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Original Article

Circulating Long Non-coding RNA ENST00000507296 Is a Prognostic Indicator in Patients with Dilated Cardiomyopathy

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Background: Long non-coding RNAs (lncRNAs) participate in the pathogenesis of cardiovascular diseases. However, whether circulating lncRNAs serve as dilated cardiomyopathy (DCM) biomarkers remains unclear. Methods: Totally, 266 controls and 818 patients were enrolled. First, microarray-based circulating lncRNA profiling was performed in 10 normal controls and 10 patients with DCM. Second, the top 20 differentially expressed lncRNAs were validated by real-time qPCR in 64 controls and 64 DCM patients. Moreover, lncRNA sequencing was performed in three human heart-derived cell types, and the correlation between circulating lncRNA levels and the severity of heart failure was evaluated in the validated population. The validated two lncRNAs were assessed in 198 DCM patients and 198 matched controls. Finally, the sensitivity and specificity of circulating lncRNA expression in DCM diagnosis were evaluated using receiver-operating characteristic curve analysis, while Cox regression and Kaplan-Meier curve analysis were further performed in 552 DCM patients. Results: Eight candidate lncRNA biomarkers were obtained after microarray screening and real-time PCR validation. Among them, five were validated in the second cohort. However, only the levels of circulating lncRNA ENST00000507296 and ENST00000532365 were significantly correlated with the cardiac function, as well as detectable in at least one of the human heart-derived cell types by lncRNA-seq. Importantly, low circulating ENST00000507296 level was associated with high event-free survival in patients with DCM. Conclusions: Circulating lncRNA ENST00000507296 was a prognostic biomarker in patients with DCM.

INTRODUCTION

Dilated cardiomyopathy (DCM) is conventionally defined by the primary presence of left ventricular (LV) dilation and reduced systolic function, which is not caused by abnormal loading conditions or coronary artery disease.[1] DCM is the most common indicator for cardiac transplantation.[2] The mortality of 3-year-treated DCM patients remains from 12% to 20%, and the major death causes are heart failure (HF) and ventricular arrhythmias. Compared with those with ischemic heart diseases, patients with DCM are usually younger.[3] Moreover, the onset of DCM can be occult (asymptomatic), and the period of diagnosis and treatment may be significantly delayed.[4] The pharmacological treatment of established DCM is in accordance with the HF guidelines, such as angiotensin-converting enzyme (ACE) inhibitors and β-blockers.[5] Currently, heart transplantation remains the only therapeutic option in patients with end-stage DCM.[6] Novel biomarkers may offer the potential for earlier disease diagnosis, with the opportunity to attenuate disease progression.[2] Therefore, identification of novel biomarkers for diagnosis and predicting prognosis for DCM is critical.

Long non-coding RNAs (lncRNAs) are characterized by transcripts longer than 200 nt in length and incapable of coding protein.[7] Recently, lncRNAs have been reported to participate in cell signaling regulation by chromatin modification,[8] structural scaffolds,[9] and RNA processing.[10] Currently, an increasing number of studies have revealed that lncRNAs played important roles in the heart. The lncRNA Fendrr bound to both Polycomb repressive complexes 2 (PRC2) and the Trithorax group/MLL protein complexes (TrxG/MLL) to modify chromatin signatures in the rodent cardiac mesoderm differentiation, and loss of Fendrr transcripts generated embryonic lethality.[11] The lncRNA Braveheart (Bvht) played pivotal roles in cardiovascular lineage commitment by activating epithelial-to-mesenchymal transition (EMT) genes (e.g., Twist) and cardiac transcription factors (e.g., MesP1, Nkx2.5, and Tbx5); also Bvht could interact with SUZ12, a core component

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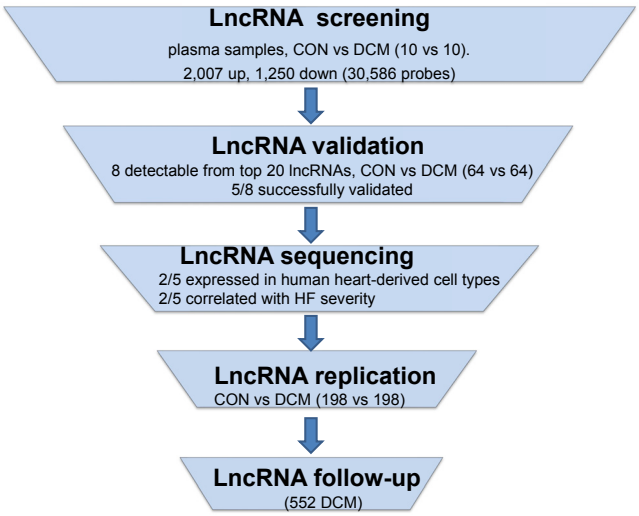

Figure 1. Schematic Diagram of Procedure of lncRNA Selection and Validation in Various Cohorts with Dilated Cardiomyopathy

The primary lncRNA microarray was performed using the plasma samples of 10 control subjects and 10 dilated cardiomyopathy (DCM) patients. Based on the fold change, eight lncRNAs in the top 20 were tested in the second populations, which included 64 control subjects and 64 DCM patients. Correlation analyses between plasma levels of validated lncRNAs and severity of heart failure, and RNA-seq in human-derived cardiac cells were performed. Two lncRNAs that have been successfully confirmed were further replicated in 198 control individuals and 198 DCM patients. In the follow-up study, which included 552 DCM patients, the final lncRNAs were evaluated for the association of the events of DCM.

of PRC2, to mediate epigenetic regulation during cardiomyocyte differentiation.[12]

Recent studies have indicated that some circulating microRNAs (miRNAs) can act as biomarkers for various cardiovascular disorders. For example, the levels of circulating miRNAs miR-1, -133a, -133b, and -499-5p were upregulated, while circulating miR-122 and -375 levels were downregulated in ST-segment elevation myocardial infarction (STEMI) patients, compared with controls.[13] In HF patients with dyssynchrony, the circulating miR-30d level was reported to be an indicator of cardiac resynchronization therapy (CRT).[14] Similarly to small non-coding RNAs, lncRNAs were stably detectable in the plasma.[15] Circulating lncRNAs could be packaged into microparticles, including microvesicles, exosomes, apoptotic microparticles, and apoptotic bodies, to avoid being degraded.[16] Recently, several studies have indicated that circulating lncRNAs can be regarded as biomarkers for various diseases. For instance, circulating long intergenic noncoding RNA-predicting cardiac remodeling, uc022bqs.1 (LIPCAR) levels were independently associated with future cardiovascular deaths in chronic HF, and higher LIPCAR levels predicted a higher risk of cardiac death.[17] The lncRNA CoroMarker could differentiate coronary artery disease patients from control subjects with an area under the curve (AUC) of 0.920.[18] Circulating lncRNAs NRON and MHRT were novel biomarkers of HF, with AUCs of 0.865 and 0.702, respectively.[19] Meanwhile, the expression level of lncRNA PCA3 in urine samples has been found to be more sensitive and specific in diagnosing prostate cancer than the universally used prostate-specific antigen (PSA) serum level.[20] Syndecan-4 was recently reported to be an indicator for adverse LV remodeling in patients with DCM.[21] Meanwhile, some studies suggested that the first, second, and third Fourier transform infrared spectroscopy (FTIR spectroscopy) indicators might be useful biophysical markers of cardiac remodeling in DCM induced by tachycardia.[22]

In our previous study, we provided a global profile of lncRNA changes in failing hearts from patients with DCM. Meanwhile, we found that cardiac-specific lncRNA RP11-544D21.2 was crucial in tube formation and migration in endothelial cells.[23] In the present study, we further explored the expression profile of circulating lncRNAs in DCM-related HF and their associations with the outcomes.

RESULTS

Study Workflow

The flowchart of this study was performed in five phases (Figure 1). The detailed procedures are presented in Materials and Methods.

Profiling of Circulating lncRNAs in the Screening Cohort

Plasma samples from 10 chronic HF patients diagnosed with DCM and 10 control subjects were recruited in the original microarray cohort. Figure 2A shows a volcano plot of plasma lncRNA profiles from control subjects and DCM patients. The scatterplot of plasma lncRNA profiles is shown in Figure 2B. lncRNA expression profiles were distinguishable between controls and DCM patients according to the hierarchical clustering, as shown in Figure 2C. Compared with the control subjects, there were 2,007 upregulated lncRNAs and 1,250 downregulated lncRNAs in chronic HF patients diagnosed with DCM (Figure 2C). The top 100 upregulated and top 100 downregulated lncRNAs based on fold change in DCM patients compared with control subjects are shown in Tables S1 and S2, respectively.

Biomarker Validation by qRT-PCR in the Validation Cohort

Based on the fold changes, we selected the top 20 (10 up- and 10 downregulated) lncRNAs to further detect their expression levels in the plasma of 64 controls and 64 DCM patients. Among these 20 differentially expressed lncRNAs, 8 lncRNAs were detectable by qPCR in plasma. These lncRNAs are highlighted in red in Tables S1 and S2. The relative expression of each lncRNA is shown in Figure 3. Consistent with the microarray assays, compared with the control subjects, there was a 2.98-fold increase of circulating ENST00000507296 in DCM patients (Figure 3A). Meanwhile, circulating ENST00000442293 and ENST00000545794 increased 1.49-fold and 1.76-fold in DCM patients, respectively (Figures 3B and 3C). On the other hand, there was a 0.54-fold decrease of circulating ENST00000532365 and a 0.60-fold decrease of HMlincRNA1548 in DCM patients (Figures 3D and 3E). The raw data of lncRNA ENST00000532365 is shown in Data S1. Another three lncRNAs showed no significant difference between the two groups

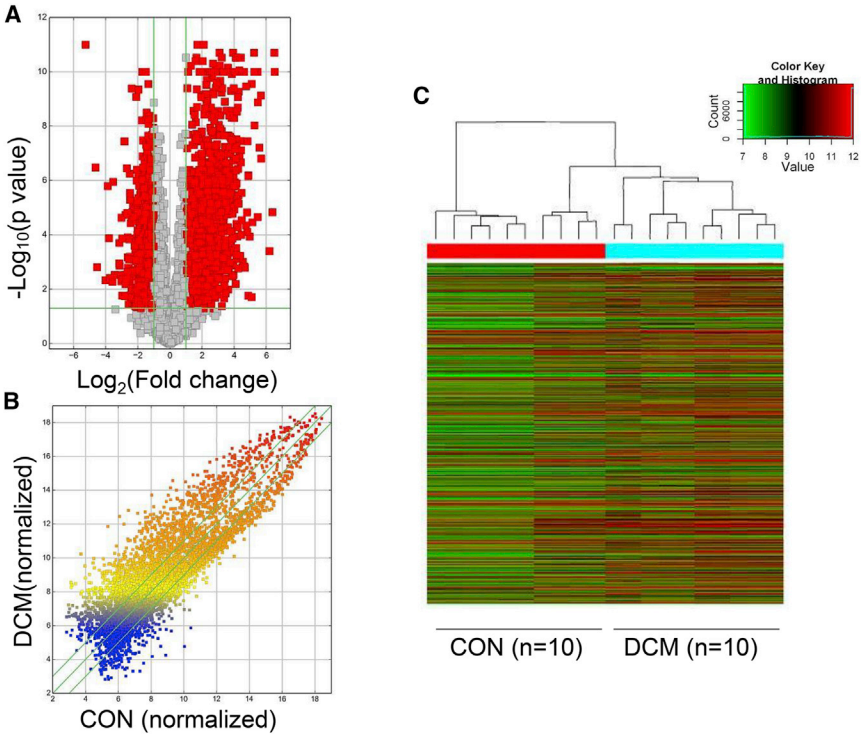

Figure 2. lncRNA Profiles in Plasma from 10 Control Subjects and 10 DCM Patients

(A) Volcano plot of corresponding p values and fold change for circulating lncRNA by comparison of the two groups (DCM versus control [CON]). (B) Scatterplots indicate the normalized signal values of the plasma samples (log_2 scaled). The lncRNAs below the bottom green line and above the top green line imply a >2.0-fold difference. (C) Hierarchical clustering on the basis of levels of circulating lncRNAs in the DCM group and CON group.

(Figures 3F–3H). Therefore, five of the eight lncRNAs were potentially biomarkers for DCM and were selected for further investigation.

Expression Patterns of lncRNAs in Three Cardiac-Derived Cell Types

To further analyze the cell sources of the successfully validated 5 lncRNAs, three kinds of human-derived primary cardiomyocyte, fibroblast, and endothelial cells were subjected to lncRNA sequencing (lncRNA-seq) (Figure 4A). As a result, only 2 (ENST00000507296 and ENST00000532365) of the 5 lncRNAs were detectable in human-derived cardiac cells (Figure 4B). Specifically, ENST00000507296 was only expressed in cardiomyocytes, compared with fibroblast and endothelial cells, while ENST00000532365 was wildly expressed in all three types of cardiac-derived cells (Figure 4B). The other 3 lncRNAs were undetectable in three kinds of human-derived cardiac cells (Figure 4B). Therefore, these data indicated that the expression levels of circulating lncRNAs ENST00000507296 and ENST00000532365 might be correlated with heart function.

Association of Circulating lncRNA Levels with the Severity of HF

The 5 successfully validated circulating lncRNA biomarker candidates were further analyzed by detecting their association with the severity of HF, which was indicated by the echocardiographic parameters left ventricular ejection fraction (LVEF) and left ventricular end diastolic diameter (LVEDD) and the plasma levels of N-terminal pro-brain natriuretic peptide (NT-proBNP), in the validation cohort by Spearman correlation analyses. Among them, ENST00000507296 and ENST00000532365, the only 2 lncRNAs expressed in human cardiac-derived cells, were significantly correlated with cardiac function. As shown in Figures 5A–5C, the circulating lncRNA ENST00000507296 level showed significant correlations with all the three parameters, and its expression level was negatively associated with the cardiac function. Meanwhile, the circulating lncRNA ENST00000532365 level also significantly correlated with all the three parameters, but its expression level was positively associated with the cardiac function (Figures 5D–5F). The other 3 lncRNAs, which were undetectable in the three kinds of human-derived cardiac cells, were not correlated with these cardiac function parameters (Figure S1).

Validation of Circulating lncRNA Expression in a Larger Replication Cohort

To further verify whether the increased expression of plasma lncRNA ENST00000507296 and the decreased expression of plasma ENST00000532365 were indicators of HF severity, we used another cohort of 198 DCM patients and 198 control subjects. As shown in Figure 6A and Figure 6B, a 4.60-fold increase of lncRNA ENST00000507296 and a 0.63-fold decrease of lncRNA ENST00000532365 were found in DCM patients, respectively, in comparison with the control subjects. Moreover, we evaluated whether the plasma contents of these lncRNAs could discriminate DCM patients from controls by applying ROC curve analyses. The lncRNA ENST00000507296 yielded a higher AUC of 0.78 than the lncRNA ENST00000532365 with an AUC of 0.61. The combination of the two lncRNAs would generate a higher AUC of 0.81 (Figure 6C). Moreover, the AUC of NT-proBNP was 0.98, since the inclusion of DCM covered many HF patients (Figure 6C).

Prediction of Events in Follow-Up Study

To further identify the associations between the selected plasma lncRNA expression levels and the risk of DCM, the final cohort study of 552 patients with DCM was conducted. In this study, five patients were lost to follow-up, 136 patients (24.6%) died from cardiac diseases, and 150 patients (27.2%) were re-hospitalized due to heart

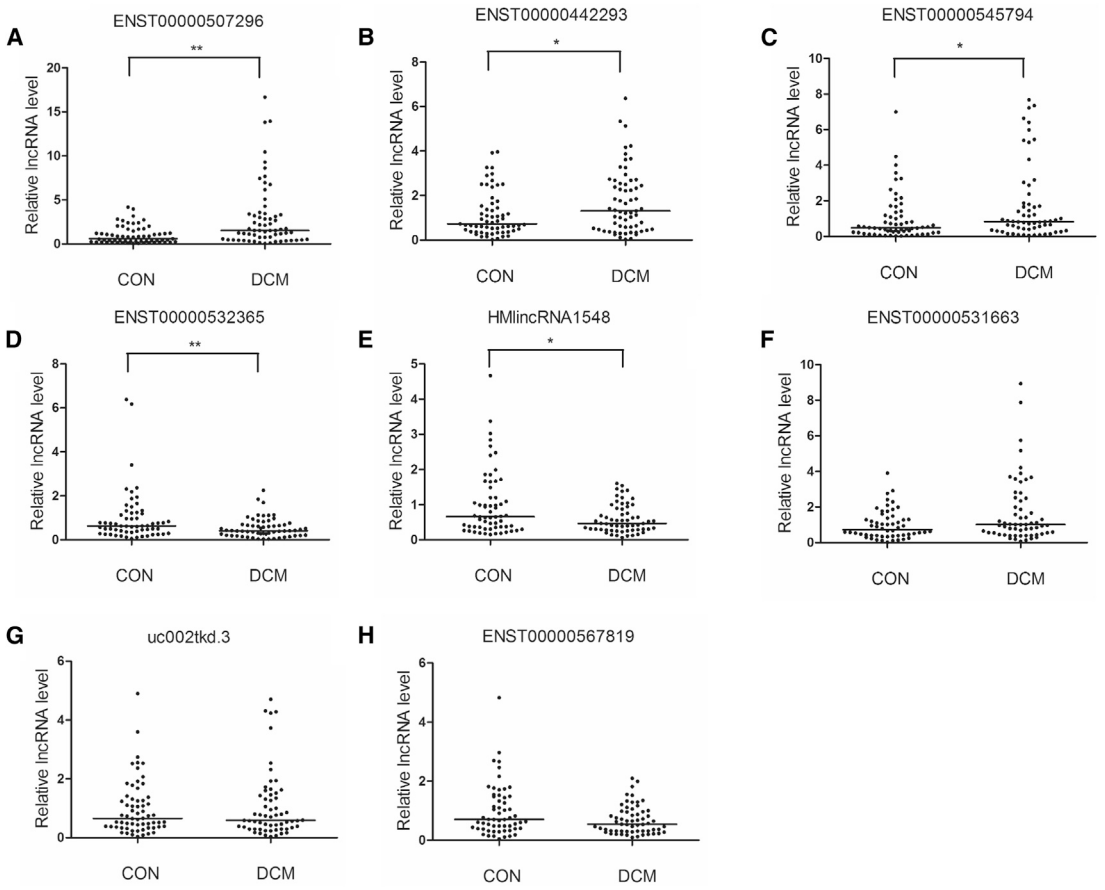

Figure 3. Circulating Expression Levels of the Screening lncRNAs in the DCM Group and Control Group

(A) Circulating expression levels of lncRNA ENST00000507296 in control subjects and DCM patients. (B) Circulating expression levels of lncRNA ENST00000442293 in control subjects and DCM patients. (C) Circulating expression levels of lncRNA ENST00000545794 in control subjects and DCM patients. (D) Circulating expression levels of lncRNA ENST00000532365 in control subjects and DCM patients. (E) Circulating expression levels of lncRNA HMlincRNA1548 in control subjects and DCM patients. (F) Circulating expression levels of lncRNA ENST00000531663 in control subjects and DCM patients. (G) Circulating expression levels of lncRNA uc002tkd.3 in control subjects and DCM patients. (H) Circulating expression levels of lncRNA ENST00000567819 in control subjects and DCM patients. Control group, n = 64; DCM group, n = 64. *$p < 0.05$ versus control; **$p < 0.01$ versus control.

diseases. The median follow-up time was 21 months, and the maximum period reached up to 60 months. The relative lncRNA expression levels were dichotomized into low and high expression by the optimal cutoff point from the ROC curve. Altogether, 522 DCM patients were analyzed using the lncRNA ENST00000507296, while 508 DCM patients were analyzed using the lncRNA ENST00000532365 (undetectable in some patients). The Kaplan-Meier survival curves showed that high lncRNA ENST00000507296 expression and low lncRNA ENST00000532365 expression were significantly associated with the endpoints of DCM (Figures 7A and 7B). After multivariable adjustments for age, sex, New York Heart Association functional classification, NT-proBNP, LVEF, LVEDD, hypertension, and diabetes mellitus, the low expression level of circulating lncRNA ENST00000507296 (heart rate [HR] = 0.729, 95% confidence interval [CI] = 0.537–0.989, p = 0.042) was significantly associated with the endpoints; however, the expression level of the lncRNA ENST00000532365 (HR = 1.169, 95% CI = 0.856–1.597, p = 0.325) was not significantly correlated with the events (Table S3). However, the Kaplan-Meier survival curves of NT-proBNP could not present a significant association with the primary event or total event (Figure S2).

DISCUSSION

DCM may be diagnosed at any age but most usually occurs by ages 30 to 40. In DCM patients, increasing age acts as an independent risk factor for death.[4] Most importantly, due to the hidden symptoms and delay of diagnosis, the prognosis for DCM is not well.

Several studies implied that, in the DCM patients with recent new onset of clinical symptoms of HF, about 25% would have spontaneous improvement, but patients might have less chance of recovery if the symptoms last more than 3 months.[24] DCM accounts for about one third of the HF cases.[25] The presenting symptoms of DCM include arrhythmias, thromboembolic events, and circulatory collapse, while

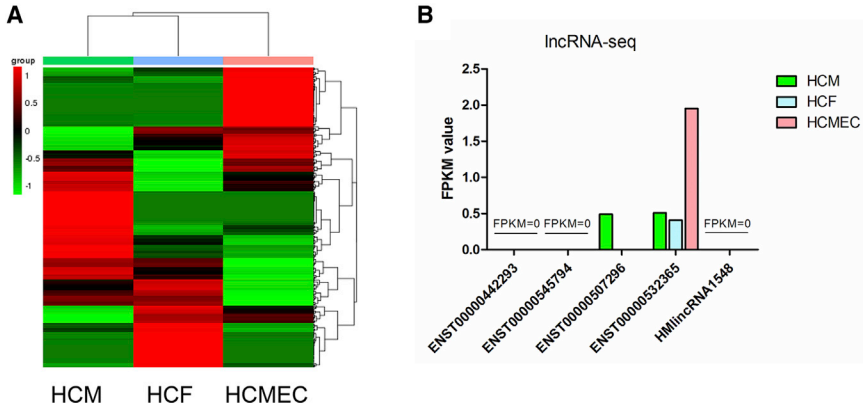

Figure 4. lncRNA-Sequencing on Samples from Three Human Heart Cells

(A) Heatmap of lncRNA expression levels in three human heart cells. (B) Expression levels of five validated lncRNAs in three human heart cells.

the most common symptoms are related to congestive HF.[26] In children with DCM, a BNP concentration greater than 300 pg/mL indicates a possibility of hospital admission because of HF, heart transplantation, or even death.[27] Nowadays, no specific treatment is available for patients with diagnosed DCM, except for drugs against HF and arrhythmia complications.[26]

In the present study, we found that lncRNAs ENST00000507296 and ENST00000532365 were able to act as biomarkers for the diagnosis of DCM-related HF. Higher circulating lncRNA ENST00000507296 levels presented a higher risk of cardiovascular events in DCM patients. Importantly, compared to other circulating lncRNAs, ENST00000507296 and ENST00000532365 were abundant in human-derived cardiac cells, which suggested a possibility of heart origin. However, in adjusted Cox proportional-hazards models including terms for HF risk factors, lower circulating lncRNA ENST00000532365 could not show the significant statistical difference due to the follow-up time and the scale of this cohort.

Accumulating evidence has demonstrated that circulating lncRNAs could act as prognostic indicators in variable types of diseases. Circulating lncRNA AK098656, which is human specific and dominant in vascular smooth muscle cells, facilitates hypertension.[28] Circulating RP11-230G5.2 and XLOC_014172 may serve as indicators of macrosomia outcome in gestational diabetes mellitus patients.[29] Meanwhile, circulating lncRNA TrAnscript could predict survival in acute kidney injury.[30]

Several studies discovered that circulating exosomal small RNAs would be significant diagnostic and prognostic biomarkers in the pathogenesis of diseases, such as multiple myeloma[31] and castration-resistant prostate cancer.[32] Recently, activated circulating myeloid-derived suppressor cells were reported to play important immunomodulatory roles in DCM.[33] The miRNAs miR-155, -636, -646, and -639 were found to have the potential to perform risk stratification in children with DCM.[34] On the other hand, miR-548c in circulating peripheral blood mononuclear cells (PBMCs) was associated with risk in DCM patients.[35] A set of CpGs as novel epigenetic biomarkers for DCM inducing HF has been identified by a multi-omics approach.[36] Some studies have discovered improved cardiac function in children diagnosed with DCM. The incidence of cardiac ameliorating varies from 16% to 63%, relying on the description of improvement and the etiology of DCM.[37–39]

Studies about the suitable endogenous control for circulating non-coding RNA are inconsistent. A previous study showed that U6 may not be a suitable endogenous control for the quantification of circulating RNAs, especially in frozen samples.[40] However, it was recently reported that U6, as well as 18S rRNA (18SrRNA), encoding glyceraldehyde-3-phosphate dehydrogenase (GAPDH), β-actin (ACTB), and β2-microglobulin (B2M), could be used for the normalization of lncRNA levels.[41] Some studies observed that the mean expression value normalization outperformed the current small non-coding RNA normalization strategy in terms of better reduction of technical variation and more accurate appreciation of biological changes.[42] In addition, cel-miR-39 could adjust the bias of the process of extracting RNA. In the present study, we applied cel-miR-39 as an exogenous normalization control. Moreover, consistent with previous studies, we found that plasma lncRNAs were stable (Figure S3).[17,43]

Nevertheless, there are some limitations in the present study. First, it is not clear whether lncRNAs ENST00000507296 and ENST00000532365 indicate cellular damage or other pathological processes in heart. Additionally, the successfully determined two lncRNAs were not highly significantly dysregulated in the heart tissues compared with our published data,[23] which prompted a more intricate regulation mechanism for DCM. Alternatively, some false-positive signals may exist in the microarray probes, and the abundance of detected lncRNAs may be inaccurate due to the limitation of microarray. Moreover, the plasma samples were from victims of accidents, not from the healthy volunteers who were controls in the screening cohort. We anticipate that a series of lncRNAs is regulated in DCM and may have a prognostic value. Next-generation sequencing technologies should be considered to provide an alternative approach, while a TaqMan probe could be applied, which may increase the specificity of this assay. In addition, expanding follow-up studies is necessary to test the utility of the lncRNA as a predictor of cardiovascular incident. Moreover, it is still not known whether the dysregulation of ENST00000507296 and ENST00000532365 could be a self-compensatory protective mechanism against DCM or not. Therefore, the function studies are crucial to identify the detailed role of dysregulated lncRNAs in DCM as well as in other cardiovascular diseases.

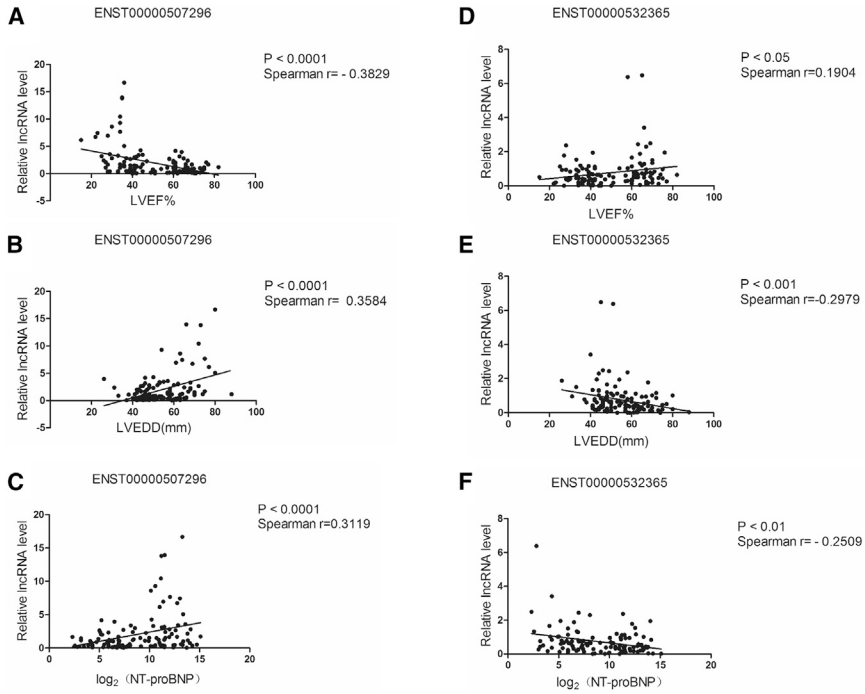

Figure 5. Correlation Analyses of lncRNA ENST00000507296 and ENST00000532365 with HF Severity

(A–C) Correlation analyses of lncRNA ENST00000507296 level with (A) LVEF, (B) LVEDD, and (C) NT-proBNP. (D–F) Correlation analyses of lncRNA ENST00000532365 level with (D) LVEF, (E) LVEDD, and (F) NT-proBNP. n = 128.

MATERIALS AND METHODS

Study Population

This study was approved by the Ethics Committee of Tongji Hospital. Written informed consent was granted by the subjects recruited in the study or by the immediate family members in accordance with the Declaration of Helsinki. Between January 2010 and October 2014, patients diagnosed with DCM or HF and control subjects were enrolled in Tongji Hospital (Wuhan, China). Diagnoses of HF and DCM were on the basis of the final diagnosis at discharge, according to the American College of Cardiology and American Heart Association (ACC/AHA) guidelines.[44]

In the screening cohort, using a test tube containing EDTA, blood samples (5 mL) were collected from 10 control donors (victims of accidents) and 10 chronic HF patients diagnosed with DCM during heart transplantation surgery. Then, plasma was obtained by centrifugation at 2,000 × *g* at 4°C for 10 min, and the supernatant was carefully transferred to a new RNase and DNase-free 1.5-mL microtube and stored at −80°C until use. The inclusion criteria for the DCM group were as follows: (1) New York Heart Association (NYHA) classes II–IV; (2) echocardiography LVEF < 50%; (3) echocardiography LVEDD > 55 mm. Exclusion criteria were coronary angiography showing the presence of more than 50% stenosis in the right coronary artery, left anterior descending artery, or left main stem.[45] The clinical characteristics of the patients and controls are shown in Table S4.

In the validation case-control study, plasma samples were obtained from 64 DCM patients and 64 control subjects. The inclusion and exclusion criteria for the DCM group were the same as for the screening cohort, and all the control subjects met the following conditions: (1) no signs or symptoms of HF, or NYHA classes I and II; and (2) echocardiography LVEF > 50% and normal LVEDD. Their clinical and demographic characteristics are provided in Table S5.

In the replication case-control study, 198 DCM patients and 198 control individuals were enrolled. Inclusion and exclusion criteria of the DCM group were in accordance with the validation population. The patient characteristics are listed in Table S6.

In the follow-up cohort study, DCM patients were included according to the inclusion criteria of the validation study. The exclusion criteria of patients were as following: (1) patients with other severe systemic diseases (e.g., renal failure or hepatic diseases) and malignant tumors; (2) congenital heart diseases or significant valvular diseases; and (3) unwillingness to provide informed consent. The endpoints of the study covered cardiovascular death, heart transplantation, implantable cardioverter-defibrillator (ICD) implantation, and hospitalization due to worsening of HF. If the patient had several events, the time of the first event was regarded as the outcome. The median follow-up time was 21 months, and the maximum period reached up to 60 months. The patient characteristics are listed in Table S7.

Study Design

The procedure of this study was indicated as the following five main steps: (1) obtaining lncRNA profiles in the screening population; (2) lncRNA testing in the validation cohort; (3) RNA sequencing (RNA-seq) of selected lncRNAs in different human-derived cardiac cell types and correlation analysis between plasma levels of validated lncRNAs and severity of HF; (4) confirmation of the selected lncRNAs in the replication population; and (5) prognosis association of lncRNAs with patients with DCM in the follow-up population.

RNA Extraction

Total RNA was extracted from 250 μL plasma with TRIzol LS Reagent (Life Technologies, Carlsbad, CA, USA) following the manufacturer's instructions. 50 pmol/L *Caenorhabditis elegans* miR-39 (cel-miR-39) was added as the spike-in control after TRIzol LS Reagent was added. The purity and quality of the total RNA were checked using the Nanodrop 2000 (Thermo Scientific, Waltham, MA, USA). The ratio of the

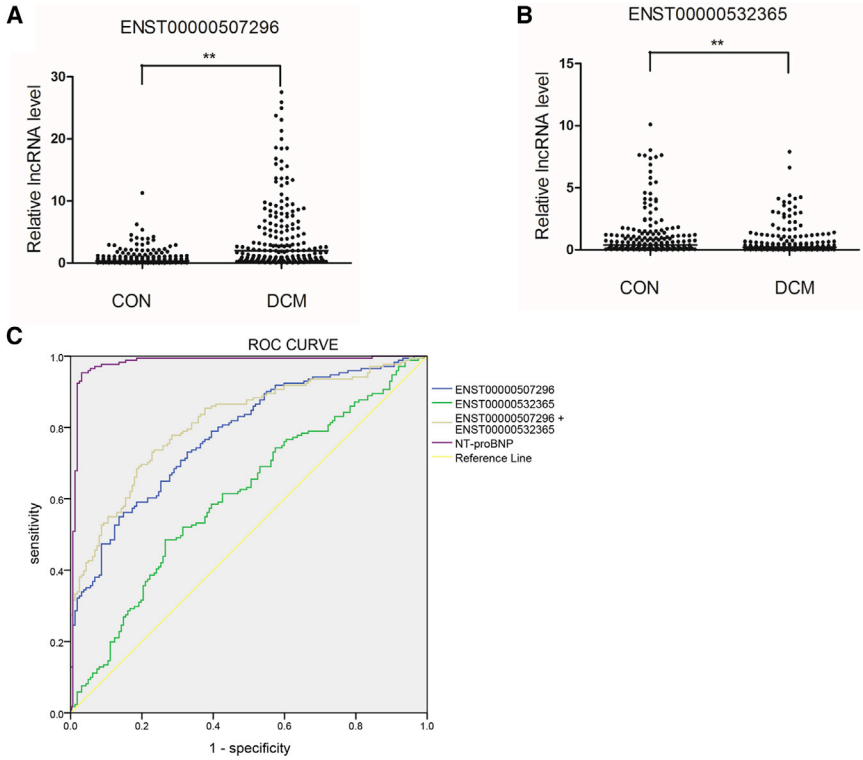

Figure 6. Circulating lncRNA ENST00000507296 and ENST00000532365 Levels and ROC Curve Analyses between DCM and Control Group

(A) Expression levels of lncRNA ENST00000507296 in DCM patients and control subjects, normalized to cel-miR-39. (B) Expression levels of lncRNA ENST00000532365 in DCM patients and control subjects, normalized to cel-miR-39. Horizontal lines indicate the median. **p < 0.01 versus control. (C) ROC curve analyses of lncRNA ENST00000507296, ENST00000532365, and NT-proBNP. The cohort included 198 DCM patients and 198 control subjects.

absorbance at 260 and 280 nm (optical density [OD] 260/280) of isolated RNA was between 1.8 and 1.9.

Microarray and Real-Time qPCR

RNA was pre-amplified and then underwent microarray analysis (Arraystar, Human lncRNA Array, v3.0). About 30,586 lncRNAs and 26,109 coding transcripts can be detected by Kangcheng Biotechnology (Shanghai, China). The microarray data gathered in this study were deposited in the NCBI Gene Expression Omnibus database under accession number GEO: GSE124401 (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE124401).

Expressions of certain lncRNAs were detected by qRT-PCR. Reverse transcription was performed according to the SuperScript III First Strand Synthesis Kit (Life Technologies, Carlsbad, CA, USA). The extracted RNA was reverse transcribed using random primers. *C. elegans* miR-39 (Riobio, Guangzhou, China) was applied as a normalization control. For each sample, the Ct values obtained for the three spiked-in cel-miR-39 samples and targeted lncRNAs were averaged to generate the relative expression levels in each plate. For different plates, cel-miR-39 of the samples were detected again to be a normalization control in this plate. The results from each plate were normalized against normalization controls. Real-time qPCR assays were conducted by the SYBR Select Master Mix (Life Technologies, Carlsbad, CA, USA) on a 7900HT FAST Real-Time PCR System (Life Technologies, Carlsbad, CA, USA). Cycling parameters were as follows: 95°C for 10 min, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. Relative expression levels between groups were calculated by the $2^{-\Delta\Delta ct}$ method, as previously described.[46] When Ct value was more than 37, the lncRNA was considered as undetectable. Gel electrophoresis, melt curve analysis, and product sequencing were used to confirm the specificity of primers. The primers are listed in Table S8.

Cell Culture

Human cardiac myocytes (HCMs; ScienCell Research Laboratories, Carlsbad, CA, USA) were from a single fetal donor, not pooled, and were maintained in cardiac myocyte medium (CMM; ScienCell Research Laboratories, catalog #6201), according to the instruction manual. CMM consists of basal medium, fetal bovine serum (FBS), cardiac myocyte growth supplement, and penicillin and streptomycin solution. Human cardiac fibroblasts-adult ventricular (HCF-av; ScienCell Research Laboratories, Carlsbad, CA, USA) were maintained in fibroblast medium-2 (FM-2; ScienCell Research Laboratories, catalog #2331), which contained fibroblast growth supplement-2 (FGS-2; Catalog #2382) and FBS. Human cardiac microvascular endothelial cells (HCMECs; ScienCell Research Laboratories, Carlsbad, CA, USA) were maintained in endothelial cell medium (ECM, catalog #1001). ECM consists of basal medium, FBS, endothelial cell growth supplement, and penicillin and streptomycin solution. All these cells were cultured and utilized as described previously.[23]

lncRNA Sequencing

lncRNA sequencing and data analyses were performed by Personal Biotechnology (Shanghai, China). The sequencing data analyzed in this study were deposited in the NCBI Gene Expression Omnibus database under accession number GEO: GSE124402 (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE124402). $p < 0.05$ was considered statistically significant.

Statistical Analysis

All data were analyzed by SPSS (v20.0; Chicago, IL, USA). Gaussian distribution was evaluated by Shapiro-Wilks and Kolmogorov-Smirnov tests. Continuous variables are described as

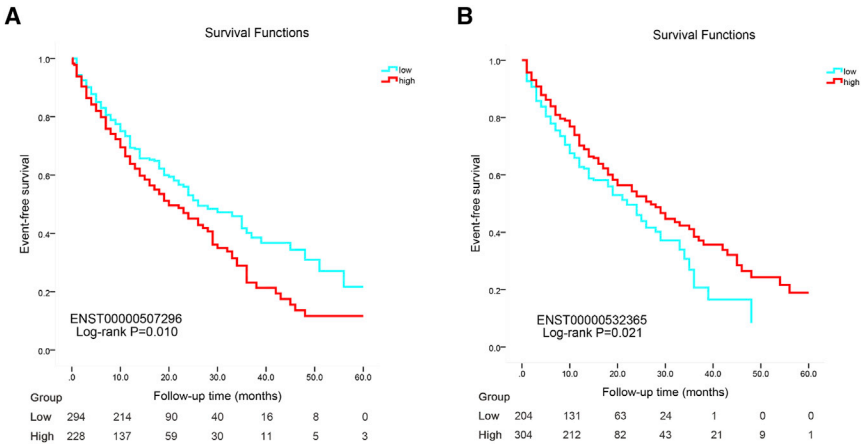

Figure 7. Kaplan-Meier Survival Curves based on Different Levels of Circulating lncRNAs in DCM

(A) Kaplan-Meier survival curves of expression levels of lncRNA ENST00000507296 and event-free survival in patients with DCM (n = 522). (B) Kaplan-Meier survival curves of expression levels of lncRNA ENST00000532365 and event-free survival in patients with DCM (n = 508). The lncRNAs were dichotomized at the optimal cutoff point of the ROC curve based on a low-versus-high expression.

means ± SD. For normal distribution data, the two-tailed Student's t test was used, while the Mann-Whitney U test was used for skewed distribution. As the distribution of plasma lncRNA expression levels was skewed, horizontal lines indicate the median. The chi-square test was used to compare categorical variables. Statistical significance was set at $p < 0.05$. Correlation analysis was assessed by Spearman correlation for non-normally distributed variables. The receiver operating characteristic (ROC) curves were used to assess lncRNAs as diagnostic tools for distinguishing DCM. The lncRNA expression values were divided into high-expression and low-expression groups based on ROC curves with Youden's index correction.[47] The Kaplan-Meier survival curve indicated survival probabilities, and the significance of differences was tested by the log-rank test. Cox proportional hazards models were used to determine lncRNAs associated with incident cases.

SUPPLEMENTAL INFORMATION

Supplemental Information includes three figures, eight tables, and one data file and can be found with this article online at https://doi.org/10.1016/j.omtn.2019.02.004.

AUTHOR CONTRIBUTIONS

X.Z. designed the study, analyzed and interpreted the data, and drafted the paper; X.N., S.Y., H.L., J.F., C.L., Y.S., Y.Z., and H.H. participated in acquiring the data; D.W.W. and C.C. designed the work and drafted the paper.

CONFLICTS OF INTEREST

The authors declare no competing interests.

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