Elevated CDR1as May Affect CACNG5 and EGFR Via hsa-miR-7-5p Sponge in Childhood Dilated Cardiomyopathy Patients

Original Article

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ABSTRACT

Background: In the present study, we mainly aimed to evaluate CDR1as and hsa-circRNA_105039 biomarkers expression in childhood dilated cardiomyopathy (DCM) and ventricular septal defects (VSD) patients. Circular RNAs (circRNAs) are non-coding RNAs that result from the back splicing of pre-mRNA. Circular RNA's exhibit enhanced stability with numerous biological functions. Thus, this circRNAs are promising targets for developing diagnostic tools and therapies for the human diseases.

Methods: Fold change of CDR1as and hsa-circRNA_105039 was detected by qRT-PCR in 101 participants. The diagnostic accuracy of CDR1as was determined using receiver operating characteristic curve analysis. To predict CDR1as/miRNAs and CDR1as/proteins interaction networks related to DCM and VSD pathogenesis, gene ontology (GO) and KEGG pathway analyses were performed.

Results: CDR1as showed significant higher fold change (FC= 2.9) in DCM group than both control and VSD groups. Experimental evidence-based GO and KEGG pathways analyses showed that CDR1as has 73 miRNAs binding sites on hsa-miR-7-5p which targets 3'UTR of mRNAs involved in MAPK signaling pathway.

Conclusion: The potential molecular mechanistic effect of the elevated CDR1as could be concluded by sponging of hsa-miR-7-5p in childhood DCM patients. Consequently, further decreasing of hsa-miR-7-5p may lead to increased dosage of CACNG5 and EGFR which involved in the MAPK signaling pathway.

Key Words: CDR1as, Circular RNAs, Dilated cardiomyopathy, hsa-circRNA_105039, MAPK signaling pathway.

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INTRODUCTION

circRNAs are circular ribonucleic acids resulting from aberrant pre-mRNA splicing by-products, and it overrepresented abundantly in blood samples and saliva. Abundance and stability of circRNAs due to its looping structure, lack of free ends, and covalent bonds between 3′ and 5′ ends. Hence, circRNAs aren't capped by m^7G caps and poly A tail making it protected from translation by cap-dependent mechanisms (**Memczak *et al.,* 2013; Salzman *et al.,* 2012; Zhang *et al.,* 2016b**). circRNAs are potential clinical noninvasive biomarkers for human embryonic development, diseases progression and prognosis (**Lee *et al.,* 2019**).

circRNAs is microRNA (miRNA) sponge molecule either inhibitory or excitatory effect on various mRNA expressions in the body causing human diseases (**Hansen *et al.,* 2013**). Furthermore, circRNAs have others modulating effects via their roles in transcription or post-transcription regulation and in proteins binding as RNA-binding proteins (RBPs) (**Yao *et al.,* 2018**). circRNAs are associated with cardiac diseases such as atherosclerotic vascular disease, coronary artery disease, cardiac fibrosis, myocardial infarction (MI), cardiac hypertrophy, and heart failure (HF) (**Fan *et al.,* 2017**).

In 2011, **Hansen** first discovered the natural circRNA as antisense transcript of the cerebellar degeneration-related protein 1 gene (CDR1, chrXq27.1) and it was called CDR1as (**Hansen *et al.,* 2011**). hsa_circRNA_105039 is spliced from filamin A (FLNA) gene, FLNA involves in actin filament-based process (**Zou *et al.,* 2017**).

CDR1as was analyzed using a three-dimensional adjustment network with related RNAs (**Guo *et al.,* 2020**). Across 32 vertebrate species, human circular exonic CDR1as harbors 74 binding sites on miR-7, out of them 63 are conserved in other species (**Memczak *et al.,* 2013**). CDR1as showed significant association with acute myocardial infarction (AMI), cardiac infarct size increasing, and cardiac hypertrophy (**Lu *et al.,* 2022; Zhang *et al.,* 2016a; Wang *et al.,* 2017**).

hsa_circRNA_105039 is associated with congenital heart defects (CHDs) in children (**Wu *et al.,* 2019**). Using induced pluripotent stem (iPS) cells were differentiated into cardiomyocytes, **Yu** and his team found that hsa_circRNA_105039 acted as a sponge for miR-17, while cyclinD2 is a direct target of miR-17 (**Yu *et al.,* 2021**). The functional importance of the hsa_circRNA_105039/miR-17/cyclinD2 axis in CHD was provided (**Yu *et al.,* 2021**).

From the above literature, we observed that both CDR1as and hsa_circRNA_105039 have a potential association with some symptoms of CHD. Therefore, CDR1as and hsa_circRNA_105039 may have a reasonable rationale for study here.

Heart disease and structural cardiac anomalies still have overrepresented in death rate worldwide. **Goina *et al.,* (2024)** pointed that cardiovascular diseases are the primary cause of death worldwide with 17.9 million deaths a year (**Goina *et al.,* 2024**). **Ding *et al.,* (2023)** found that out of 790 children with CHD; 81(10.25%) had CHD alone, and 709(89.75%) had CHD with at least one comorbidity (**Ding *et al.,* 2023**). In 1005 Egyptian pediatric CHD patients aged 1 day to 12 years, **Al-Fahham and Ali, (2021)** found that the most common acyanotic CHD was isolated VSD, while the most common cyanotic CHD was tetralogy of Fallot (**Al-Fahham and Ali, 2021**). However, there aren't many reliable techniques for prognostic assessment and early diagnosis.

So the development of suitable therapy is highly demanded. In this study, we aimed to detect the expression of CDR1as and hsa_circRNA_105039 quantities in plasma of children (aged 3–5 years) with DCM and VSD, and to analysis the relevant miRNA and proteins ontology (GO) data. Using this approach, we hoped to identify potential noninvasive diagnostic markers for VSD and DCM in pediatric patients in context of the current research limitations. Notably, both VSD and DCM are not studied with CDR1as yet.

METHODS:

Ethics approval and consent to participate: All children's parents or guardians had given their written informed consent. Protocol of this study was ethically approved by medical research ethics committee (MREC).

Patients and healthy controls: A total of 101 participants were grouped into 25 VSD patients (group 1), 30 Childhood DCM patients (group 2), and 46 participants as controls with cardiac diseases-free signs e.g. chest pain, shortness of breath, irregular heart rhythms, cyanosis, and observed structured cardiac abnormalities by echocardiography or syndromic CHD features by the clinical investigations. In order to reduce heterogeneity, controls were matched with cases on the basis of age and sex. All participants were selected from the cardiology division, Cairo University, Children's Hospital (Abu El Rish), and the Clinical Genetics Outpatient Clinic of the National Research Centre during the period from September 2021 to April 2022. We excluded other CHD or acquired heart diseases like myocarditis.

Study design: Here, a case-control study design was adopted. 101 participants (55 patients and 46 controls) were selected. The study was divided into two stages; the first stage concerned with identify the quantity of CDR1as and hsa_circRNA_105039 expressions in plasma of 101 participants using duplicated qRT-PCR runs, and followed by the second stage concerning with gene ontology (GO) and KEGG pathway analyses to evaluate function of the differentially expressed circRNAs and its proposed molecular mechanistic effect. Based on the qRT-PCR data analysis, ROC curve-based risk assessment analysis was conducted to assess the sensitivity and specificity toward diseases' prediction.

Clinical investigations: For each participant, full medical history was taken including age, sex, family history of similar diseases, developmental history, and history of recurrent chest infections. Full medical examination was done with special emphasis on cardiac examination. Diagnosis was confirmed with conventional echocardiography done by single expert cardiologist according to the recommendations of American society of echocardiography (**Lai *et al.,* 2006**).

Blood sampling and processing: Blood samples had been taken into a sterile vacutainer tube with anti-coagulant factor (pot. EDTA) after the agreement signature on the consent form. All the blood samples were centrifuged at 3000 rpm for 15 minutes to separate the plasma. Then, the plasma was aliquoted in the RNase-free microfuge tubes and store at −80 centigrade.

RNA extraction and qRT-PCR: Total plasma RNAs was isolated using Direct-zol™ RNA MiniPrep (Zymo Research) according to manufacturer's protocol. Each RNA sample was quantified with a spectrophotometer (NanoDrop 1000, Thermo Scientific, Wilmington, Delaware). 20 ng of the isolated RNAs were converted to complementary DNAs (cDNAs) by miScript II RT Kit (Qiagen). qRT-PCR reactions were conducted in 96-well plates with 120 ng/ul of the total cDNA and

miScript SYBR Green PCR Kit (Qiagen) using ROCH 480II lightcycler instrument. qRT-PCR was performed in duplicate for all samples. Primer sequences were as follows: CDR1as (forward); ACGTCTCCAGTGTGCTGA, CDR1as (reverse); CTTGACACAGGTGCCATC, hsa-circRNA_105039 (forward); GGAGAATGAGGACGGCACTT, hsa-circRNA_105039 (reverse); CCTTCGGGATCCGTCACTT.

qRT-PCR data analysis: The data were analyzed using the relative fold change (FC) by $2^{-\Delta\Delta Ct}$ equation and transformed log2FC values. Differential FC was considered significant at |log2FC| ≥1. Relative gene expression level was normalized to a nonendogenous synthetic noncoding RNA-16-2.

Gene ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEEG) analyses: To better understand the biological function and the relevant mechanistic pathway of CDR1as, the putative target miRNAs and proteins were predicted under confidence interval 95% using CircInteractome (**Dudekula *et al.*, 2016**) at p <0.05. miRNA/mRNA network was build using experimentally validated miRNA-target interactions derived from miRTarBase database (**MIRTARBASE, Accessed on May 15, 2022**). To gain deep insight into the biological functions, GO and KEGG analyses were done (**Kanehisa *et al.*, 2021, GENEONTOLOGY, Accessed on Aug 29, 2022**).

Statistical analysis:

Values were expressed as mean±SD or median according to skewness and kurtosis of the values, and outliers' values were excluded. Median-based tests were used for median expression comparison. ROC curve was analyzed to assess specificity and sensitivity of CDR1as. p <0.05 value considered significant. The statistical analysis was done by PASW (**IBM, Accessed on May 29, 2022**) statistics (SPSS Inc. Released 2009. PASW Statistics for Windows, Version 18.0. Chicago).

RESULTS

Clinical characteristics of patients: A total of 30 DCM and 25 VSD patients were enrolled in this study, beside 46 age-matched cardiac diseases free participants as controls. The diagnosis of DCM and VSD patients was confirmed by echocardiography. All DCM and VSD patients were sporadic without positive family history of the disease. No syndromic VSD or DCM, and extracardiac defects were observed. No significant differences in age at diagnosis and gender were detected as shown in table 1.

Significant increased CDR1as in plasma of DCM patients:

Measurement of CDR1as in plasma of DCM and VSD patients showed that relative expression of CDR1as was significantly higher in DCM patients than controls with FC= 2.88 and log2FC= +1.53. Although that CDR1as expression was higher in VSD patients than controls, but log2FC fall out the significant level (log2FC= +0.76). A second studied hsa-circRNA_105039 showed no difference in expression among two patients groups and controls. Details of CDR1as expression in DCM patients were listed in supplementary (Table 1).

Table 1: Patients and controls characteristics:

Groups	*n*	Age a (months) Mean±SD	Gender (%)[a]
DCM Patients	30	47.4± 2.2	Male; 60% Female; 40%
VSD patients	25	53.1±2.7	Male; 59% Female; 41%
Control	46	56.5 ± 3	Male: 54.5% Female; 45.5%

[a]: No statistically significant difference was found between the patients and controls groups for ages and gender, p-value= 0.979, and 0.087 respectively.

Clinical utility of plasma CDR1as levels in predicting DCM in children:

To assess the efficiency of CDR1as to be a discriminator diagnostic biomarker for childhood DCM patients, we performed ROC curve analysis. As shown in figure (1), ROC curve analysis showed that CDR1as could distinguish childhood DCM patients from healthy controls by sensitivity= 82.8% and the specificity= 73.6% with an AUC of 0.872 at p value= 0.01. Therefore, CDR1as could act as a suitable biomarker for DCM diagnosis in children.

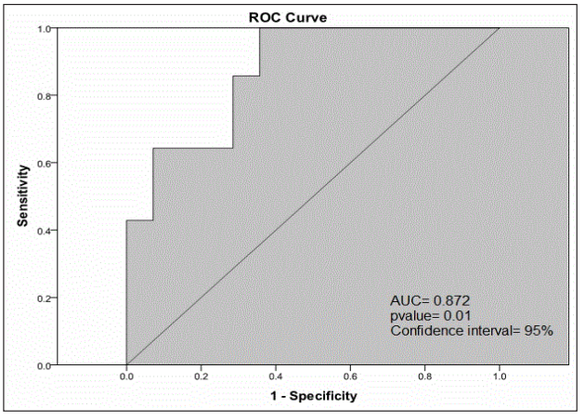

Figure 1: ROC curve analysis using CDR1as to distinguish DCM patients.

Prediction of the CDR1as/miRNA and CDR1as/protein interaction networks related to dilated cardiomyopathy and cardiac muscles contraction KEGG pathways:

GO analysis was performed to describe the CDR1as in context of its molecular functions (MF), biological

processes (BP), and pathways. RNP and miRNA prediction tools were used to identify CDR1as binding site on proteins and miRNAs. Fisher's exact test was used to exclude probable overlap between CDR1as and GO annotation list than that would be expected by chance. P <0.05 was considered significant.

Using circular RNA interactome (CircInteractome) tool, it was predicted that most abundant CDR1as-binding site proteins are AGO2 and FUS with 43 and 26 biding sites respectively as shown in figure (2), and most abundant miRNA binding sites are hsa-miR-7-5p with 73 binding sites as shown in figure (3).

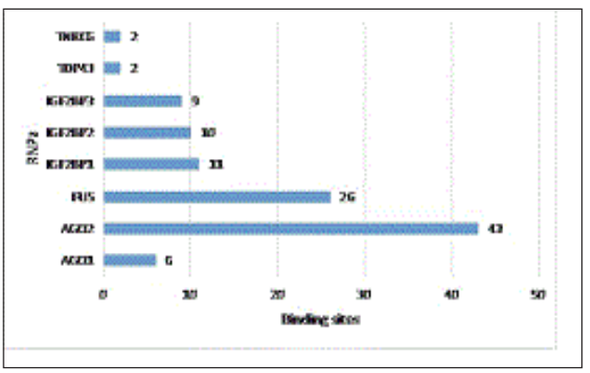

Figure 2: CDR1as-binding protein sites analysis.

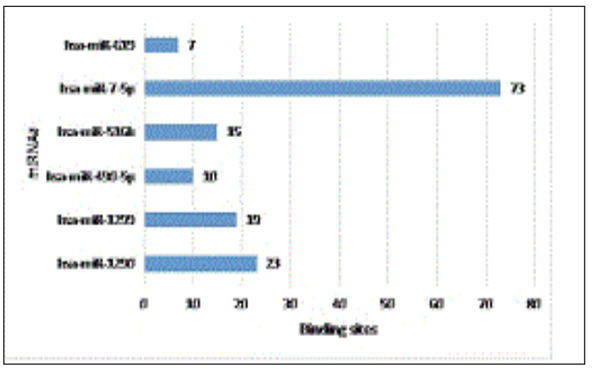

Figure 3: CDR1as-binding miRNAs sites analysis.

Experimental evidence data of interaction between CDR1as-binding site miRNAs and 3'UTR of the targeted genes by miRNAs was derived by miRTarBase database under highest confidence score 0.95 and binding probability =1, in which CACNG5 (calcium channel, voltage-dependent, gamma subunit 5) and EGFR (epidermal growth factor receptor) were targeted by hsa-miR-7-5p. Both CACNG5 and EGFR are included in DCM and cardiac muscles contraction pathways according to KEGG database, as illustrated in table (2). Subsequent GO-pathway analysis showed that the common KEGG pathway for CACNG5 and EGFR is MAPK signaling pathway (KEGG_04010). All CDR1as-binding site proteins aren't included in the KEGG DCM and cardiac muscles contraction pathways.

Table 2: Experimentally validated target genes of hsa-miR-7-5p:

miRNAs	Target gene	MANE [a] Transcript	Binding Energy	Binding Probability	KEGG pathways
hsa-miR-7-5p	EGFR	NM_005228	-20.5	1	DCM & Cardiac muscle contraction
	CACNG5	NM_145811	-18.4	1	

[a]: Matched annotation transcript in NCBI and Ensemble.

DISCUSSION

The risk estimation of the relevant social and economic aberrations due to childhood diseases is more effective than adult diseases. The pediatric DCM patients had worse cardiovascular prognosis (**Puggia *et al.*, 2016**). Recently, it was reported that although pediatric DCM and adult DCM have somewhere similar clinical characteristics, but there are exponential evidence presenting unique biomarker profile for pediatric DCM compared to adult one, and unresponsiveness of childhood DCM to adult DCM medications (**Puggia *et al.*, 2016, Gropler *et al.*, 2022**). All the above make the strong demand to develop new non-invasive biomarkers for pediatric DCM that we tried to achieve it here.

circRNAs are potential biomarkers for diagnosis of the diseases (**Siede *et al.*, 2017; Zhou and Yu, 2017**). Although that there are studies describing the relationship between circRNAs and cardiac diseases (**Siede *et al.*, 2017**), but no studies have been observed to examine the CDR1as in childhood VSD and DCM.

In the present study, expression level of CDR1as and hsa-circRNA_105039 circRNAs were quantized. Then, the target miRNAs and proteins were predicted. GO analysis and KEGG pathway analyses showed that MAPK signaling pathway was the consensus pathway in childhood DCM. The molecular mechanistic effect for CDR1as was conducted.

Several studies pointed that dysregulated CDR1as has implicated in multiple human diseases, including cardiac one. CDR1as has multiple binding sites on miR-7, so it has powerful sponging for miR-7 (**Belter *et al.,* 2022; Wang *et al.,* 2012; Salmena *et al.,* 2011; Fang *et al.,* 2012**). The current study showed significant relative up-expression of CDR1as in plasma of childhood DCM without VSD patients using qRT-PCR technique. Subsequent altered miRNAs due to elevated CDR1as expression were computationally conducted here. We found that CDR1as has 73 binding sites on hsa-miR-7-5p, and consequently high sponge rate of hsa-miR-7-5p by CDR1as is potentially predicted.

The downregulation of miR-7 due to it is intensely absorbed by CDR1as, where many studies have shown that miR-7 was degraded by CDR1as. Therefore, conceptual of alter the miR-7 target genes is more acceptable (**Tang *et al.,* 2019**). Here, we showed that hsa-miR-7-5p has highest binding capacity sites for CDR1as by 73 binding sites, and followed by hsa-miR-1290, hsa-miR-1299, hsa-miR-516b, hsa-miR-490-5, and hsa-miR-619. Many studies showed that CDR1as has binding sites for other miRNAs beside miR-7 that are; miR-671, miR-1299, miR-876-5p, and miR-135a (**Sang *et al.,* 2018; Yang *et al.,* 2017; Li *et al.,* 2018**). From the above, we supposed that elevated expression of CDR1as will be lead to sponge and decrease of hsa-miR-7-5p expression. **Huang *et al.,* (2021)** pointed that down-regulated hsa-miR-7-5p expression was detected in coronary atherosclerotic heart disease using qRT_PCR, the authors concluded that hsa-miR-7-5p may contribute to CHD pathogenesis (**Huang *et al.,* 2021**). We identified multiple binding sites for CDR1as on AGO2 and FUS proteins, but those two proteins weren't supported by our GO and KEGG pathways analyses and the previous literature.

Our miRNAs/mRNAs network analysis showed that hsa-miR-7-5p has binding sites on 3'UTR of MANE transcripts of CACNG5 and EGFR (Binding energy ≤-18.4 and Binding probability= 1 at P <0.05). CACNG5 encodes type II transmembrane AMPA receptor regulatory protein (TARP) that is composed of subunits GluA1–4 controlling sodium, calcium and potassium ions transmission (**Diering and Huganir, 2018**). Patch-clamp and ion-fluorescence studies (**Coppini *et al.,* 2013; Ulus *et al.,* 2013; Coppini *et al.,* 2019**) on isolated cardiomyocytes specimens from myocardial tissues of hypertrophic cardiomyopathy (HCM) patients and from septal specimens of controls were studied. These studies revealed that the action potential duration (APD) was significantly prolonged in hypertrophic cardiomyocytes, and APD was due to calcium and sodium ion change abnormalities. **Molina-Navarro *et al.,* (2013); Fayez *et al.,* (2022)** stated that cardiac ion channel genes were significantly involved in DCM pathogenesis (**Molina-Navarro *et al.,* 2013; Fayez *et al.,* 2022**). Epidermal growth factor receptors (EGFRs) consider important players in cardiac development and cardiac remodeling (**Li *et al.,* 2017**). The above results partially support our results regarding contribution CACNG5 and EGFR in DCM pathogenesis.

KEGG analysis for MANE transcripts of CACNG5 and EGFR were performed, the analysis showed that MAPK signaling pathway is an enriched pathway. Moreover, **Huang *et al.,* (2019)** concluded to 23 differential dysregulated miRNAs were identified in plasma of DCM patients, and those miRNAs associated with MAPK signaling pathway. The same authors pointed that FGFR4, FGFR1 and ACNA1 were hub target mRNAs in MAPK signaling pathway in the studies DCM patients (**Huang *et al.,* 2019**).

The MAPK signaling pathway contains three cascades that are extracellular signal-regulated kinases 1 and 2 (ERK1/2), c-Jun N-terminal kinases 1 and 2 (JNK1/2), and p38. The kinases that are involved in MAPK singling pathway have essential contributions in regulating heart failure and cardiac hypertrophy (**Liao *et al.,* 2001**). **Hong *et al.,* (2018)** reported that the inactivation of the MAPK signaling pathway led to myocardial fibrosis and left ventricular hypertrophy in rats with dilated cardiomyopathy (**Hong *et al.,* 2018**).

So, CDR1as/miR-7-5p/CACNG5-EGFR axis may be a potential molecular mechanism for childhood DCM pathogenesis. Our supposition was supported by the literature findings, where **Geng *et al.,* (2016)** revealed that in vivo CDR1as overexpression increased cardiac infarct size (**Geng *et al.,* 2016**). Also, **Chen *et al.,* (2020)** found that CDR1as is mechanistically involved in the chronic heart failure pathogenesis pointing that CDR1as sponge miR-7, and its overexpression was induced in the infarcted heart in mice (**Chen *et al.,* 2020**).

Wu *et al.,* (2019) pointed that hsa_circRNA_105039 was significant downregulated in plasma of 40 VSD and 10 ASD patients (**Wu *et al.,* 2019**). On contrast, the present study showed that hsa_circRNA_105039 wasn't dysregulated in plasma of 25 VSD patients. No obvious justifications for the inconsistency of the current hsa_circRNA_105039 result with what was mentioned in the **Wu *et al.,* (2019)** study, but the characteristics and ethnicity of the patients may be different, and the ages of the patients were younger than those we recruited (**Wu *et al.,* 2019**).

Using text mining about enriched miRNAs in VSD, we found others miRNAs related to VSD, like miR-181c, miR-498, miR-379-5p, miR-409-3p, miR-433, hsa-let-7e-5p, miR-155-5p, miR-222-3p, and miR-487b (**Liu *et al.,* 2015; Wehbe *et al.,* 2019**), hsa-miR-146a-5p (**Jin *et al.,* 2021**), and miR-1-3p, miR-1b and miR-293-5p (**Yang *et al.,* 2022**). Moreover, **Wang *et al.,* 2023** found that both CACNG5 and EGFR were absent in differential mRNA expression profiling that made in amniotic fluid from fetuses with VSD (**Wang *et al.,* 2023**). These miRNAs are

not targeted by CDR1as and hsa_circRNA_105039 in the current study.

Research limitations of the current study are; (i) CDR1as/miRNAs and CDR1as/proteins interaction networks were conducted by computational analysis. Although that miRNA/mRNA network was derived from experimentally validated data, but the functional validation of these dysregulated miRNAs is still under consideration, (ii) DCM and VSD patients were selected from eligible sample of the Egyptian children.

CONCLUSION

Circulating CDR1as could consider noninvasive circRNA diagnostic marker for childhood DCM patients based on our ROC curve analysis. Beside that, it could be used to differentiate between DCM and VSD patients. CDR1as/hsa-miR-7-5p/CACNG5/EGFR is potential axis in DCM pathogenesis. In addition, GO and KEGG pathways analyses unveiled the potential roles of MAPK signaling pathway in childhood DCM. Here, the interaction networks of miRNAs and proteins should be confirmed experimentally. Finally, this study completes the CHD-relevant circRNAs, and it may provide potential targets for the future treatment of childhood DCM. Also, it gave wide insights into the DCM pathogenesis. Moreover, the study was excluded that has-circRNA_105039 to be differential biomarker for DCM and VSD patients.

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ETHICS APPROVAL AND CONSENT TO PARTICIPATE

“This study was performed in line with the principles of the Declaration of Helsinki. Approval was granted by the Ethics Committee (Date December 2020../No20177).”

“Written informed consent was obtained from the parents”.

CONFLICT OF INTEREST

There are no conflicts of interest.

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AUTHORS` CONTRIBUTIONS

NNE and AF contributed to the study conception and design. LAI collected the clinical data and conducted the investigations. HTE supervised the full clinical and manuscript revisions. NNE and THA performed the molecular investigations. AF performed Material preparation, data collection and analysis. AF wrote the first draft of the manuscript. All authors read and approved the final manuscript.” all authors have approved the manuscript for submission.

COMPETING INTERESTS

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