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Original Article

Diagnostic value and clinical significance of lncRNA *NEAT1* combined with miR-425-3p in children with viral myocarditis

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ABSTRACT

Background. Viral myocarditis (VMC) is common in children. Previous studies have reported the clinical value of nuclear paraspeckle assembly transcript 1 (*NEAT1*) and microRNA-425-3p (miR-425-3p) in certain diseases, but not in VMC. This article was designed to investigate the expression of long noncoding RNA (lncRNA) *NEAT1* and miR-425-3p in the serum of patients with VMC and their clinical significance.

Methods. We assessed VMC and healthy patients and analyzed differences in the expression levels of *NEAT1* and miR-425-3p. The correlation and targeting relationship between the two were reported by Spearman correlation analysis and luciferase reporter assay. ROC curves were plotted to reflect the diagnostic effect of both. In addition, according to the 12-month prognostic effect grouping, patients with VMC were separated into a group with good vs. poor prognosis, and the difference in the expression levels of *NEAT1* and miR-425-3p between the two groups were analyzed. The ability of the two markers in the prognosis of VMC was further analyzed by multiple logistic regression.

Results. *NEAT1* expression was up-regulated in VMC and miR-425-3p expression was down-regulated, and there was a negative correlation and targeting link between the two. The diagnostic efficacy of both *NEAT1* and miR-425-3p was higher than that of a single indicator. High expression of *NEAT1* and low expression of miR-425-3p were found in VMC patients with poor prognosis. Both were independent influencers of VMC prognosis.

Conclusion. *NEAT1* and miR-425-3p expressions were affected by VMC and had important clinical implications for VMC, indicating for the first time the clinical function of *NEAT1* and miR-425-3p in VMC.

Key words: NEAT1, miR-425-3p, viral myocarditis, diagnosis, prognosis.

Viral myocarditis (VMC), is a myocardial disease caused by viral invasion of cardiac tissue, resulting in myocardial cell degeneration, necrosis, and interstitial inflammatory changes.[1] The clinical manifestations of the disease are variable, and the preclinical symptoms are not obvious. Clinical studies have demonstrated that most VMC patients recover well after treatment, but continued progression can cause further damage to cardiomyocytes, leading to myocardial fibrosis, arrhythmias, heart failure, and even sudden death.[2-4] This type of VMC has a poor prognosis, with an annual survival rate of only 50% within 5 years.[5] Although the short-term mortality rate of fulminant myocarditis has been further reduced with the improvement of diagnostic and therapeutic modalities at present, there is a general lack of effective clinical

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treatments for VMC, and current treatments are mainly focused on controlling the complications caused by VMC.[6] Therefore, early diagnosis and prognostic monitoring are particularly important to reduce the mortality rate of VMC and improve the treatment outcome.

Long noncoding RNAs (lncRNAs) are long-chain RNAs that are not translated into proteins and have diverse biological activities.[7] Publications have reported that lncRNAs maternally expressed gene 3 (*MEG3*), regulator of reprogramming (*ROR*), and metastasis-associated lung adenocarcinoma transcript 1 (*MALAT1*) can be involved in the development of myocarditis.[8-10] LncRNA nuclear paraspeckle assembly transcript 1 (*NEAT1*) is associated with the mechanism of action of lipopolysaccharide-induced myocardial injury.[11] *NEAT1* expression is upregulated in the peripheral blood of patients with myocardial infarction and has the potential to predict the development of myocardial infarction.[12] This disease is associated with myocardial damage closely related to VMC, but there is no relevant literature on the relationship between *NEAT1* and VMC. As competing endogenous RNAs (ceRNAs) of lncRNAs, microRNAs (miRNAs) likewise play important roles in VMC, for instance miR-21-5p and miR-1-3p.[13] miR-146b concentration is elevated in VMC patients and is associated with myocardial injury, independently predicting patient prognosis.[5] In myocardial injury tissues of the coxsackievirus type B3-induced VMC mouse model, reduced miR-425-3p expression levels suggest that miR-425-3p is associated with VMC.[14] The function of miR-425-3p on VMC was only researched in the mouse model with VMC.[14] However, its expression and clinical value in patients with VMC have not been previously reported. Therefore, this paper focuses on the clinical importance of *NEAT1* and miR-425-3p in VMC.

In this manuscript, we collected serum samples and clinical data from 108 patients with VMC and assessed the levels of *NEAT1* and miR-425-3p to explore the possibility as both diagnostic biomarkers as well as their prognostic value, and to provide references for early clinical diagnosis and therapeutic effects.

Materials and methods

Collection of research objects

Cases of children with VMC admitted to Xingtai People's Hospital from May 2019 to May 2022 were chosen for the study. All of them met the diagnostic criteria for VMC formulated by the Chinese Medical Association.[15] Specifically, the basis for the diagnosis of VMC involves clinical diagnostic findings and pathogen measurements. Clinical diagnostic bases include cardiac insufficiency, cardiogenic shock, or heart-brain syndrome; cardiac enlargement; electrocardiographic changes; and elevated creatine kinase-myocardial band (CK-MB) or positive cardiac troponin (cTnI or cTnT). Diagnosis of pathogens consists primarily of virus detection. Exclusion criteria included myocardial injury caused by congenital heart disease and rheumatic heart disease; metabolic diseases; and antiviral drugs and immunotherapy before admission. A further 102 cases of children who underwent health check-ups during the same time frame at our hospital were chosen for the purpose of constituting the control group. The clinical examination data of all subjects were collected, and all samples were collected and investigated with the informed consent of the guardian and signed for confirmation. The study was reviewed and approved by the ethics committee of Xingtai People's Hospital (Approval number: 2018-XPH-14).

Follow-up and prognosis outcome judgment

All patients received the routine treatment of VMC. Conventional treatment included antiviral therapy and intravenous gammaglobulin. Other measures included monitoring patients using electrocardiograms and oxygen therapy. All patients were followed up for 12 months and the prognosis within 12 months was summarized. Poor prognosis was defined as the

presence of recurrent myocarditis, readmission with arrhythmia, rehospitalization with heart failure, cardiac transplantation, and death. The remaining patients were included in the good prognosis group.

Measurement of serum NEAT1 and miR-425-3p level

Three ml of venous blood from VMC patients at admission was collected and centrifuged at 1800 r/min (centrifugal radius 15 cm) for 10 min to separate serum.

Extraction of total RNA from serum was used Trizol LS (ThermoFisher Scientific, California, America). The absorbance of the extracted solution was determined by an ultraviolet spectrophotometer, and the ratio of optical density (OD) 260/OD280 was calculated. RNA with a ratio between 1.8 and 2.0 was up to standard. Total RNA was reverse transcribed into complementary DNA (cDNA) using the first strand of cDNA synthesized premixed reagents (TIANGEN, Beijing, China) for *NEAT1* and miRNA First Strand cDNA Synthesis Kit (Vazyme, Nanjing, China) for miR-425-3p. The expression of *NEAT1*, miR-425-3p, glyceraldehyde 3-phosphate dehydrogenase (GAPDH), and U6 was detected by a Talent fluorescence quantitative detection kit (TIANGEN, Beijing, China) on a BioRad fluorescence quantitative PCR instrument (BioRad, Hamburg, Germany). *GAPDH* and *U6* were detected as housekeeping genes. Cycle threshold (CT) values were obtained after the assay, and relative expression was obtained using $2^{-\text{deltadeltaCT}}$.

Levels of cTnI, CK-MB, and high-sensitivity C-reactive protein (hs-CRP)

The cTnI levels were detected by enzyme-linked immunosorbent assay (ELISA) using a Human cTnI ELISA Kit (Abcam, Cambridge, UK). CK-MB levels were detected using a DXC600 automatic biochemical analyzer. hs-CRP level was detected using a Human CRP ELISA Kit (Abcam, Cambridge, UK).

Detection of double luciferase reporter gene

NEAT1 wild-type vector (WT-*NEAT1*) and mutant vector (MUT-*NEAT1*) were constructed. miR-425-3p mimics, inhibitors, and negative controls were purchased from ThermoFisher Scientific (California, America). and WT-*NEAT1* and MUT-*NEAT1* were co-transfected with miR-425-3p mimic, inhibitors, and controls, respectively, into 293T cells. After 48 hours, luciferase activity was detected following the luciferase activity assay kit instructions (Biolab, Beijing, China).

Statistical analysis

GraphPad Prism (Version: 7.0) and IBM SPSS statistics (Version: 24) were used for the statistical analysis of data. Continuous variables were tested with the Kolmogorov-Smirnov inspector, and the data was confirmed to conform to normal distributions. Then the continuous variables were tested by t-test. The correlation between lncRNA and miRNA was identified by the Spearman method. The $\chi 2$ test was used for discrete data. Luciferase reported results were tested by two-way ANOVA followed by post hoc Bonferroni's test. The diagnostic value of *NEAT1*, miR-425-3p, and the combination of the two for VMC was determined using the subject's work characteristics (ROC) curve. Combined ROC curves were constructed using the predicted probability acquired through multivariate logistic regression of miR-425-3p and *NEAT1*. Multiple logistic analysis was used to analyze the influencing factors in the prognosis of VMC patients. The level of statistical significance was set at $p < 0.05$ in all statistical tests.

Results

Comparison of clinical symptoms between VMC and control groups

The clinical characteristics of all recruited patients and controls were gathered and estimated. There was no noteworthy

differentiation between the two groups of participants in relation to their age, gender, and BMI ($p > 0.05$, Table I). Patients in the VMC group possessed higher cTnl CK-MB, and hs-CRP levels ($p < 0.001$, Table I).

Serum levels of NEAT1 and miR-425-3p in VMC

The relative concentration of *NEAT1* was elevated in the VMC group, reflecting that *NEAT1* was involved in the development of VMC ($p < 0.001$, Fig. 1A). Diminished relative quantification of miR-425-3p was found in the VMC group ($p < 0.001$, Fig. 1B). A negative correlation between *NEAT1* and miR-425-3p was detected. As depicted in Fig. 1C-D, *NEAT1* and miR-425-3p were negatively correlated in both control group (R: -0.750, $p < 0.001$) and VMC patients (R: -0.777, $p < 0.001$), suggesting that there may be some correlation between the two.

Verification of the targeting relationship of miR-425-3p with NEAT1

The binding site of miR-425-3p to *NEAT1* was predicted using the RNAhybrid website (Fig. 2A). The luciferase intensity was significantly decreased in the miR-425-3p mimic and WT-*NEAT1* co-transfected group, and significantly increased in the miR-425-3p inhibitor and WT-*NEAT1* co-transfected group ($p < 0.001$, Fig. 2B). The variance in luciferase activity among groups co-transfected with MUT-*NEAT1* was not deemed statistically significant ($p > 0.05$, Fig. 2B). These results suggest that miR-425-3p might be a ceRNA of *NEAT1*.

Diagnostic significance of NEAT1 and miR-425-3p

The ROC curve showed that serum *NEAT1* predicted VMC patients with ROC of 0.875, sensitivity of 73.15%, and specificity of 87.25% (Fig. 3). miR-425-3p predicted patients with poor prognosis with a ROC of 0.832, sensitivity of 79.63%, and specificity of 77.45% (Fig. 3). The ROC, sensitivity, and specificity of the two combined were 0.901, 80.60%, and 87.30%, respectively (Fig. 3). The comparison of the area under the curve indicated that the combined diagnostic efficacy was better than the two single diagnostic indexes.

Comparison of basic data of VMC patients with different prognostic outcomes

There was no significance in the difference in age, BMI, and gender between the good and poor prognosis groups ($p > 0.05$, Table II). The cTnI, CK-MB, and hs-CRP levels of the poor prognosis group were all elevated ($p < 0.001$, Table II).

Serum levels of NEAT1 and miR-425-3p in VMC patients with different prognostic outcomes

The serum *NEAT1* levels in the good prognosis group were significantly lower than those in the poor prognosis group ($p < 0.001$, Fig. 4A). The

Table I. Demographics and baseline information of healthy and VMC groups.

Clinical features	VMC group (n = 108)	Control group (n = 102)	*p*-value
Age (year)	5.63 ± 2.15	6.22 ± 2.12	0.923
BMI (kg/m^2)	14.46 ± 1.98	14.33± 2.28	0.321
Duration (day)	4.86 ± 1.53	/	/
Gender (male/female)	50/58	52/50	0.497
cTnI (μg/L)	0.49 ± 0.11	0.06 ± 0.01	< 0.001
CK-MB (U/L)	33.48 ± 5.81	8.91 ± 2.18	< 0.001
hs-CRP (mg/L)	4.32 ± 0.88	1.17 ± 0.19	< 0.001
NEAT1	1.00 ± 0.29	1.47 ± 0.30	< 0.001
miR-425-3p	1.00 ± 0.31	0.59 ± 0.26	< 0.001

Note: BMI: Body-mass index; CK-MB: Creatine kinase myocardial band; cTnI: Cardiac troponin I; hs-CRP: Hypersensitive C-reactive protein; VMC: Viral myocarditis.

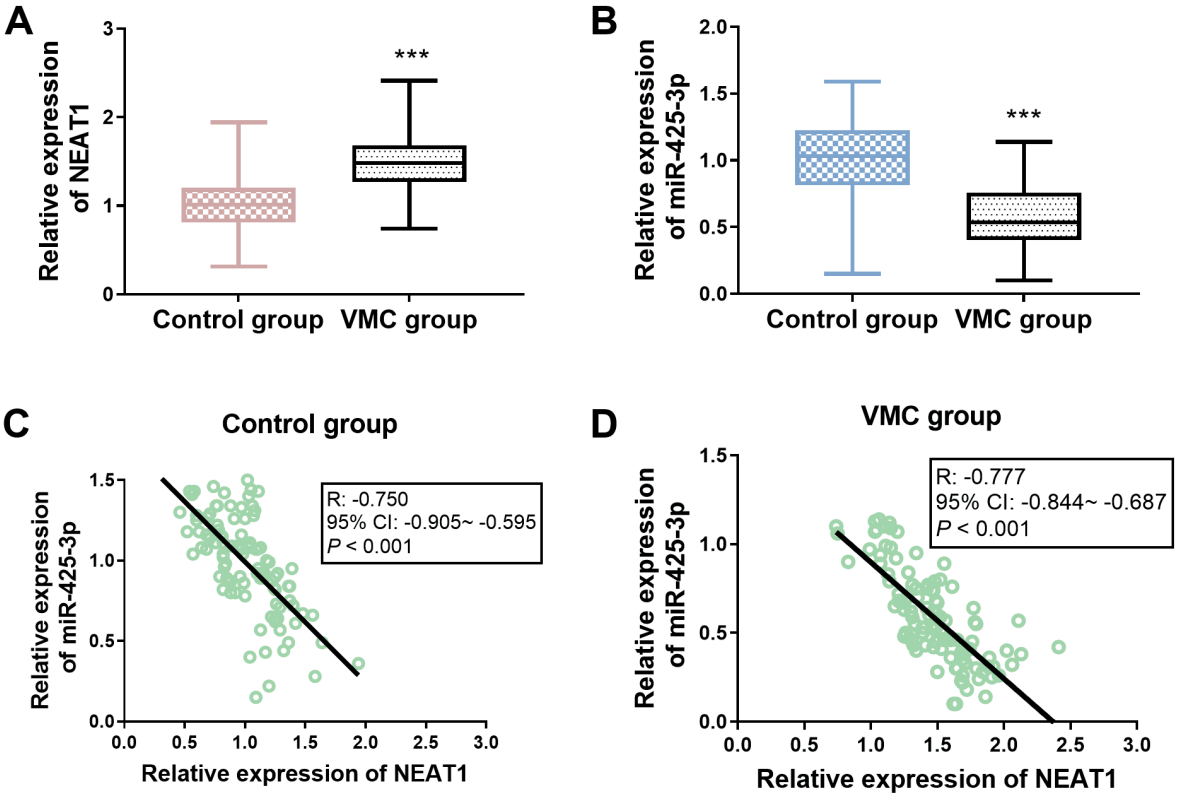

Fig. 1. (A) The concentration of NEAT1. **(B)** Reduced miR-425-3p in VMC patients. **(C)** Negative relationship between NEAT1 and miR-425-3p in control group. **(D)** Negative relationship between NEAT1 and miR-425-3p in VMC group. ***p < 0.001, compared to control group. VMC: viral myocarditis.

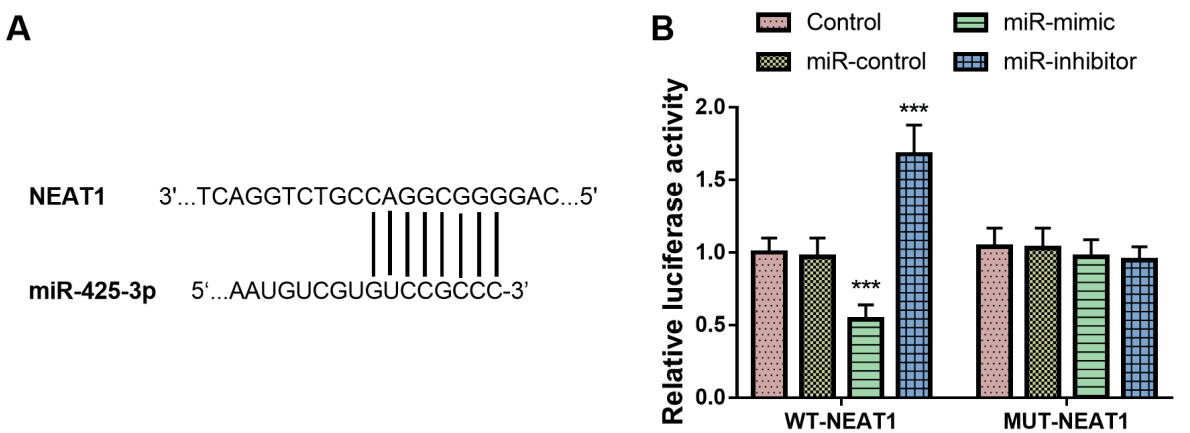

Fig. 2. miR-425-3p targeted NEAT1. **(A)** Targeted bases between *NEAT1* and miR-425-3p. **(B)** Luciferase reporter assay certified targeted interconnection between *NEAT1* and miR-425-3p. ***P < 0.001, compared to control group.

declined levels of miR-425-3p were observed in the poor prognosis group ($p < 0.001$, Fig. 4B). These results displayed that the concentration of *NEAT1* and miR-425-3p was influenced by the prognosis of VMC.

Possibility of NEAT1 and miR-425-3p as independent predictors of VMC

A multiple logistic regression model was developed to analyze the independent influencing factors. Baseline characteristics

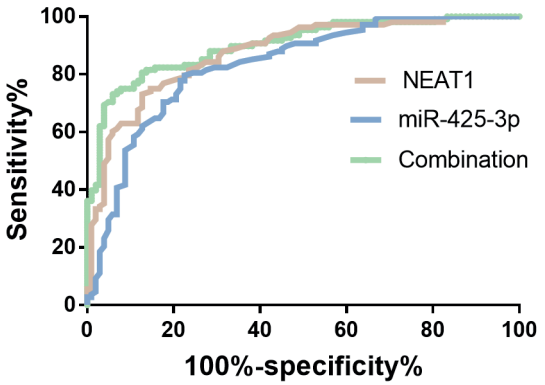

Fig. 3. Diagnostic significance of NEAT1, miR-425-3p, and their combination.

were categorized into 9 variables that were all entered into logistic regression models. The model's goodness of fit was evaluated with the Hosmer-Lemeshow test (p = 0.228). The results showed that elevated cTnI (odd ratios: 3.883, 95% confidence interval: 1.275-11.825), elevated CK-MB (odd ratios: 3.671, 95% confidence interval: 1.194-11.285), elevated *NEAT1* (odd ratios: 9.714, 95% confidence interval: 3.204-29.453) and reduced miR-425-3p (odd ratios: 0.159, 95% confidence interval: 0.049-0.512) were independent risk factors for poor prognosis in VMC patients (all $p < 0.05$, Table III).

Table II. Demographics and baseline information of patients with VMC.

Clinical features	Good prognosis group (n = 62)	Poor diagnosis group (n = 46)	p-value
Age (year)	6.13 ± 2.29	6.35 ± 1.90	0.599
BMI (kg/m^2)	14.68 ± 2.09	14.18 ± 1.79	0.201
Duration (day)	4.88 ± 1.44	4.84 ± 1.67	0.191
Gender (male/female)	31/31	19/27	0.437
cTnI (μg/L)	0.43 ± 0.09	0.57 ± 0.09	< 0.001
CK-MB (U/L)	30.87 ± 5.12	36.99 ± 4.79	< 0.001
hs-CRP (mg/L)	4.04 ± 0.73	4.69 ± 0.94	< 0.001
NEAT1	1.37 ± 0.31	1.61 ± 0.23	< 0.001
miR-425-3p	0.73 ± 0.23	0.40 ± 0.17	< 0.001

Note: BMI: Body mass index; CK-MB: Creatine kinase myocardial band; cTnI: Cardiac troponin I; hs-CRP: Hypersensitive C-reactive protein; VMC: Viral myocarditis.

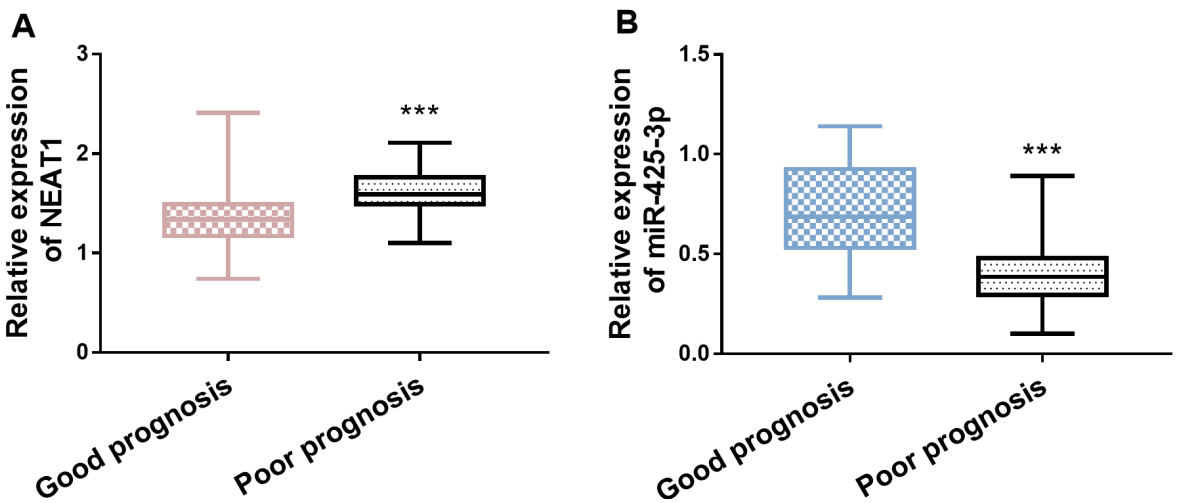

Fig. 4. (A) Elevated NEAT1 levels in poor prognosis group **(B)** Declined miR-425-3p levels in poor prognosis group ***p < 0.001, compared to good diagnosis group.

miR-425-3p: microRNA-425-3p; NEAT1: nuclear paraspeckle assembly transcript 1.

Discussion

VMC has high morbidity and mortality.[16] At present, the diagnosis of VMC is mainly based on the history of viral infection, clinical features, and serum myocardial enzyme levels, but the sensitivity of these is low.[17] The gold standard for clinical diagnosis of VMC is endomyocardial biopsy, but it is difficult to obtain an accurate diagnosis in the early stage of the disease.[5,18] Therefore, screening for appropriate markers is necessary for the clinical management of VMC.

LncRNAs are closely related to heart diseases.[19] LncRNA *HIF1A* antisense RNA 1 (*HIF1A*-AS1), *AK085865*, and *GBP9* are both influenced by the VMC and participate in the development of VMC, indicating the potential roles of lncRNAs in VMC.[20-22] *NEAT1* is involved in a variety of illnesses, including heart failure and myocardial infarction. Wang et al.[23] show that the expression level of *NEAT1* is significantly increased in the mice of myocardial infarction after coronary heart disease, and the high expression of *NEAT1* together with the diminished miR-22-3p may become targets for myocardial infarction. Ge et al.[24] report that NEAT1 is improved in patients with heart failure and can facilitate the progression of fibrosis in vitro models, indicating that NEAT1 is associated with the pathological process of heart dysfunction. All these articles suggest that NEAT1 might be involved in heart disorders. The present study determined that the relative expression level of serum NEAT1 was improved in the VMC group and further elevated in VMC patients with poor prognosis, suggesting that NEAT1 might be involved in the development of VMC. Elevated serum expression levels of *NEAT1* are valuable for the diagnosis of VMC. In addition, *NEAT1* can be used as an independent biomarker to detect the disease progression of VMC. Taken together, these findings pinpointed that the expression of *NEAT1* was affected by the development of VMC and could predict the diagnosis and prognosis of VMC.

miRNAs have been proven to regulate the expression process of host and virus genes, which plays a certain role in many diseases.[25] In children with VMC, the concentration of miR-155 and miR-381 is regulated by VMC, indicating these miRNAs are closely linked to VMC.[26,27] The results of this study verified that the content of miR-425-3p in the VMC group was decreased and its expression level further declined in patients with a poor prognosis. The ROC curve showed that miR-425-3p was a prognostic marker. The multiple logistic regression analysis model showed that the decrease of miR-425-3p was an independent indicator for adverse prognosis in patients with VMC. Our findings further certified the diagnostic and prognostic significance of miR-425-3p for patients with VMC. MiR-425-3p is a member of the miRNA gene cluster which has been studied a lot. In myocardial tissues of mice with VMC, the concentration of miR-425-3p is down-regulated, reflecting that miR-425-3p might be a mediator in VMC.[14] The reduced miR-425-3p expression is observed in patients with heart failure and it can serve as a biomarker, lending evidence that miR-425-3p is associated with the occurrence of heart diseases.[28] In addition, the targeting relationship between *NEAT1* and miR-425-3p was further confirmed. miR-425-3p concentration changes altered the luciferase intensity of *NEAT1*-WT, suggesting that, miR-425-3p is a downstream regulator of *NEAT1*. The value of the combined diagnosis of NEAT1 and miR-425-3p was also evaluated by the ROC, which showed that the combined diagnosis significantly improved the predictive efficacy. However, there are some limitations in this study, such as a lack of in-depth mechanism research, a relatively small sample size, and a single research center.

To sum up, the concentration of NEAT1 in the serum of patients with VMC was up-regulated, while the expression of miR-425-3p was down-regulated. There was a negative relationship between NEAT1 and miR-425-3p and miR-425-3p was possibly a target of NEAT1. The combination of NEAT1 and miR-425-3p had a good diagnostic value for VMC. Both NEAT1 and miR-425-3p were independent prognostic biomarkers.

Ethical approval

The study protocol was approved by The Ethics Committee of Xingtai People's Hospital (Ethical approval number: 2018-XPH-14, 02 April 2018) and followed the principles outlined in the Declaration of Helsinki. In addition, informed consent has been obtained from the participants involved.

Author contribution

The authors confirm contribution to the paper as follows: study conception and design: JG, HH; data collection: LQ, QG, DZ; analysis and interpretation of results: GM, KZ, SW; draft manuscript preparation: JG, HH. All authors reviewed the results and approved the final version of the manuscript.

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Conflict of interest

The authors declare that there is no conflict of interest.

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