

## Original Article

# Circulating miR-665 and miR-30c-1-3p, the candidate markers of congestive heart failure with qi-deficiency-blood-stasis syndrome

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Received November 22, 2015; Accepted April 1, 2016; Epub June 15, 2016; Published June 30, 2016

**Abstract:** According to the Chinese medicine (CM) theory, qi-deficiency-blood-stasis syndrome might be one of major patterns of congestive heart failure (CHF), but the underlying mechanism is yet not clear. Here, we aim to investigate the role of microRNAs (miRNAs) in the regulation of CHF in the presence of qi-deficiency-blood-stasis syndrome. We tested miRNAs expression microarray on the serum samples from CHF patients (three patients with qi-deficiency-blood-stasis syndrome, and three without), the significant different expressed miRNAs (DE-miRNAs) were screened and further quantified by RT-PCR in tissue samples. Target genes of these DE-miRNAs were analyzed using the software and miRNAs database, which could predict the relations between these DE-miRNAs and CHF mediated by qi-deficiency-blood-stasis syndrome. A total of 9 DE-miRNAs were confirmed, among them, miR-665 and miR-30c-1-3p draw specific attention. Because pathway analysis revealed that both miR-665 and miR-30c-1-3p target on the zinc finger protein 460 and 880, which translocated into nuclear and activated GATA4 transcription factor, the later was important for cardiac development survival and hypertrophy, therefore the upregulation of DE-miRNAs might be a biomarker to predict the progression of CHF. The occurrence and development of CHF may result from certain DE-miRNAs and their target genes participating in several signal pathways. MiR-665 and miR-30c-1-3p may be the candidate markers for the risk of congestive heart failure in Qi-deficiency-blood-stasis Syndrome.

**Keywords:** Congestive heart failure, Chinese Medicine, miR-665, miR-30c-1-3p, qi-deficiency-blood-stasis syndrome

## Introduction

Congestive heart failure (CHF) is a common disease often occurs at the end stage of coronary heart diseases. Traditional Chinese medicine plays a superior role in the treatment of chronic diseases, including myocardial ischemia-induced CHF. Inspection, auscultation & olfaction, inquiry and palpation are the four ways of diagnosis. In clinic, we found that different CHF patients would show different symptoms. According to the characteristic of coating liquid on the tongue, a patient come to hospital could be diagnosed with or without qi-deficiency-blood-stasis or deficiency of both qi and yin syndrome, and the former is one of the most com-

mon syndrome patterns in CHF, which is characterized by certain regularities of distribution and differentiation [1].

However, both to Chinese Medicine (CM) and Western medicine, clinical practice of intervention for CHF at the early stage was poor by the limitation of specific cardiovascular markers to identify individual who is at high risk of CHF or its CM syndrome pattern.

In the recent years, circulation levels of microRNA (miRNA) are indicated as one of the suitable risk factors for the early diagnosis and intervention of CHF. Since patients with qi-deficiency-blood-stasis syndrome are the most common

## miR-665 and miR-30c-1-3p being candidate markers of congestive heart failure

**Table 1.** Incidence distribution of CM syndromes in 1694 CHF patients

Syndrome	Case load	Ratio (%)
Qi-deficiency-blood-stasis	367	21.67
Heart blood stasis and obstruction	356	21.02
Deficiency of both qi and yin	272	16.06
Qi-stagnation-blood-stasis	224	13.22
Yang-deficiency-water-diffusion	151	8.91
Phlegm blocking Heart vessels	132	7.79
Phlegm-stagnation	58	3.42
Heart-yang-deficiency	49	2.89
Heart-qi-deficiency	48	2.83
Phlegm-heat obstructing Lung	25	1.48
Lung-Kidney deficiency	12	0.71
Total	1694	100

CM pattern in CHF, in this study we investigate the association between circulating miRNAs and CHF in patients with or without qi-deficiency-blood-stasis syndrome.

### Materials and methods

#### Research objects

Clinical cases were collected from Longhua Hospital affiliated to Shanghai University of Traditional Chinese Medicine from December 2013 to February 2014 according to diagnosis criteria of CHF, inclusion and exclusion criteria, and diagnosis standard of CHF syndromes established by our preliminary study [1]. In this study, 3 CHF cases with qi-deficiency-blood-stasis syndrome and 3 cases with deficiency of both qi and yin syndrome were collected. According to requirements of clinical medical ethics, the chosen people had been told the contents of this study, and signed informed consent form.

#### Primary instrument

TY8824 Axon GenePix 4000B Chip scanner (Axon Instruments, U.S.A), hybridization instrument (Hybridization System 4, NimbleGen Company, U.S.A), miRCURY LNA™ microRNA Array (Exiqon).

#### Serum specimen

3 ml Fasting venous blood was collected application disposable vacuum tubes. After stewing

for 2 hours in 4°C and centrifuging in 3000 r/min for 10 minutes, the supernatant were drawn in centrifuge tube and cryopreserved in -80°C for standby application.

#### Operation process

The main operation procedures are RNA isolation, RNA quality test, miRNA labeling, and miRNA array hybridization. MiRNA array scanning and analysis is by GenePix pro V6.0 to read the raw intensity of the image. We use Median Normalization Method to obtain "Normalized Data". After normalization, the statistical significance of differentially expressed miRNA was analyzed by T-test. Unsupervised hierarchical clustering and correlation analysis was performed on miRNA data.

#### Biological information analysis of miRNA

DE-miRNAs screening contains significant DE-miRNAs that fold Change  $\geq 2.0$  and  $P$ -value  $\leq 0.05$ . Heat map shows hierarchical clustering result on the significant DE-miRNAs. Scatter plot indicates that the miRNA expression differences between the 2 samples. We integrate miRNA targets from Targetscan, Miranda, and Mirbase. The genes with target sites for at least two co-expressed miRNAs will be identified as a potential cooperative target gene set.

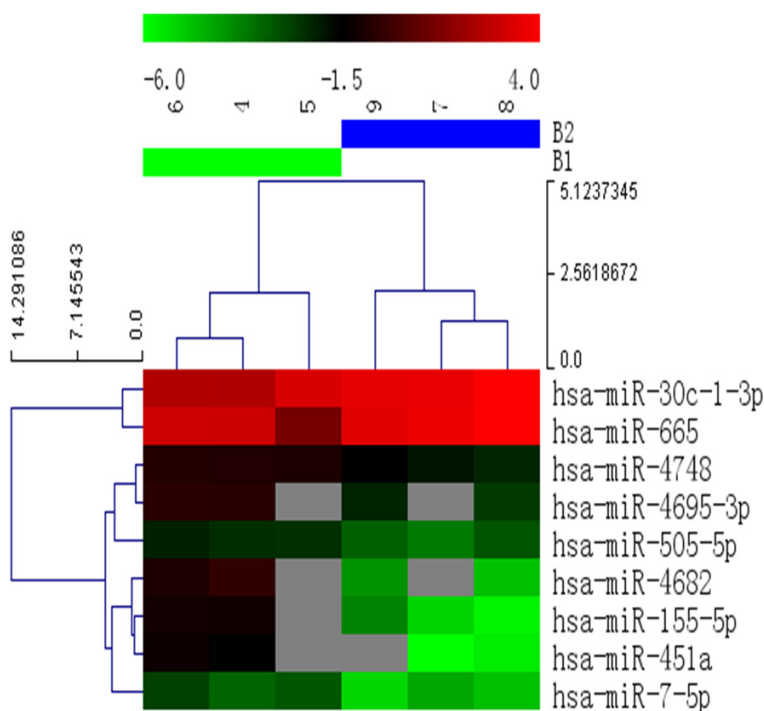
#### Statistical analysis

Statistical analysis was performed using SPSS-17.0 software. miRNAs that intensities  $\geq 30$  in samples were chosen for calculating normalization factor. All data were expressed as mean  $\pm$  s.e.m. Statistical significance between groups was analyzed by two-way hierarchical clustering in statistical test. A value of  $P < 0.05$  was considered statistically significant.

### Results

#### Characteristics of CHF patients with qi-deficiency-blood-stasis syndrome

In our preliminary clinic case review of CHF, we found that the incidence of qi-deficiency-blood-stasis syndrome was the highest compared with other syndrome patterns. In 1694 CHF patients, there are 367 patients with qi-deficiency-blood-stasis syndrome, which accounted for 21.67%. The incidence distribution of qi-deficiency-blood-stasis syndrome and other



**Figure 1.** From the hierarchical clustering for normalized data of DE-miRNAs in CHF patients with qi-deficiency-blood-stasis syndrome compared with CHF patients with deficiency of both qi and yin syndrome, there are total nine DE-miRNAs.

miR-665 and the green one is miR-30c-1-3p.

*Relations between miR-665/miR-30c-1-3p and Qi-deficiency-blood-stasis syndrome*

To investigate the relations between miR-665/miR-30c-1-3p and qi-deficiency-blood-stasis syndrome, we compared the expression intensities of qi-deficiency-blood-stasis syndrome with deficiency of both qi and yin syndrome in **Table 3**. In this table, No. 1, 2, 3 means CHF patients with deficiency of both qi and yin syndrome, and No. 4, 5, 6 are the patients with qi-deficiency-blood-stasis syndrome. Just as shown in **Table 3**, the expression intensities of miR-665 and miR-30c-1-3p are much higher in the patients with qi-deficiency-blood-stasis syndrome.

**Table 2.** Up-regulated DE miRNAs of Qi-deficiency-blood-stasis syndrome

ID	Name	Fold change	P-value
145768	hsa-miR-665	2.30	0.04
42702	hsa-miR-30c-1-3p	2.09	0.04

standardized patterns is summarized in **Table 1**.

*DE-miRNAs in CHF patients with Qi-deficiency-blood-stasis syndrome*

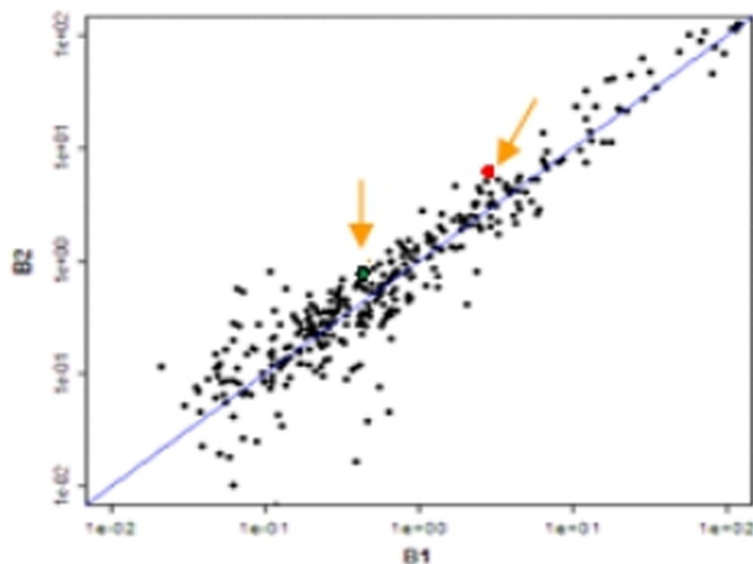
Hierarchical clustering for normalized data of DE-miRNAs in CHF patients with qi-deficiency-blood-stasis syndrome compared with CHF patients with deficiency of both qi and yin syndrome (**Figure 1**), two miRNAs, miR-665 and miR-30c-1-3p were significantly up regulated (**Table 2**); and seven miRNAs were down regulated. Among them, we paid attention to the 2 up regulated miRNAs, because both of them targeted on zinc finger proteins (ZFP).

The scatter plot of qi-deficiency-blood-stasis syndrome with deficiency of both qi and yin syndrome is shown in **Figure 2**. The red point is

**Discussion**

In the present study, we revealed qi-deficiency-blood-stasis syndrome was the major patterns of CHF, it usually resulted in the poor prognosis of heart failure and higher mortality rate. Therefore, providing stable and significant prognostic information in addition to other established biomarkers would be helpful for the early diagnosis and treatment of disease. In this study, we screened the miRNAs database in CHF patients, and we found 9 DE-miRNAs in patients with qi-deficiency-blood-stasis syndrome compared with those without qi-deficiency-blood-stasis syndrome. Among them, miR-665 and miR-30c-1-3p which are the up-regulated DE-miRNAs, target on the zinc finger protein 460 and 880, which are important for cardiac development survival and hypertrophy.

MicroRNAs (miRNAs) are small, endogenous and non-coding RNAs that are 21-25 nucleotides in length. Plenty of studies have reported the circulation miRNAs as the potential biomarkers in many diseases associated with cell apoptosis and multiple stress disorders [2-4]. Some miRNAs are closely related to cardiovascular diseases including acute coronary syn-



**Figure 2.** From the scatter plot of qi-deficiency-blood-stasis syndrome with deficiency of both qi and yin syndrome, the red point is miR-665 and the green one is miR-30c-1-3p.

**Table 3.** Up-regulated DE miRNAs of qi-deficiency-blood-stasis Syndrome

Name	Expression Intensity					
	1	2	3	4	5	6
miR-665	4006.5	1525.5	4389	6601.5	9774	5837
miR-30c-1-3p	2711.5	5550	2950.5	6058	10016	6211.5

dromes (ACS), atherosclerosis and hypertrophic cardiomyopathy [5-8]. For example, the plasma levels of miR-133 and miR-208b were elevated at acute phase of ACS and highly related to mortality over six months [9]. Increased miR-155 resulted in macrophage infiltration and enhanced atherogenesis in ApoE knockout mice [10]. The upregulation of miR-208a was attributed to the suppression of thyroid hormone receptor-associated protein 1 (THRAP1) and myostatin, both targets of miR-208a and negative regulators of muscle development of hypertrophy [11].

Nowadays, studies on inner material basis of syndromes became the focus in Chinese Medicine. However, till now there was few studies focus on the miRNAs biomarkers for CHF with qi-deficiency-blood-stasis syndrome. This greatly hindered the precision medical treatment of Chinese Medicine in the early diagnosis of heart failure. At present, most studies on

miRNAs prediction of cardiovascular diseases mainly focus on exploring the biological marker of syndromes in coronary heart disease [12], but did not add predictive value over the syndromes of CHF itself.

In our study, we found that miR-665 and miR-30c-1-3p were significantly increased in serum of patients with qi-deficiency-blood-stasis syndrome, and both were positively correlated with qi-deficiency-blood-stasis syndrome. To our best knowledge, this was the first report to analyze the association between serum miRNA levels with TCM treatment of CHF. Most importantly, we revealed that the common target of miR-665 and miR-30c-1-3p was zinc protein, which played a critical role in cardiac development. Zinc protein such as Zac1, cooperated with Nkx2-5, the later binding to the ANF promoter, which promoted DNA

amplification and cardiac myocyte maturation and differentiation [13].

Another zinc finger protein 460 and 880, which translocated into nuclear and activated GATA4 transcription factor, the later was important for cardiac development survival and hypertrophy. The upregulation of miR-665 and miR-30c-1-3p in CHF patients with qi-deficiency-blood-stasis syndrome suggested that the important zinc proteins might be greatly inhibited in these patients which attributed to the defect of cardiac survival in the process of heart failure, and the underlying mechanism for qi-deficiency-blood-stasis mediated upregulation of miR-665 and miR-30c-1-3p needed further investigation. Meanwhile, the upregulation of miR-665 and miR-30c-1-3p might be useful biomarkers to predict the progression of CHF.

In summary, we revealed in this study that the occurrence and development of CHF might

result from certain DE-miRNAs and their target genes participating in several signal pathways. MiR-665 and miR-30c-1-3p might be the candidate markers for the prediction of the risk of qi-deficiency-blood-stasis syndrome in CHF.

#### Acknowledgements

This study was sponsored by the National Science Foundation of China (30873398, 81300097), Major Clinical Research Project of Army (2006021003), and supported by Training Plan on Excellent Academic Leader of Shanghai Health System (XBR2011070), National Science & Technology Pillar Program during the 12th Five-year Plan Period, (2012BAI25B05) also in part supported by Development Project of Shanghai Peak Disciplines -Integrated Chinese and Western Medicine, and Shanghai Committee of Science and Technology grants (13ZR1406800).

#### Disclosure of conflict of interest

None.

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