex rearrangements at 5p/ 5q, complex critical congenital heart defects and intrauterine growth retardation, and low birth weight. Birth weight less than 1.5 kg with a critical congenital heart defect was known to be less likely to survive hospital discharge (Kim et al., 2021). Early observations of 5p deletion syndrome reported a 9.7% (32 of 341 individuals) mortality in childhood in 1978 with 90% of deaths within the first year (Niebuhr, 1978). The mortality rate decreased to 6.4% in the report in 2006 with 64% of deaths in the first year of life (Mainardi et al., 2006). Mainardi et al. also observed a higher mortality rate with unbalanced translocations that include a 5p deletion than the individuals with terminal deletions (18.5% vs. 4.8%) (Mainardi et al., 2006).

The etiology of CHD is multifactorial, and both epidemiologic studies and patient cohorts with chromosomal microarray testing have reported approximately 11%–18% of patients with CHD also have an identifiable syndromic genetic diagnosis (Helm et al., 2021), while 15%–21% of subjects with isolated CHD had LVOTO (Helm et al., 2021; Hoang et al., 2018). Genes known to be involved in cardiac expression are located on 5p (Supplementary Tables S3 and S4), including *DNAH5* (5p15.33, in patients 2, 4), NDUFS6 (5p15.33, in patients 1–4), IRX4 (5p15.33, in patient 1–4), and ADAMTS16 (5p15.32, in patient 2–4) (Pervolaraki et al., 2018). *ADAMTS16* p.H357Q variant is reported to be an inheritable human bicuspid aortic valve-related gene variant resulting from the fibronectin/ focal adhesion kinase (FAK) signal-mediated over-proliferation with extracellular matrix remodeling interruption (Lin et al., 2024). Patients 2 and 4 had a deletion in the *DNAH5* gene, a disease-causing variant related to laterality defects resulting from immotile cilia that lack dynein arms (Nöthe-Menchen et al., 2019). Patients 1–4 had a deletion in the region containing both *SDHA* and *NDUFS6*, complexes related to mitochondrial oxidative phosphorylation, which are highly expressed in the left ventricle (Pervolaraki et al., 2018). Intrinsic mitochondrial defects contribute to the different prognoses of single ventricle CHD, especially for HLHS (Xu et al., 2022). Patients 1–4 also had a deletion in the region containing *IRX4*, a homeobox gene with an expression in the ventricular myocardium (Bruneau et al., 2000). We observed the enrichment of five transcription factor targets (Subramanian et al., 2005) that are associated with heart development or defects (Supplementary Table S5) from the analysis of the affected genes within the 5p deletions in our patients. e.g., *DLX6* (Distal-Less Homeobox 6) directly regulates Basic helix-loop-helix transcription (bHLH) factor *HAND2*, which plays a crucial role for the development of the cardiac outflow tract (Holler et al., 2010). *FOXJ2*, a member of the Fork Head transcription factors family. Previous reports showed that there is a right/left heart difference in expression for *FOXJ2* (Philip-Couderc et al., 2008). *FOXJ2* also regulates Connexin-43 and E-Cadherin which may be associated with hypertrophic heart (Martin-de-Lara and Sanchez-Aparicio, 2008). These clues may explain how 5p deletion contributes to LVOTO.

The cases in this report suggest that the previous thought that 5p minus patients only have simple cardiac defects like ASD, VSD, and PDA, is not the case. There is a case report of a child with 5p minus and 20q duplication with Ebstein anomaly; the authors make a similar argument that this rare, complex congenital heart phenotype such as Ebstein has not ever been reported with 5p minus, though the 20q duplication may be part of the phenotype, too (Olivella et al., 2020). However, 5p deletion in the presence of other CNVs that are related to cardiac development may be associated with more complex hemodynamic significant cardiac defects. The correlation with LVOTO will be useful for clinical prognosis prediction on 5p deletion patients, as congenital heart defects are one of the most common causes of 5p deletion death (Nguyen et al., 2015). Significant hemodynamic complex congenital heart defects such as LVOTO increase the risk of mortality and morbidity. Further research is needed to elucidate an association between these genes and specific CHDs. In addition, further study of gene enhancers that may regulate gene expression from a distance or epigenetic regulation may provide additional insight into the critical regions for left heart formation and function on the short arm of chromosome 5.

Conclusion

We present data suggesting an association of LVOTO and 5p deletion with high mortality in the presence of additional CNVs. Discovering new genotype-phenotype correlations for rare CHDs, including expanding the associations with known syndromes, is an important ongoing process requiring large patient cohorts with deep phenotyping such as that collected by the CCVM Consortium.

Learning objectives

1. An association is suggested between LVOTO and 5p deletion, with high mortality occurring in patients with 5p deletion and other CNVs.
2. Expanding the phenotypic spectrum of known syndromes to include rare CHD requires multi-institution collaboration and expert-detailed heart phenotype evaluation.

Data availability statement

The original contributions presented in the study are included in the article/Supplementary Material, further inquiries can be directed to the corresponding author.

Ethics statement

The studies involving humans were approved by University of Pittsburgh/ Indiana University Institutional review Boards. The

studies were conducted in accordance with the local legislation and institutional requirements. Written informed consent for participation was obtained from the participants or the participant's legal guardians/next of kin in accordance with the national legislation and institutional requirements.

Author contributions

KM: Writing–original draft, Writing–review and editing. SY: Data curation, Methodology, Validation, Writing–review and editing. CL: Conceptualization, Supervision, Writing–review and editing. XX: Data curation, Formal Analysis, Methodology, Writing–review and editing. JJ: Methodology, Visualization, Writing–review and editing. LH: Project administration, Writing–review and editing. SB: Data curation, Writing–review and editing. CM: Data curation, Validation, Writing–review and editing. SL: Data curation, Validation, Writing–review and editing. VG: Data curation, Validation, Writing–review and editing. JH: Data curation, Validation, Writing–review and editing. KLM: Data curation, Validation, Writing–review and editing. SW: Conceptualization, Data curation, Formal Analysis, Funding acquisition, Methodology, Resources, Supervision, Validation, Writing–review and editing. J-HI: Conceptualization, Data curation, Formal Analysis, Methodology, Supervision, Writing–review and editing.

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Conflict of interest

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fgene.2024.1451746/full#supplementary-material

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Cleft palate, congenital heart disease, and developmental delay involving *MEIS2* heterozygous mutations found in the patient with attention deficit hyperactivity disorder: a case report

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This case is the first reported patient with a *MEIS2* gene mutation who primarily exhibits pronounced inattention as the main manifestation and is diagnosed with ADHD, requiring methylphenidate treatment. It is characterized by unique clinical features that set it apart from previously reported cases with mutations in the *MEIS2* gene. Here, we report a female child with a diagnosis of ADHD and comorbidities. She received treatment with methylphenidate, starting at a dose of 18 milligrams per day, which was gradually increased to 45 milligrams per day based on her attention performance, while also undergoing physical and language rehabilitation training. In addition, the parents involved the child in reading and retelling stories at home every day. After 2 years of treatment, the scale results indicated that the child still had a moderate degree of attention deficit. Therefore, she underwent whole exome sequencing (WES) showing that her *MEIS2* gene carries a *de novo* frameshift mutation (c.934_937del, p. Leu312Argfs*11). After comparing the patient's features with those of other patients who also had the *MEIS2* mutation, we discovered that the patient's cleft palate, heart abnormalities, and minor facial dysmorphism were all extremely comparable. A broad forehead, elongated and arched eyebrows, and a tent-shaped upper lip were examples of mild facial dysmorphic traits. Subtypes with phenotypes such as cleft palate, cardiac anomalies, or facial malformations were presented in all previously reported cases of *MEIS2* mutations. Furthermore, less common characteristics include ADHD, learning difficulties, hearing loss, recurring respiratory infections, asthma, rhinitis, enuresis, and dental cavities. This case further supports the critical role of genetic testing in patients with ADHD who exhibit a suboptimal

response to methylphenidate and present with multiple comorbidities. Furthermore, this case report expands the clinical symptom spectrum associated with *MEIS2* gene mutations, providing a broader understanding of the condition.

KEYWORDS

ADHD, *MEIS2*, cleft palate, congenital heart defect, developmental delay, case report

1 Introduction

ADHD is one of the most prevalent neurodevelopmental disorders, affecting approximately 5%–10% of children and adolescents worldwide (1). The disorder is characterized by persistent inattention, hyperactivity, and impulsivity, leading to significant challenges in all aspects of life, including academic performance, social interactions, and overall daily functioning (2). In addition, approximately 43% of childhood ADHD persists into adulthood (3). With regard to the cause of the disease, research suggests that it is due to the interaction of genetic and environmental factors, but that genetics play a considerable role. Based on the results of family and twin studies, the estimated heritability of ADHD approximates 80% (4, 5). The fact that ADHD frequently co-occurs with other diseases such as learning disabilities, anxiety disorders, and oppositional defiant disorder complicates both the diagnosis and treatment of ADHD. However, the advancement of genetic testing technologies has made it possible to determine the genesis of neurodevelopmental disorders exhibiting intricate clinical manifestations.

The *MEIS2* gene encodes a transcription factor belonging to the three-amino-acid-loop extension (TALE) protein superclass (6) and is an important transcription factor controlling embryonic development and cell differentiation. *MEIS2* is involved in the development of the heart and craniofacial region based on phenotypes linked to the overexpression or deletion of the gene in animal models (7–9), limb growth and patterning (10, 11), and axial skeletal patterning (12). The function of *MEIS2* in mouse palatal development has been thoroughly studied. Molecular and genomic analyses revealed that *MEIS2* directly regulates important osteogenic genes, and specific inactivation of the *MEIS2* gene in cranial neural crest cells resulted in complete cleft palate or submucous cleft and complete loss of palatal bone (9). In addition, Desiderio et al. (13) looked into the possibility that when crossing mouse strains to successfully deactivate *MEIS2* in the neural crest, the resulting cleft palate in the newborn pups would be consistent with earlier findings that *MEIS2* is essential for the development of the mouse's cranial and cardiac neural crest cells (7).

Additionally, *MEIS2* has a role in nearly every facet of the development of the central nervous system, such as neural tube patterning, proliferation of neural progenitor cells, acquisition of cell destiny, maturation of neurons, neurite outgrowth, and synaptogenesis (14–17), and is required for the survival and function of different neuronal populations (18, 19). Furthermore, *MEIS2* regulates the development of striatal neurons (20) and the maintenance of retinal progenitor cell pools (16). In the meantime, *MEIS2* is required for inner ear formation and proper morphogenesis of the cochlea (6).

At least 17 distinct mutations in the *MEIS2* gene have been linked to neurodevelopmental abnormalities in humans (21–23), highlighting the crucial role this gene plays in neuronal differentiation. Furthermore, *MEIS2* has been reported to be a susceptibility gene for obsessive-compulsive behaviors in humans. Somatic mutations that produce *de novo MEIS2* binding motifs are discovered in putative enhancer regions in the brains of people with autism spectrum disorders (24, 25). The characteristic features of a heterozygous missense mutation in the *MEIS2* gene locus or a 15q14 microdeletion are a triad of cleft palate, congenital heart defects, and intellectual disability, which is referred to as *MEIS2* syndrome (26).

This is the first report of an ADHD patient with a mutation in the *MEIS2* gene (c.934_937del, p. Leu312Argfs*11). Her primary clinical manifestations are attention deficit and developmental delay, and she also presents with issues involving multiple organ systems, including the respiratory, urinary, cardiac, oral, ear, and nasal systems. Additionally, her attention deficit did not improve significantly after treatment with methylphenidate. This case further extends the clinical phenotype of mutations in the *MEIS2* gene and confirms the importance of genetic testing in finding the etiology of ADHD in patients who do not respond well to methylphenidate treatment and have comorbidities.

2 Materials and methods

The study was approved by the Ethics Committee of West China Second Hospital of Sichuan University. In addition, written informed consent was obtained from the patient's parents before whole-exome sequencing was performed.

Peripheral blood samples (2–4 ml) from the pre-certified patient and her parents were collected into ethylenediaminetetraacetic acid (EDTA) anticoagulated blood sample tubes. Genomic DNA from this patient and parents was extracted from the blood according to

Abbreviations

ADHD, attention deficit hyperactivity disorder; WES, whole-exome sequencing; TALE, three-amino-acid-loop extension; EDTA, ethylenediaminetetraacetic acid; BWA, Burrows-Wheeler Aligner; NIH, National Institutes of Health; HGMD, Human Genetic Mutation Database; ACMG, American College of Medical Genetics and Genomics; SNVs, single nucleotide variants; VtSD, ventricular septal defect; PFO, patent foramen ovale; WISC-IV, Wechsler Intelligence Scale for Children-Fourth Edition; DSM-5, Diagnostic and Statistical Manual of Mental Disorders, Fifth Edition; SNAP-IV-26, Swanson, Nolan, and Pelham, Version IV–26 items; WFIRS-P, Weiss Functional Impairment Rating Scales-Parent Report; CBCL, Child Behavior Check List.

the manufacturer's instructions (QIAamp DNA Blood Minikit). And WES was performed using the Illumina NovaSeq6000 platform. Sequencing reads were then aligned to the human reference genome GRCh38/HG38 and variants annotated using Burrows-Wheeler Aligner (BWA) software. Reads were aligned and locally recalibrated using GATK. A series of principles were used to screen for pathogenic mutations based on variant annotation, as follows: (1) Filtering for variants that are not seen to be carried by normal humans or have a carrier rate of less than 5% in databases such as gnomAD, 1,000 Genomes Project, and others. (2) Disease-causing mutation sites were evaluated using databases such as the Online Mendelian Inheritance in Man (OMIM) database, the National Institutes of Health (NIH), the Human Genetic Mutation Database (HGMD), and ClinVar. (3) Protein function prediction using software such as SIFT, PolyPhen2, and CADD. According to the American College of Medical Genetics and Genomics (ACMG) classification guidelines, the obtained single nucleotide variants (SNVs) were categorized into five categories, including pathogenic, possibly pathogenic, of uncertain significance, possibly benign, and benign. Finally, validation was performed using first-generation Sanger sequencing technology and samples from family members.

3 Case description

We report a 10-year-old girl who presented to the Department of Pediatric Rehabilitation, West China Second Hospital, Sichuan University, with what her parents described as inattention and developmental delay. The infant was born at 37 weeks of gestation, undersized for gestational age, weighing 2,100 g (below the 10th percentile), and measuring 47 cm (10th–25th percentile) in length. At birth, her parents noticed that she had a cleft palate, which was surgically corrected when she was one year old. A ventricular septal defect (VtSD), a patent foramen ovale (PFO), and mild tricuspid regurgitation were all detected by cardiac ultrasonography. Following that, the VtSD resolved on its own, and the foramen ovale has not yet closed. In addition, she developed full-mouth caries, asthma, rhinitis, enuresis, recurrent respiratory infections, and otitis media-related hearing loss. At the time of our initial evaluation, the child was 5 years and 2 months old, with a low bone age (equal to 4.9 years), a height of 105 cm (−2 SD), and a weight of 14.7 kg (between −2 and −3 SD). The patient also showed delayed motor development, beginning to walk at 18 months. She can no longer perform continuous movements, descends stairs slowly, and her coordination is weak. She also lags behind youngsters her age in fine motor skills, such as button fastening.

She also exhibits delayed language development. At 2 years and 10 months of age, she was only able to speak a few simple words, and her pronunciation was not accurate. Now at ten years old, her vocabulary in language communication is less than that of her peers, and she frequently experiences dysfluency in speech, which affects normal communication. During literacy reading, she skips words, and her logical thinking is poor. At school, multiple subject teachers have reported that she exhibits significant inattention during class, being able to concentrate for only about 20 min per lesson. Concurrently, she has learning difficulties and exhibits procrastination when doing homework, requiring parental supervision to complete tasks. Additionally, the child has poor social skills. Physical examination reveals mild facial dysmorphia, including a large forehead, extended and arched eyebrows, and a tent-shaped upper lip. These features are individually difficult to recognize.

The Wechsler Intelligence Scale for Children—Fourth Edition (WISC-IV) is used to assess children's level of intelligence, and the patient's total IQ score on the test was 92, which is at the normal level of intelligence for her age group. Based on the Diagnostic and Statistical Manual of Mental Disorders, Fifth Edition (DSM-5), the child was diagnosed with ADHD. The Swanson, Nolan, and Pelham, Version IV-26 items (SNAP-IV-26) consist of three parts: inattention, hyperactivity/impulsivity, and oppositional defiant. The scale uses a four-point scoring system ranging from 0 to 3, where 0 indicates the complete absence of such symptoms, and 3 represents the very frequent occurrence of these symptoms. It is used to assess the severity of ADHD symptoms, evaluate the effectiveness of treatment, and the degree of symptom improvement. The child showed moderately abnormal attention deficits using the SNAP-IV-26 test, while hyperactivity/impulsivity and oppositional defiance scores were at normal levels.

The Weiss Functional Impairment Rating Scales-Parent Report (WFIRS-P) is designed to assess the degree of impairment in the daily functioning of children and adolescents, encompassing areas such as family, school, life skills, self-management, social activities, and risky behaviors. The WFIRS-P assessment of the ADHD child revealed functional impairments across domains including family, school, life skills, and engagement in risky activities. Furthermore, the Child Behavior Check List (CBCL) categorizes behavioral problems into six primary behavioral symptom factors: social withdrawal, depression, sleep disturbances, somatic complaints, aggressive behavior, and destructive behavior. It aims to assess children's behavioral and emotional issues within the family, school, or community setting. The child's CBCL showed that she scored higher than normal in both social withdrawal and depression, implying that the child's performance in these specific domains tended to show symptoms of social withdrawal and depression more than most children of the same age. This may indicate that the child is having difficulty with social interactions, may feel isolated or unwilling to socialize with others, and may exhibit traits associated with depression in her emotional state, requiring close attention to the child's mental health.

The child's brain magnetic resonance imaging (MRI) at 9 months of age revealed delayed myelination maturation in comparison to peers. The most recent MRI of the brain revealed no anomalies. The child's blood routine, liver and kidney function, blood electrolytes, electrocardiogram, and electroencephalogram all show no abnormalities. Because the child had a history of recurrent cough, respiratory infections and wheezing, combined with allergic rhinitis, she needed to be alerted to the possibility of bronchial asthma. As a result, she

underwent pulmonary ventilation testing that showed small airway airflow obstruction and impaired pulmonary ventilation.

4 Diagnostic inventory

The child was diagnosed with ADHD, cleft palate, VtSD, PFO, mild tricuspid regurgitation, delayed motor and language development, hearing loss, recurrent respiratory infections, asthma, rhinitis, enuresis, and dental caries based on her medical history, clinical symptoms, auxiliary examinations, and scale assessment results.

5 Patient's progress report

Given the clear diagnosis of ADHD in the child, she was treated with methylphenidate, starting at a dose of 18 mg/day, which was gradually increased to 36 mg/day based on her attentional performance, while also undergoing physical and language rehabilitation training. Additionally, the pediatrician advised the parents to engage the child in reading and retelling stories at home daily. The parents were fully involved in the treatment process. During the first year of treatment, the parents reported a positive impact, and the child's teacher noted an improvement in her attention span and the speed of completing homework. However, in the second year of treatment, both parents and the teacher reported that the child had difficulty concentrating again and exhibited learning difficulties, prompting an increase in the methylphenidate dose to 45 mg/day. This led to difficulties in falling asleep at night and poor sleep quality, so the dose was reduced back to 36 mg/day (Table 1). After two years of regular treatment, the child still met the diagnostic criteria for ADHD. Therefore, this suggests that methylphenidate was not effective in treating the child, and given her comorbid conditions, the possibility of inherited metabolic diseases should be considered. To further investigate the etiology and guide treatment, after obtaining informed consent from the parents, we conducted whole exome sequencing.

TABLE 1 Changes in methylphenidate dosage and attention deficit scores over time.

Times	Dosage (mg/day)	SNAP-IV-26 (attention deficit scores)
May 2022 to December 2022	18	17
January 2023 to February 2024	36	18
March 2024 to April 2024	45	21
May 2024 to August 2024	36	untested

(SNAP-IV-26) Swanson, Nolan, and Pelham, Version IV-26 items.

6 Results

WES was performed to further elucidate the causes of multiple diseases in our patient. A heterozygous frameshift mutation in the *MEIS2* gene (NM_170674.5: exon9: c.934_937del, p. Leu312Argfs*11) was identified through further genetic testing (Table 2). The *MEIS2* gene is a *de novo* mutation, which means it is not present in the genetic sequence of the parents but emerges as a new mutation in the child. This variant is the only *de novo* protein coding variant found in this case. It has been confirmed by Sanger sequencing. This variant has been reported in the study by Verheije et al. (26) and has (27) been recorded in the ClinVar database. Furthermore, according to ACMG criteria (28), the c.934_937del variant in this patient is classified as pathogenic.

7 Discussion

We report a case of *de novo* heterozygous frameshift mutation in the *MEIS2* gene that resulted in developmental delays and a phenotypic overlap of cardiac, craniofacial, and other abnormalities. The clinical presentation of this case included cleft palate, VtSD and PFO, ADHD, delayed motor and language development, mild facial dysmorphic features, learning difficulties, hearing loss, recurrent respiratory infections, asthma, rhinitis, enuresis, and dental caries. The clinical presentation of this patient shared some common clinical features with other previously described patients (21, 22, 26, 29–34), but also included some less common characteristics. Table 3 summarized the clinical phenotypes and genetic findings of this case and previously reported cases with *MEIS2* gene variants.

The diagnosis and treatment of ADHD are further complicated by the fact that ADHD often co-exists with other disorders such as learning difficulties, anxiety disorders, and oppositional defiant disorder. Currently, the exact etiology of ADHD remains undetermined, but researchers generally agree that ADHD is the result of a combination of genetic and environmental factors (35, 36). With the advent of innovative genomics technologies, genetic evaluation of patients with complex clinical presentations has become possible. We report the patient diagnosed with ADHD, who also has a variety of other diseases, presenting with complex clinical manifestations. In addition, her treatment

TABLE 2 Mutation site details.

Gene name	Location	Gene mutation information	Mutation type	Variant classification (ACMG)	Disease	Zygosity type	Variant origin
MEIS2	Chr15:36950364_36950367	NM_170674.5: exon9: c.934_937del, p. Leu312Argfs*11	Frameshift mutation	Pathogenic	*MEIS2* syndrome	Heterozygote	*de novo*

TABLE 3 The clinical phenotypes and genetic findings of this case and previously reported cases with *MEIS2* gene variants.

Publication	Case number	Sex	Age (yrs)	*MEIS2* variant	Cleft palate	Heart defects	Intellectual disability/delayed development	Dysmorphic features
Louw et al. (29)	1	F	5	c.998_1000del p.Arg333del	CP	AtSD type II, VtSD, CA, LVOTO	Moderate ID Severely delayed gross motor, verbal development	Bitemporal narrowing, arched and laterally extended eyebrows, mild upslanting palpebral fissures, deep-set eyes, a tented upper lip, thin upper vermilion, full lower vermilion, broad first ray of hands and feet, a gap between the first and second toes, and syndactyly of toe II–III
Fujita et al. (30)	1	F	2	c.611C > G p.Ser204*	CP	AtSD, VtSD	Severe ID delayed motor development, speech delay	Large forehead, mild trigonocephaly, sparse eyebrow, deeply set eyes, large and low-set ears, full cheeks and thin upper lip vermilion
Srivastava et al. (32)	1	F	NR	c.955A > G p.Arg319Gly	BU	AtSD, VtSD	ID hypotonia	Minor dysmorphisms
Douglas et al. (31)	1	M	0.75	c.905C > T p.Pro302Leu	BU	VtSD	ID Developmental delay, fine and gross motor, speech	Micrognathia, full cheeks
	2	M	5	c.992G > A p.Arg331Lys	CP, BU	VtSD	Severe ID Global developmental delay, gross motor, speech and language delay	Broad face, full cheeks, plagiocephaly, deeply set eyes, eyelid ptosis, narrow nose with pointed tip, small nasal alae, short philtrum, eversion of lower lip, protruding ears with simple helix on right
	3	F	5	c.1004 T > C p.Val335Ala	CP	No	ID Global developmental delays, fine and gross motor, speech	NR
	4	F	12	c.965A > T p.Gln322Leu	CP	No	Severe ID Developmental delay, no verbal speech	Full lips, downturned corners of mouth, simple structure of ears, mild facial asymmetry
Verheije et al. (26)	1	M	5	c.978G > A p.Trp326*	CP	VtSD	Mild ID Psychomotor development delay, fine and gross motor, speech	Mild eversion of the lower eyelids, fine arched eyebrows, and a small chin, prominent metopic suture
	2	M	10	c.639 + 1G > A Splice variant	CP	No	Mild/Moderate ID Developmental delay moderately delayed speech	Ptosis of the left eye, sagging of the lower eyelids, and square-shaped ear helices
	3	M	4	c.640-2A > G Splice variant	SMCP	No	Moderate ID Developmental delay, motor and speech delay	Epicanthic folds, hypoplastic alae nasi, prominent ears, and a frontal cow lick
	4	F	20	c.829C > T p.Gln277*	CP	Mitral valve insufficiency	Mild ID Motor, cognitive and speech development delay	Broad forehead with bitemporal narrowing
	5	M	13	c.868dupA p.Ile290Asnfs*40	BU	No	Mild ID Walk independently at 18 months old, speak at two years of age, speak sentences consisting of 2–3 words at three and a half years	Prominent forehead, epicanthic folds, hypertelorism, long eyelashes with distichiasis, bulbous beaked nose with short alae nasi, thin upper lip, retrognathia, short neck, hypoplastic right nipple, and fifth finger clinodactyly, atypical medial eyebrow flare, micrognathia mild posterior rotation of ears
	6	F	5	c.978-2A > G Splice variant	CP	No	Mild ID Mild developmental delay, speech delay	Fine arched eyebrows and hypoplastic alae nasi
	7	F	18	c.383delA p.Lys128Serfs*19	NR	TOF Ebstein anomaly	No ID Mild psychomotor developmental delay	No
	8	F	11	c.934_937del p.Leu312Argfs*11	SMCP	AtSD type II, VtSD Ebstein anomaly	Mild ID Motor and speech development delay	High and broad forehead, fine arched eyebrows, hypoplastic alae nasi, short philtrum, and low-set dysplastic ears with a right-sided ear pit, bilaterally overriding toes
	9	M	1	c.998G > A p.Arg333Lys	No	AtSD, LPVS, VtSD	Profound ID Profound developmental delay	High and small forehead with a high frontal hairline, frontal bossing and temporal narrowing, short palpebral fissures, hypertelorism, a depressed nasal bridge with

(*Continued*)

TABLE 3 Continued

Publication	Case number	Sex	Age (yrs)	*MEIS2* variant	Cleft palate	Heart defects	Intellectual disability/delayed development	Dysmorphic features
								anteverted nares, full cheeks, a smallmouth, short neck and limbs in comparison with the trunk
Giliberti et al. (22)	1	M	8	c.520C > T p.Arg174*	HP	AtSD, VtSD	ID Psychomotor and speech delay	Large forehead, low frontal hairline, thick hair, thin and laterally extended eyebrows, large nasal tip with anteverted and hypoplastic nostrils, M-shaped upper lip, high palate, tapering fingers, sandal gap bilaterally
Santoro et al. (33)	1	M	15	c.27_28del p.His10Leufs*84	CP	No	Moderate-Severe ID delayed psychomotor development	Brachycephaly, broad and sloping forehead, full eyes with upslanting palpebral fissures, thin upper vermilion, eyebrows widening medially and gradually thinning laterally, bulbous nasal tip and thin philtrum, upturned mouth corners, wide and lowly inserted columella, M-shaped/tented upper lip, and full lower vermilion, retrognathia, large and low-set ears, broad thumbs and great toes, scoliosis with sloping left shoulder, pectus excavatum, camptodactyly of toe II, and syndactyly of toes II–III
Gangfuß et al. (21)	1	M	10	c.998G > A p.Arg333Lys	CP	AtSD, PDA	ID Motor and speech development severely delayed	Thin and arched eyebrows, thin lip vermillion and prominent nasal tip with short alae nasi, and large, protruding, ears with enlarged fossa triangularis and hypoplastic antihelix
Barili et al. (34)	1	M	10	c.998G > A p.Arg333Lys	CP	AtSD, TOF	Moderate-Severe ID delayed psychomotor development absent speech at 5 years of age	Finely arched eyebrows,broad forehead, moderately shortened philtrum and tented upper lip
Current case	1	M	10	c.934_937del p.Leu312Argfs*11	CP	VtSD, PFO	No ID Developmental delay, fine and gross motor, speech	Broad forehead, elongated and arched eyebrows, tent-shaped upper lip

Publication	Case Number	Behavioral problems	Other features
Louw et al. (29)	1	ASD	Congenital lobar emphysema, severe feeding problems (gastro-esophageal reflux, oral aversion, aerophagia, and achalasia)
Fujita et al. (30)	1	No	Serious feeding difficulty with severe gastro-esophageal reflux, severe hypermetropia, severe constipation
Srivastava et al. (32)	1	ASD	Stereotyped hand movements and impaired sleep pattern, seizures, bruxism when awake, intense eye communication
Douglas et al. (31)	1	No	Delayed myelination, Bilateral conductive hearing loss, short stature, failure to thrive, poor weight gain, mild hypotonia, sacral dimple, dermatitis
	2	Autism	Staring spells, dysphagia, drooling, chewing difficulties, low facial muscle tone, choking, vomiting, epigastric hernia, hypotonia, broad thorax with widely spaced, inverted nipples, elongated tailbone, genu valgum, toe-walking, wide based gait, with poor coordination, scoliosis, based gait, with poor coordination, scoliosis, long, tapered fingers, deep set nails, puffy hands, planovalgus, sacral dimple, dermatitis, mild eczema
	3	ASD, short attention span, stereotypic behavior	Prominence of CSF spaces, hypotonia, hypertonia and spasticity in legs leading to abnormal gait, hyperextensible joints
	4	Autism, self-injurious and repetitive behaviors (clapping, hand flapping, chest thumping)	Febrile, grand mal, drop seizures, staring spells, hearing improved with myringotomies, chronic constipation, facial muscle hypotonia
Verheije et al. (26)	1	No	No
	2	ASD (temper tantrums, aggressive behavior, and short attention span)	Gastro-esophageal reflux, conductive hearing loss, sleep apnea, mild asymmetry of the lateral ventricles
	3	ASD	Poor suck, hypermetropia, strabismus, cryptorchidism, and difficulties with co-ordination
	4	No	Limited movement with intermittent hyperextension of the trunk, mild myopia with adequate vision, intermittent hearing impairment, minimal billowing and insufficiency of the mitral valve

(*Continued*)

TABLE 3 Continued

Publication	Case Number	Behavioral problems	Other features
	5	No	Cryptorchidism, retractile right testicle, phimosis/meatal stenosis rep, precocious adrenarche and evaluated, borderline/low testosterone levels after stimulation, recurrent ear infections and possible hearing loss, ocular melanocytosis and iris nevus, pre-glaucoma
	6	No	Learning difficulties
	7	No	Feeding difficulties
	8	No	Severe feeding problems
	9	Restless behavior	Duodenal stenosis, bilateral inguinal hernia, permanent respiratory insufficiency, hypothyroidism, transitory pancreatitis, nephrocalcinosis, feeding difficulties, recurrent vomiting, failure to thrive, high temperatures without infections, bilateral ventriculomegaly and brain atrophy, severe muscular hypotonia of the trunk and hypertonia of the limbs, poor head control, cannot roll over, cannot interact and grasp, strained breathing
Giliberti et al. (22)	1	ASD	Undescended right testicle, stereotypic hand and trunk movements, ectasia and gliosis of Virchow-Robin areas
Santoro et al. (33)	1	No	Minimal mitral regurgitation, brain MRI revealed the presence of UBOs and hypoplasia of the corpus callosum, cafe-au-lait spots, inguinal freckling and cutaneous neurofibromas, severe constipation, CT scan highlighted the early partial closure of both coronal cranial sutures
Gangfuß et al. (21)	1	ASD, (auto-)aggression, lack of distance, aggressive behavior	Feeding difficulties, tracheomalacia (including stenosis of left main bronchus), recurrent pulmonary infections, dystrophy, muscular hypotonia, hypermobile joints, and a mild right convex thoracic scoliosis
Barili et al. (34)	1	NR	Brain anomalies
Current case	1	ADHD	Poor coordination, mild tricuspid regurgitation, recurrent respiratory infections, asthma, rhinitis, enuresis, dental caries, hearing loss due to otitis media, learning difficulties, brain MRI showed a delayed level of mature myelination, moderately impaired lung function

ASD, Autism spectrum disorder; F, Female; M, Male; yrs, Years; NR, No report; BU, Bifid uvula; SMCP, Submucous cleft palate; AtSD, Atrial septal defect; VtSD, Ventricular septal defect; CA, Coarctation of the aorta; LVOTO, Left ventricular outflow tract obstruction; CSF, Cerebrospinal fluid; LPVS, Left pulmonary vein stenosis; TOF, Tetralogy of Fallot; PFO, Patent foramen ovale; PDA, Patent ductus arteriosus; MRI, Magnetic resonance imaging; UBOs, Unidentified bright objects; CT, Computerized tomography.

with methylphenidate showed no significant improvement in attention deficit. In search of the etiology, she underwent WES, which revealed a mutation in the *MEIS2* gene. This further illustrates that genetic testing is an important clinical tool for identifying the causes in ADHD patients with multiple comorbidities and poor response to methylphenidate treatment.

To date, a total of 21 patients carrying *MEIS2* gene mutations have been reported, with 20 patients detected through whole-exome sequencing, and only one patient in the study by Verheije et al. (26) identified using targeted sequencing. This suggests that the phenotype associated with *MEIS2* gene mutations is not easily recognizable. Among the reported patients, most of them had intellectual disability, oral-facial clefts (18/21), similar facial malformation characteristics (19/21), and developmental delay (20/21), suggesting that intellectual disability, oral-facial clefts, facial malformations, and developmental delay are common characteristics. However, the patient we reported did not have an intellectual disability. Approximately half of the patients have cardiac defects (14/21), with ventricular septal defect being the most common feature. However, in three cases, more severe cardiac defects were reported, including CA, TOF, and Ebstein's anomaly. This is because these three severe cases carried a frameshift mutation or an intronic deletion of a single highly conserved amino acid (Arg333) (Patient by Louw et al. and Patients 7 and 8 by Verheije et al.) (26, 29). Our patient shared the same *MEIS2* gene frameshift mutation with the patient 8 reported by Verheije et al. (26), yet the cardiac defects manifested only as a mild VtSD and PFO, with the ventricular septal defect resolving spontaneously over time.

ASD has been identified in some of the patients with *MEIS2* gene mutations that have been documented thus far (9/21). But attention deficits are uncommon in patients with the *MEIS2* gene mutation. The patient in this case report had attention deficits that met the diagnostic criteria for ADHD, a diagnosis that had never before been made in a case with a mutation in the *MEIS2* gene. However, two additional patients with *MEIS2* gene mutations have shown comparable results. Douglas et al. (31) and Verheije et al. (26) reported a 5-year-old girl and a 10-year-old boy, respectively, both diagnosed with ASD and showing short attention spans. Overall, more patients carrying *MEIS2* gene mutations are needed to better define the genotype-phenotype correlations.

Recently, Verheije et al. (26) described a patient with the same frameshift mutation (c.934_937del, p. Leu312Argfs*11). This patient had right ventricular hypoplasia, VtSD, PFO, and tricuspid valve abnormalities, which resulted in severe cyanosis at birth. She also had severe eating issues, delayed motor and language development, a moderate intellectual handicap, a cleft soft palate, and a slight indentation of the hard palate, as well as facial malformations. This patient and the case we reported had comparable clinical symptoms, including delayed motor and verbal development, cleft palate, VtSD, PFO, and facial

malformations. In addition, our patient had ADHD, recurrent respiratory infections, learning difficulties, hearing loss, asthma, rhinitis, enuresis, and dental caries. However, she did not exhibit intellectual disability, which further expands the spectrum of clinical phenotypes caused by the same *MEIS2* gene mutation.

Research has demonstrated that the human, mouse, and chicken *MEIS2* gene regulates specific areas of the developing brain, indicating its significance in neurocognitive development (27, 37, 38). In chicken and mouse heart tissue, DeLaughter et al. (39) showed how the *MEIS2* gene contributes to the conversion of endothelial cells to endocardial mesenchyme. While research indicates that *MEIS2* gene knockout mice are embryonically deadly, conditional knockout mice display aberrant development of the heart, cranial nerves, and craniofacial bones (7), providing evidence to further support that a phenotype of general developmental abnormalities was caused by a disruption of normal human *MEIS2* gene function. Furthermore, research has shown a connection between the *MEIS2* gene and neurodegenerative illnesses and intellectual disability (40, 41).

Additionally, research has demonstrated that the *MEIS2* gene has a role in the etiology of human malignancies (42, 43). Abnormal expression of *MEIS2* significantly impacts neuroblastoma cell proliferation and tumorigenicity (44). Wan et al. (45) first demonstrated that *MEIS2* acts as a metastasis promoter in colorectal cancer. Xiao et al. (46) found that *MEIS2* functions as a tumor suppressor in breast cancer development. A recent study revealed that targeting *MEIS2* expression inhibits proliferation and invasion of prostate cancer cells (47). High levels of *MEIS2* are correlated with poor survival rates in patients with liver cancer (48). *MEIS2* expression is highly downregulated in thyroid cancer patients, as demonstrated by Wen et al. (49) suggesting that *MEIS2* may be a target for early diagnosis and targeted therapy in these individuals. *MEIS2* inhibits the expression of genes specific to the ciliary marginal zone and optic disc and increases the expression of genes specific to retinal progenitor cells (16). *MEIS2* plays a role in acute myeloid leukemia (AML)-ETO-positive leukemia (50). Conversely, high expression of *MEIS2* is associated with improved prognosis in ovarian cancer patients (51). Therefore, *MEIS2* can be considered as one of the genes involved in neurodevelopmental disorders and cancers, such as those related to the RAS pathway genes or BAF complex genes (37, 52).

Our report demonstrates the importance of genetic testing to find the etiology of ADHD in patients with multiple comorbidities who are poorly treated with methylphenidate. In addition, this case further extends the clinical phenotype of mutations in the *MEIS* gene.

Data availability statement

The original contributions presented in this study are included in this article/Supplementary material, further inquiries can be directed to the corresponding author.

Ethics statement

The studies involving humans were approved by West China Second Hospital of Sichuan University. The studies were conducted in accordance with the local legislation and institutional requirements. Written informed consent for participation in this study was provided by the participants' legal guardians/next of kin. Written informed consent was obtained from the individual(s), and minor(s)' legal guardian/next of kin, for the publication of any potentially identifiable images or data included in this article.

Author contributions

FS: Conceptualization, Data curation, Formal Analysis, Investigation, Methodology, Software, Writing – original draft. JL: Conceptualization, Data curation, Formal Analysis, Writing – original draft. DL: Conceptualization, Formal Analysis, Writing – original draft. HZ: Supervision, Writing – review & editing.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Case Report: Efficacy and safety of recombinant growth hormone therapy in a girl with Loeys–Dietz syndrome

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Background: Loeys–Dietz syndrome (LDS) is a clinically and genetically heterogeneous, autosomal dominant aortic aneurysm syndrome with widespread systemic involvement. We present the case of a 16.5-year-old girl with LDS type 2 (LDS2) caused by a heterozygous pathogenic variant, c.1582C>T (p.Arg528Cys), in the transforming growth factor-beta receptor type 2 (TGFBR2) gene who was treated with recombinant growth hormone (rGH) due to coexisting GH deficiency (GHD). This case report (observational study) presents the efficacy of rGH therapy and the safety aspects of this treatment, including aortal imaging follow-up (echocardiography, ECHO). To our knowledge, this is the first investigation of the effects of long-term rGH treatment on aortic dimensions in an LDS patient.

Case summary: LDS was recognized in the patient in the 2nd year of life. After the 3rd year of life, growth deceleration was observed. At age 6, GHD was recognized [the maximum GH after stimulation 7.2 ng/ml; insulin-like growth factor-1 (IGF-1), 35 ng/ml; *N*: 84–447]. At age 6.5 years, rGH was initiated (height standard deviation score, htSDS −2.4), which continued for up to 14.25 years (htSDS-1.4). Her height at 16.5 years was 155 cm. The dose of rGH was 0.025–0.028 mg/kg/day. After the age of 16 months, widening of the aortic root was observed via echocardiography. At nearly 16 years, due to dilated aortic root (Z score +5.95), the girl underwent a plastic operation on the aorta, which had a satisfactory outcome. The patient's current status is stable, but the management of patients with LDS requires multidisciplinary cooperation due to the many coexisting comorbidities.

Conclusions: Although aortic dilatation occurs in most LDS patients, the possible influence of GH therapy on aortic size must be considered. However, whether IGF-1, the main biochemical marker of GH activity, can be independently associated with increased aortic diameter has not been determined. In addition to its growth-promoting effect, the wide influence of GH on the human body, metabolic status, and muscle strength is also significant. The extremely low IGF-1 level before rGH therapy in the present patient and the strict monitoring of the IGF1/IGFBP3 ratio during rGH administration seem to be safe and beneficial for therapy.

KEYWORDS

Loeys-Dietz syndrome, TGFβR2, recombinant growth hormone, cardiovascular system, short stature

1 Introduction

Loeys–Dietz syndrome (LDS) was first described in 2005 (1). Clinical research that led to the diagnosis of LDS focused on probands with three main perturbations: hypertelorism, cleft palate, and aortic or arterial aneurysms. LDS is a disease with an autosomal dominant inheritance, and approximately 70% of cases result from a *de novo* pathogenic variant (2). The disorder is genetically heterogeneous and caused by mutations in the genes of the transforming growth factor *β* (*TGF β*) signaling pathway: *TGFβR1* (LDS1), *TGFβR2* (LDS2), *SMAD3* (LDS3), *TGFβ2* (LDS4), *TGFβ3* (LDS5) and *SMAD2* (LDS6). TGFβ is a cytokine that plays an important role in many physiological processes. It participates in angiogenesis, apoptosis, and regulation of the amount of extracellular matrix protein. TGF-β pathway dysfunction leads to cardiovascular abnormalities and craniofacial and musculoskeletal manifestations. Mutations in *TGFβR2* are the most frequent cause of LDS (3–6).

Clinical findings in four major systems characterize LDS: vascular, craniofacial, skeletal, and cutaneous. In the vascular system, the most frequent disorder is dilatation of the aortic root, which, if unnoticed, results in aortic dissection and rupture (7). The most common skeletal findings are pectus deformities, joint laxity and hypermobility. Craniofacial manifestations include micrognathia, retrognathia, shallow orbits, hypertelorism, and bifid uvula with or without cleft palate. Craniosynostosis and blue sclerae are also reported in LDS (1, 8). LDS is also associated with immunologic-related disorders: affected individuals exhibit food allergies, and LDS patients have a high incidence of asthma, rhinitis, and eczema (3). Gastroenteric manifestations include inflammatory bowel disease and nutritional problems (9, 10). The clinical features and complications of a subject with genetically confirmed LDS are summarized in Figure 1 (1, 11, 12). Due to multisystem disorders, some patients with LDS may present short stature and impaired development. Because this syndrome was first described nearly 15 years ago, the clinical experience is relatively poor, and possible therapeutic options still need to be discussed.

We present the case of a 16.5-year-old girl with LDS2 caused by the heterozygous pathogenic variant c.1582C>T (p.Arg528Cys) in the *TGFβR2* gene who was treated with recombinant growth hormone (rGH) for nearly eight years due to coexisting growth hormone deficiency (GHD). rGH therapy is used mainly in GHD patients. GH plays a key role in heart development and plays a positive role in maintaining the structure of the vascular endothelium. GH has a positive effect on aortic wall distensibility (13). Childhood GHD may restrict cardiac growth, and rGH treatment improves body size and cardiac mass, especially during the first year of therapy (14). However, whether IGF-1, the main biochemical marker of GH action (15), can be independently associated with increased aortic diameter has not been determined. Retrospective studies concerning rGH therapy in patients with Turner syndrome (TS), another genetic syndrome characterized by short stature and aortic dilation, do not suggest a cardiovascular risk (10, 16, 17).

This case series (observational study) reports the efficacy of rGH therapy and the safety of this treatment, including aortal imaging follow-up - echocardiography (ECHO). The safety of substitutional rGH therapy in LDS must be determined, especially given that cardiovascular complications are the most frequent anomalies in this syndrome. The benefits of rGH for LDS in addition to growth promotion, such as any positive effect on the skeleton, the stability of the joints, and the strength of the muscles, should be mentioned. To our knowledge, this is the first investigation of the effects of long-term rGH treatment on aortic dimensions in patients with LDS.

2 Materials and methods

2.1 Genetic analysis

Molecular genetic testing included polymerase chain reaction (PCR) amplification and bidirectional Sanger sequencing of all exons and flanking intronic regions of the *TGFβR1* and *TGFβR2* genes. The analytic methods used were previously described by Matyas et al. (18) and Jamsheer et al. (19).

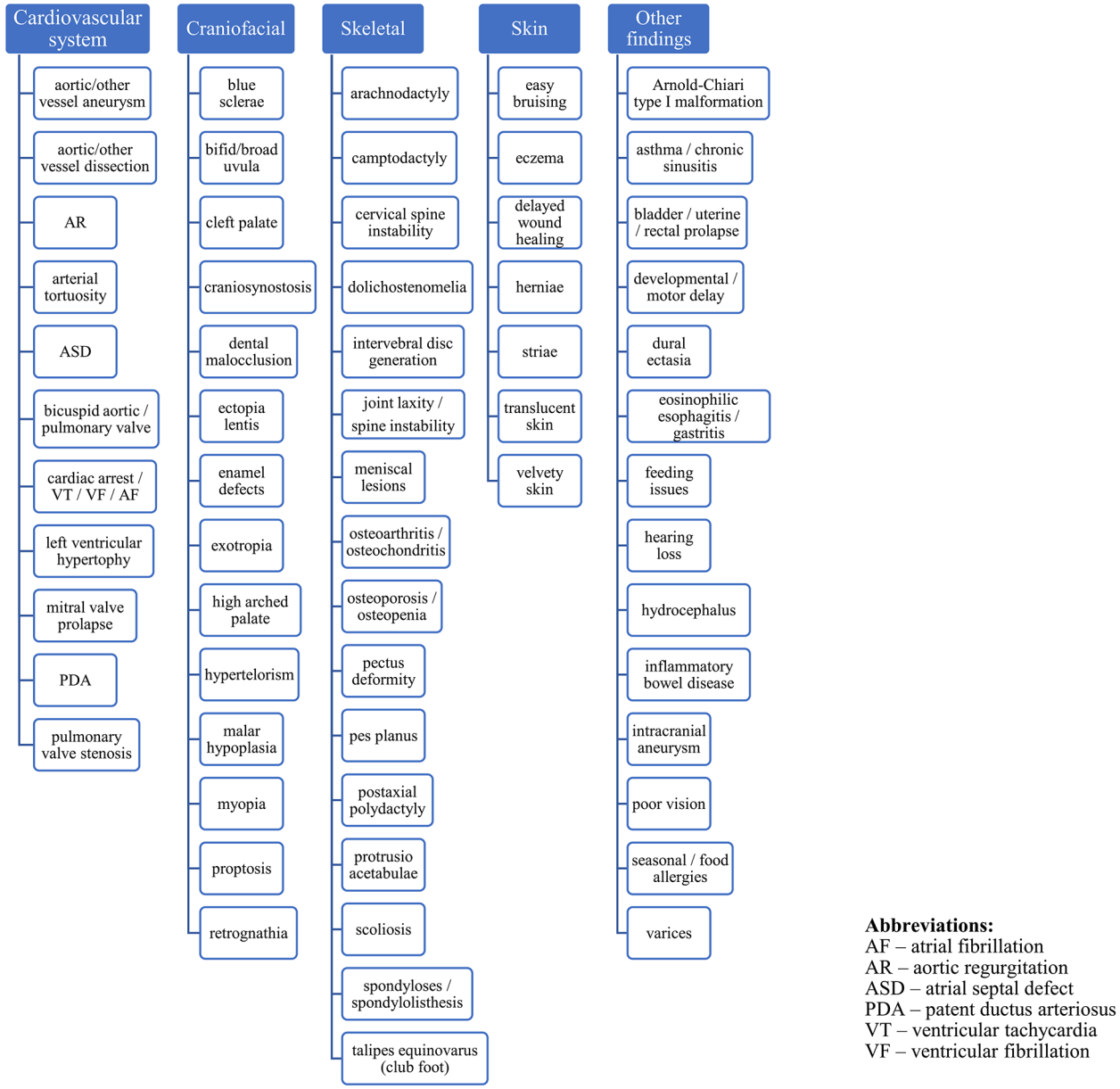


FIGURE 1
Clinical characterization of the LDS.

2.2 Endocrinology

We calculated height standard deviation score (htSDS) for chronological age according with Polish references (20). We estimated bone age according to Greulich and Pyle (21).

2.3 Cardiology

ECHO examinations were performed during visits to the hospital cardiology clinic or outpatient clinic (throughout the patient's lifetime). Specialists in pediatric cardiology performed the surgeries.

ECHO was performed using a convex transducer. The aortic root and ascending aorta sizes were measured from the parasternal long-axis view. The sinus, sinotubular junction, and ascending aorta were measured in diastole using the leading-edge to leading-edge technique, and the obtained values were subsequently analyzed. Z scores were calculated using data developed by Gautier et al. (22). BSA was calculated using the DuBois formula.

Cardiac magnetic resonance (CMR) and 3D dynamic magnetic resonance angiography (MRA) were performed with a 1.5-tesla scanner (Siemens, Avanto) using a matrix coil for body and cardiac

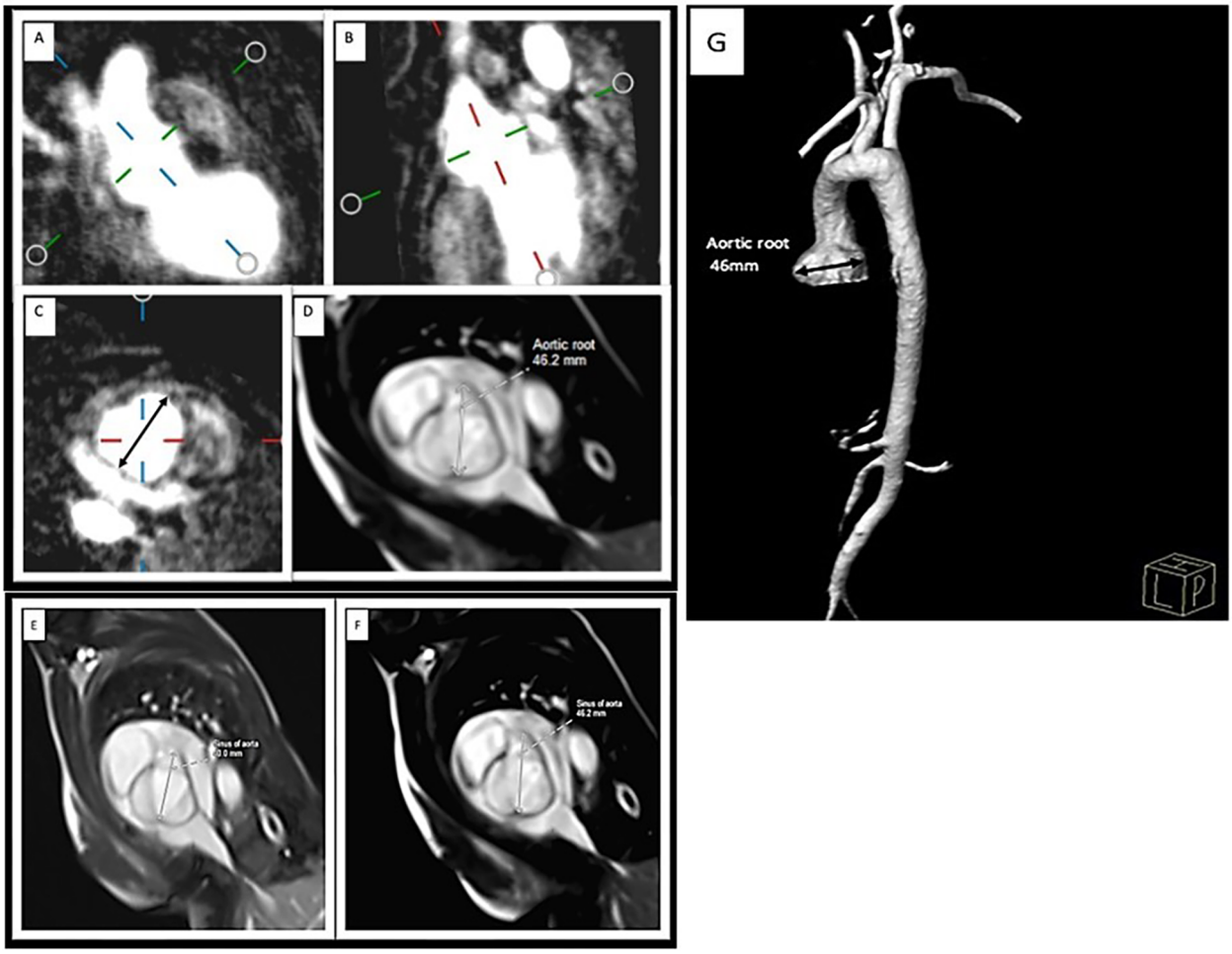


FIGURE 2
Measurement of the aortic root MRA: **(A,B)** perpendicular plane, **(C)** diameter of the ascending aorta in the transverse plane. CMR: **(D)** SSFP sequence of the aortic root. Aortic root diameter progression on CMR: **(E)** initial study (40 mm) and **(F)** examination after 11 months (46.2 mm). 3D reconstruction of the MRA image of the aorta in LDS: aortic sinus aneurysm. **(G)**.

applications combined with a spinal coil. All the sequences were electrocardiogram (ECG)-gated during breath-holding on expiration. Angio-MR was performed using dynamic time-resolved angiography with interleaved stochastic trajectories (TWIST) after administering a contrast agent (0.1 mmol/kg, gadobutrol) followed immediately by a 20 ml saline flush. The duration of contrast agent injection was calculated after the administration of 1 ml of contrast agent. The sequence parameters were TR/TE 2.3/0.87 ms, field of view, 500 × 312.5 mm, slice thickness 1.5 mm, gap 0 mm, matrix size 384 × 223, and in-plane resolution 1.30 × 1.30 mm. The TWIST sequence was used for aortic measurements. The aortic diameter was measured at nine levels: the aortic root, sinotubular junction, ascending aorta, origin of the brachiocephalic artery, first transverse segment, second transverse segment, isthmic region, descending aorta, and thoracoabdominal aorta at the level of the diaphragm (Figure 2). All measurements were obtained with the dedicated software Medis Suite MR 3.0. The results for the aorta were compared against the ranges developed by Kaiser et al. (23). A standardized Z score for aortic diameter at each segment was calculated with an electronic calculator developed by Kaiser et al. (23), in which the diameter of each segment of the aorta and BSA were used.

3 Case report

The girl was born at 39 weeks of gestation by C-section and had a birth weight of 2,800 g, length of 51 cm, and an Apgar score of 10/10. Her parents (unrelated) and older brother (born three years before) did not present any health problems. Based on parental height, the target height was 154 cm.

Physical examination after delivery revealed cleft palate (surgery at 1.5 years old), craniosynostosis (premature closure of sagittal suture), enlarged fontanelle, blue sclerae, hypertelorism, micrognathia, arachnodactyly (fingers and toes), camptodactyly (5th fingers on both hands), joint hypermobility, hyperextensible skin, and atopic dermatitis. A head ultrasound showed hydrocephalus. Subsequent ultrasound examinations showed that the hydrocephalus had improved. There were no indications for

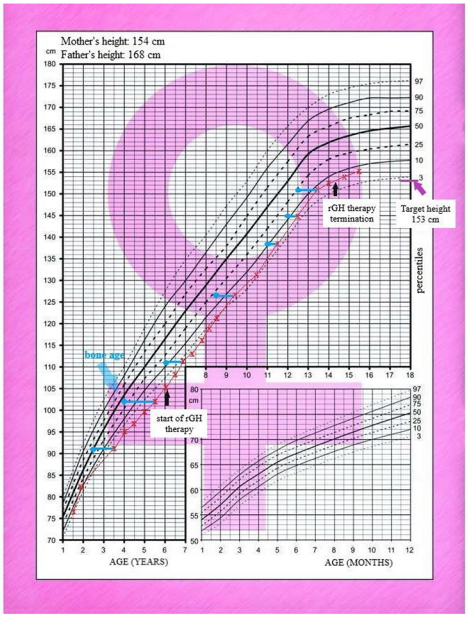
A. Laboratory results evaluation in the presented patient during the qualification for recombinant growth hormone treatment.

Hormonal parameter	Patient	Reference range
TSH [μU/ml]	2.606	0.47 - 3.40
fT4 [ng/dl]	1.13	0.89 - 1.37
ACTH 7.00 a.m. [pg/ml]	**17**	94 - 260
Cortisol 7.00 a.m. [ng/ml]	156	37-194
IGF-1 [ng/ml]	35	15.0 - 65.0
IGFBP-3 [ng/ml]	**2851**	3400 - 4554
max GH after sleep onset [ng/ml]	**5.0**	> 10
max GH after clonidine [ng/ml]	**7.2**	> 10
max GH after glucagon	**3.6**	> 10
PRL (ng/ml)	18.14	3.24 - 29.12
PTH [pg/ml]	17.4	15.0 - 65.0
ALP [IU/l]	142	108 - 360
Ca [mmol/l]	2.5	2.1 - 2.6
Mg [mg/l]	20.7	18.2 - 23.1
P [mg/dl]	4.72	4.5 - 5.52
Na [mmol/l]	140	132 - 145
K [mmol/l]	4.71	3.1 - 5.1
25(OH)D3 [ng/ml]	25.7	7.6 - 75.0
IgA tTG-Ab and IgG Ttg-Ab	negative	negative

B. IGF-1 and IGFBP-3 follow-up values during rGH therapy.

Year of rGH therapy	IGF-1 [ng/ml] Concentration and reference range	IGFBP-3 [ng/ml] Concentration and reference range
1	194 (79-432)	4698 (3400-4554)
2	193 (79-432)	4744 (3156-4839)
3	**140** (175-445)	5053 (3156-4839)
4	196 (175-445)	6081 (3334-4544)
5	292 (175-445)	5058 (3257-5158)
6	329 (202-1101)	6309 (3144-5031)
at the end of rGH therapy	424 (202-1101)	7321 (3771-5249)

C. Growth chart in the presented patient.



Abbreviation:

*The abnormal results are marked in bold.
TSH - thyroid-stimulating hormone
fT4 – free thyroxine
ACTH – adrenocorticotropic hormone
IGF-1 – insulin-like growth factor 1
IGFBP-3 – insulin-like growth factor binding protein-3
PRL – prolactin
PTH – parathyroid hormone
ALP – alkaline phosphatase
Ca – calcium
Mg – magnesium
P – phosphorus
Na – sodium
K – potassium
25(OH)D3 – 25-hydroxy-vitamin D
tTG-Ab - anti-tissue transglutaminase antibody
IgA – Immunoglobulin A
IgG - Immunoglobulin G
rGH - recombinant growth hormone
IGF-1 – insulin-like growth factor 1
IGFBP-3 – insulin-like growth factor binding protein-3

FIGURE 3
Laboratory evaluation of the patient during the qualification for rGH treatment. **(A)** IGF-1 and IGFBP-3 follow-up values during rGH therapy. **(B)** Patient growth chart. **(C)**.

neurosurgical intervention. The girl had left-sided conductive hearing loss. At 16 months, slight widening of the aortic root was diagnosed on ECHO. Due to dysmorphic features and congenital defects, the patient was consulted by a geneticist at age 2 years. The analysis revealed a normal female karyotype. By molecular analysis of the entire coding sequences of the *TGFβR1* and *TGFβR2* genes, the heterozygous pathogenic variant c.1582C>T (p.Arg528Cys) in the *TGFβR2* gene was detected. Genomic DNA was isolated from peripheral mononuclear blood cells. The results confirmed the clinical diagnosis of LDS, indicating type 2 specifically. The mutation c.1582C>T was not detected in the leukocyte DNAs of the unaffected parents, but (germline) mosaicism cannot be excluded. Haplotype analysis using 8 short tandem repeat markers on 6 different chromosomes confirmed paternity and thus *de novo* occurrence of c.1582C>T (19).

At 3 years of age, a slow growth rate was observed, as her height was below the 3rd percentile. The patient was referred to an endocrinology clinic. On admission to the Department of Pediatric Endocrinology, the girl was 3.5 years old. Her height was 91 cm (htSDS −2.57), and her weight was 11 kg [<3th centile; body mass index (BMI) 13.4 kg/m^2] (Figure 3C). Her skeletal age was 2 years and 6 months. According to the diagnostic results, the maximum GH secretion after sleep onset was 5.0 ng/ml. In stimulation tests with clonidine and glucagon, the maximum GH secretion was 7.2 and 3.6 ng/ml, respectively; therefore, partial GHD was diagnosed. On magnetic resonance imaging (MRI) of the head, we excluded a growing lesion process.

At 6 years and 2 months, the girl was still below the 3rd centile for a height of 105.5 cm, with an htSDS of −2.4 (Figure 3C). Her bone age was 4 years. Qualification for rGH therapy was initiated. Figure 3A shows the laboratory results obtained during the qualification time. When the patient was 6.5 years old, the decision to start rGH therapy was made, but intensive supervision of the cardiovascular system was needed. She was administered rGH as a subcutaneous injection. The starting dosage regimen for rGH was 0.025 mg/kg/day. The dose was adjusted during treatment according to the clinical response and laboratory data. The rGH dose was between 0.025 and 0.028 mg/kg/day, and the maximum dose was reached at 12 years. When she turned 7 years old, she reached the 3rd centile of height. She began menstruating at age 13. At 14 years and 3 months, with a satisfactory height of 154.3 cm (htSDS −1.4, equal to parental height), we terminated the rGH therapy. Her height at 16.5 years was 155 cm. During rGH therapy, the girl and her parents did not report any symptoms that might suggest side effects during the treatment. Figure 3B shows her IGF-1 and IGFBP-3 follow-up values during rGH therapy. Figure 3C shows the growth chart of the patient.

Due to dilation of the aortic root, the patient underwent regular ECHO examinations (Table 1). From the 7th year of life, therapy with 10 mg/d propranolol was started. CMR and 3D dynamic MR angiography confirmed the presence of aortic root dilatation (Figure 2). No myocardial late contrast enhancement and no contractility disorders of the left ventricle on CMR were diagnosed. Due to the diagnosis of an aneurysm in the ascending aorta, the patient was qualified for plastic surgery of the aortic root at 15 years and 8 months. The surgical outcome was satisfactory.

The coexisting problems that influenced the patient's quality of life were several operations on lower limb deformities during LDS and

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TABLE 1 Diameters of the aortic root (AoR) and ascending aorta (AoA) on ECHO in the patient.

	Age [years]	AoR		AoA	
		Diameter [cm]	Z-score	Diameter [cm]	Z-score
Initiation of rGH therapy At 6 6/12	3 2/12	2.3	+3.41		
	7 5/12	2.8	+4.01	1.7	+0.68
	8 9/12	2.9	+3.71	1.7	+0.03
	13	3.1	+2.78	2.1	+0.57
Discontinuation of rGH therapy At 14 3/12	13 10/12	3.5	+3.85	2.1	+0.31
	14 5/12	4.1	+5.48	2.1	+0.18
	15 8/12	4.3	+5.95	2.2	+0.45
	15 9/12 after surgery	2.5	-0.71	2.2	+0.0
	16 10/12	2.3	-1.12	2.1	+0.02

severe atopic dermatitis and asthma. At 15 years of age, the girl was diagnosed with epilepsy with focal seizures. She started treatment with oxcarbazepine due to localized changes and generalized discharges recorded in the interictal electroencephalogram (EEG), accounting for her medical history (slurred speech, loss of consciousness). Through consultation with a psychologist, it was found that the patient's ability to think abstractly and logically was below the norm for her age. She had no memory disorders, but her general course of psychomotor development was inharmonious. Supplementary Figure 1 shows a photograph of the patient at age 9 months (A) and at the present time (B).

4 Discussion

LDS is a sporadic genetic syndrome. Current data show fewer than 4,000 patients with confirmed LDS. To our knowledge, our proband is the first patient with LDS and GHD in whom rGH therapy was introduced (11, 12). The patient in the present case was diagnosed with LDS at the early age of two years due to many disorders, such as craniofacial dysmorphism, mild aortic root dilatation, hydrocephaly, and skin and musculoskeletal disturbances (19). Because of her short stature, diagnostics were performed, and an early diagnosis of GHD was established. The laboratory results indicated partial GHD but the peripheral markers of GH action, especially IGF-1, were significantly below the normal range. The influence of coexisting comorbidities and the nutritional status of patients (BMI up to 12 years below the normal range) on IGF-1 generation should be considered. In contrast, the marked increase in IGF-1 after rGH initiation indicates that the organism has a good reaction and readiness to generate growth factors. The patient achieved satisfactory final growth after rGH therapy. More than 2 years after rGH therapy termination, the patient's height at 16.5 years was 155 cm, equal to the target height based on her parents' height. This finding suggested that the girl had recovered her growth potential at that time. A well-documented factor influencing the response to therapy is the child's age at the start of rGH treatment. Based on clinical observations, it is recommended to start rGH therapy as soon as the child has not shown a spontaneous catch-up process. This enables a better response to rGH therapy and, accordingly, a greater final height (24, 25). In addition to the growth-promoting action of rGH, the wide anabolic influence on the human body, including body composition, bone mass density, muscle mass, and strength, seems to be beneficial in patients suffering from joint hypermobility and low muscle mass. GH therapy positively influenced body composition, as shown through the patient's psychomotor development.

The right balance between the need for the substitution of GH and the safety aspects of the therapy in the presented patient must be noted. GH, physiologically, plays a key role in the development of the heart during fetal development and plays a positive role in maintaining the structure and function of the normal adult heart by stimulating cardiac growth and heart contractility. GH acts directly on myocardial growth by inducing the mRNA expression of specific proteins and facilitating cardiomyocyte proliferation (16, 26). Moreover, GH may directly act on endothelial cells through the promotion of the expression and activity of endothelial NO synthase (eNOS) (27). Patients with GHD exhibit cardiac atrophy with lower LV mass, ejection fraction, and cavity dimensions; lower cardiac output; greater peripheral vascular resistance; and lower functional capacity than healthy controls of the same age, sex, and height (28). Additionally, GHD patients exhibit accelerated development of atherosclerosis. As a result, they have a high rate of cardiovascular mortality (29). GH was found to influence collagen metabolism and change the mixture of fibrous elements in the aortic wall. GH increases muscle strength by increasing muscle mass without affecting contractile force or fiber composition (30). Therefore, GH contributes to improved myocardial performance, increasing physical activity.

Although aortic dilatation occurs in most LDS patients, the possible influence of GH therapy on aortic size must be considered. Our patient experienced a widening of the aortic root since the age of 16 months. While the aortic root increased during

the administration of growth hormone, the Z-scores did not exhibit consistent increases over time. The highest increase in the diameter was observed at the age of 14 years. There are no data on the long-term effect of rGH therapy on the cardiovascular system in patients with LDS. The best experience of long-term rGH effects and safety is described in TS, where aortic dilatation may occur in early childhood (31). However, the genetic etiology of aortopathy is still under study. There are contrasting data on the influence of rGH treatment on aortic diameter in TS patients. Laroussi et al. (32) suggested that rGH therapy in TS patients had a nonsignificant influence on the aortic dimension. However, aortic dilation was observed more often in patients treated with rGH than in untreated patients (8/25 patients - 32% vs. 3/17–17.6%). A nonsignificant difference in the ascending aorta Z score was noted (32). Another study concluded that neither the history of rGH treatment nor the length of GH treatment affected the aortic diameter in patients with TS (17). Aortic root dilation in children should lead to the suspicion of connective tissue abnormalities, including LDS. Generally, patients with LDS types 1 and 2 have similar risks of aortic dissection, but males have a greater risk of aortic complications than females (33–36). Based on the follow-up of consecutive patients, the incidence of aortic root dilation ranges from 0.11–0.67 cm/year, with the most significant progression occurring in patients with LDS type 2 (36, 37). There are no clear guidelines for treating aortic aneurysms and dilatation in LDS. Surgical intervention is often recommended when the aortic diameter exceeds 4.0–4.6 cm for the aortic root and abdominal aorta, exceeds 5.0 cm for the descending thoracic aorta, or shows rapid growth (>0.5 cm/year) in any location (7, 12). Jondeau et al. (35) considered preventive replacement of the aortic root at a diameter of 45 mm or 40 mm in females with low BSA, TGFβR2 mutation, or severe extra-aortic features. Certain phenotypic factors are associated with an increased risk of aortic dissection: the presence of a TGFβR2 mutation, female sex, the presence of aortic tortuosity, hypertelorism, and translucent skin (35). The inherent weakness of the aortic wall may warrant earlier intervention, depending on the patient's family history or an evaluation of the risks and benefits of surgery (7). However, it can be assumed that a supraphysiologic increase in the IGF-1 concentration due to rGH therapy could contribute to an increase in aortic diameter. Other reports have suggested that GH does not impact aortic or ventricular size when adjusted for height or body surface area. In our own experience, strict control of plasma IGF-1 and IGFBP-3 levels ensures that they are similar to normal values for age and sex and ensures the safety of rGH therapy. The IGFBP-3 level is pivotal for the bioactivity of circulating IGF-1 (38). Blum et al. (39) found IGFBP-3 to be a more authentic discriminator of GH-dependent parameters than IGF-1 was in IGF generation tests. Careful follow-up of therapy is also necessary because concern arises with the oncogenic potential of GH. However, long-term studies in GHD children have not shown any increase in the incidence of tumors (40). Therefore, we can also suggest a significant role for the IGF-1/IGFBP-3 ratio during rGH treatment in patients with LDS syndrome (16, 41). The dose should be modified according to the IGF-1 level during treatment. In the treated patient, the IGF-1 level at the start of rGH therapy was significantly lower than normal. Her IGF-1 level was normalized during follow-up, and her IGFBP-3 level remained within or slightly above the normal range. These various biochemical results confirm the proper dosing of rGH. Indeed, additional studies are needed in the future to verify the relationship between the rGH treatment and aortic dilation progression in LDS.

5 Conclusions

This case report shows the results of long-term rGH therapy in a girl with LDS as a substitution therapy, which allowed us to reach a final height close to mid-parental height. The wide influence of GH on the human body, metabolic status, stabilization of the vascular wall, and muscle strength is also significant, in addition to its growth-promoting action. Because most patients with LDS experience aortic aneurysms, regular follow-up of aortic diameter is required. We do not suspect that rGH therapy had a negative impact on the dimensions of the aorta in our patient, but additional studies in the future are needed. A low IGF-1 at the GHD diagnosis and strict monitoring of the IGF-1/IGFBP-3 ratio during rGH administration were crucial in the presented patient. This is the first investigation of the effects of long-term rGH treatment on aortic dimensions in LDS.

5.1 Patient consent

Written informed consent was obtained from the patient's parent. Documentation was recorded in the patient's medical records.

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Ethics statement

Ethical approval was not required for the study involving humans in accordance with the local legislation and institutional requirements. Written informed consent to participate in this study was not required from the participants or the participants' legal guardians/next of kin in accordance with the national legislation and the institutional requirements. Written informed consent was obtained from the individual(s), and minor(s)' legal guardian/next of kin, for the publication of any potentially identifiable images or data included in this article.

Author contributions

KD: Conceptualization, Data curation, Formal Analysis, Investigation, Methodology, Project administration, Software,

Supervision, Validation, Visualization, Writing – original draft, Writing – review & editing. AJ: Formal Analysis, Investigation, Resources, Software, Validation, Writing – review & editing. MB: Formal Analysis, Investigation, Software, Validation, Writing – review & editing. WB: Formal Analysis, Investigation, Resources, Software, Validation, Writing – review & editing. MP-W: Formal Analysis, Investigation, Resources, Software, Writing – review & editing. JR-T: Formal Analysis, Investigation, Resources, Software, Validation, Writing – review & editing. AT: Conceptualization, Investigation, Writing – original draft. JB: Conceptualization, Investigation, Writing – original draft. ZA: Conceptualization, Investigation, Writing – original draft. MN: Conceptualization, Data curation, Formal Analysis, Funding acquisition, Investigation, Methodology, Resources, Software, Supervision, Validation, Writing – review & editing. MO-M: Conceptualization, Data curation, Formal Analysis, Funding acquisition, Investigation, Methodology, Project administration, Resources, Software, Supervision, Validation, Visualization, Writing – original draft, Writing – review & editing.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as potential conflicts of interest.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcvm.2024.1377510/full#supplementary-material

SUPPLEMENTARY FIGURE 1
Photographs of the patient at age 9 months (**A**) and at 16.5 years (**B**).

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Increased cardiac macrophages in *Sorbs2*-deficient hearts: revealing a potential role for macrophage in responding to embryonic myocardial abnormalities

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Macrophages are known to support cardiac development and homeostasis, contributing to tissue remodeling and repair in the adult heart. However, it remains unclear whether embryonic macrophages also respond to abnormalities in the developing heart. Previously, we reported that the structural protein Sorbs2 promotes the development of the second heart field, with its deficiency resulting in atrial septal defects (ASD). In analyzing RNA-seq data, we noted an upregulation of macrophage-related genes in *Sorbs2*$^{-/-}$ hearts. Immunostaining and lineage-tracing confirmed an increase in macrophage numbers, underscoring a macrophage response to myocardial abnormalities. Partial depletion of macrophages led to downregulation of genes involved in lipid metabolism, muscle development and organ regeneration, alongside upregulation of genes associated with DNA damage-induced senescence and cardiomyopathy. Additionally, a non-significant increase in septal defects in macrophage-depleted *Sorbs2*$^{-/-}$ hearts suggests a potential reparative function for macrophages in maintaining structural integrity. Valve formation, however, remained unaffected. Our findings suggest that embryonic macrophages might sense abnormalities in embryonic cardiomyocytes and could adaptively support cardiac structure and function development in response to myocardial abnormalities.

KEYWORDS

macrophage, Sorbs2, cardiac septal defect, valve formation, adaptive response

Introduction

Cardiac morphogenesis initially involves the coordinated actions of progenitor cells, which give rise to diverse cell types within the heart and drive the initial stages of heart formation, establishing the basic structure and organization of the heart (Van Vliet et al., 2012). However, myocardial development is also essential for cardiac morphogenesis,

providing the contractile force needed for circulation and shaping the developing heart (Taber et al., 2010). Cardiomyocyte differentiation initiates the expression of sarcomeric proteins such as actin and myosin. These proteins subsequently assemble into the complex and highly ordered sarcomere, the basic structural and functional unit of myofibrils. Over time, more accessory proteins are added into the rudimentary assemblies to form a mature muscle contractile apparatus (Guo and Pu, 2020). Mutations in major sarcomeric genes are commonly associated with cardiomyopathy but can also lead to abnormal non-syndromic congenital heart defects such as ASD (Yasuhara and Garg, 2021). Sorbs2 (sorbin and SH3 domain-containing 2) is an accessory protein located at the Z disk and intercalated disk in cardiomyocytes, crucial for sarcomere organization and the structural integrity of the intercalated disk (Ding et al., 2020; Wang et al., 1997). Knockout of *Sorbs2* causes arrhythmogenic and dilated cardiomyopathies (Ding et al., 2020; McLendon et al., 2020). *Sorbs2* deficiency also leads to incomplete penetrance of ASD (Liang et al., 2021).

Beyond the intrinsic structural components within cardiomyocytes, other cell types in the myocardial microenvironment, such as fibroblasts and immune cells, contribute to cardiac morphogenesis and maintenance (Ding et al., 2022). In the embryonic heart, macrophages initially present in the subepicardial space later spread to deeper layers (Gula et al., 2021), including the bulbar and atrioventricular cushions (Shigeta et al., 2019). During mammalian heart development, macrophages participate in coronary vessel development, lymphangiogenesis, and cardiac valve shaping (Cahill et al., 2021; Leid et al., 2016; Shigeta et al., 2019). In mature hearts, cardiac macrophages contribute to electrical conduction, maintain homeostasis, and respond to pathological conditions to affect post-injury repair and remodeling (Moskalik et al., 2022). However, the macrophage response to pathological conditions in the embryonic heart remains unclear.

We previously reported that *Sorbs2* is essential for atrial septum development, with *Sorbs2* knockout causing ASD in about 40% of embryos (Liang et al., 2021). Interestingly, RNA-seq data from E10.5 *Sorbs2*$^{-/-}$ embryos revealed upregulated macrophage gene expression. To determine whether this upregulation is due to increased macrophages in the heart, we used immunofluorescent staining and macrophage lineage-tracing to evaluate macrophage number and distribution in E12.5 hearts. Results showed an increase in macrophages within embryonic hearts. Partial ablation of cardiac resident macrophages significantly altered the cardiac transcriptome at E12.5. Although we did not observe valve malformation, there was a non-significant increase in septal defect penetrance. Collectively, our results indicate that cardiac macrophages respond to structural gene mutations and might play a reparative role in myocardial morphogenesis and function.

Results

Increased expression of macrophage-related genes in *Sorbs2*$^{-/-}$ hearts

In analyzing the transcriptomic data of E10.5 embryos (Liang et al., 2021), we noted upregulation of macrophage-related genes, such as *C1q1*, *Adgre1*, and *Mrc1*, in *Sorbs2*$^{-/-}$ mutants (Figure 1A). However, *Sorbs2* is not expressed in macrophages but is highly expressed in embryonic hearts (Supplementary Figure S1). We hypothesized the upregulation of macrophage-related genes occurs within the heart. Since macrophages start to populate hearts as early as E9.5 (Epelman et al., 2014), we collected E12.5, E15.5 and E18.5 ventricles to perform RNA-seq (Supplementary Tables S1–3). We selected genes significantly downregulated in E12.5 mutant hearts [$\log_2$ (fold change) <-0.58, $p < 0.05$] to perform GO analysis and found these genes enriched in pathways related to the electron transport chain and mitochondrial translation elongation (Figures 1B, C), suggesting that *Sorbs2* positively regulates myocardial maturation. Using the same threshold, we selected genes significantly upregulated in E12.5 mutant hearts to perform GO analysis. Interestingly, these genes are enriched in pathways regulating immune response (Figure 1B). Upon closer examination, we observed differential expression of macrophage marker genes such as *Cx3cr1* and *Lyz2* (Figure 1C), suggesting that macrophages may be activated in *Sorbs2*$^{-/-}$ hearts. These changes in gene expression patterns persisted throughout E18.5 (Figure 1C). Taken together, our RNA-seq results revealed that *Sorbs2* deficiency impairs myocardial maturation and triggers a response in macrophages.

Increased number of macrophages in *Sorbs2*$^{-/-}$ hearts

The increased expression of macrophage marker genes in *Sorbs2*$^{-/-}$ embryonic hearts prompted us to examine macrophage numbers in mutant hearts. To this end, we performed whole-mount immunostaining on E12.5 hearts, using an antibody against the pan macrophage marker F4/80. Indeed, it revealed increased macrophages in the ventricles of E12.5 *Sorbs2*$^{-/-}$ hearts (Figure 2A). In section analysis, macrophages were mainly distributed under the epicardium and in the outer layer of the myocardium (Figure 2B). Consistently, sections of *Sorbs2*$^{-/-}$ heart displayed increased macrophage counts (Figures 2B, C).

Next, we used macrophage lineage-tracing to further validate this observation. *Cx3cr1* is a marker of embryonic heart macrophage (Leid et al., 2016). We bred *Cx3cr1*^{CreERT2} and *Rosa26*^{nTmG} alleles into *Sorbs2*$^{-/-}$ mice. Tamoxifen-induced CreERT-mediated recombination in the *Rosa26* locus led to EGFP expression in *Cx3cr1*-lineage macrophages, confirming increased macrophages in the ventricular walls of E12.5 *Sorbs2*$^{-/-}$ hearts (Figures 2D, E). These data indicate that increased expression of macrophage-related genes results from the increased number of macrophages in *Sorbs2*$^{-/-}$ hearts.

Macrophage-specific CreERT-induced DTA significantly reduced cardiac macrophages

Macrophages are vital residents of the developing heart. During heart development, tissue resident macrophages regulate coronary vessel formation and lymphatic network development (Cahill et al., 2021; Leid et al., 2016). They are also essential for the developmental remodeling of cardiac valves (Shigeta et al., 2019). The increased macrophages in *Sorbs2*$^{-/-}$ hearts led us to question whether

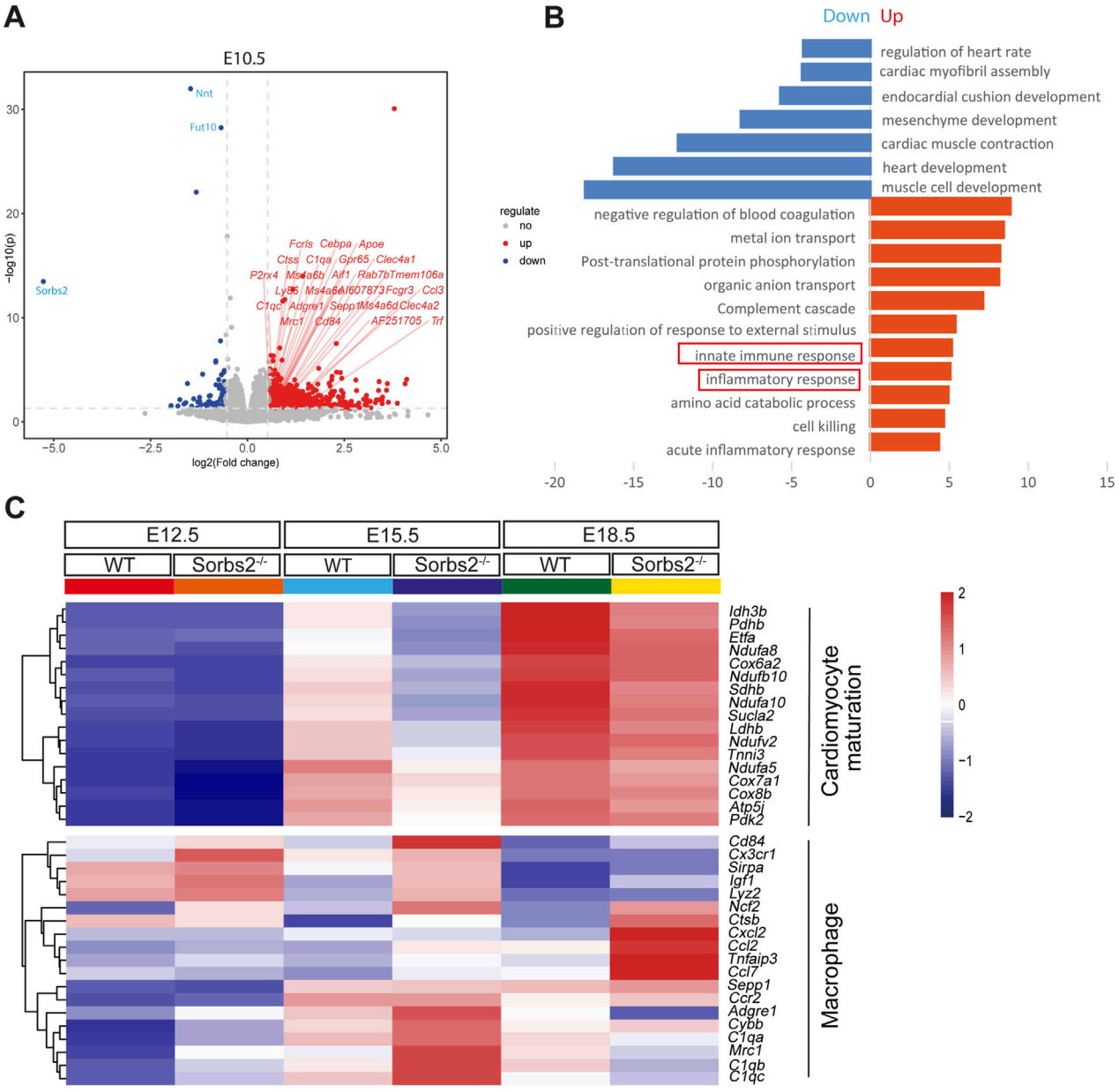

FIGURE 1
Increased expression of macrophage-related genes in *Sorbs2*$^{-/-}$ embryonic heart **(A)** Volcano plot illustrating differentially expressed genes (DEGs) at embryonic day 10.5. Each point corresponds to an individual gene. Genes with statistically significant differential expression ($p < 0.05$ and an absolute $\log_2$ (fold change) > 0.58) are highlighted. Red, upregulated genes. Blue, downregulated genes. Gray, non-significant genes. Macrophage-related genes are marked with red slashes. **(B)** Enrichment analysis of DEGs in E12.5 embryonic ventricles (homozygous vs. wild type). **(C)** Heatmap visualizing gene expression in embryonic ventricles at E12.5, E15.5, and E18.5. Color tints indicate expression levels. Genes in the upper section are involved in cardiomyocyte maturation, while those in the lower section are linked to macrophages.

macrophages might play an unknown role in the abnormal embryonic hearts.

To this end, we took a cell depletion approach with the *Rosa26*^{DTA} alleles, which encodes cytotoxic diphtheria toxin A (DTA) after Cre-induced recombination removes the STOP element, therefore killing cells that express DTA (Ivanova et al., 2005). Tamoxifen was administered to *Cx3cr1*$^{CreERT2/+}$; *Rosa26*$^{DTA/+}$ mice at E9.5 and E11.5 through oral gavage, and hearts were collected at E12.5 for F4/80 immunostaining to check the efficiency of macrophage depletion (Figures 3A, B). Results showed that macrophages were decreased in *Cx3cr1*$^{CreERT2/+}$; *Rosa26*$^{DTA/+}$ hearts (Figure 3C). The quantification indicated that macrophage numbers significantly decreased and the reduction ratio was about 40% (Figure 3D).

Transcriptomic changes induced by macrophage depletion

We obtained *Cx3cr1*$^{CreERT2/+}$; *Rosa26*$^{DTA/+}$;*Sorbs2*$^{-/-}$ mice to examine the effect of macrophage depletion on *Sorbs2*$^{-/-}$ hearts.

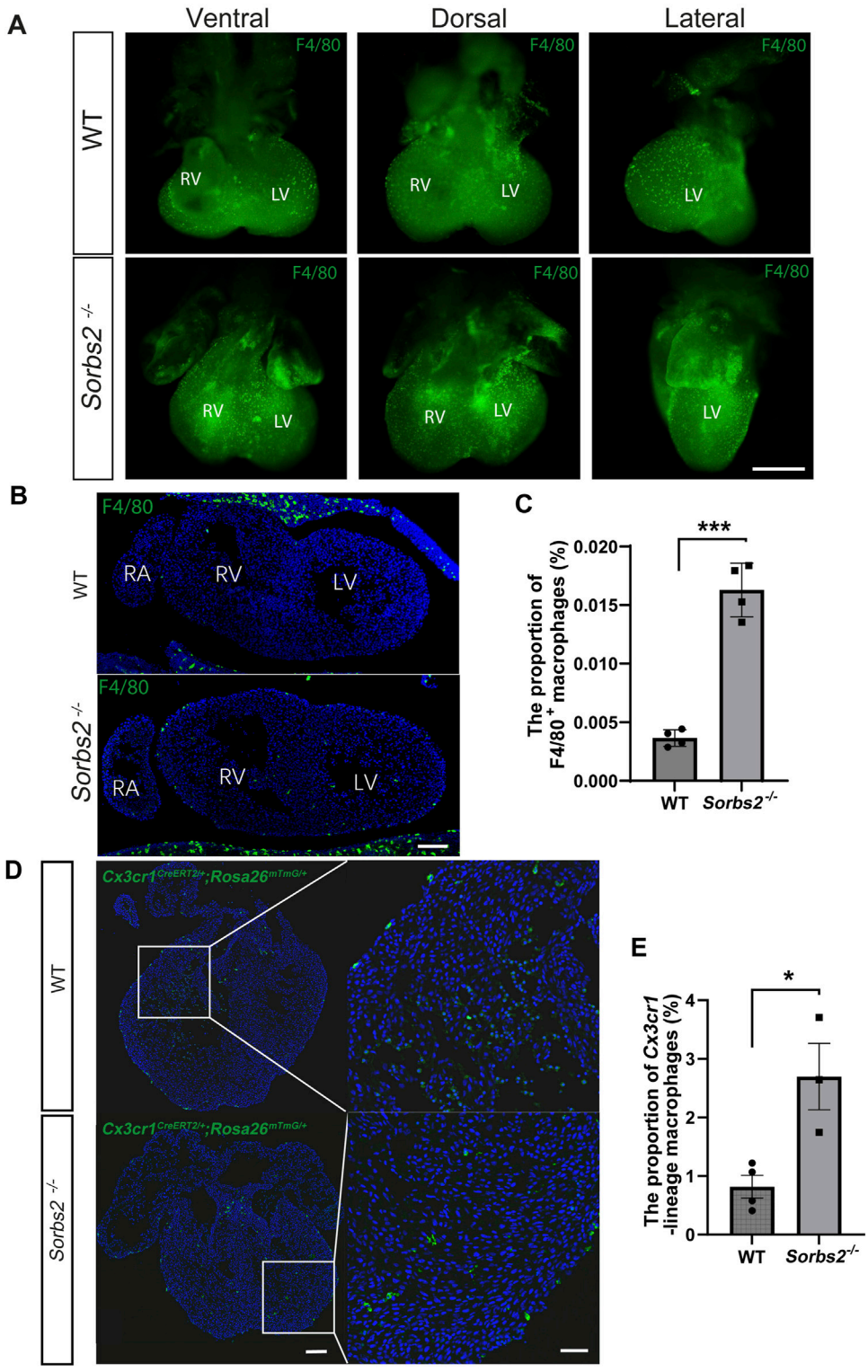

FIGURE 2
Increased number of macrophages in embryonic *Sorbs2*$^{-/-}$ hearts **(A)** Whole-mount immunostaining of E12.5 embryonic hearts. Samples were stained with anti-F4/80 antibody (green) to visualize macrophages. RV, right ventricle. LV, left ventricle. Scale bar, 500 μm. **(B)** Representative images of E12.5 heart sections immunostained with anti-F4/80 antibody (green) and DAPI (blue). RV, right ventricle. LV, left ventricle. RA, right atrium. Scale bar, 100 μm. **(C)** Quantification of F4/80^{+} macrophages (n = 4 per group). *, $p < 0.001$. Nested ANOVA test. **(D)** Representative images of *Cx3cr1*-lineage macrophages (green) in E12.5 hearts. Scale bar, 100 μm for the low magnification and 50 μm for the high magnification. **(E)** Quantification of *Cx3cr1*-lineage macrophages (n = 4 for WT group, n = 3 for *Sorbs2*$^{-/-}$ group). *, $p < 0.05$. Nested ANOVA test.

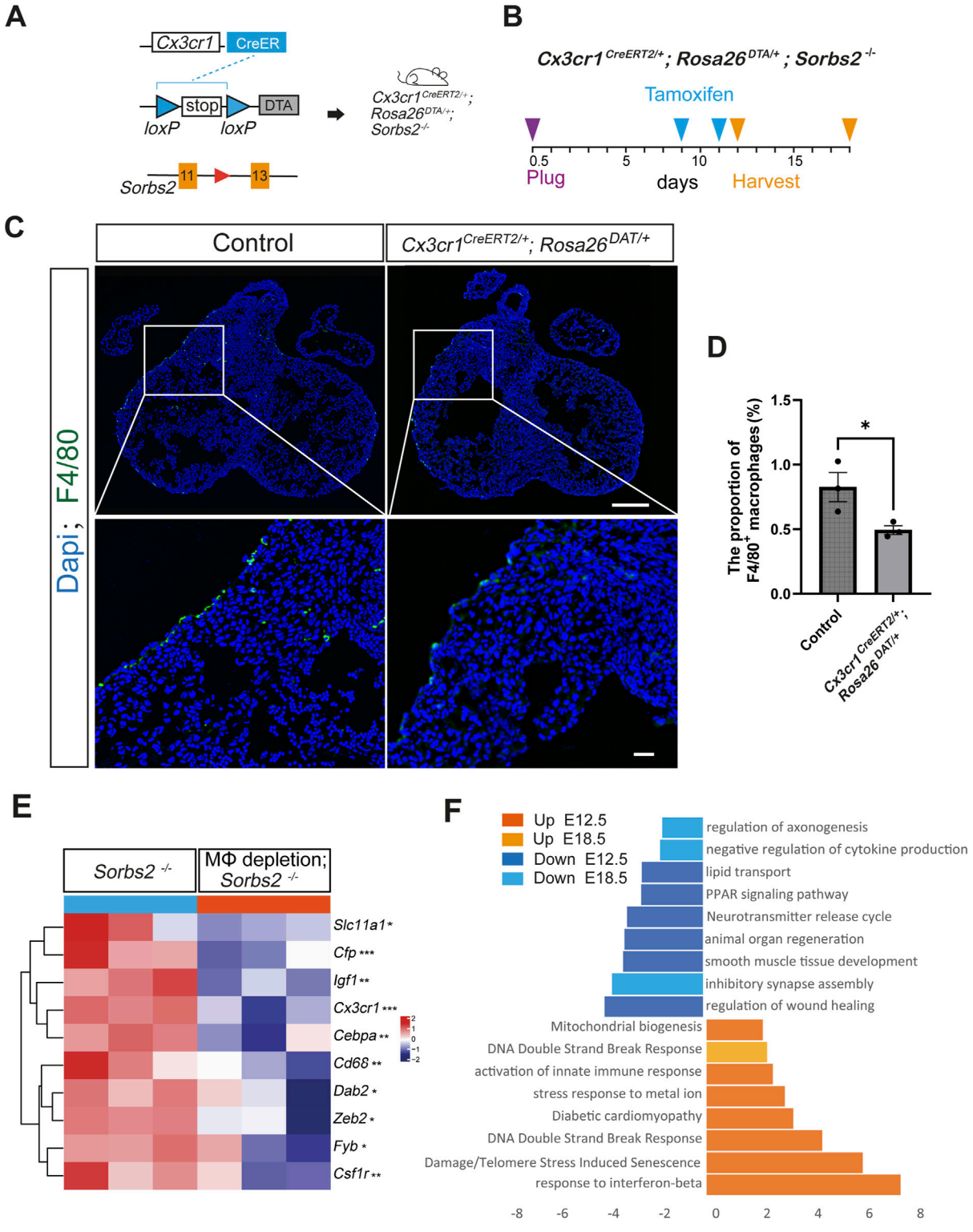

FIGURE 3
Transcriptomic changes in macrophage-depleted *Sorbs2*$^{-/-}$ hearts **(A)** Strategies for macrophage depletion in *Sorbs2*$^{-/-}$ mice. **(B)** Experimental design. Tamoxifen was administered at E9.5 and E11.5 via oral gavage. Hearts were harvested at E12.5 or E18.5. **(C)** Representative immunofluorescent images of E12.5 hearts stained with anti-F4/80 antibody (green) and DAPI (blue). Scale bar, 200 μm for the low magnification and 400 μm for the high magnification. **(D)** Quantification of F4/80^{+} macrophages (n = 3 per group). *, $p < 0.05$. Nested ANOVA test. **(E)** Heatmap illustrating expression levels of macrophage-related gene in E12.5 heart ventricles. *, $p < 0.05$. **, $p < 0.01$. ***, $p < 0.001$. MΦ, macrophage. **(F)** Enrichment analysis of DEGs in embryonic ventricles at E12.5 and E18.5 (*Cx3cr1*$^{CreERT2/+}$; *Rosa26*$^{DTA/+}$; *Sorbs2*$^{-/-}$ vs. *Sorbs2*$^{-/-}$).

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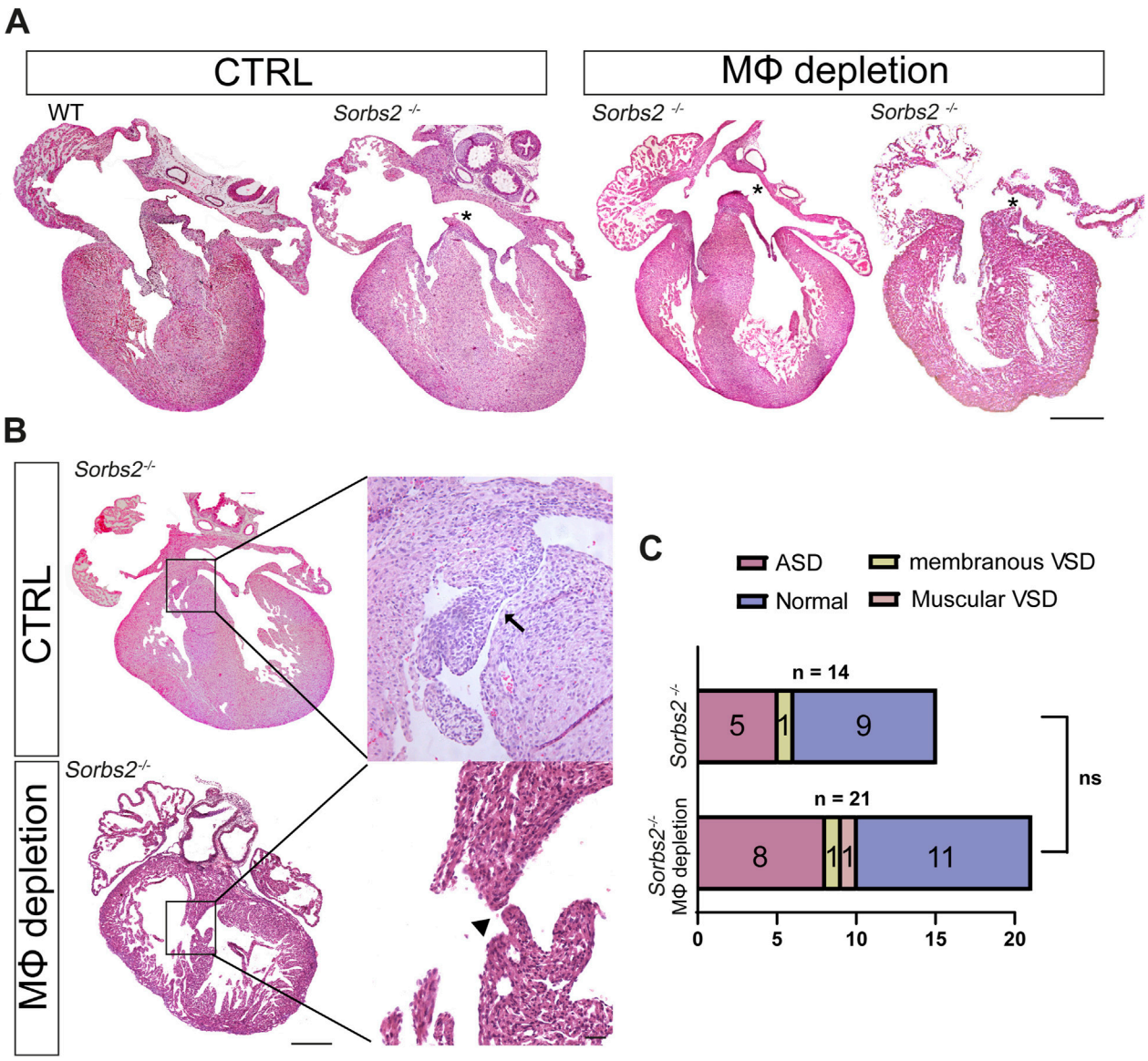

FIGURE 4
Septal defects in macrophage-depleted and non-depleted *Sorbs2*$^{-/-}$ hearts **(A)** Hematoxylin and eosin (HE)-stained paraffin sections of E18.5 hearts. Asterisk indicates ASD. Scale bar, 200 μm. **(B)** HE-stained paraffin sections of E18.5 hearts. Boxed areas are magnified to highlight VSDs. Arrow, membranous VSD. Arrowhead, muscular VSD. Scale bar, 200 μm for the low magnification and 500 for the high magnification. **(C)** Penetrance of ASD and VSD in macrophage-depleted and non-depleted *Sorbs2*$^{-/-}$ hearts. Ns, non-significant. Chi-square test.

We collected E12.5 ventricles for RNA-seq (Supplementary Table S4). Results showed that macrophage marker genes, such as *Cx3cr1*, *Cd68* and *Csf1r*, were significantly reduced compared with control littermate embryos (Figure 3E), verifying reduced macrophages. Pathway analysis showed downregulated genes involved in organ regeneration, lipid metabolism, muscle development, and neurotransmitter signaling, while upregulated genes were associated with innate immune response activation, DNA damage-induced senescence, diabetic cardiomyopathy, and mitochondrial biogenesis (Figure 3F). These results suggest that macrophage depletion impairs cardiac metabolism and causes cardiomyocyte damage in mid-gestation stage. By E18.5, transcriptomic changes between macrophage-depleted and non-depleted groups were minimal (Supplementary Table S5), indicating a transient effect of our macrophage depletion strategy, though we noted continued downregulation in neural development genes and upregulation in DNA damage response genes (Figure 3F).

Partial macrophage depletion slightly increased the penetrance of structural cardiac defects in *Sorbs2*$^{-/-}$ hearts

As previously reported, about 40%–60% *Sorbs2*$^{-/-}$ mice died within 1 week after birth, with about 40% presenting atrial septal defect (ASD) (Zhang et al., 2016; Liang et al., 2021). We wondered whether the macrophage increase in *Sorbs2*$^{-/-}$ hearts is an attempt to

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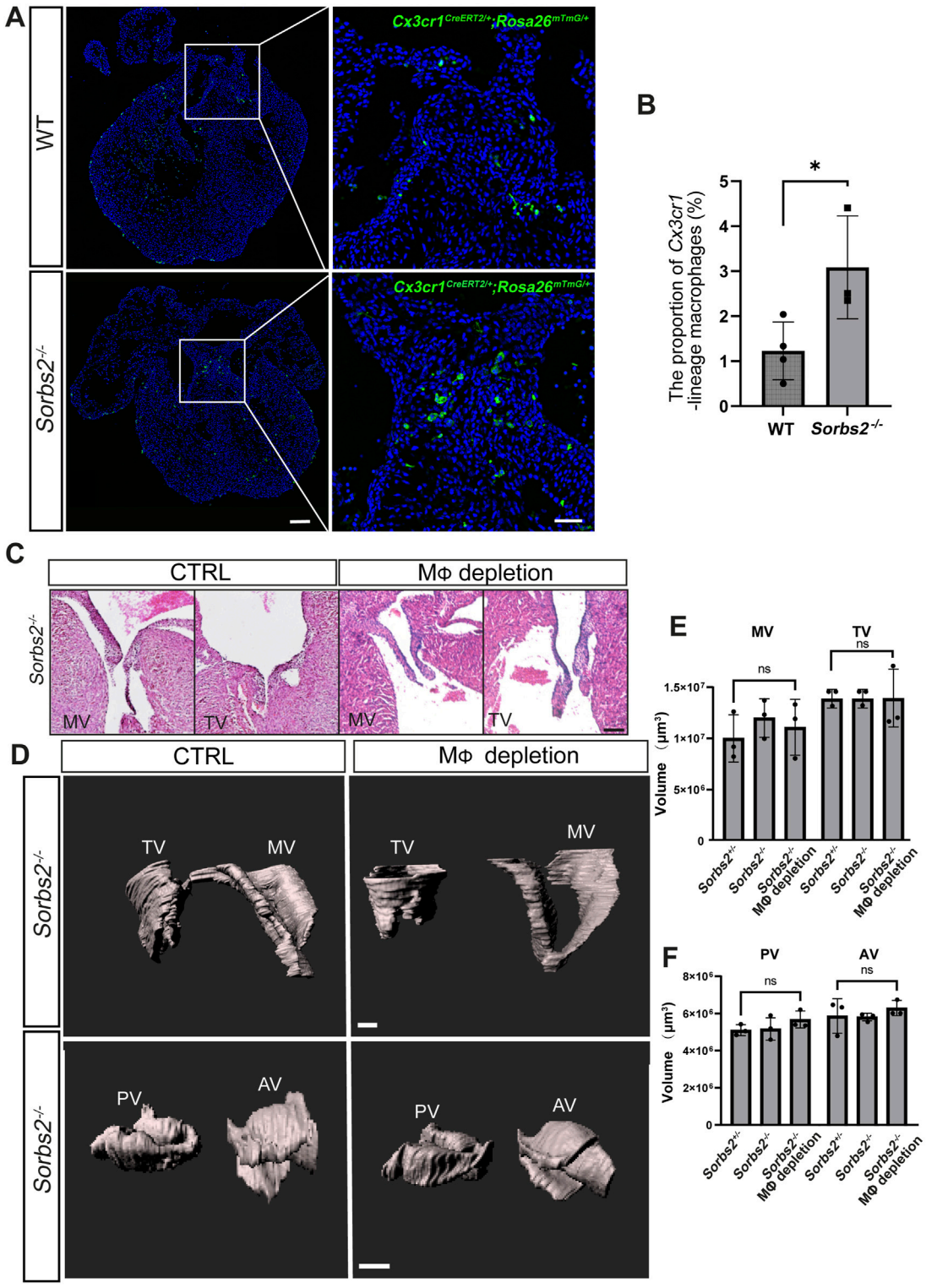

FIGURE 5
Morphological analysis of valves in macrophage-depleted and non-depleted *Sorbs2*$^{-/-}$ hearts **(A)** Representative images of *Cx3cr1*-lineage macrophages (green) in the endocardial cushion of E12.5 hearts at E12.5d. Scale bar, 100 μm for low magnification and 500 μm for high magnification. **(B)** Quantification of *Cx3cr1*-lineage macrophages in the endocardial cushion (n = 4 for the WT group, n = 3 for the *Sorbs2*$^{-/-}$ group). *, $p < 0.05$. Nested ANOVA test. **(C)** HE-stained paraffin sections of E18.5 hearts showing the mitral and tricuspid valves. MV, mitral valve. TV, tricuspid valve. Scale bar, 50 μm. **(D)** Representative 3D reconstructions of E18.5 mitral, tricuspid, pulmonary, and aortic valves through manual surface rendering in Imaris. MV, mitral valve. TV, tricuspid valve. PV, pulmonary valve. AV, aortic valve. Scale bar, 100 μm for the top row, 150 μm for the bottom row. **(E)** Quantification of the mitral and tricuspid valve volume (n = 3 per group). Ns, non-significant. One-way ANOVA test. MV, mitral valve. TV, tricuspid valve. **(F)** Quantification of the pulmonary and aortic valve volume (n = 3 per group). Ns, non-significant. One-way ANOVA test.

repair the structural defect caused by *Sorbs2* deficiency. To this end, we collected embryos at E18.5 to check cardiac structure defects. Genotype distribution ratios were consistent with Mendel's law, suggesting no embryo loss during embryonic development (Supplementary Table S6). In Tamoxifen-administered hearts, all 8 macrophage-depleted WT and *Sorbs2*$^{-/-}$ hearts exhibited no structural abnormalities, whereas we observed ASD in *Sorbs2*$^{-/-}$ hearts as reported previously (Figure 4A). None of *Sorbs2*$^{-/-}$ hearts showed conotruncal defects, but we noted membranous ventricular septal defect (VSD) in both macrophage-depleted and non-depleted *Sorbs2*$^{-/-}$ hearts (Figures 4B, C). Among 21 macrophage-depleted *Sorbs2*$^{-/-}$ embryos, 8 showed ASD, 1 showed membranous VSD, and 1 showed muscular VSD (Figures 4B,C). In contrast, among the 14 macrophage-non-depleted *Sorbs2*$^{-/-}$ embryos, 5 showed ASD and 1 showed membranous VSD, with one embryo exhibiting both ASD and VSD (Figure 4C). Compared to macrophage-non-depleted *Sorbs2*$^{-/-}$ embryos, there was a trend toward increased penetrance of cardiac defects in macrophage-depleted *Sorbs2*$^{-/-}$ embryos, though the difference was not significant (Figure 4C). This non-significant increase in cardiac defect penetrance, particularly the occurrence of one muscular VSD in macrophage-depleted *Sorbs2*$^{-/-}$ embryos, suggests that macrophages might play a repairing role in cardiac development.

Ablation of cardiac resident macrophages did not affect valve development

A previous report shows that cardiac resident macrophages are required for valve formation (Shigeta et al., 2019). We also noted an increase in the number of *Cx3cr1*-lineage macrophages in the endocardial cushions of E12.5 hearts (Figures 5A, B). Therefore, we harvested E18.5 embryos to perform a morphological analysis of valves. In histological sections, we did not observe any obvious morphological abnormality in mitral and tricuspid valves of both macrophage-depleted and non-depleted *Sorbs2*$^{-/-}$ hearts (Figure 5C). To obtain a whole view of cardiac valves, we used light sheet fluorescence microscopy to reconstruct a three-dimensional visualization of valves. Surface rendering was applied to delineate and calculate the volumes of the mitral, tricuspid, pulmonary and aortic valves. We did not detect any obvious abnormality in cardiac valves (Figure 5D). Quantification of valve volume showed no significant differences in any of cardiac valves between macrophage-depleted and non-depleted *Sorbs2*$^{-/-}$ groups, and nor in comparisons between *Sorbs2*$^{+/-}$ and *Sorbs2*$^{-/-}$ groups (Figures 5E, F).

Discussion

Our study sheds light on a previous unknown role of macrophages in the embryonic heart under conditions of structural gene mutation, specifically the Sorbs2 knockout model. We observed increased macrophage numbers in the embryonic hearts of *Sorbs2*$^{-/-}$ mice. This response likely represents an adaptive reaction to structural abnormalities in the myocardium, suggesting that macrophages may have a supportive role in cardiac morphogenesis under compromised conditions.

Through macrophage depletion, we observed a profound transcriptomic shifts indicating impaired metabolic and developmental pathways in E12.5 *Sorbs2*$^{-/-}$ hearts. Downregulation of genes related to lipid metabolism, animal organ regeneration, and muscle development in macrophage-depleted hearts suggests that macrophages contribute to the metabolic support required for myocardial maturation. In mice, the completion of the placenta formation occurs at E12.5 and subsequently and the partial pressure of oxygen in the fetal circulation increases (Hemberger et al., 2020; Slaats et al., 2020). Meanwhile the heart muscle experiences substantial thickening and mitochondrial morphology and function, crucial for substrate oxidative phosphorylation, undergo a process of maturation at this time (Barak et al., 2019; Porter et al., 2011). On the molecular level, heart glucose uptake decreases after E12 and the expression of glycolytic enzymes, including *Glut1*, *Pdk1*, and *Ldha*, becomes decreased along ventricular myocyte thickening during mid-to late-gestational stages (Menendez-Montes et al., 2016; Nakano et al., 2017). Our data indicate that increased macrophages attenuate the impacts on lipid metabolism and muscle development induced by *Sorbs2* deficiency. Additionally, the observed increase in DNA damage response genes and cardiomyopathy hints at increased cellular stress in macrophage-depleted hearts, further highlighting their protective role in managing cellular stress and maintaining cardiac integrity. Therefore, macrophages might be safeguards for the metabolic shift and myocardial growth at this stage.

While macrophage depletion did not significantly increase the penetrance of septal defects, the observed trend still implies that macrophages might play a reparative role in the presence of structural cardiac abnormalities. The appearance of VSD in macrophage-depleted *Sorbs2*$^{-/-}$ hearts, which were not seen in non-depleted *Sorbs2*$^{-/-}$ hearts, supports the idea that macrophages may help mitigate certain developmental defects in compromised embryonic hearts. These findings point to a potential role for cardiac macrophages as adaptive responders to structural gene mutations. Given the established role of macrophages in cardiac regeneration and repair (de Couto, 2019), our primitive findings could extend this role to include compensatory repair during morphogenesis.

In contrast, macrophage depletion did not significantly impact valve formation or volume, despite prior reports indicating their role in valve development (Shigeta et al., 2019). This discrepancy could be due to the partial and/or transient nature of macrophage depletion in our study. New depletion strategies that achieve complete and consistent macrophage removal could help resolve this inconsistency. Although partial macrophage depletion has provided initial insights into a possible compensatory role of macrophages during embryonic myocardial development, the incomplete depletion may have limited our ability to observe the full impact of macrophage absence on heart development. Further studies with more targeted macrophage ablation techniques, or using models that allow for more complete and temporally controlled macrophage depletion, would clarify these effects in responding to myocardial abnormality and contributing to different aspects of cardiac morphogenesis.

A significant limitation of our study is the incomplete understanding of the mechanisms underlying the observed

increase in macrophages in *Sorbs2*$^{-/-}$ hearts. Although our findings indicate an upregulation of macrophage-related genes and an increase in macrophage numbers, we have not addressed that it is due to increased macrophage recruitment or proliferation. It remains unclear whether the increase in macrophages results directly from changes within cardiomyocytes due to *Sorbs2* deficiency or from secondary signals generated by other cells or altered extracellular matrix components in the myocardial environment. Identifying the sources and nature of these signals would provide valuable insights into how structural gene mutations influence immune cell behavior.

In conclusion, our findings highlight the adaptive role of cardiac macrophages in response to structural gene mutations. While macrophages are known to be vital for normal heart development, our study provides evidence that they may also mitigate developmental defects in structurally compromised hearts. Future work should investigate the specific signaling pathways that mediate macrophage responses to myocardial abnormalities, as well as potential therapeutic strategies for modulating macrophage activity to support heart development in congenital heart disease.

Methods

Mice

The mouse strains utilized in this study comprise *Sorbs2*$^{-}$ (Liang et al., 2021), *Cx3cr1*^{CreERT2} (Xu et al., 2020), *Rosa26*^{DTA} (Ivanova et al., 2005) and *Rosa26*^{mTmG} (Muzumdar et al., 2007). The *Cx3cr1*^{CreERT2} allele was a gift from Dr. Bo Peng's lab (Fudan University, Shanghai, China). All strains were backcrossed with C57BL/6 to ensure the consistent genetic background. Tamoxifen was administered at E9.5 and E11.5 through oral gavage. Mice were maintained under specific pathogen-free conditions in the animal facility at Shanghai Children's Medical Center. All animal procedures adhered to the guidelines set by the Institutional Animal Care and Use Committee of the Shanghai Children's Medical Center, affiliated with the Shanghai Jiao Tong University School of Medicine.

Histological analyses

For the preparation of frozen sections, dissected embryonic hearts were fixed in 4% paraformaldehyde for 20 min at 4°C, followed by equilibration in a 30% sucrose PBS solution overnight at 4°C.Subsequently, the hearts were embedded in 100% OCT compound within Cryomolds. The prepared blocks were immediately frozen at −80°C. The sample blocks were sectioned into 10 μm thin slices using a Leica CM3050S cryostat. For paraffin sections, the dissected embryonic hearts were fixed in 4% paraformaldehyde for 24 h at 4°C. The fixed hearts were rinsed with PBS, then subjected to a graded ethanol series (30%, 50%, 70%, 80%, 95%, 100%) for complete dehydration. Typically, each ethanol step was maintained for 30 min, followed by the embedding process. Upon completion of dehydration, the hearts were soaked in xylene for 30 min, followed by overnight paraffin infiltration. Finally, the hearts were processed for embedding in paraffin. The sample blocks were sectioned into 5 μm thin slices.

The sections were dewaxed with xylene and subsequently rinsed with ethanol. The sections were stained with a hematoxylin dye solution for a duration varying between 5 and 20 min, followed by a rinse with running water. The sections were then subjected to a 30-second differentiation process using a differentiation solution and subsequently rinsed with running water for 5 min. The slides were then immersed in eosin dye for 2 min. This was followed by conventional procedures for dehydration, clearing, and mounting.

Immunofluorescent staining

The frozen sections were permeabilized in 0.5% Triton X-100/phosphate-buffered saline (PBS) for 20 min, followed by blocking in 3% bovine serum albumin/PBS for 1 h. The sections were then stained with F4/80 antibodies (1:400, ab16288; Abcam). The nuclei were subsequently stained with 4′,6-diamidino-2-phenylindole (DAPI). Fluorescent images were captured using a high-resolution fluorescence microscope.

Whole-mount immunostaining

Initially, the samples are fixed in 4% paraformaldehyde/phosphate-buffered saline (PFA/PBS), followed by a sequential dehydration and rehydration process. Subsequently, the samples are treated with proteinase K and then inactivated with hydrogen peroxide (H_2O_2). Thereafter, blocking is performed, followed by the incubation with primary and secondary antibodies, and subsequent multiple washes. Afterwards, the Elite ABC reagent is prepared and incubated. Following staining with DAB reagent, the samples are transferred to PBS, fixed again in 4% PFA, and finally dehydrated and stored in 100% methanol.

RNA-Seq

Total RNA of E12.5, E15.5 and E18.5 cardiac ventricles were isolated using TRizol reagent (Thermo Fisher Scientific; 15596018). Library preparation and transcriptome sequencing on an Illumina HiSeq platform were performed by Novogene Bioinformatics Technology Co., Ltd. to generate 100-bp paired-end reads. HTSeq v0.6.0 was used to count the read numbers mapped to each gene, and fragments per kilobase of transcript per million fragments mapped (FPKM) of each gene were calculated. We used FastQC to control the quality of transcriptome sequencing data. The expression level of each gene under different treatment conditions was obtained by HTSeq-count after standardization. The differentially expressed genes were analyzed by DESeq2 package (version 1.42.0). Functional enrichment of differentially expressed genes was analyzed on Metascape website. Heatmaps were created by the Pheatmap package (version 1.0.12) in R (version 4.3.1). scRNA-seq data for embryonic hearts (GSE150817) were retrieved from the Gene Expression Omnibus (GEO) database. Seurat toolkit (version 4.3.0) was used for scRNA-seq analysis. After data integration, batch effect elimination, normalization, and scaling, different cell populations were identified based on existing references. Gene expression was plotted using normalized read counts.

Three-dimensional visualization of embryonic heart valves

For three-dimensional visualization of the embryonic heart valves, E18.5 embryos were harvested in PBS, fixed overnight in 10% formaldehyde and 2.5% glutaraldehyde, rinsed twice in PBS, and dehydrated through a graded series of alcohol (50%, 75%, 90%, and 100% twice) for 30 min per step at room temperature. The hearts were then transferred into specially designed glass tubes containing 100 µL of BABB solution (1:2 benzyl alcohol: benzyl benzoate) for complete clearing. A custom-developed device was used to mount the heart within the Zeiss Lightsheet Z.1 microscope chamber filled with 87% glycerol (RI = 1.45). 3D images were captured using the 561 nm laser line and detection optics 5x/0.16 (n = 1.45). The reconstruction of the image stacks was analyzed with Imaris 10.0 software. Surface rendering was applied to delineate and calculate the volumes of the mitral and tricuspid valves.

Statistical analysis

Statistical significance was performed using a two-tailed Student's t test, or nested ANOVA test as appropriate. Statistical significance is indicated by *, where $p < 0.05$, **, where $p < 0.01$, and ***, where $p < 0.001$.

Data availability statement

The original contributions presented in the study are publicly available. This data can be found here: RNA-seq data have been deposited in the NCBI's Gene Expression Omnibus under accession GSE284404.

Ethics statement

The animal study was approved by the Institutional Animal Care and Use Committee of the Shanghai Children's Medical Center, affiliated with the Shanghai Jiao Tong University School of Medicine. The study was conducted in accordance with the local legislation and institutional requirements.

Author contributions

BH: Formal Analysis, Investigation, Methodology, Visualization, Writing–original draft. XL: Formal Analysis, Investigation, Methodology, Visualization, Writing–original draft. SX: Investigation, Visualization, Writing–original draft. QG: Investigation, Writing–original draft. JY: Investigation, Writing–original draft. HS: Methodology, Writing–review and editing. MZ: Methodology, Writing–review and editing. FL: Investigation, Writing–review and editing. ZZ: Conceptualization, Funding acquisition, Project administration, Supervision, Writing–review and editing.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Generative AI statement

The author(s) declare that Generative AI was used in the creation of this manuscript. We used ChatGPT to polish our writing.

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The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fgene.2024.1525931/full#supplementary-material

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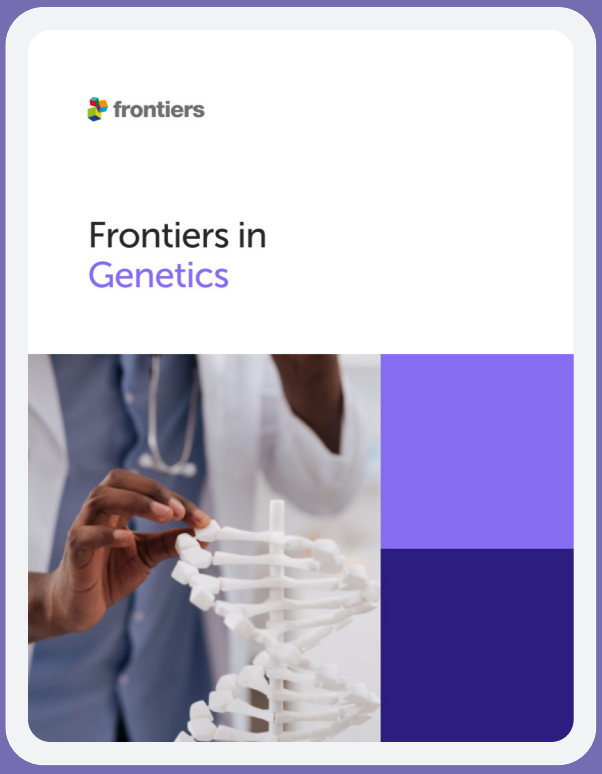

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