Combination of peripheral blood mononuclear cell miR-19b-5p, miR-221, miR-25-5p, and hypertension correlates with an increased heart failure risk in coronary heart disease patients

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Abstract

Objective: The aim of this study was to explore the differences in microRNA (miRNA) profiles in peripheral blood mononuclear cells (PBMCs) between coronary heart disease (CHD) patients with and without heart failure (HF) and to assess the values of differentially expressed miRNAs (DEMs) regarding HF risk in CHD patients.

Methods: Six CHD patients with HF and six age- and gender-matched CHD patients without HF were enrolled in the exploration stage, and 44 CHD patients with HF and 42 age- and gender-matched CHD patients without HF were recruited in the validation stage. Peripheral blood samples were collected from all the participants, and PBMCs were separated for miRNA detection. miRNA microarray and quantitative polymerase chain reaction were performed to assess the miRNA expression.

Results: In the exploration stage, heat map analysis showed that CHD patients with HF could be distinguished from those without HF using PMBC miRNA expressions; 63 downregulated DEMs and 84 upregulated DEMs in PBMCs were identified in CHD patients with HF using volcano map, and top 8 DEMs were selected based on their p values. In the validation stage, PBMC miR-221, miR-19b-5p, and miR-25-5p were found to be markedly dysregulated in CHD patients with HF. Multiple logistic regression analysis showed PBMC miR-221, miR-19b-5p, miR-25-5p, and hypertension to be the independent predictive factors for HF in CHD patients. A receiver operating characteristic curve demonstrated that area under curve of the combination of miR-221, miR-19b-5p, miR-25-5p, and hypertension was 0.871 (95% CI: 0.794-0.944).

Conclusion: CHD patients with and without HF could be differentiated according to PBMC miRNA profiles, and the combination of PBMC miR-19b-5p, miR-221, miR-25-5p, and hypertension correlates with an increased HF risk in CHD patients. *(Anatol J Cardiol 2018; 20: 100-9)*

Keywords: miRNA, profile, risk, heart failure, coronary heart disease

Introduction

The prevalence of coronary heart disease (CHD), a major cause of mortality worldwide, has been increasing in developed countries due to aging of the population, physical inactivity, and unhealthy diet habits (1, 2). Heart failure (HF), affecting more than 2% individuals around the world, is a grave outcome in CHD patients (3). Moreover, high prevalence of HF as well as high hospital admission and mortality rates associated with HF has made it a compelling problem in the management of CHD patients (4). CHD patients with HF can exhibit several typical symptoms such as breathlessness, orthopnea, paroxysmal nocturnal dyspnea, and fatigue, whereas a considerable proportion of HF patients are misdiagnosed as having exacerbation of chronic obstructive pulmonary disease, atypical pneumonia, or other diseases with alike symptoms, which has led to diagnosis-related problems in clinical practice (5). Thus, there is a dire need of biomarkers for predicting HF risk in CHD patients.

microRNA (miRNA) is a class of short, single-stranded, non-coding RNAs that regulate gene expression through either translational repression or mRNA degradation at the post-

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transcriptional level (6). In human diseases, the pathological role of miRNA has been well established by extensive studies, which have shown that miRNA mediates pathogenic processes through regulating cellular activities such as cellular proliferation, differentiation, and migration (7). Aberrantly expressed miRNAs have been identified by increasing number of studies to be of high potential in the diagnosis and prognosis of CHD patients (8, 9). Meanwhile, growing number of studies focusing on the role of miRNA in HF have reported that circulating miRNAs participate in the etiology of HF and are the potential biomarkers in HF patients (10, 11). For instance, plasma miR-132 expression is independently associated with disease severity and hospitalization rate of HF patients (12). Furthermore, miRNAs have been reported to be related with the pathogenesis of HF; for example, circulating miR-30d could regulate cardiomyocyte apoptosis in HF patients (13). However, the value of miRNAs for predicting HF risk in CHD patients still needs to be further investigated.

Thus, our study aimed to explore the difference in PBMC miRNA profiles between CHD patients with and without HF and to assess the values of differentially expressed miRNAs (DEMs) regarding HF risk in CHD patients.

Methods

Study design

This study comprised an exploration stage and a validation stage (Fig. 1). In the exploration stage, six CHD patients with HF and six age- and gender-matched CHD patients without HF were enrolled from Renmin Hospital of Wuhan University, 4 mL peripheral blood was collected from 12 patients, and peripheral blood mononuclear cells (PBMCs) were subsequently isolated for miRNA microarray detection. In the validation stage, 44 CHD patients with HF and 42 age- and gender-matched CHD patients without HF were recruited. PBMC samples were collected from 86 patients for quantitative polymerase chain reaction (qPCR) assay of eight candidate DEMs selected from the microarray. This study was approved by the Ethics Committee of Renmin Hospital of Wuhan University, and all the participants signed the informed consent.

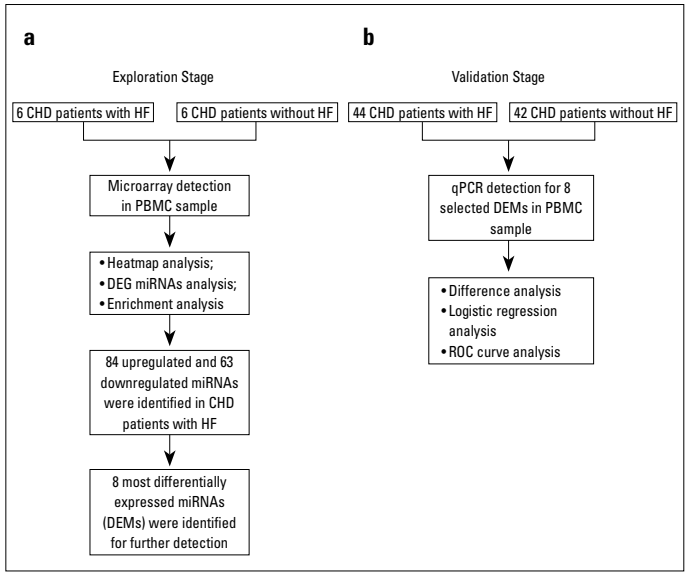

Figure 1. Study flow

RNA extraction

Blood samples were collected from all patients and stored in ethylenediaminetetraacetic acid (EDTA)-3K tubes. Subsequently, serum was extracted after centrifugation at the speed of 3000 rpm for 15 min, and the Ficoll solution was added to the remaining part of the blood sample for PBMC extraction. Subsequently, the samples were centrifuged at the speed of 2000 rpm for 20 min, and PBMC was then well prepared for RNA extraction. Total RNA was extracted from PBMC of each patient using the Trizol kit (Invitrogen, Carlsbad, CA, USA), and the quantity as well as the quality of total RNA was evaluated using a spectrophotometer, which was followed by reverse transcription and amplification of total RNA through reverse transcription PCR.

Microarray detection

miRNA microarray was performed following manufacturer's protocol (LC Sciences, USA); 500 ng of total RNA was extracted from 1.0–1.5 mL blood sample and was used for microarray detection, which was marked by biotin-labeled DNA molecule and hybridized, and then washed on the GeneChip Fluidics Station 450 platform.

Data preprocessing of microarray

The signal intensity of each chip varies from one another due to different essential backgrounds of chips; thus, for eliminating the calculation error of miRNA expression, raw data was normalized using Robust Multichip Average (RMA), which is an algorithm used for creating an expression matrix from Affymetrix data. To be exact, the raw values of signal intensity were background corrected, log2 transformed, and subsequently quantile normalized using RMA method. Thereafter, the normalized data was calculated using a linear model.

DEMs screening in microarray

In the microarray assay, DEMs were compared using R package limma. Benjamini and Hochberg procedure was performed to adjust the p values, and clinical significance was defined as a difference of 2.0 folds {absolute [log2 (fold change)]>1.0}; volcano map and heat map analysis were used to distinguish information between the two groups (version 1.0.2, available at http://cran.r-project.org/web/packages/pheatmap/index.html).

Enrichment analysis

To assess the similarity in DEMs regarding their correlations with pathological processes and pathways of HF, the annotation of DEMs was performed using miRNA enrichment analysis and annotation (miEAA) database, including Kyoto Encyclopedia of

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Supplementary Table S1. Primer sequences in quantitative polymerase chain reaction

miRNA	Forward primer	Reverse primer
miR-222	5'-ACACTCCAGCTGGGAGCTACATCTGGCTACTG-3'	5'-TGTCGTGGAGTCGGCAATTC-3'
miR-221	5'-ACACTCCAGCTGGGAGCTACATTGTCTGCTGG-3'	5'-TGTCGTGGAGTCGGCAATTC-3'
miR-455-3p	5'-ACACTCCAGCTGGGGCAGTCCATGGGCATATA-3'	5'-TGTCGTGGAGTCGGCAATTC-3'
miR-25-5p	5'-ACACTCCAGCTGGGAGGCGGAGACTTGGGCAA-3'	5'-TGTCGTGGAGTCGGCAATTC-3'
miR-133a	5'-ACACTCCAGCTGGGAGCTGGTAAAATGGAACC-3'	5'-TGTCGTGGAGTCGGCAATTC-3'
miR-19b-5p	5'-ACACTCCAGCTGGGAGTTTTGCAGGTTTGCAT-3'	5'-TGTCGTGGAGTCGGCAATTC-3'
miR-320c	5'-ACACTCCAGCTGGGAAAAGCTGGGTTGAGAGG-3'	5'-TGTCGTGGAGTCGGCAATTC-3'
miR-532-3p	5'-ACACTCCAGCTGGGCCTCCCACACCCAAGGC-3'	5'-TGTCGTGGAGTCGGCAATTC-3'

Genes and Genomes (KEGG) pathway database and Gene Ontology (GO). Fisher's exact test was used to differentiate over-represented miRNA-related items for the enrichment analysis of DEMs and their precursors.

qPCR determination

qPCR was performed to assess the relative expression of the top eight DEMs that were identified in the exploration stage. Total RNA was reverse transcript into cDNAs using Transcript First-strand cDNA synthesis superMix (TransGen Biotech, Beijing, China). Following that, SYBR Premix Ex Taq kit (Takara, Dalian, China) was used for the detection of DEMs. U6 was used as an internal reference, and then, the relative expression of eight DEMs was calculated using $2^{-\triangle\triangle t}$ method. The primer sequences have been listed in Supplementary Table S1.

Statistical analysis

Statistical analyses were performed using SPSS 22.0 (IBM, USA), R software (MathSoft, USA), and GraphPad Prism 6 (GraphPad Software, USA). Data was mainly described as mean±standard deviation, median, and (25^{th}–75^{th}) or count (percentage). Comparison of baseline characteristics was performed using t-test or χ^2 test. In the validation stage where qPCR was performed, comparison of candidate DEMs was performed using Wilcoxon rank sum test. Univariate logistic regression model was used to analyze the factors predicting HF risk in CHD patients, and factors with a p value <0.1 were further analyzed using multiple logistic regression model. Receiver operating characteristic (ROC) curve was performed to assess the value of candidate factors affecting HF risk in CHD patients. P value <0.05 was considered significant.

Results

Baseline characteristics of patients in the exploration stage

As listed in Table 1, the mean age of CHD patients without and with HF was 62.67±6.31 and 62.33±8.31 years, respectively (p=0.939). There were five males and one female among CHD pa-

Table 1. Characteristics of six coronary heart disease patients with heart failure and six coronary heart disease patients without heart failure in exploration stage

Parameter	CHD without HF (n=6)	CHD with HF (n=6)	*P* value
Age (years)	62.67±6.31	62.33±8.31	0.939
Gender (Male/Female)	5/1	6/0	0.296
BMI (kg/m^2)	25.11±2.68	25.19±5.78	0.977
Hypertension (n/%)	4 (67)	5 (83)	0.505
Diabetes (n/%)	0 (0)	2 (33)	0.121
Smoke (n/%)	1 (17)	1 (17)	1.000
TG (mmol/L)	1.57±0.45	1.71±1.09	0.765
TC (mmol/L)	3.46±0.91	4.25±0.80	0.141
HDL-C (mmol/L)	1.11±0.17	1.18±0.33	0.652
LDL-C (mmol/L)	2.64±0.86	2.85±0.93	0.693

Data was mainly presented as mean±standard deviation or count (percentages). Comparisons were made using t-test or χ^2 test. *P*<0.05 was considered significant.
BMI - body mass index; CHD - coronary heart disease; HDL-C - fasting high-density lipoprotein cholesterol; HF - heart failure; TG - triglyceride; TC - total cholesterol; LDL-C - fasting low-density lipoprotein cholesterol

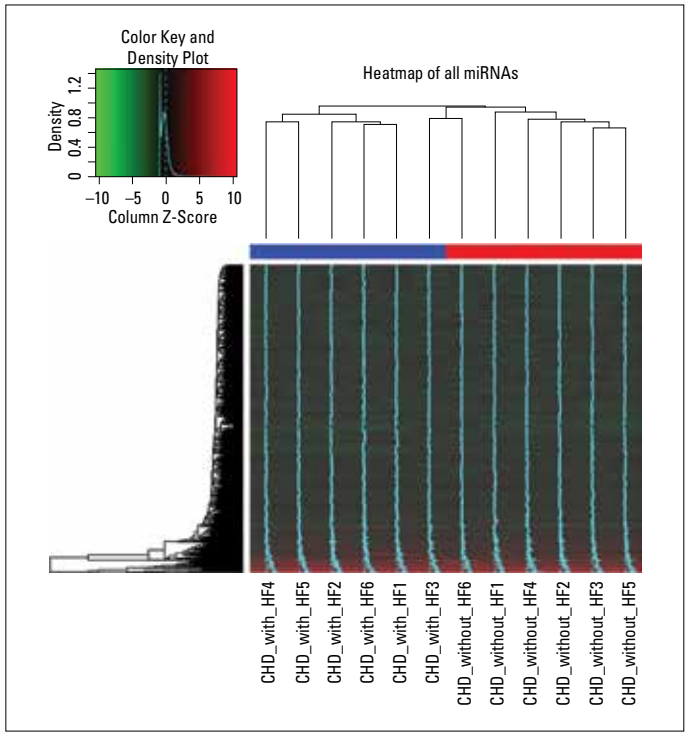

Figure 2. Heat map analysis of all miRNAs. The heatmap analysis of miRNAs expressions in CHD patients with and without HF

tients without HF, whereas all CHD patients with HF were males (p=0.296). In addition, the mean body mass index (BMI) was 25.11±2.68 kg/m^2 in CHD patients without HF and was 25.19±5.78 kg/m^2 in CHD patients with HF (p=0.977). Among CHD patients without HF, 4 (67%) had hypertension, and among CHD patients with HF, 5 (83%) had hypertension (p=0.505). Diabetes mellitus was not seen in CHD patients without HF, whereas there were 2 (33%) patients with diabetes mellitus among CHD patients with HF (p=0.121). The number of patients with smoking history was one (17%) each among CHD patients without and with HF (p=1.000).

DEM analysis

Heat map analysis was performed to evaluate the differences in PMBC miRNA aggregates, which showed that CHD patients without and with HF could be differentiated according to PBMC miRNA expressions (Fig. 2). Furthermore, as presented in Figure 3a, 63 downregulated miRNAs and 84 upregulated miRNAs in PBMCs were identified in CHD patients with HF. The upregulated and downregulated miRNAs were then analyzed using heat map analysis, which showed that CHD patients without and with HF could be differentiated according to upregulated and downregulated miRNA expressions (Fig. 3b).

Enrichment analysis

As presented in Figure 4, the enrichment analysis of PBMC DEMs comprised two areas including KEGG pathway and GO. The analysis in KEGG pathway database showed that DEMs in PBMCs mainly correlated with the pathways related to heart development and inflammation mediated by chemokines and cytokines (Fig. 4a). Regarding the associations of DEMs with patho-

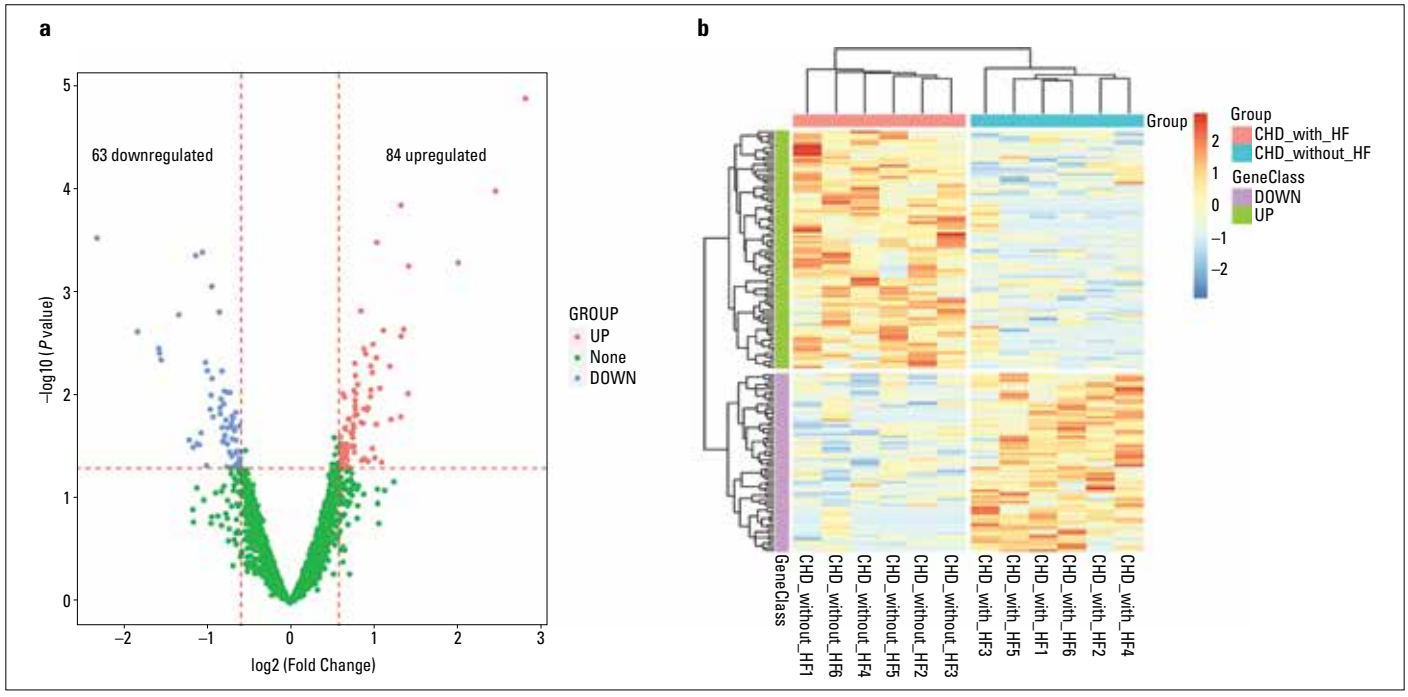

Figure 3. Differential analysis of DEMs. Volcano map was performed to evaluate the downregulated and upregulated miRNAs in CHD patients with HF compared with those in CHD patients without HF (a), and the expressions of upregulated and downregulated miRNAs in CHD patients with and without HF were assessed using heat map (b). DEMs were compared using R package limma, and Benjamini and Hochberg procedure was performed to adjust P values; clinical significance was defined as a difference of 2.0 folds {absolute [log2 (fold change)]>1.0}

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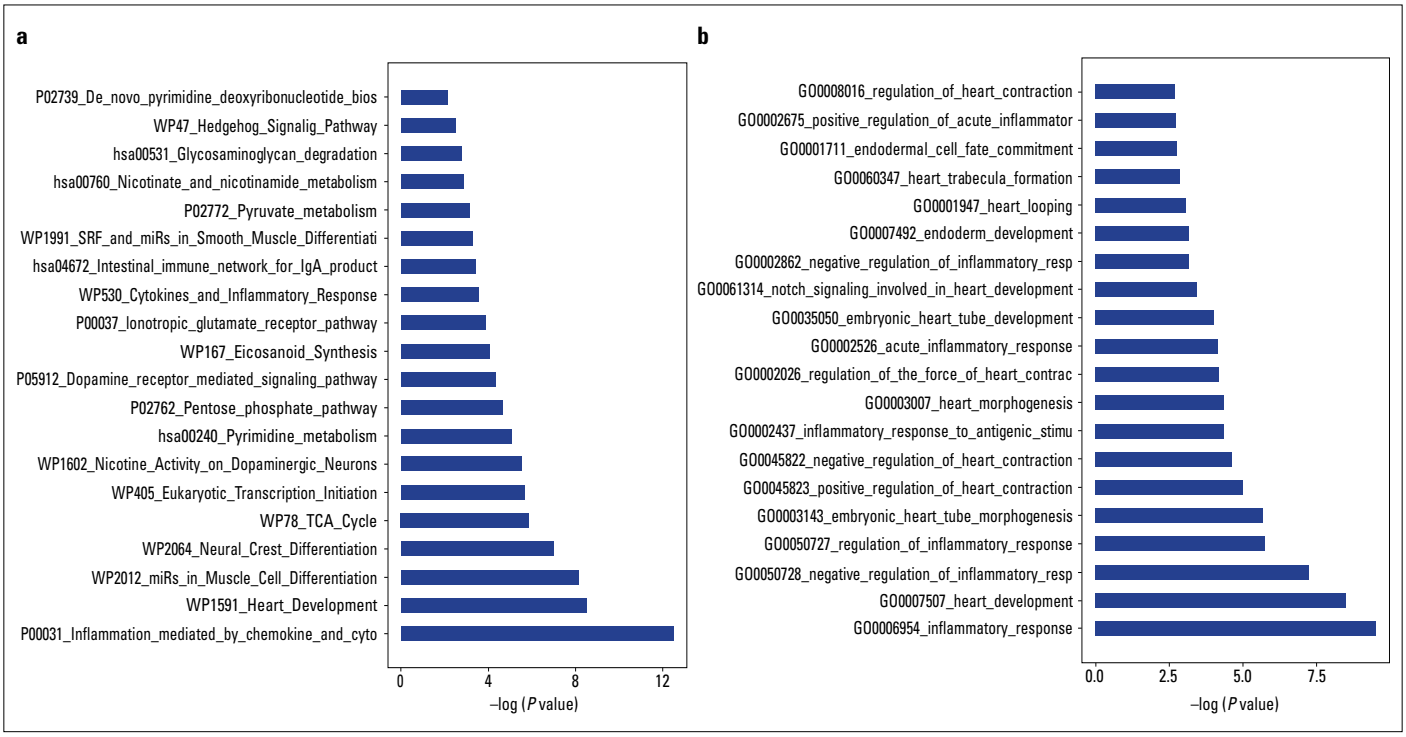

Figure 4. Enrichment analysis. Enrichment analysis was performed to evaluate the similarity in DEMs regarding their associations with the pathways (a) and pathological processes (b). Fisher's exact test was performed to differentiate overrepresented miRNA-related items for the enrichment analysis of DEMs and their precursors

Table 2. Top eight differentially expressed miRNAs in coronary heart disease patients with heart failure compared with those in coronary heart disease patients without heart failure in microarray

DEMs	LogFC	AveExpr	*P* value	Adjusted *P* value	Trend
miR-222	2.8221	1.9220	<0.001	<0.001	UP
miR-221	2.4604	1.5479	<0.001	0.003	UP
miR-455-3p	-2.3139	3.2409	<0.001	0.007	DOWN
miR-25-5p	2.0152	1.1624	<0.001	0.008	UP
miR-133a	-1.8275	2.2795	0.002	0.025	DOWN
miR-19b-5p	-1.5690	3.6314	0.004	0.031	DOWN
miR-320c	-1.5636	1.2685	0.004	0.032	DOWN
miR-532-3p	-1.5431	2.5045	0.005	0.035	DOWN

Eight DEMs were selected according to the absolute value of LogFC. Comparison was completed by R package limma.
AveExpr - average of expression level; CHD - coronary heart disease; DEMs - differentially expressed miRNAs; HF - heart failure; LogFC - $\log_2$ (fold change)

logical HF-related processes, the analysis performed in the GO database displayed that DEMs were predominantly correlated with heart development and inflammatory responses (Fig. 4b). These results indicate that DEMs might be primarily present in the cardiomyocytes in CHD patients.

Top eight DEMs in CHD patients with HF

Top eight DEMs in PBMCs were selected in CHD patients with HF according to their P values; they comprised three upregulated miRNAs (Table 2) (miR-222, miR-221, and miR-25-5p) and five downregulated miRNAs (miR-455-3p, miR-133a, miR-19b-5p, miR-320c, and miR-532-3p).

Patient characteristics in the validation stage

In the validation stage, the mean age of CHD patients without HF was 59.88±8.89 years, and they included 37 males and 5 females; the mean age of CHD patients with HF was 61.98±9.34 years, and they included 39 males and 5 females (Table 3). There was no difference in terms of age (p=0.290) and gender ratio (p=0.938) between the groups, whereas the mean value of BMI

Table 3. Characteristics of 44 coronary heart disease patients with heart failure and 42 coronary heart disease patients without heart failure in validation stage

Parameter	CHD without HF (n=42)	CHD with HF (n=44)	*P* value
Age (years)	59.88±8.89	61.98±9.34	0.290
Gender (Male/Female)	37/5	39/5	0.938
BMI (kg/m^2)	24.58±3.52	26.42±4.35	0.035
Hypertension (n/%)	28 (67)	39 (89)	0.014
Diabetes (n/%)	7 (17)	10 (23)	0.481
Smoke (n/%)	16 (38)	19 (43)	0.631
TG (mmol/L)	1.79±0.72	1.94±0.88	0.396
TC (mmol/L)	4.14±1.13	4.15±1.23	0.986
HDL-C (mmol/L)	1.21±0.26	1.16±0.26	0.376
LDL-C (mmol/L)	2.51±0.64	2.91±1.11	0.041

Data was mainly presented as mean±standard deviation or count (percentages). Comparisons were made using t-test or χ^2 test. *P*<0.05 was considered significant.
BMI - body mass index; CHD - coronary heart disease; HDL-C - fasting high-density lipoprotein cholesterol; HF - heart failure; TG - triglyceride; TC - total cholesterol; LDL-C - fasting low-density lipoprotein cholesterol

Table 4. Univariate and multiple logistic analyses of factors for heart failure risk in coronary heart disease patients

	Univariate logistic regression				Multiple logistic regression			
	P value	OR	95% CI		*P* value	OR	95% CI	
			Lower	Higher			Lower	Higher
miR-222	0.104	1.354	0.940	1.950	-	-	-	-
miR-221	0.004	1.333	1.095	1.623	0.012	1.417	1.080	1.861
miR-455-3p	0.242	0.587	0.240	1.434	-	-	-	-
miR-25-5p	<0.001	2.281	1.472	3.536	0.001	2.157	1.353	3.438
miR-133a	0.209	0.685	0.380	1.235	-	-	-	-
miR-19b-5p	0.003	0.245	0.097	0.623	0.005	0.154	0.042	0.565
miR-320c	0.112	0.499	0.212	1.175	-	-	-	-
miR-532-3p	0.365	0.863	0.627	1.187	-	-	-	-
Age	0.288	1.026	0.978	1.076	-	-	-	-
Gender (Male)	0.938	1.054	0.282	3.941	-	-	-	-
BMI	0.040	1.129	1.005	1.267	0.072	1.157	0.987	1.356
Hypertension	0.018	3.900	1.259	12.081	0.016	6.354	1.406	28.713
Diabetes	0.482	1.471	0.502	4.309	-	-	-	-
Smoke	0.631	1.235	0.521	2.925	-	-	-	-
TG	0.392	1.263	0.741	2.153	-	-	-	-
TC	0.986	1.003	0.699	1.441	-	-	-	-
HDL-C	0.373	2.123	0.406	11.098	-	-	-	-
LDL-C	0.045	1.658	1.010	2.722	0.075	1.954	0.935	4.084

Univariate and Multiple logistic regression models were used to analyze the factors at baseline in predicting HF risk in CHD patients. *P*<0.05 was considered significant.
BMI - body mass index; CHD - coronary heart disease; HDL-C - fasting high-density lipoprotein cholesterol; HF - heart failure; OR - odds ratio; TG - triglyceride; TC - total cholesterol; LDL-C - fasting low-density lipoprotein cholesterol

in CHD patients with HF was higher than that in CHD patients without HF (26.42±4.35 kg/m^2 vs. 24.58±3.52 kg/m^2, p=0.035). Furthermore, the number of patients with hypertension was greater among CHD patients with HF than among those without HF [39 (89%) vs. 28 (67%), p=0.014]. Additionally, the mean fasting high-density lipoprotein cholesterol levels in CHD patients without and

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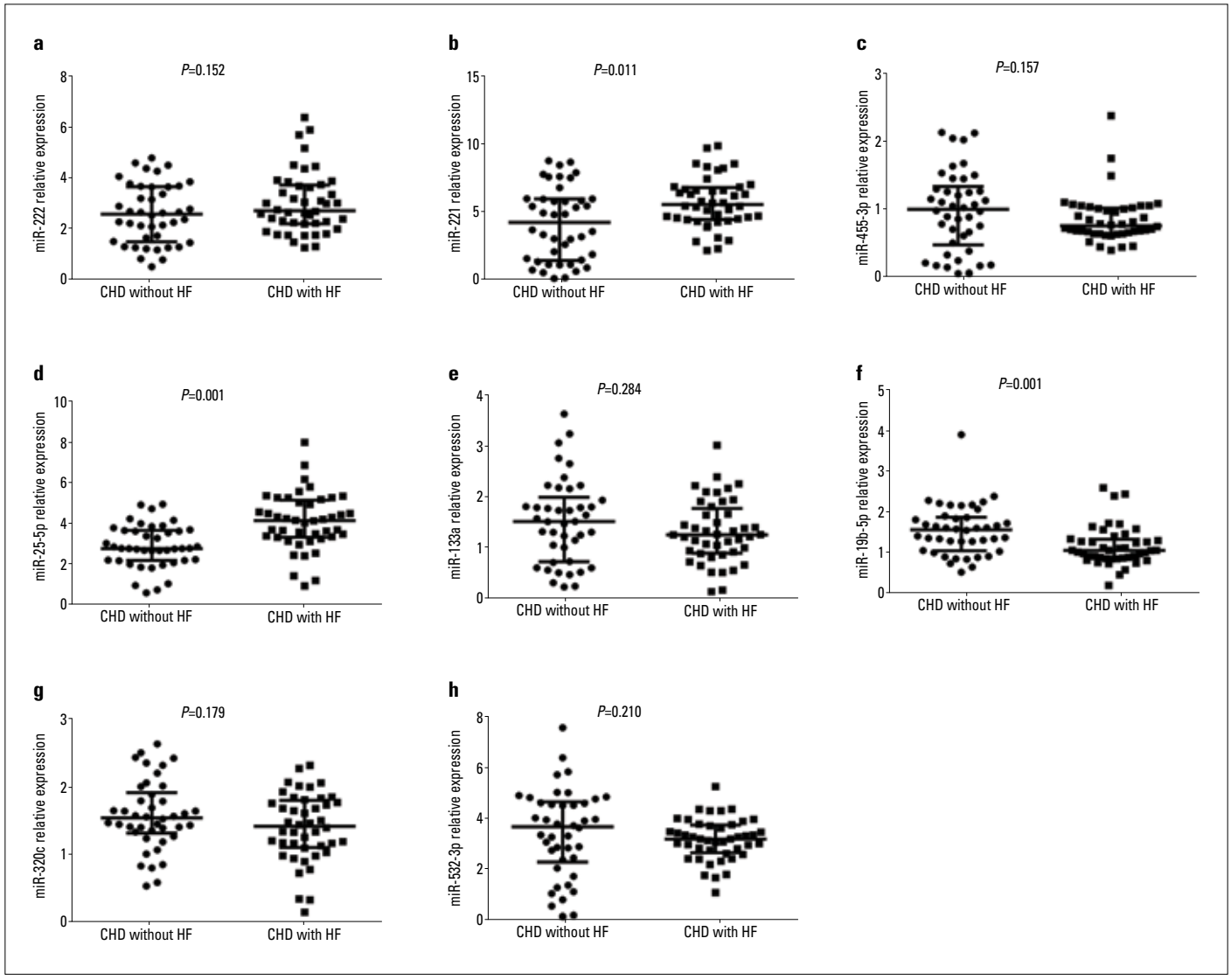

Figure 5. Relative expression of eight DEMs in the validation stage. The relative expressions of eight DEMs were evaluated using qPCR in the validation stage, which included the expressions of miR-222 (a), miR-221 (b), miR-445-3p (c), miR-25-5p (d), miR-133a (e), miR-19b-5p (f), miR-320c (g), and miR-532-3p (h). Comparisons between the two groups were made using Wilcoxon rank sum test. *P*<0.05 was considered significant

with HF were 1.16±0.26 mmol/L and 1.21±0.26 mmol/L, respectively (p=0.376); fasting low-density lipoprotein cholesterol (LDL-C) levels were increased in CHD patients with HF compared with those in CHD patients without HF (2.91±1.11 mmol/L vs 2.51±0.64 mmol/L, p=0.041). Other information regarding disease history and the values of laboratory indexes are listed in Table 3.

Difference analysis of eight DEMs in the validation stage

As shown in Figure 5, in the validation stage, PMBC miR-221 (p=0.011) and miR-25-5p (p<0.001) were strikingly upregulated in CHD patients with HF and miR-19b-5p (p=0.001) was downregulated in CHD patients with HF. No difference regarding the expressions of PBMC miR-222 (p=0.152), miR-455-3p (p=0.157), miR-133a (p=0.284), miR-320c (p=0.179), and miR-532-3p (p=0.210) was found between CHD patients with and without HF.

Analysis of factors predicting HF risk in CHD patients

In the univariate logistic regression model, increased expressions of PBMC miR-221 (p=0.004) and miR-25-5p (p<0.001) were correlated with higher HF risk, whereas upregulated PBMC miR-19b-5p (p=0.003) was associated with a lower HF risk in CHD patients (Table 4). Regarding predictive value of baseline characteristics, CHD patients with hypertension (p=0.018), an increased BMI value (p=0.040), and increased LDL-C levels (p=0.045) were at an increased risk for developing HF. All factors with a p value <0.1 were included in the multiple logistic regression analysis (Table 4), which showed that higher PBMC expressions of miR-221 (p=0.012) and miR-25-5p independently correlated with an increased HF risk, whereas higher PBMC miR-19b-5p (p=0.005) expression levels were independently associated with a decreased HF risk in CHD patients. Furthermore, hypertension (p=0.016) was an independent predictive factor for HF risk in CHD patients.

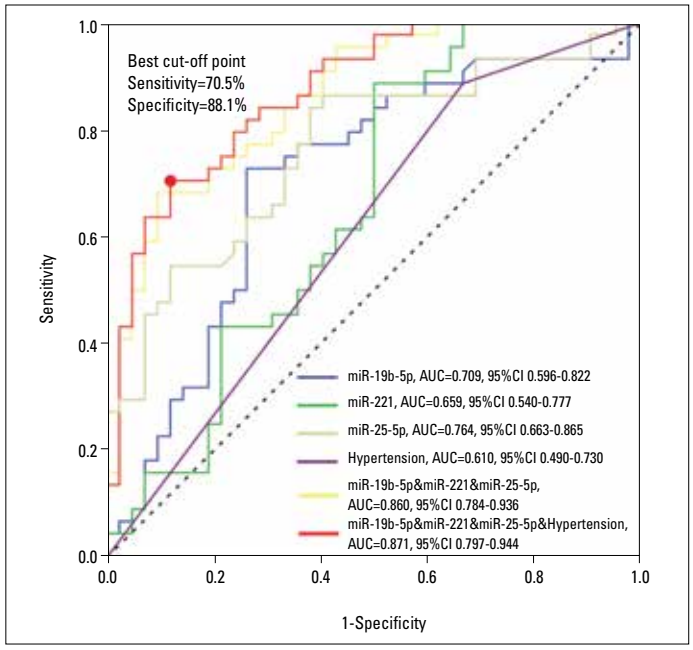

Figure 6. ROC curve analysis of three DEMs and hypertension. ROC curve analysis was performed to evaluate the predictive value of miR-19b-5p; miR-221; miR-25-5p; hypertension; a combination of miR-221, miR-19b-5p, and miR-25-5p; and a combination of miR-221, miR-19b-5p, miR-25-5p, and hypertension for HF risk in CHD patients

ROC curve analysis

ROC curve analyses were performed to assess the diagnostic value of the independent predictive factors for HF risk in CHD patients determined using multiple logistic regression models. As displayed in Figure 6, the areas under curve (AUCs) of PBMC miR-19b-5p, miR-221, and miR-25-5p were 0.709 (95% CI: 0.596–0.822), 0.659 (95% CI: 0.540–0.777), and 0.764 (95% CI: 0.663–0.865), respectively. In addition, AUC of hypertension was 0.610 (95% CI: 0.490–0.730). On combining PBMC expressions of miR-19b-5p, miR-221, and miR-25-5p, the ROC curve showed a high AUC of 0.860 (95% CI: 0.784–0.936), and on combining PBMC expressions of these three miRNAs with hypertension, AUC was as high as 0.871 (95% CI: 0.794–0.944), suggesting that a combination of these three miRNAs as well as a combination of these three miRNAs with hypertension possesses great value for predicting HF risk in CHD patients.

Discussion

The results of our study showed the following: (1) CHD patients with and without HF could be differentiated according to PBMC miRNA expressions; 63 downregulated miRNAs as well as 84 upregulated DEMs in PBMC were identified in CHD patients with HF. Further enrichment analysis revealed that the miRNAs were mainly correlated with pathways related to heart development and inflammation mediated by chemokines and cytokines in PBMC. And the miRNAs were also correlated with heart development as well as inflammatory responses; (2) Top eight DEMs in PBMC were selected in CHD patients with HF, among which miR-221, miR-25-5p, and miR-19b-5p were independent predictive factors for HF risk in CHD patients, and their combination had a good predicting value for HF risk in CHD patients. These results indicate that miRNAs probably participate in the pathogenesis of HF in CHD patients via regulating heart development and inflammation in PBMC; this provides an in-depth understanding of the etiology of HF in CHD patients. Moreover, further analysis in our study revealed that several DEMs are potential biomarkers for predicting HF risk in CHD patients, suggesting that those miRNAs can be applied in clinical practice; however, this needs to be further validated in future clinical studies.

Recently, accumulating studies have revealed the potential of miRNAs for being diagnostic or prognostic biomarkers for CHD (8, 14, 15). While for HF, the investigation on the roles played by miRNAs in pathogenesis is not as sufficient as for CHD, several miRNAs have been identified as potential biomarkers for HF risk. For instance, circulating miR-150-5p is found to be strikingly downregulated in acute HF patients and was elucidated to be associated with maladaptive remodeling, disease severity, and outcomes (16). Another study revealed that the transitory form of miR-22-3p could be a biomarker for predicting worse clinical outcomes in chronic HF patients (17). Given the fact that CHD is regarded as a traditional risk factor for HF, which is reported to be a common complication that may lead to fatal outcomes in CHD patients, there is a dire need of biomarkers for predicting HF risk in CHD patients. However, there are only few investigations exploring the value of miRNA as a predictive factor for HF risk in CHD patients.

As a heterogeneous disease, multiple conditions could be the risk factors for HF, namely CHD, hypertension, diabetes mellitus, familial history, and so on, which lead to cardiac injuries, result in myocardial dysfunctions, and finally cause cardiac structural damage. The pathogenesis of HF is mediated by multiple mechanisms, and so far, several mechanisms have been established including neurohormonal activation and inflammation.

miR-221 belongs to the miR-221/222 family and plays pivotal roles in the pathogenesis of various diseases, including cancers and inflammatory diseases (18). Recent studies have elucidated that miR-221 can also mediate heart development through multiple mechanisms. In their study on mice models with cardiac-specific high miR-221 expression levels, Su et al. (19) observed cardiac dysfunction and HF, and they demonstrated that miR-221 suppresses autophagy and promotes HF via regulating p27/cyclin-dependent kinase/mammalian target of rapamycin axis. In their previous study, miR-221 was found to further cardiac hypertrophy *in vitro* by regulating p27, which is a cardiac hypertrophic inhibitor (20). The probable reason of the upregulated PBMC miR-221 levels discovered in our study may be that miR-221 contributes to the processes related to cardiac injury (19, 20). miR-19b, abundantly expressed in heart tissue, is a member of miR-17/92 cluster (21). Lately, it has been reported that in rat models, miR-19b could reduce the H202-induced apoptosis of H9C2 cardiomy-

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ocytes through targeting phosphatase and tensin homolog, and losing myocytes is a cause of cardiac damage (22). Qin et al. (23) showed that upregulation of miR-19b boosts the proliferation and differentiation of P19 cell model of cardiac differentiation, probably through Wnt/β-catenin signaling pathway. Another study showed that miR-19b is downregulated in HF patients and is correlated with elevated myocardial collagen cross-linking (24). In our study, miR-19b-5p was shown to be downregulated in PMBC in CHD patients with HF, which could be explained by the protective role of miR-19b for cardiac function shown by the previous studies (22-24). Another dysregulated miRNA found in our study was miR-25, which was found to be markedly upregulated in CHD patients with HF. MiR-25 belongs to the miR-106b-25 cluster; it is located on chromosome 7q22.1 and contributes to various pathological processes that are mostly related to cancers and diseases like diabetic nephropathy (25-27). Interestingly, miR-25 is also reported to be involved in the pathogenesis of HF. For instance, Wahlquist et al. (28) reported that downregulating miR-25 expression could be a therapeutic strategy for patients with HF, and an increased level of miR-25 is observed in patients with HF; these results are in line with ours. Additionally, Wahlquist et al. (28) showed that miR-25 is mainly expressed in cardiomyocytes of transverse aortic constriction (TAC)-induced failing hearts of mice, and it postpones the calcium uptake kinetics; furthermore, AAV9-mediated upregulated miR-25 levels *in vivo* lead to the loss of contractile function. These results reported in their study suggest that miR-25 could promote HF, which might explain the increased PBMC levels of the mature form of miR-25 in our study. In addition, multiple logistic regression revealed that PBMC miR-221, miR-19b-5p, miR-25-5p, and hypertension were independent predictive factors for HF risk in CHD patients, and ROC curve displayed that the combination of miR-221, miR-19b-5p, and miR-25-5p as well as the combination of these three DEMs with hypertension had a great diagnostic value for CHD patients with HF. The diagnostic value could be explained based on the following: 1) miR-221, miR-19b-5p, and miR-25-5p are involved in the pathogenesis of HF, and PBMCs have been reported to be associated with the pathogenesis of HF; therefore, PMBC expressions of miR-221, miR-19b-5p, and miR-25-5p were observed to have a good predictive value for HF risk in CHD patients (29). 2) Hypertension is a classic risk factor for HF (30).

The results of our study suggested that miRNAs could be utilized as diagnostic biomarkers for HF risk in CHD patients in clinical practice. Nonetheless, despite the fact that the results of our study were encouraging, there were still some unanswered questions. First, it is still not clear through which pathways the DEMs found in our study regulate heart development or via regulating which inflammatory cytokines and chemokines these DEMs mediate inflammation. Second, the diagnostic value of DEMs needs more validation. To answer the above questions, more *in vitro* and *in vivo* experiments should be conducted, and the diagnostic value of miRNAs should be validated by more clinical studies with a larger sample size.

Study limitations

There were some limitations of our study that should not be ignored: 1) The sample size was limited in the validation stage. 2) We did not examine the miRNA expression in heart tissue; although miRNAs are more specifically expressed in the tissue, the blood sample is easier to collect and circulating miRNA expression testing is more applicable in clinical practice. Thus, further study that evaluates the correlations of miRNAs with HF in CHD patients should enlarge the sample size. 3) There might be some false-positive results in the microarray analysis, which could have resulted from multiple reasons; for example the contamination of the PCR product and so on. Therefore, future studies should control the quality of microarray analysis by means of replication etc. 4) The blood samples in our study were collected in the EDTA-3K tubes and not in the paxgene RNA blood tubes, which might result in an influence on the RNA integrity in our samples. However, the miRNAs evaluated in our study were those with known sequences and short length; therefore, the quality and quantity of miRNAs could be largely preserved, and there are other studies as well in which EDTA tubes were used to collect blood samples for miRNA detection (31, 32).

Conclusion

In conclusion, CHD patients with and without HF could be differentiated according to PBMC miRNA profiles, and a combination of PBMC miR-19b-5p, miR-221, and miR-25-5p as well as a combination of these three miRNAs with hypertension correlates with an increased HF risk in CHD patients.

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