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Original Article

Correlation research on plasma miRNA-17-5p and miRNA-20a with prognosis for pulmonary arterial hypertension

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Abstract: Objective: The aim of this study was to measure changes in plasma miRNAs in patients with pulmonary arterial hypertension (PAH), investigating their correlation with severity and prognosis of PAH. Methods: Plasma total RNA from 112 PAH patients and 100 healthy controls was selected using the microarray method. Contents of miRNA-17-5p and miRNA-20a were determined, adopting real-time quantitative polymerase chain reaction (PCR). Concentrations of miRNA-17-5p and miRNA-20a in plasma of PAH patients were measured. Results: Significant differences in plasma concentrations of miRNAs between the control group and case group could be seen in 116 cases, according to results of plasma miRNA concentration determination. Upregulation of miRNA-17-5p and miRNA-20a was the most remarkable in the PAH group. Results of ROC curve analysis indicated that concentrations of plasma miRNA-17-5p and miRNA-20a were correlated with 2-year survival rates of PAH patients. miRNA-17-5p and miRNA-20a levels were shown to be important predictive factors of PAH survival rates, confirmed by Cox regression analysis. Age, cardiac index, WHO classification, 6-minute walking distance, disease duration, and red blood cell distribution width may also be serve as predictive factors of survival rates. Multivariate analysis, incorporating these co-variants, verified that miRNA-17-5p and miRNA-20a levels were independent predictive factors of 2-year survival rates of PAH. Conclusion: Increases in plasma miRNA-17-5p and miRNA-20a levels are correlated with poor prognosis in PAH patients.

Keywords: miRNA-17-5p, miRNA-20a, prognosis, pulmonary artery hypertension

Introduction

Research regarding microRNAs (miRNAs) has become a focus in recent years. MicroRNAs are a class of non-protein-coding RNA molecules, involved in expression of multiple indispensable genes and cell evolution. They can regulate transcriptional and post-transcriptional expression. Downregulation of miRNAs in miRNA binding 3' terminal untranslated regions expressed by genes and effective combination with the Watson-Crick matching algorithm have contributed to prediction of target genes of miRNAs [1]. They may be involved in the genesis and development of multiple diseases in pulmonary vascular disease pathways. This research aimed to investigate miRNAs induced by pulmonary arterial hypertension (PAH).

Morbidity and mortality of PAH in China have increased rapidly recently, becoming a major disease threatening the health of Chinese citizens. PAH is a fatal disease induced by excessive proliferation of pulmonary artery endothelial cells (PAECs) and pulmonary artery smooth muscle cells (PASMCs). Proliferation gives rise to changes in pulmonary vascular structure, manifesting as dysfunction of PAECs and PASMCs, which is the pathogenesis of PAH. Proliferation further leads to increased pulmonary vascular resistance, resulting in right ventricular heart failure [2-4]. Evidence has indicated that miRNAs plays an important role in pulmonary vascular growth and development, as well as disease formation. Moreover, destruction of pulmonary vascular homeostasis and regulation of such miRNAs participate in the pathogenesis of PAH [5-7].

Bone morphogenetic protein receptor II (BMPR2), hypoxia, STAT3, and Apelin-APJ pulmonary vascular cellular signal pathways are clo-

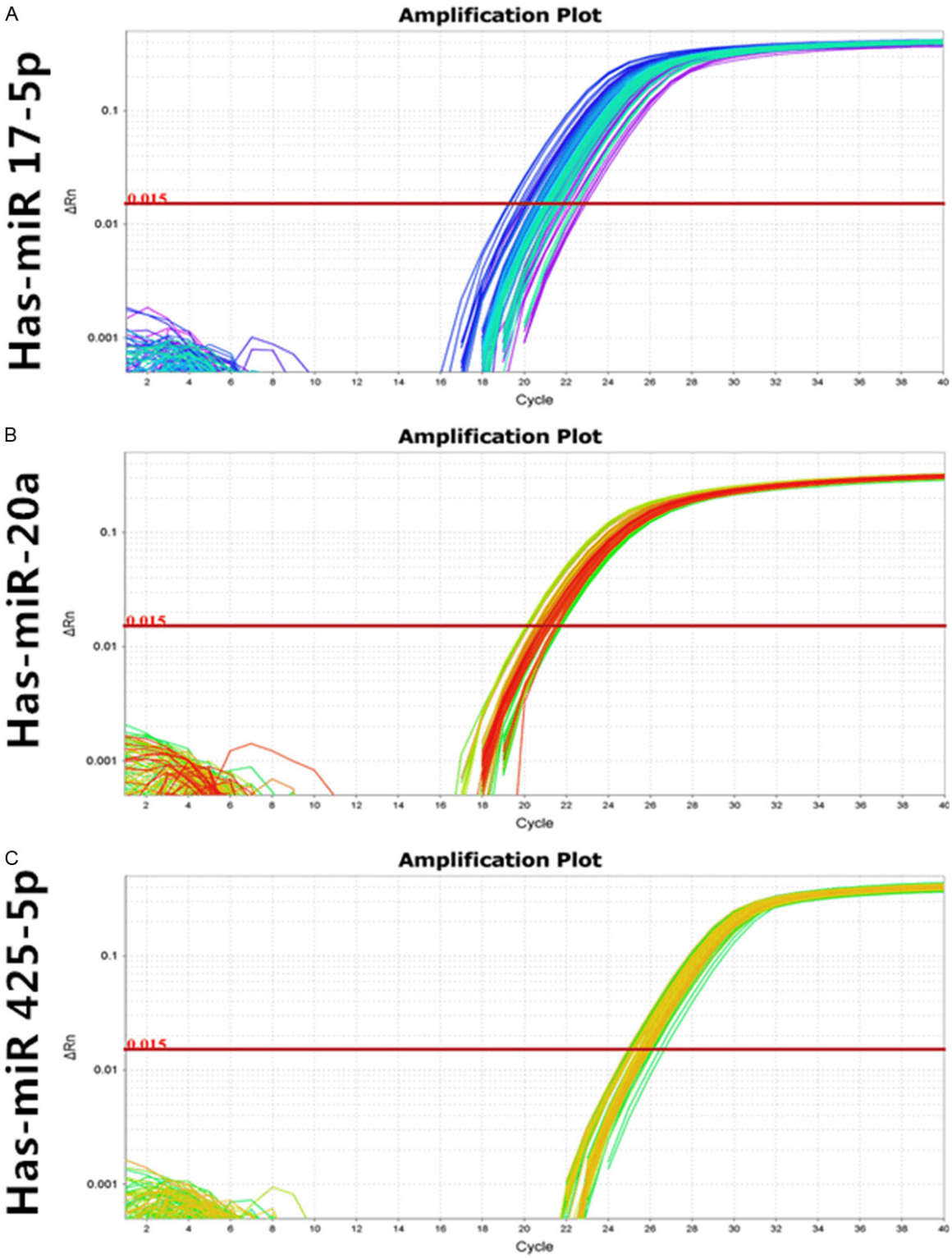

Figure 1. Quantile normalization, with background subtraction and elimination of extreme outlier values, was performed. qPCR was performed with Applied Biosystems (Carlsbad, CA); MicroRNA assays were run on an Applied Biosystems Step OnePlus machine.

sely correlated with the genesis and development of PAH [4, 8]. Research has suggested that the contents of miRNA-204 in PAH pulmonary tissues and PASMCs are reduced. In addi-

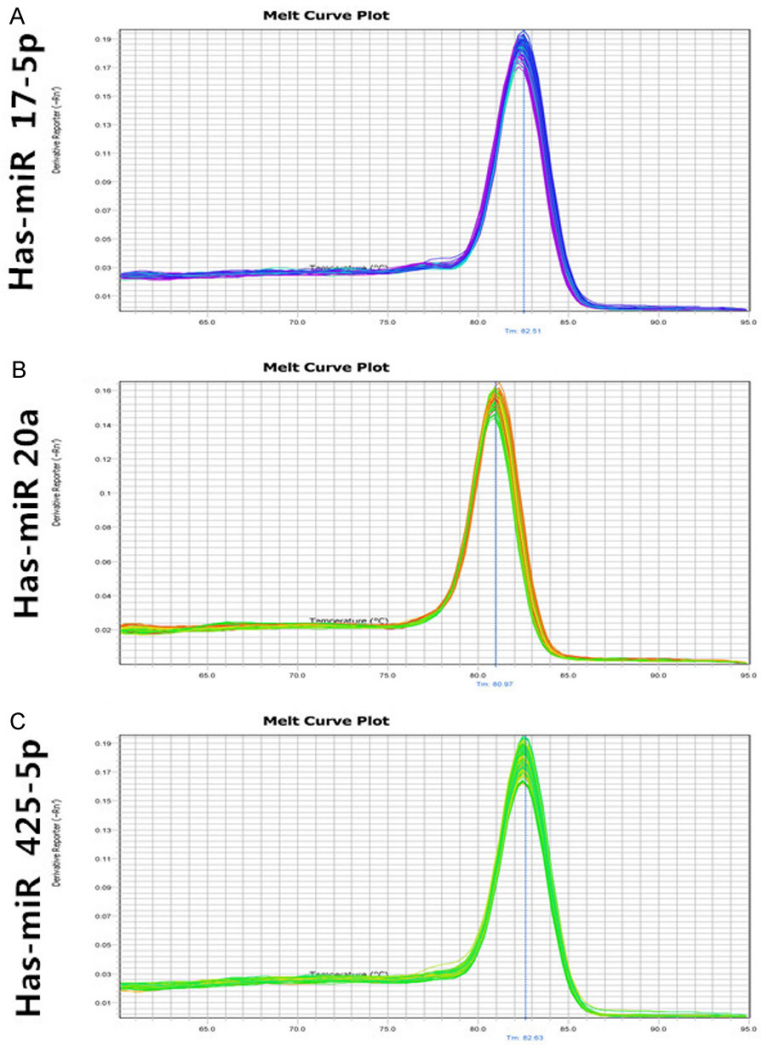

Figure 2. Quantile normalization, with background subtraction and elimination of extreme outlier values, was performed. qPCR was performed with Applied Biosystems (Carlsbad, CA). MicroRNA assays were run on an Applied Biosystems Step OnePlus machine.

tion, it was discovered in an animal model that miRNA-204 is related to cell proliferation. The miR-17/92 family is also expressed in PAH, which can downregulate (BMPR)-2 downstream region through IL-6 signal pathways [9, 10]. miR-21 can be induced by BMP-Smad signal pathways and has been reported to be downregulated in blood cells and pulmonary tissue samples of PAH patients [11, 12]. Correlation analysis of miRNAs with PAH suggests that miRNA changes and regulation may be involved in the pathophysiological process of PAH [13].

The current study probed into the relationship between miRNA changes in peripheral blood of PAH patients and PAH severity, as well as the relationship with patient survival rates, aiming to determine whether it could serve as a potential biological indicator. Current research has indicated that miRNA-17-5p and miRNA-20a are related to cancer, dilated cardiomyopathy, congenital heart disease, and strokes. However, reports concerning the relationship of miRNA-17-5p and miRNA-20a with PAH have been scarce. This research aimed to examine the relationship of miRNA-17-5p and miRNA-20a with susceptibility and prognosis of PAH, further illuminating the pathogenesis of PAH.

Methods

Subjects

Patients, from January 2010 to December 2013, were selected for the present PAH case control study. A total of 116 PAH patients were enrolled in the case group, all meeting the diagnostic criteria of PAH. Diagnostic criteria of PAH were formulated according to the latest guidelines [13]. Additionally, 100 healthy subjects were selected as the control group. Differences in sex and age between two groups were not statistically significant. All subjects provided informed consent. They were investigated by strictly-trained investigators. A uniform questionnaire was presented and quality control was randomly carried out.

Samples

A total of 5 mL fasting peripheral blood was collected from all objects of the study, with 1 mL sent to the laboratory for immediate determination of blood biochemical indexes using an automatic biochemical analyzer (Europa XL-200, Germany). These indexes included blood glucose, total cholesterol, and triglycerides. The remaining 4 mL was treated with hep-

Table 1. Clinical characteristics of study subjects

Condition	Control	COPD with PAH	Significance of differences, *P* value
Number	*60*	112	
M (F)	*32 (28)*	59 (53)	*P*=0.366
Age	*65.3*	65	*P*=0.378
Height (cm)	*168±4.7*	169±*4.9*	*P*=0.452
Weight (kg)	*67±7.1*	*68±6.7*	*P*=0.231
BMI (kg/m^2)	*23.1±2.1*	*23.6+1.9*	*P*=0.169
Pack year smoking	*10±5*	*15+6.2*	*P*=0.048
FEV1 (%, pred)	*80±5*	*60±7*	*P*=0.035
RV/TLC (%)	*25±5*	*35±6*	*P*=0.076
DL_{CO} (%, pred)	*20±3*	15±*5*	*P*=0.057
$PaCO_2$ (mmHg)	*30±3*	*41±5*	*P*=0.038
PaO_2 (mmHg)	*70±5*	*64±6*	*P*=0.059
6 MWD (m)	*600±30*	*390±100*	*P*=0.011
PAH therapies			
None			
Phosphodiesterase-5 inhibitor		*12*	
Endothelin receptor antagonist		*10*	
Prostacyclin analog		20	
RDW, %	*16.5±5.9*	15.2 (2.4)	*P*=0.324
NT-proBNP, pg/ml	*1003±23.1*	7089±*607*	*P*=0.057
MiR-17-5p	*0.000432*	0.000702	*P*=0.021
MiR-20a	*0.000498*	0.000786	*P*=0.031
Pulmonary arterial pressure	*20±5*	45±*25*	*P*=0.025

Definition of abbreviation: 6 MVD=6-minute walk distance; PAH=pulmonary arterial hypertension; RDW=red blood cell distribution width. Smoke, hypertension history, diabetes history, miR-17-5p, miR-20a, and CHD family history in the case group were significantly higher than those in the control. Difference was significant *(P*<0.05).

arin anti-coagulation and stored in a refrigerator at -80°C. A total of 106 PAH patients were followed up, for up to 24 months, via telephone interviews. Survival conditions of patients were studied on December 20th, 2015, with death being the primary endpoint. Six patients lost to follow-up. Major reasons were incorrect telephone numbers, refusal to take part continuously, and changes in the original address or unclear address. Differences in basic demographic information (age, sex, smoking, and drinking) between patients lost to follow-up and the research group showed no statistical significance. This research was approved by Institutional Review Board and was carried out in accordance with the Declaration of Helsinki. All subjects provided written informed consent.

miRNA analysis

Tiangen kit was adopted for extraction. Whole blood genome DNA was extracted first. Total RNA was then isolated from plasma, circulatory blood cells, and pulmonary tissues. PCR, as well as end plate reading and analysis, was carried out using high-throughput real-time fluorescent quantitative PCR detection system 7900HT. This system was compatible with 96-well, 384-well, and Taqman low density expression profile microarrays. A "genome MEPA" chip was adopted for miRNA detection (FEBIT Biomedical Co., Ltd, Heidelberg, Germany). Nonhousekeeping gene miRNAs were normalized and verified. Next, 5 nmol/L caenorhabditis elegans miR39 (cel-mir-39) was added int the samples, recognized to be a marker for normalized measurement of RNA reagents. To maximally detect miRNA information, microarrays were adopted to detect total RNA in plasma sample of subjects, while miRNA contents were measured by real-time quantitative PCR (qPCR). Quartile method, background subtraction, and elimination of extreme outliers were adopted for quantity statistics. Fluorescent quantitative PCR detection was carried out using a biological system (Carlsbad, CA) and microRNA detection was implemented adopting the Applied Biosystem analyzer (**Figures 1**, **2**).

Statistical analysis

Chi-squared test and t-test were adopted for analysis of general information between the two groups. MicroRNAs with maximum differences between the case group and control group were considered to have research value through screening analysis. Moreover, 95%

Table 2. Demographics and miR-17-5p correlation

Clinical variable	miR 17-5 p<0.000689	miR 17-5 p>0.000689	Correlations (Rho, *P* value)
M (F)	*35 (25)*	28 (24)	P=0.162
Age	*63.5*	66	0.167, P=0.098
Height (cm)	*169±5.1*	168±*4.8*	-0.035, P=0.121
Weight (kg)	*68±6.1*	*67±6.5*	-0.028, P=0.244
BMI (kg/m^2)	*23.3±2.3*	*23.4+1.9*	0.098, P=0.105
Pack year smoking	*14±6*	*16+3*	0.112, P=0.096
FEV1 (%, pred)	*64±5*	*63±6*	-0.067, P=0.152
RV/TLC (%)	*33±5*	*36±5*	0.136, P=0.102
DL_{CO} (%, pred)	*20±3*	15±*5*	-0.034, P=0.078
$PaCO_2$ (mmHg)	*38±5*	*41±6*	0.097, P=0.101
PaO_2 (mmHg)	*64±5*	*61±4*	-0.197, P=0.089
6 MWD (m)	*460±30*	*330±50*	-0.201, P=0.032
PAH therapies			
None	*35*	*34*	-0.012, P=0.198
Phosphodiesterase-5 inhibitor	*7*	*6*	-0.098, P=0.165
Endothelin receptor antagonist	*6*	*4*	-0.096, P=0.112
Prostacyclin analog	*12*	8	-0.108, P=0.072
RDW, %	*15.4±5.7*	15.1±3.6	-0.032, P=0.132
NT-proBNP, pg/ml	*6008±303*	7345±*400*	0.201, P=0.103
Pulmonary artery pressure	*30±8*	*45±9*	0.102, P=0.035

Definition of abbreviation: 6 MVD=6-minute walk distance; PAH=pulmonary arterial hypertension; RDW=red blood cell distribution width. Demographics and miR-17-5p Correlations in cohort. MicroRNA-17-5p there was no correlation between plasma levels and WHO class, baseline hemodynamic measures, treatment, N-terminal pro-brain natriuretic peptide (NT-proBNP), and red cell distribution width (RDW). miRNA-17-5p and miRNA-20a levels correlated weakly with 6 MWD, age, and disease duration.

confidence intervals (CI) were calculated, adopting multivariate logistic regression analysis. OR values represent relative risks. Differences in follow-up durations mainly manifested as date of major cardiovascular event, final date of follow-up, and date of diagnosis. Lost cases were excluded from examined data. ROC curves were adopted to evaluate the relationship between the decrease in all-cause mortality and biomarkers, as well as the relationship between biomarkers and prognosis. Kaplan Meier method was used for survival analysis. In addition, correlation analysis of miRNAs with patient mortality was carried out. Multivariate Cox regression analysis was utilized to calculate hazard ratios (HR) and 95% CI. All statistical tests were double-probability tests. Differences with P<0.05 are statistically significant. All data were processed using SPSS 19.0.

Results

Clinical characteristics of study subjects

As seen in **Table 1**, differences in age and sex between the case group and control group showed no statistical significance (P>0.05). Differences in systolic pressure, diastolic pressure, and body mass index (BMI) between two groups were not statistically significant (P> 0.05). Smoking rate in the case group was remarkably higher than that in control group, with differences showing statistical significance (P<0.01). Differences in capacity for alcohol between the two groups were not statistically significant (P> 0.05). Furthermore, hypertension history, diabetes history, and family history of coronary heart disease in the case group were all markedly higher than those in the control group, with differences showing statistical significance (P< 0.01). Hypertension history in the case group was notably higher than that in control group, but differences in systolic pressure and diastolic pressure between the two groups were not significant. This might be related to long-term use of antihypertensive drugs.

miRNA-17-5p and miRNA 20a correlation with PAH

Differences in plasma distributions of miRNA-17-5p and miRNA-20a were not statistically significant. Hemodynamic parameters, therapeutic methods, N-terminal pro-brain natriuretic peptide (NT-proBNP), and red blood cell distribution width (RDW) were all research contents (**Table 2**). Correlation of miRNA-17-5p and miRNA-20a levels with 6 MWD, age, and course

Table 3. Demographics and miR-20a correlation

Clinical variable	miR 20a<0.000732	miR 20a>0.000732	Correlations (Rho, *P* value)
M (F)	*30 (28)*	29 (25)	P=0.153
Age	*65.2*	64.4	-0.167, P=0.098
Height (cm)	*168±5.3*	169±4.6	0.046, P=0.108
Weight (kg)	*68±5.9*	*67±6.1*	-0.028, P=0.202
BMI (kg/m^2)	*22.1±2.8*	*23.4+2.1*	0.098, P=0.098
Pack year smoking	*15±3*	*16+5*	0.115, P=0.088
FEV1 (%, pred)	*65±6*	*64±5*	-0.067, P=0.148
RV/TLC (%)	*34±5*	*35±6*	0.136, P=0.094
DL_{CO} (%, pred)	*20±4*	16±4	-0.104, P=0.078
$PaCO_2$ (mmHg)	*39±5*	*42±4*	0.097, P=0.101
PaO_2 (mmHg)	*63±6*	*62±5*	-0.197, P=0.089
6 MWD (m)	*470±30*	*342±52*	-0.201, P=0.029
PAH therapies			
None	*35*	*34*	-0.012, P=0.198
Phosphodiesterase-5 inhibitor	*8*	*6*	-0.088, P=0.125
Endothelin receptor antagonist	*7*	*5*	-0.096, P=0.102
Prostacyclin analog	*10*	*7*	-0.095, P=0.069
RDW, %	*15.3±5.8*	15.1±4.2	-0.032, P=0.132
NT-proBNP, pg/ml	*6032±357*	7323±398	-0.193, P=0.093
Pulmonary arterial pressure	*33±10*	*48±9*	0.122, P=0.038

Definition of abbreviation: 6 MVD=6-minute walk distance; PAH=pulmonary arterial hypertension; RDW=red blood cell distribution width. Demographics and miR-20a Correlations in cohort. MicroRNA-17-5p there was no correlation between plasma levels and WHO class, baseline hemodynamic measures, treatment, N-terminal pro-brain natriuretic peptide (NT-proBNP), and red cell distribution width (RDW). miRNA-17-5p and miRNA-20a levels correlated weakly with 6 MWD, age, and disease duration.

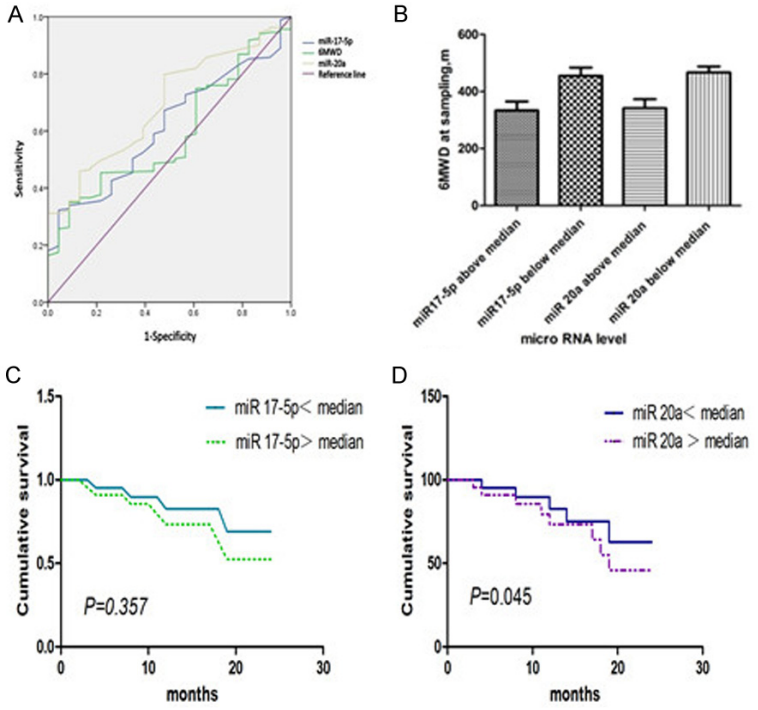

Figure 3. A. The best-performing cutoffs derived from ROC analysis were also assessed, MicroRNA-17-5p more than 0.000689 performed strongly (76.5% specificity, 76.8% specificity) in predicting 2-year survival, and identified patients with higher mortality in both the total PAH population. B. The distances of 6MVD were positively correlated with the content of microRNAs. C, D. Patients with high MicroRNA-17-5p and microRNA-20a levels (below the group median) had lower 6-minute walk distances and significantly poorer survival over time.

of disease (**Tables 2** and **3**) was poor.

miRNA-17-5p, miRNA-20a, and survival

According to the ROC curve, miRNA-17-5p and miRNA-20a levels were correlated with 2-year survival rates of PAH patients and 6 mwd. They were independent predictors of PAH survival rates (**Figure 3A**). miRNA-17-5p and miRNA-20a levels in patients were increased,

Table 4. Cox regression survival analysis

Variable	Single-Variable Model		Multivariable Model	
	Hazard Ratio (95% CI)	Sig	Hazard Ratio (95% CI)	Sig
Age, 10 yr	0.786 (0.567-1.232)	0.078		
Sex	0.631 (0.478-1.123)	0.106		
Mean pulmonary artery pressure	0.963 (0.932-1.098)	0.093		
In (Cardiac index), L/min/m^2	0.342 (0.089-0.821)	0.023		
6 MWD, 100 m	0.567 (0.135-0.912)	0.012	0.578 (0.345-0.876)	0.042
Therapies	-	0.452		
Treated vs. untreated	1.321 (0.765-1.564)	0.421		
Disease duration, yr	1.232 (1.023-1.508)	<0.001		
RDW, %	1.308 (1.102-1.587)	0.032	1.328 (1.234-1.463)	0.022
In (miR-17-5p)	0.563 (0.231-0.876)	0.021	0.589 (0.365-0.821)	0.012
In (miR-20a)	0.469 (0.198-0.856)	<0.001	0.478 (0.301-0.767)	<0.001

Definition of abbreviation: 6 MVD=6-minute walk distance; RDW=red blood cell distribution width. miRNA-17-5p and miRNA-20a levels as an independent predictor of survival (Hazard ratio, 0.589, P=0.012; Hazard ratio, 0.478, P<0.01).

while 6 mwd was shortened, indicating a close relationship to shortened survival times (**Figure 3C** and **3D**).

The cutoff value was also evaluated by ROC analysis. A miRNA-17-5p content of greater than 0.000689 was an important indicator for predicting 2-year survival rates (with a specificity and sensitivity of 76.5% and 76.8%, respectively). Mortality in the PAH group was also higher (**Figure 3A**). A miRNA-20a content of greater than 0.000732 could be used to predict 2-year survival rates of PAH patients, with a specificity and sensitivity of 91.3% and 58.8%, respectively. It could be regarded as a high-risk subgroup (**Figure 3A**). It was determined through Cox regression survival analysis that miRNA-17-5p and miRNA-20a could be treated as independent predictors of survival rates of PAH patients. Age, EF value, WHO functional classification, 6 MWD, course of disease, and RDW could also predict survival rates (**Table 4**). RDW and 6 MWD could independently predict survival rates of PAH, while in a multi-factor model incorporating microrna-17-5p and miRNA-20a levels, microrna-17-5p and miRNA-20a levels were independent predictive factors of survival rates of PAH patients (hazard ratio 0.589, P=0.012; hazard ratio 0.478, P<0.01). Bivariate analysis indicated that miRNA-17-5p and miRNA-20a were independent predictors, after adjusting for age, EF value, time of disease onset, and NT-proBNP (**Table 4**).

Discussion

MicroRNAs are a type of highly conserved non-coding small RNA single strand molecules, with lengths of about 21 to 24 nucleotides. Complementary pairing with its target mRNA contributes to identifying its target mRNA, regulating expression of target genes through degrading mRNA and inhibiting translation of its protein. Therefore, miRNAs are involved in vital processes, such as cell growth, development, differentiation, and proliferation. They target multiple protein coding genes, participate in regulating numerous cell growth and development stages, and play an important role in maintaining pulmonary vascular hemostasis. Abnormal miRNA expression may be the pathogenesis of a variety of diseases, including PAH.

BMPR2 signal pathways play a crucial role in maintaining pulmonary vascular hemostasis. Interference of such signal pathways and BMPR2 gene mutations may lead to PAH [11, 14]. Though BMPR2 dysfunction is considered a clinical characteristic of PAH, few studies have evaluated the mechanisms associated with BMPR2 dysfunction. No studies examining whether miRNAs participate in the course of BMPR2 and PAH are currently available. Studies concerning miRNA-BMPR2 signal pathways have suggested that IL-6 mediates STAT3 activation and induces expression of cell miR-17-92 gene family [15]. Coding miR-17-5p and miR-20a through corresponding miRNA gene families can specifically downregulate expression of BMPR2 proteins [16]. Consequently, there are two research groups evaluating the potential of these two miRNA-inhibitors (miR-17 and miR-20a) in treating PAH. In a rodent model, intravenous injections of miR-17 inhibitor have been shown to be a means of improving experimen-

tal PAH. Furthermore, content of cyclin-dependent kinase inhibitor 1A (P21), a known target gene of miR-17, is increased in mouse and rat pulmonary tissues after treatment with miR-17 inhibitors. Moreover, it shows an increasing trend in human PASMCs. Research has also indicated that treatment with miR-20a inhibitors can improve right ventricular hypertrophy and pulmonary vascular remodeling in PAH model mice. Additionally, it can induce BMPR2 expression through downstream signal pathways of BMPR2 in pulmonary tissues and PASMCs [7].

Hypoxia and exposure to chronic hypoxia environments are causes of PAH, while PASMC proliferation and vascular remodeling lead to PAH formation. Chronic hypoxia is an important reason responsible for PAH. An effective animal model of hypoxia-induced pulmonary vascular remodeling is available [17]. Studies have discovered that BMPR2 protein, in the case of hypoxia (but not under hypoxia downregulated by BMPR2 gene), may reveal the involvement of miRNAs [18].

Research on differences in plasma miRNA of PAH suggests that increases in microrna-17-5p and miRNA-20a levels are correlated with survival rates of PAH patients. Furthermore, it is an independent predictor, apart from 6 MWD, WHO functional classification, and other clinical indicators.

The following conclusions can be drawn from the current study. Plasma miRNA-17-5p and miRNA-20a levels are related to survival rates of PAH patients. The significance of miRNA-17-5p and miRNA-20a should be further investigated. More correlation studies are needed to investigate the relationship of other miRNAs with PAH. Research concerning the regulatory relationship between miRNAs and PAH formation has unveiled a brand-new view for investigators. The current study has established a foundation for the effects of miRNAs on ion channels in PAECs and PASMCs. MicroRNAs have the characteristics of a simple molecular structure, small molecular weight, and ease of synthesis and modification. They will certainly become the new generation drug for treatment of PAH.

Disclosure of conflict of interest

None.

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