Plasma Levels of microRNA-221 (miR-221) are Increased in Patients with Acute Pulmonary Embolism

Authors' Contribution:
Study Design A
Data Collection B
Statistical Analysis C
Data Interpretation D
Manuscript Preparation E
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Background: The aim of this study was to measure and compare the plasma levels of the microRNA (miRNA), miR-221, in patients with acute pulmonary embolism (PE) with healthy individuals and to evaluate the potential role of miR-221 as a diagnostic biomarker for acute PE.

Material/Methods: In blood samples collected from 60 patients with acute PE and 50 healthy volunteers, plasma levels of microRNA were identified using a microRNA microarray, and miR-221 expression was detected using real-time quantitative reverse transcription polymerase chain reaction (qRT-PCR). Brain natriuretic peptide (BNP) and troponin I were measured using an automated immunoassay analyzer. D-dimer levels were measured with an enzyme-linked immunosorbent assay (ELISA).

Results: From the evaluation of 32 differentially expressed plasma miRNAs, miR-221 was significantly upregulated in the plasma of patients with acute PE compared with normal individuals (P<0.05). Correlation analysis showed that plasma miR-221 levels in patients with acute PE were positively correlated with levels of BNP (r=0.842, P<0.05), troponin I (r=0.853; P<0.05), and D-dimer (r=0.838; P<0.05). The receiver operating characteristic (ROC) area under the curve (AUC) for plasma miR-221 was 0.823 (95% CI, 0.757–0.906) (P<0.05), compared with the AUC for D-dimer of 0.768 (95% CI, 0.727–0.853), the AUC for troponin I of 0.713 (95% CI, 0.646–0.868), and the AUC for BNP of 0.648 (95% CI, 0.601–0.723).

Conclusions: Plasma levels of miR-221 were significantly increased in patients with acute PE when compared with healthy individuals.

MeSH Keywords: **MicroRNAs • Pulmonary Embolism • Troponin I**

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CLINICAL RESEARCH

Background

Acute pulmonary embolism (PE), or pulmonary thromboembolism, is a common medical condition associated with high morbidity and mortality that requires rapid diagnosis and treatment [1,2]. Acute PE is the third most common cause of acute cardiovascular death, after acute myocardial infarction (MI) and stroke [3]. However, due to the variable clinical symptoms and signs, the diagnosis of acute PE is often missed or delayed [4]. Currently, the gold standard for diagnosis of acute PE is pulmonary angiography, which is an invasive diagnostic procedure with the potential for serious complications. Computed tomography pulmonary angiography (CTPA) is the most commonly used imaging method to diagnose acute PE. However, CTPA requires the use of intravenous contrast medium, and cannot be used for patients with renal insufficiency and or who adversely react to the contrast agent.

MicroRNAs (miRNAs) of up to 22 nucleotides in length are a class of highly conserved non-coding small RNAs that control protein expression levels by regulating gene expression by translational inhibition or mRNA degradation [5,6]. A previously published study has shown that miRNAs participate in a series of biological processes, including cellular proliferation, differentiation, metabolism, and apoptosis [7]. Circulating miRNAs have also been shown to exist in a stable form that can be detected and measured in plasma, and so could represent potential diagnostic biomarkers [8].

The expression levels of miR-221 are increased in the plasma of patients with acute myocardial infarction (MI), which most commonly results from acute coronary artery thrombosis associated with atherosclerosis [9]. Also, in vascular smooth muscle cells from patients with pulmonary arterial hypertension, miR-221 has been shown to promote pulmonary artery smooth muscle cell proliferation [10]. However, the expression levels and functional role of miR-221 in acute PE remains to be determined.

Therefore, the aim of this study was to measure and compare the plasma levels of miR-221 in patients with acute PE with healthy individuals and to evaluate the potential role of miR-221 as a diagnostic biomarker for acute PE.

Material and Methods

Patients

Between January 2016 and January 2017, 60 patients who presented with acute pulmonary embolism (PE) were recruited into the study from the First Affiliated Hospital of China Medical University. Acute PE was diagnosed by computed tomography pulmonary angiography (CTPA) [11]. Risk stratification was

Table 1. Clinical characteristics of acute pulmonary embolism (APE) patients and normal control (NC).

Variable	APE (n=60)	Control (n=50)
Male	35 (58.33%)	28 (56.00%)
Age	55.83±7.52	55.15±7.02
Risk stratification		
High	10	/
Intermediate	18	/
Low	32	/
Main clinical symptoms		
Dyspnea	28	/
Thoracic pain	44	/

conducted according to the European Society of Cardiology (ESC) recommendations, using the following groupings [12]: high-risk (n=10), intermediate-risk (n=18), and low-risk (n=32). The normal control (NC) group consisted of 50 healthy volunteers who underwent a routine physical examination at the hospital. The baseline clinical data of patients and controls enrolled in the study are shown in Table 1. The exclusion criteria for participants in the present study included heart failure, acute coronary syndrome, cerebrovascular disease, chronic obstructive heart disease (COPD), contraindication to fibrinolysis, or known bleeding abnormalities. The study was approved by the Research Ethics Committee of the First Affiliated Hospital of China Medical University. All study participants gave written informed consent before enrolment in the study.

Blood and serum samples

Venous blood samples were collected in K2-EDTA tubes. The samples were centrifuged at 4°C using a two-step protocol at 820×g for 10 min, then at 16000×g for 10 min. The cell-free plasma was stored at –80°C until the plasma samples were required for analysis.

RNA extraction

The total RNA from 400 µl plasma was extracted using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) and purified using an RNeasy Mini kit (Qiagen, Hilden, German) according to the manufacturer's instructions. Briefly, 10 nM synthetic *C. elegans* miR-39 (RiboBio, China) was spiked-in to each sample after the addition of denaturing solution (Ambion, Austin, TX, USA) for normalization of the between-sample variation. RNA concentrations were determined by measuring the sample absorbance at 260 nm with the ultraviolet spectrophotometer (Thermo Fisher Scientific, Inc., Waltham, MA, USA).

MicroRNA (miRNA) microarray

MicroRNA (miRNA) expression was compared between plasma samples from patients with acute PE and plasma samples from healthy individuals, who were the normal controls (NC), using the GeneChip® miRNA 2.0 Array, which included 15,644 mature miRNA probes from miRBase V15 (Affymetrix, Santa Clara, CA, USA). Array hybridization and wash were performed by GeneChip® Hybridization, a Wash and Stain Kit, and a GeneChip Eukaryotic Hybridization Control Kit (Affymetrix, Santa Clara, CA, USA) in a 645 Hybridization Oven (Affymetrix, Santa Clara, CA, USA), and using Fluidics Station 450 (Affymetrix, Santa Clara, CA, USA), according to the manufacturer's instructions. After hybridization, microarrays were analyzed with the GeneChip Scanner 3000 7G (Affymetrix, Santa Clara, CA).

Real-time quantitative reverse transcription polymerase chain reaction (qRT-PCR)

Real-time qRT-PCR was performed with a SYBR Green PCR SuperMix Kit (TransGen Biotech, Beijing, China), using specific primers:
miR-221, 5'-CAGCATACATGATTCCTTGTGA-3' and 5'-CTTTGGTGTTTGAGATGTTTG-3'.

Relative levels of gene expression were evaluated relative to GAPDH and calculated using the $2^{-\Delta\Delta Ct}$ method.

The measurement of biochemical markers

Plasma brain natriuretic peptide (BNP) and troponin I concentrations were determined using a chemiluminescent immunoassay method with the Immulite 2500 (Siemens Medical Solutions, Erlangen, Germany). D-dimers were measured with an enzyme-linked immunosorbent assay (ELISA).

Statistical analysis

Data were analyzed using SPSS version 17.0 software and GraphPad software. The normal distribution measurement data were calculated as the mean ±SD, and the t-test was used for comparison between groups. Categorical variables were compared using the chi-squared (χ^2) test. The correlation analysis of continuous variables was performed using the Spearman rank correlation method. The diagnostic efficacy of the plasma miR-221 levels were analyzed with the receiver operating characteristic (ROC) curve, and the area under the curve (AUC) was calculated. A P-value <0.05 was considered to be statistically significant.

Results

Expression profiles of microRNAs (miRNAs) in the plasma of patients with acute pulmonary embolism (PE)

A total of 32 differentially expressed plasma microRNAs (miRNAs) were evaluated in the plasma samples from patients with acute pulmonary embolism (PE), as well as healthy individuals who were the normal controls (NCs) (Figure 1A). As shown in Figure 1B, miR-221 was significantly upregulated in the plasma of patients with acute PE compared with the NCs (a 4-fold difference in plasma concentration was considered to be significant). Also, plasma miR-221 levels were significantly increased in patients with acute PE who were categorized as being intermediate-risk and high-risk patients (Figure 1C).

Correlation between plasma miR-221 levels and clinical parameters of patients with acute PE

Plasma levels of brain natriuretic peptide (BNP), troponin I, and D-dimer were detected, and correlation analysis showed that the relative plasma levels of miR-221 in patients with acute PE were positively correlated with the plasma concentrations of BNP (r=0.842; P<0.05), troponin I (r=0.853; P<0.05), and D-dimer (r=0.838; P<0.05) (Figure 2).

Increased plasma level of miR-221 was a diagnostic biomarker for acute PE

The receiver operating characteristic (ROC) curve analysis was performed to evaluate the possible role of plasma miR-221 as a diagnostic biomarker for acute PE. The ROC area under the curve (AUC) for plasma miR-221 was 0.823 (95% CI, 0.757–0.906) (P <0.05), compared with the AUC for D-dimer of 0.768 (95% CI, 0.727–0.853), the AUC for troponin I of 0.713 (95% CI, 0.646–0.868), and the AUC for BNP of 0.648 (95% CI, 0.601–0.723) (Figure 3). In this study, the results of the measurement of plasma levels of miR-221 in patients with acute PE, compared with normal individuals (NCs) indicated that miR-221 might be a biomarker for acute PE following thromboembolism.

Discussion

Peripheral venous blood samples are the preferred source for the detection of biomarkers of disease, as blood samples are routinely taken from patients, and most established biomarkers evaluated by this method have shown consistent and repeatable results [13]. Acute pulmonary embolism (PE) can present with nonspecific clinical symptoms and signs and may be difficult to diagnose at an early stage [12]. There have been some recent studies to identify peripheral blood components as potential biomarkers for the diagnosis of acute PE. For example,
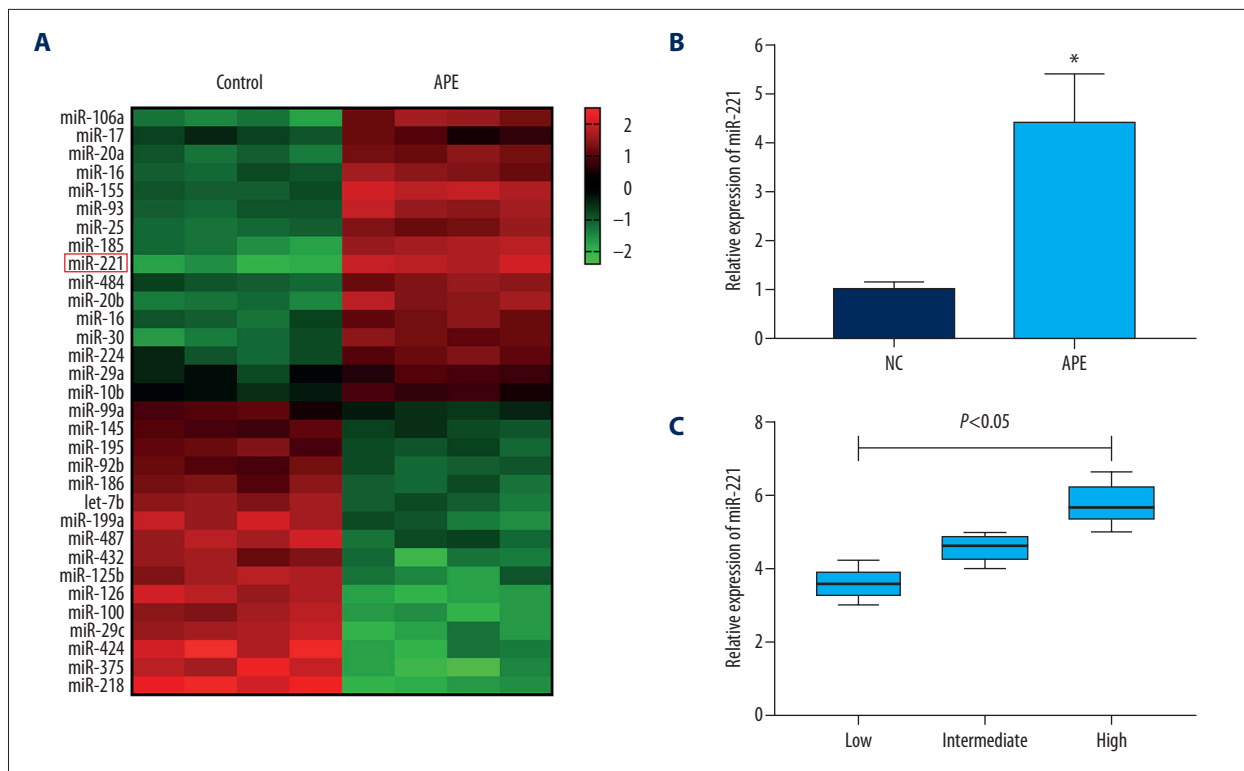
Figure 1. Expression profiles of microRNAs (miRNAs) in the plasma of patients with acute pulmonary embolism (PE). (**A**) A total of 32 differentially expressed plasma microRNAs (miRNAs) were evaluated in plasma samples from patients with acute pulmonary embolism (PE) and normal individuals, or normal controls (NC). (**B**) miR-221 is shown to be significantly increased in the plasma of patients with acute PE compared with NCs. (**C**) miR-221 levels are shown to be significantly increased in patients with acute PE of intermediate-risk and high-risk. (* P<0.05).

Insenser et al. identified haptoglobin as a potential diagnostic biomarker for acute PE [14], and Selimoglu Sen et al. evaluated serum levels of apelin-13 as a new biomarker to improve the diagnosis of patients with acute PE [15].

Given that microRNAs (miRNAs) are stable in serum and can be measured reliably [8], increasing numbers of specific miR-NAs are being studied for screening and monitoring human disease. Previously published studies have shown that miR-NAs could be potential biomarkers for the diagnosis of acute PE, notably the study by Zhou et al. who showed that plasma miR-28-3p could be used as a non-invasive and stable diagnostic biomarker [16]. Xiao et al. showed that plasma levels of miRNA-134 were significantly increased in patients with acute PE when compared with healthy controls or patients without acute PE, indicating that plasma miR-134 could be a diagnostic biomarker for acute PE [17].

In the present study, we evaluated 32 differentially expressed plasma miRNAs in patients with acute PE and found that miR-221 was significantly increased when compared with healthy individuals. Also, the increased plasma levels of miR-221 were associated with an increased risk of PE. The symptoms of acute

PE include chest pain and shortness of breath and are similar presenting symptoms in acute myocardial infarction (MI), which means that clinically distinguishing between these two diseases can be difficult in the acute stage [3,18]. An increase in plasma markers of acute cardiac damage, brain natriuretic peptide (BNP) and troponin I, can support the diagnosis of acute MI [19]. Also, the D-dimer test used in the diagnosis of venous thromboembolism (VTE) and acute PE [20]. The correlation analysis performed in the present study showed that the relative plasma levels of miR-221 in patients with acute PE were positively correlated with the plasma concentrations of BNP, troponin I, and D-dimer. The receiver operating characteristic (ROC) area under the curve (AUC) analysis showed that miR-221 could distinguish between patients with acute PE and healthy people when compared with levels of D-dimer, troponin I, and BNP. Therefore, the findings of the present study support the potential for miR-134 as a plasma biomarker to diagnose acute PE. However, these preliminary findings require further research with larger controlled clinical studies.

This study had several limitations. The study was performed at a single center and had a small sample size. The study was preliminary in nature, and although the findings were significant,
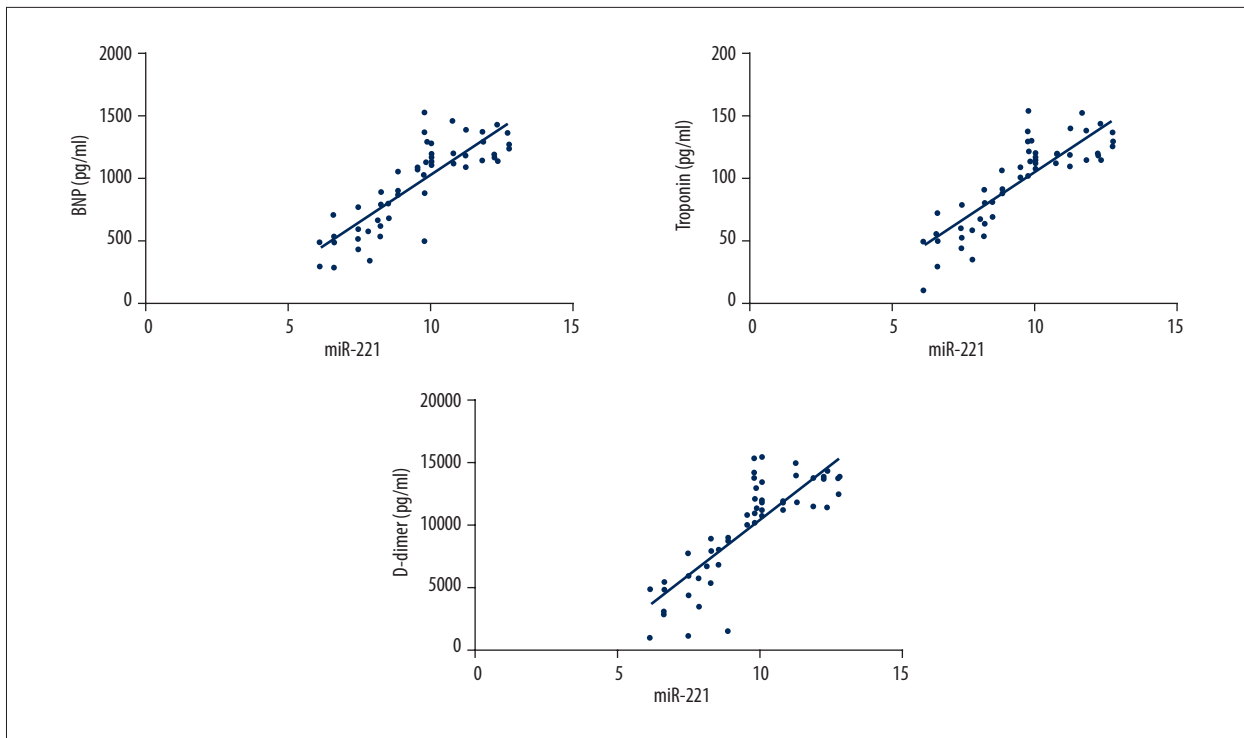
Figure 2. The correlation between plasma levels of miR-221 and clinical parameters of patients with acute pulmonary embolism (PE). Correlation analysis was performed for miR-221 levels in patients with acute pulmonary embolism (PE) compared with normal controls (NC) and shows that the relative miR-221 levels in patients with acute pulmonary embolism (PE) are positively correlated with the plasma concentrations of brain natriuretic peptide (BNP) (r=0.842; P<0.05), troponin I (r=0.853; P<0.05), D-dimer (r=0.838; P<0.05).
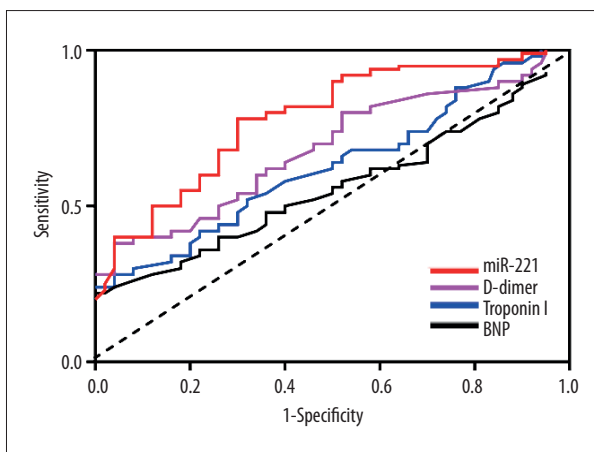
Figure 3. Plasma levels of miR-221 as a diagnostic biomarker in patients with acute pulmonary embolism (PE). Analysis in patients with acute pulmonary embolism (PE) showed receiver operating characteristic (ROC) area under the curve (AUC) for plasma miR-221 is 0.823 (95% CI, 0.757–0.906) (P <0.05), compared with the AUC for D-dimer of 0.768 (95% CI, 0.727–0.853), the AUC for troponin I of 0.713 (95% CI, 0.646–0.868), and the AUC for brain natriuretic peptide (BNP) of 0.648 (95% CI, 0.601–0.723).

there remains insufficient evidence to confirm the specific role of miR-221 in acute PE. Further studies on the evaluation of plasma levels of miR-221 require large study sample sizes performed in multiple centers. A further limitation of this study was that we evaluated a total of only 32 differentially expressed plasma miRNAs in patients with acute PE. Therefore, it was not possible to determine whether or not miR-221 is specifically released in the initial phase of acute PE or whether there are other microRNAs involved in the progression and prognosis of PE, as previous research has shown that microRNAs show a restricted temporal and spatial expression pattern [21].

Conclusions

The findings of this study have shown that plasma levels of the microRNA (miRNA), miR-221, were significantly increased in patients presenting with acute thromboembolic pulmonary embolism (PE) when compared with healthy individuals. The findings of this study support that miR-221 should be studied further as a potential diagnostic biomarker in acute pulmonary embolism (PE), and other diseases associated with thrombosis and thromboembolism.

Conflict of interest

All authors declare no conflict of interest.

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