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Peripheral blood miRNAs as a biomarker for chronic cardiovascular diseases

SUBJECT AREAS:
DIAGNOSTIC MARKERS
MIRNAS
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2 December 2013Accepted
22 April 2014Published
22 May 2014

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Vascular diseases are the most prevalent diseases worldwide. This study intended to analyze peripheral blood miRNA levels and their correlation with NT-pro-BNP and cTN-I in patients with atherosclerosis or pre-atherosclerotic conditions to build a dynamic correlation between vascular diseases and their biomarkers. Serum NT-pro-BNP and cTN-I levels were measured by their respective ELISA kits. The miRNA levels were assayed by quantitative PCR. Unique miRNA signatures were identified for both atherosclerosis and pre-atherosclerosis. The levels of miR-92a, 126, 130a, 222, and 370 levels were decreased in the peripheral blood of pre-atherosclerotic subjects. In atherosclerosis, miR-21, 122, 130a, and 211 were significantly increased whereas miR-92a, 126, and 222 were markedly decreased. Serum levels of NT-pro-BNP and cTN-I correlated with each other and increased with the progression of atherosclerosis. Moreover, the levels of cTN-I and NT-pro-BNP were positively correlated with miR-21 and negatively correlated with miR-126. Integrating specific pattern of miRNA levels with NT-pro-BNP and/or cardiac troponin may improve the diagnosis of cardiovascular diseases.

Cardio-cerebrovascular disease (CVD) is the number one cause of mortality and morbidity worldwide¹. Obesity, diabetes, hyperlipidemia, and hypertension increasingly contribute to CVD mortality as the prevalence of those disorders increase over time². Atherosclerosis is the most prominent indication among all CVDs, which is a chronic condition caused by lipid-induced inflammation of the vessel wall resulting from a complex interplay among endothelial cells, smooth muscle cells, macrophages, and leukocytes³. Atherosclerosis can remain asymptomatic for decades⁴ during the accumulation of fatty materials such as cholesterol and triglyceride on an artery wall until lumen stenosis is so severe that it restricts blood supply downstream to a level resulting in ischemia. The advancement of atherosclerosis are chronic, slowly progressive and cumulative. Diabetes, hyperlipidemia, and hypertension are the major risk factors promoting atherosclerosis⁴.

B-type natriuretic peptide (NT-pro-BNP) is a useful biomarker of acute heart failure (HF) in emergency settings⁵. The serum NT-pro-BNP level is correlated with the stage of HF^{5,6} and is associated with age, race⁷, and body mass index⁸. Moreover, it has been shown that serum NT-pro-BNP level is also increased in chronic renal dysfunction patients^{9,10}.

Cardiac troponin I (cTN-I) is a prognostic indicator for acute myocardial infarction (AMI)^{11,12}. It has been shown that serum cTN-I level is elevated in patients with chronic pulmonary hypertension¹³, rhabdomyolysis¹⁴, and diabetic ketoacidosis¹⁵, indicating that the changes of NT-pro-BNP and cTN-I levels occurred before acute cardiovascular events.

MicroRNAs (miRNAs) belong to a class of small non-coding RNA molecules. MiRNAs bind to the 3'-untranslated regions of messenger RNAs to block translation and/or to promote mRNA degradation¹⁶. MiRNA has been shown to regulate physiological and pathophysiological process including cardiac regeneration¹⁷ and atherosclerosis¹⁸. Circulating miRNAs have emerged as biomarkers for different diseases^{19–22}. As most studies focused on the association between changes of miRNAs with a clearly defined stage of a disease, we intended to investigate both physiological and miRNA changes of patients with atherosclerosis or with conditions leading to atherosclerosis.

Results

Changes of NT-pro-BNP and cTN-I in patients with pre-atherosclerotic conditions or atherosclerosis. NT-pro-BNP and cTN-I serum concentration increased in atherosclerotic (Athero) patients and pre-atherosclerotic (pre-Athero) patients (patients with hyperlipidemia, hypertension, and or/diabetes). The



average serum NT-pro-BNP level was increased to 42.77 pg/ml and 49.17 pg/ml in pre-atherosclerotic and atherosclerotic patients respectively from 30.15 pg/ml in normal controls ($p < 0.05$ Athero vs control and pre-Athero vs control; $p < 0.05$ Athero vs pre-Athero) (Fig. 1A). The serum cTN-I concentration was increased from 10.24 ng/ml in controls to 12.02 ng/ml in pre-atherosclerotic patients and 13.14 ng/ml in atherosclerotic patients ($p < 0.05$ vs control and $p < 0.05$ vs pre-athero) (Fig. 1B).

The serum levels of NT-pro-BNP and cTN-I were highly correlated with each other in the subjects overall ($R^2 = 0.8229$; Fig. 2A). When stratified by healthy controls, pre-atherosclerotic, and atherosclerotic groups, the highest correlation between NT-pro-BNP and cTN-I was seen in healthy individuals ($R^2 = 0.9593$, Fig. 2B), and became weaker in pre-atherosclerotic ($R^2 = 0.7046$, Fig. 2C) and atherosclerotic ($R^2 = 0.716$, Fig. 2D) patients.

Signatures of miRNA expression in pre-atherosclerotic and atherosclerotic patients. The profiles of a selected group of miRNAs in peripheral blood were significantly different in patient group with atherosclerosis or without atherosclerosis but having hypertension, diabetes, and/or hyperlipidemia from that of healthy individuals (Fig. 3). Specifically, miR-92a, miR-126, miR-130a, miR-222, and miR-370 were markedly reduced in pre-atherosclerotic patients, with miR-126 and miR-130a reduced more than 50% (Fig. 3A). However, The levels of miR-21, miR-122, miR-130a, and miR-211 in peripheral blood significantly increased whereas those of miR-92a, miR-126, and miR-222 markedly decreased in atherosclerotic patients (Fig. 3B).

The peripheral blood levels of miR-21 and miR-122 in patients with hyperlipidemia, diabetes, and/or hypertension were comparable to that of the control group but significantly increased with atherosclerosis (Fig. 4) though miR-21 showed a trend of increasing with the emergency of atherosclerosis. On the other hand, the level of miR-130a was significantly increased in the atherosclerosis group whereas it was deeply depressed in pre-atherosclerotic patients ($p < 0.01$ Athero vs control, pre-Athero vs control, and Athero vs pre-Athero) (Fig. 4).

Levels of miR-21 and miR-126 are well correlated with NT-pro-BNP and cTN-I levels. The correlation between the expression of individual miRNA and NT-pro-BNP or cTN-I level was analyzed. A negative correlation was identified between miR-126 with cTN-I ($R^2 = 0.6758$; Fig. 5A) and NT-pro-BNP ($R^2 = 0.8317$; Fig. 5B) levels whereas a positive correlation between miR-21 with cTN-I level ($R^2 = 0.7066$; Fig. 5C) and NT-pro-BNP level ($R^2 = 0.8601$; Fig. 5D) among atherosclerotic patients (Fig. 5B and 5D) was observed.

Discussion

A miRNA expression signature emerged from patients both with atherosclerosis or without atherosclerosis but diagnosed with one or more disorders of hyperlipidemia, diabetes mellitus, and hypertension. The levels of NT-pro-BNP and TN, considered markers for acute coronary artery diseases and heart failure, were increased in those patients.

The dysregulation of miRNA has been widely reported in different cardiac and vascular diseases^{18, 21–23}. The peripheral blood levels of miR-92a, miR-126, and miR-222 in this study were markedly decreased in both atherosclerotic and pre-atherosclerotic patients compared to healthy controls though the decrease of miR-92a and miR-222 in pre-atherosclerotic patients was not as significant as that in atherosclerotic patients. Endothelial miR-126 inhibited VCAM-1 protein levels during inflammation²⁴ to protect against the onset of atherosclerosis. MicroRNA-92a regulated Krüppel-Like factors 4 and 2 in arterial endothelium and contributed to regional atherosusceptibility and protection in vivo²⁵ and its circulating level was found significantly lower in patients with coronary artery disease²⁶. The opposite effects of miR-221/222 on the proliferation, migration, and apoptosis of endothelial cells and vascular smooth muscle cells allowed them to promote neointimal formation while inhibit re-endothelialization after vascular injury²⁷. The levels of miR-221 and miR-222 were decreased whereas levels of miR-21, miR-130a, and some other miRNAs in patients with peripheral arterial disease²⁸. Interestingly, plasma level of liver specific miR-122 was increased significantly after cardiogenic shock²⁹.

There have not been many efforts to establish the association between specific miRNA and NT-pro-BNP or troponin to develop a multi-factor diagnostic panel for various vascular diseases. In the present study, a stronger association was found between NT-pro-BNP with miR-126 and with miR-21 in atherosclerotic patients while cTN-I showed good association with those two microRNAs as well. However, due to the limited number of patients with hypertension, diabetes, and/or hyperlipidemia, it was impossible to further stratify the data from the pre-atherosclerotic group. A correlation between cTN-I and peripheral blood miR-133a, miR-208a, and especially miR-133b was reported during early myocardial injury and recovery in patients after heart transplantation³⁰, indicating that circulating miRNA and cTN-I levels could serve as an indicator of cardiac injury. The levels of cTN-T, miR-499, along with other risk factors such as hypercholesterolemia, diabetes, and smoking were able to predict the mortality of patients with acute myocardial infarction³¹. The incorporation of microRNA levels has been shown to improve the diagnostic power of NT-pro-BNP³². Those data demonstrated the possibility to identify the correlations between specific miRNA

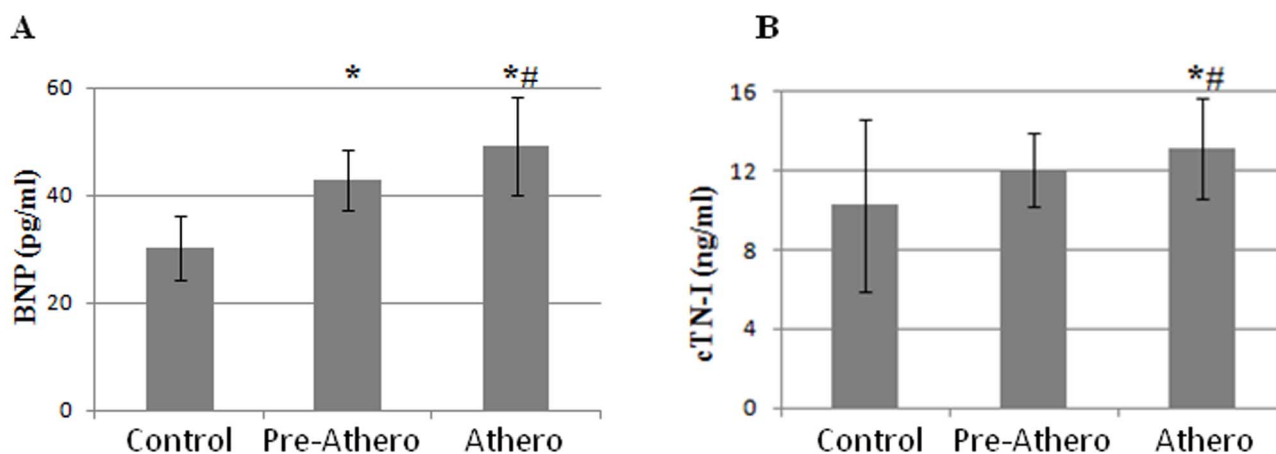


Figure 1 | The serum NT-pro-BNP and cTN-I levels were increased in both atherosclerotic and pre-atherosclerotic patients. Serum levels of NT-pro-BNP (A) and cTN-I (B) from cardiovascular disease patients and healthy controls were analyzed by ELISA kits.

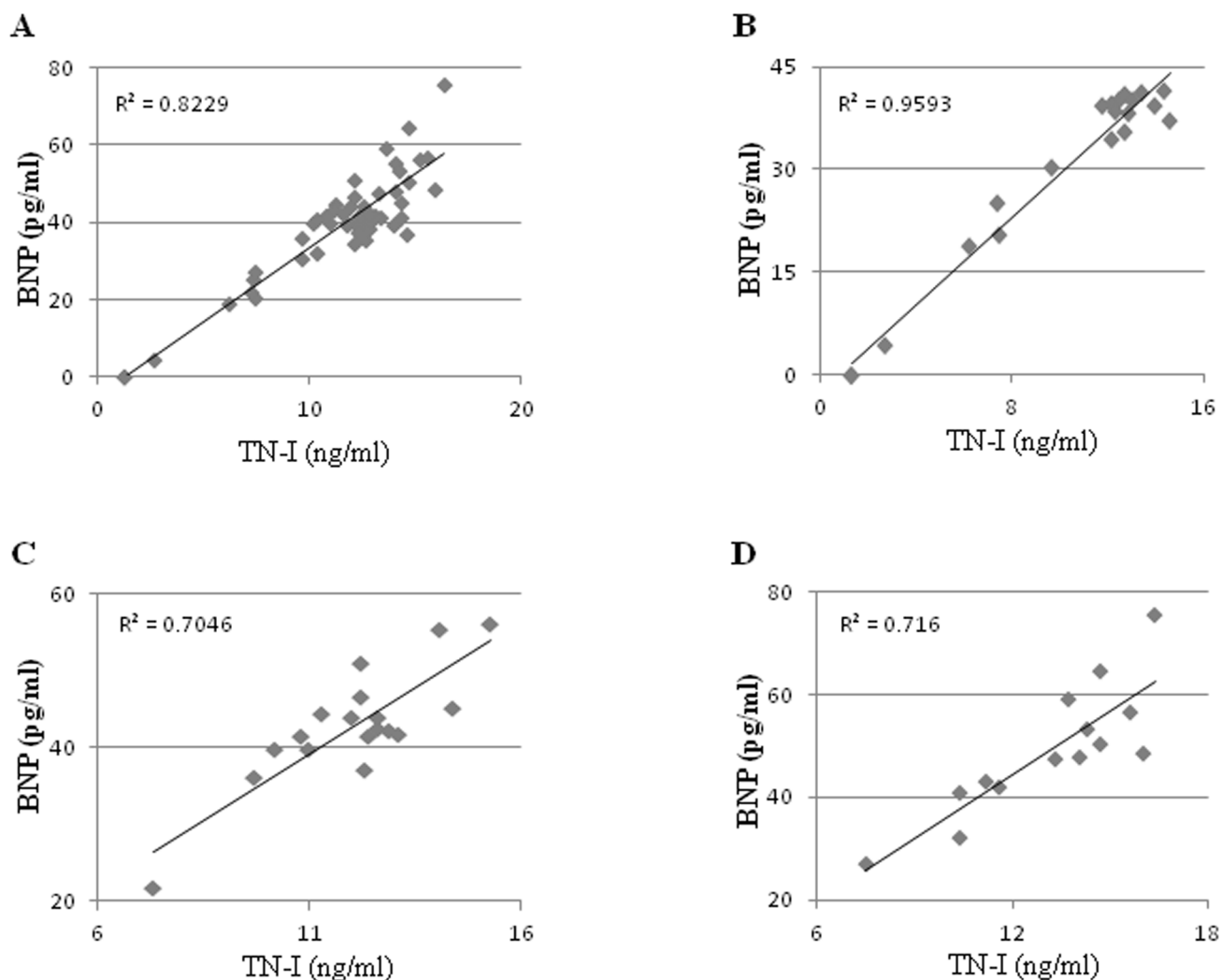


Figure 2 | The serum NT-pro-BNP and cTN-I levels were positively correlated. Strong correlation between serum NT-pro-BNP and cTN-I levels was found in all study subjects (A), controls (B), pre-atherosclerotic (C), and atherosclerotic (D) groups.

signature with NT-pro-BNP and/or cardiac troponin and to improve the diagnostic power.

In conclusion, the peripheral miRNA signature was different in patients with atherosclerosis or with pre-atherosclerotic conditions.

Plasma NT-pro-BNP and cTN-I levels showed a trend of increase with the progression of atherosclerosis and correlated with each other. Meanwhile, strong association of miR-21 and miR-126 with NT-pro-BNP and cTN-I was identified in patients with atherosclerosis,

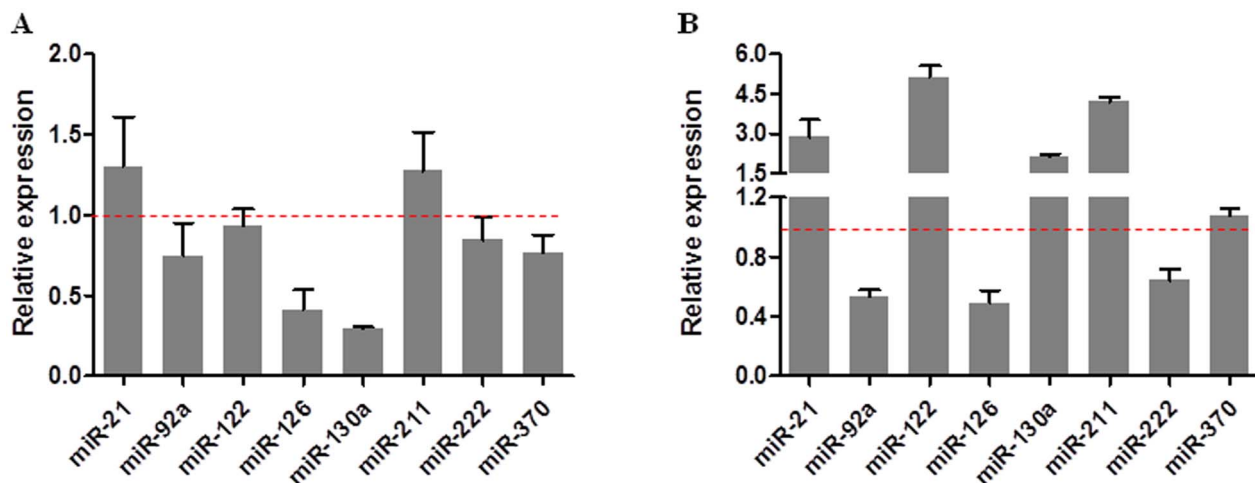


Figure 3 | Unique peripheral blood miRNA signatures distinguish pre-atherosclerosis and atherosclerosis. The miRNA level in peripheral blood was assayed by reverse transcription quantitative real-time PCR. The pattern of miRNA of pre-atherosclerotic (A) and atherosclerotic (B) patients were distinct.

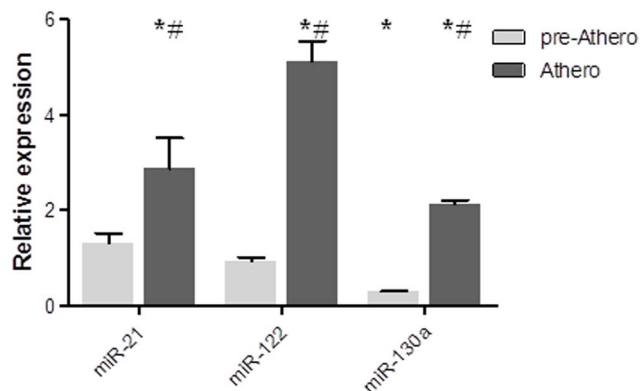


Figure 4 | The changes of miR-21, miR-122, and miR-130a associated with pre-atherosclerotic conditions and atherosclerosis. *: $p < 0.05$ vs control; #: $p < 0.05$ vs pre-atherosclerosis.

indicating that integration of miRNA signature with other parameters like NT-pro-BNP and cTN could significantly improve the diagnostic power of vascular diseases.

Methods

Study Subjects. Subjects, 20 healthy control, 20 patients with atherosclerosis, and 20 patients with one or more indications of hypertension, hyperlipidemia, and diabetes mellitus but without atherosclerosis detected, were recruited from January 2013 through June 2013 at the affiliate hospital of Jilin Medical University. All procedures conformed with the Declaration of Helsinki and approved by the institutional review

Table 1 Nucleotide sequence	
miR-21	TGCCGCCAACACCAGTCGAT
miR-92a	TGCCGCAGGTGGGATCGGT
miR-122	TGCCGCAACGCCATTATCACA
miR-126	TGCCGCTCGTACCGTGAGTAA
miR-130a	TGGCCAATGTTAAAAGGGCAT
miR-211	TGCCGCGCAGGGACAGCAA
miR-222	TGCCGCAGTACATCTGGCT
miR-370	TGCCGCGCTGCTGGGGTG
hU6	TGCTGGATGACACGCCAAATTC

board of Jilin Medical University. A signed consent form was obtained from each participant. All hypertension, hyperlipidemia, and diabetes patients undergone coronary calcium scoring by CT, carotid intimal media thickness measurement by ultrasound, lipoprotein subclass analysis, and HbA1c, hsC-reactive protein, and homocysteine measurements to detect the existence of atherosclerosis.

Blood Sample Processing. Fasting blood (2 ml) was drawn from each participant into blood collection tube containing sodium heparin (Nihoo International, Wenzhou, China) and put on ice immediately and processed within 3 hr after drawing. One milliliter of blood was transferred into a eppendorf tubes and centrifuged at 1000 rpm for 5 min. 200 μ l serum was set aside for NT-pro-BNP and cTN-I analyses. The total RNA was isolated from 1 ml whole blood sample using Trizol reagent (TransGen, Nanjing, China) according to manufactures' protocols.

Serum NT-pro-BNP and cTN-I level measurement. Serum NT-pro-BNP and cTN-I levels were analyzed with ELISA kits from Yupingbio (Shanghai, China) according to kit protocols. Briefly, 50 μ l of standards and samples (1:4 diluted with sample diluents) was added into assay strips followed by mixing in 100 μ l of HRP-conjugated detecting antibody. The strips were sealed and incubated at 37 $^{\circ}$ C for 60 min, washed

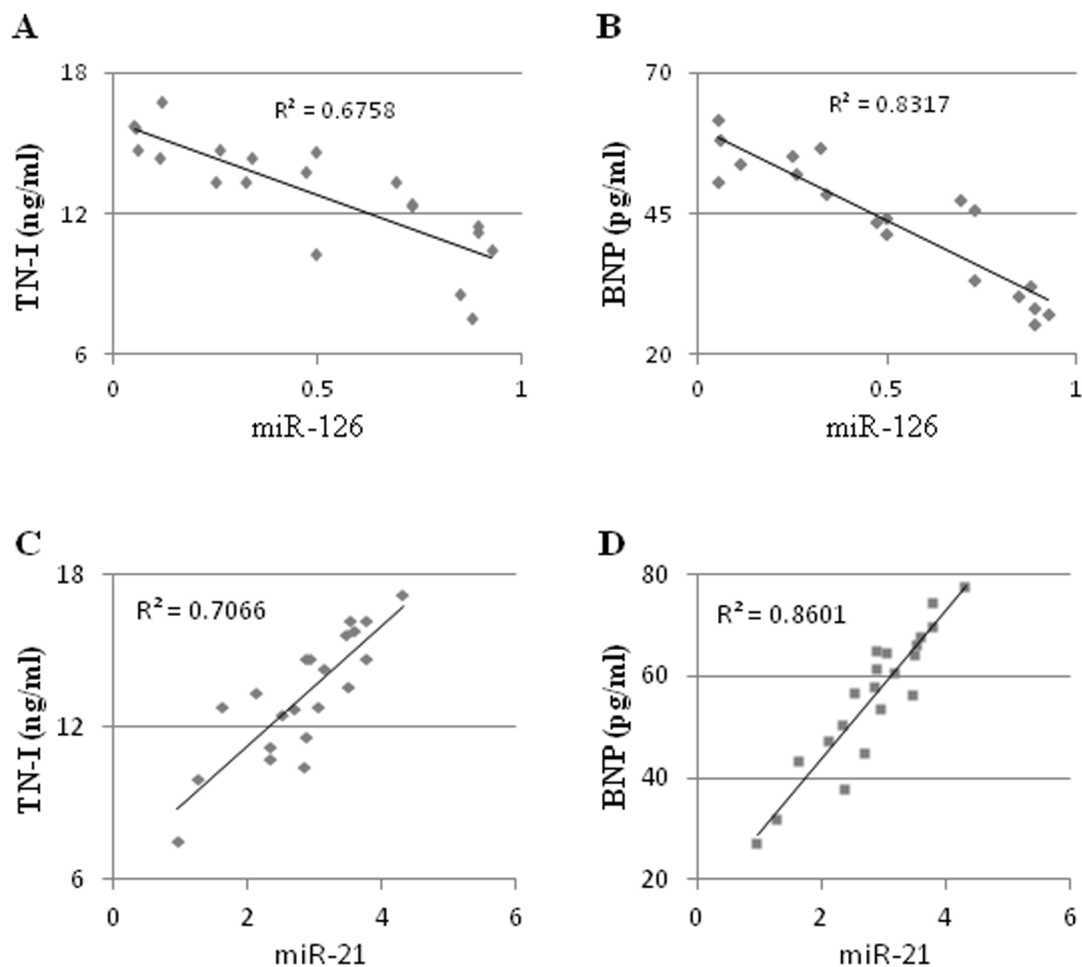


Figure 5 | The miR-21 and miR-126 levels were strongly associated with NT-pro-BNP and cTN-I in atherosclerosis. MiR-126 was negatively correlated with cTN-I (A) and NT-pro-BNP (B) levels while miR-21 was positively associated with cTN-I (C) and NT-pro-BNP (D) levels.



5 times with 1 ml per well of washing solution, patted dry, added 50 µl each of substrates A and B followed by incubating at 37 °C for 15 min and then stopped with 50 µl of Stop Solution and read at 450 nm within 15 min. Sample NT-pro-BNP and cTN-I concentrations were calculated against its own standard curve.

Real-time quantitative analysis of miRNA. The first strand cDNA of small RNAs was synthesized using a one step simultaneous polyadenylation and reverse transcription system from Chi Biotechnology (Jiangyin, China) according to manufacturer's instruction. A panel of 8 miRNAs (Table 1) linked to cardiovascular diseases were analyzed with MirCount™ system (Chi Biotechnology, Jiangyin, China) following supplier's protocol. The relative expression levels were calculated by 2^{-ΔΔCt} method with U6 as internal control.

Statistical analyses. The correlation between different factors, linear regression, and co-efficiency were analyzed by Graphpad Prism 5. The difference between groups was analyzed by t-test with either Graphpad Prism 5 or Excel. It was considered statistically significant if $p < 0.05$.

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Acknowledgments

The authors would like to thank Dr. Shi Lei (Chi Biotechnology) for critical reading of the manuscript. This work was supported by grants from the Jilin Provincial Health Department Project (2011ZC029), National Natural Science Foundation of China (31100566; 81273421; 81202953; 81100808), Scientific research of Jilin province college talent cultivation project of Chunmiao (2013-352), and Jilin City Science and Technology Project (201032244).

Author contributions

Y.J. and J.H.C. conceived the study and wrote the manuscript; H.Y.W., H.M.C., C.Y.W., L.Z., H.W., L.L. and Y.L. generated data and reviewed the manuscript.

Additional information

Competing financial interests: The authors declare no competing financial interests.

How to cite this article: Jiang, Y. *et al.* Peripheral blood miRNAs as a biomarker for chronic cardiovascular diseases. *Sci. Rep.* **4**, 5026; DOI:10.1038/srep05026 (2014).



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