Author's response to reviews

Title: Expression of circulating miR-486 and miR-150 in patients with acute myocardial infarction

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Author's response to reviews: see over
Dear editor,

Thank you for your letter and for the referees’ comments concerning our manuscript entitled “Expression of circulating miR-486 and miR-150 in patients with acute myocardial infarction (MS: 3712465221621530) ”. Those comments are all valuable and very helpful for revising and improving our paper, as well as the important guiding significance to our researches. We have studied referees’ comments carefully and have made revision which marked in red in the paper. The main corrections in the paper and the responds to the referees’ are as following. We hope the corrections will meet with approval.

Sincerely yours,

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Referee 1:

1. Discretionary Revisions

According to miRBase (www.mirbase.org), which is the biggest miRNA database, over 2,500 mature microRNAs are cloned in humans so far. Why did the authors focus on the miR-486 and miR-150? Did the authors conduct any screening experiments previously? Or is there any reason?

Response: We had performed a miRNA microassay chip analysis of AMI patients and healthy controls before this study and the results showed significant changes in the level of plasma miR-486 and miR-150 in AMI patients. Moreover, some studies have revealed that circulating miR-486 and miR-150 were involved in cardiovascular
diseases including AMI. Thus we investigated the roles of plasma miR-486 and miR-150 in AMI patients.

2. Major Compulsory Revisions

In Patient characteristics section of Methods, Paragraph 2: In this study, the patients with significant renal dysfunction were excluded. This exclusion criterion is not clear. This exclusion criterion should be described accurately (e.g. eGFR or hemodialysis) because it is reported that some circulating miRNAs upregulated in AMI patients are excreted via urine (Gidlof et al. Cardiology. 2011;20:493-500).

Response: We have made correction according to the referee’s comments. The patients with kidney failure (GFR < 15 mL/min/1.73m² or on dialysis) were excluded.

3. Major compulsory Revisions

From first to third section in Methods: It is better to describe the further detailed methods for the total RNA preparation from the patients and for the quantification of the miRNAs. From where was the patient’s blood taken (e.g. vein or artery)? How did the authors isolate the plasma from whole blood? How much was the mass or volume of total RNA to reverse transcription of the miRNAs. Also, the assay ID or catalog number of the Taqman human miRNA assay kits used for quantifying the miR-486 and miR-150 should be described, because it is uncertain which mature miRNA (-5p or -3p) level was evaluated in each miRNA. I think these descriptions are very important because the detectable miRNA levels vary depending on the used method.

Response: The referee’s comments are very important. We have amended the manuscript, details as follows: Five milliliters venous blood samples of patients with AMI were collected in EDTA anticoagulant tubes at admission. Samples were centrifuged at 3000×g for 10 min at 4°C, then the supernatant was isolated and centrifuged at 12,000×g for 10 min at 4°C. Plasma was collected and stored at -80°C until RNA extraction. RNA samples were diluted with DEPC-water to ensure a constant starting concentration of 180 ng/µL for each reverse transcription reaction.
The levels of expression of miR-486-3p or miR-150-3p were quantified using qRT-PCR using TaqMan human microRNA assay kits (Applied Biosystems, Part no. 4366596) according to the manufacturer’s instructions. The 15µL RT reaction mix contains 0.3 µL of 100mM dNTPs, 3 µL of MultiScribe Reverse Transcriptase (50 U/µL), 1.5 µL of 10×RT buffer, 0.19 µL of RNase inhibitor (20 U/µL), 6 µL of RT primer, 3µL of RNA sample and 1.01 µL of Nuclease-free water. For RNA from plasma samples, the concentration was diluted to 180ng/µL. The reagent mixes were incubated at 16 °C for 30 min, 42 °C for 30 min and 85 °C for 5 min, the RT products were stored at –20 °C until used for qRT-PCR. The 20 µL PCR reaction mix includes 1.0µL 20×TaqMan MicroRNA Assays, 0.16 µL RT product, 10 µL TaqMan Universal Master Mix II, No AmpErase UNG (2×) (Part no. 4440040), 8.84 µL Nuclease-free water. The PCR cycles consisted of an initial denaturation at 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s and 60 °C for 60 s.

4. Major Compulsory Revisions

In RNA extraction section of Methods, the authors depicted that total RNA was extracted from plasma using miRNeasy Mini Kit (Qiagen). However, this Kit is optimized for extraction of total RNA not from plasma or serum but from cells or tissues. From where the total RNA you extracted was derived? Did you extract the total RNA from residual cells in plasma? Also, it is known that miRNAs in plasma or serum in exosomes and HDL as well. Does the extracted total RNA contain exosomes-derived and HDL-derived miRNAs?

Response: We are very sorry for the mistake. Total RNA was extracted from plasma using an miRNeasy Serum/Plasm Kit (Qiagen, NO. 217184). Total RNA we extracted was derived from plasma. At present, it is well recognized that circulating miRNAs are packaged in microparticles or associated with RNA-binding proteins or lipoprotein complexes (HDL). Unfortunately, we did not make further investigation to differentiate whether the miRNAs was exosomes-derived or HDL-derived. We will make more investigation on it in our following studies.
5. Major Compulsory Revisions

In qRT-PCR section of Methods: To evaluate the levels of circulating miR-486 and miR-150, U6 snRNA was used. While it is well established that U6 snRNA as an internal control for circulating miRNAs. To the best of my knowledge, spike-in method using cel-miR-39 is common in these days.

Response: Spike-in method using cel-miR-39 for evaluating the levels of circulating miRNAs that you recommend is really excellent. While U6 snRNA is also used widely as an internal control and we just had the internal control U6 snRNA during the experiments in our lab. We will follow your suggestion and adopt the spike-in method in the future studies.

6. Minor Essential Revisions

In Figure 2, the color of diagonal line should be changed to purple. Also, it is better to change the ROC curve colors of miR-486, miR-150, and combined to blue, green, and red, respectively. Because to be consistent.

Response: The referee’s comments are very important. According to your suggestion, we have changed the colors of diagonal line and the ROC curve in Figure 2.

7. Major Compulsory Revisions

The authors indicated that the AUC values calculated by the combination of the two miRNAs levels were improved compared with that of miR-486 or miR-150 in Figure 2 and Figure 4. Are the improvements of AUC value between the combined data and the each miRNA statistically significant?

Response: In AMI patients, whether the differences of AUC value between miR-486 and the combined miRNAs or the differences of AUC value between miR-150 and the
combined miRNAs were statistically significant ($Z=2.179, P<0.05; Z=2.971, P<0.01$).
In NSTEMI patients, there was also statistical significance in AUC value between miR-486 or miR-150 and the combined miRNAs ($Z=2.231, P<0.05; Z=2.399, P<0.05$).

8. Minor Essential Revisions

In the last section of Results and Figure 4: Can you change the order of Figure 4A-4F?
The order of Figure 4A-4F in the main text is different to the order of panels in Figure 4. That results in complicated. Also, it is better to change the colors of diagonal line (to purple) and ROC curves (to blue and green) to be consistent in Figure 4A, 4B, 4D, and 4E.

Response: Thanks for your suggestion. We have revised the manuscript and changed the Figure 4, according to your suggestion. “ROC curve analysis of miRNAs showed the AUC of plasma miR-486 and miR-150 in STEMI patients to be 0.695 and 0.639, respectively (Fig. 4A, Fig. 4B), which was lower than in NSTEMI patients (0.782, 0.734) (Fig. 4D, Fig. 4E). There was also a higher AUC value, 0.845, for the combination of miR-486 and miR-150 in the NSTEMI group than in the STEMI group (Fig. 4C, Fig. 4F).”

Additional minor issues not for publication:
1. Generally, the AUC value is indicated up to 1.0.
Response: Thanks for your suggestion. We have revised the AUC value in the manuscript.

2. In qRT-PCR section of Methods, Page 7, line 12 (line 100): I think Taqman human microRNA Assay kit is supplied from Applied Biosystems.
Response: We are very sorry for the mistake. We have revised it. “The levels of expression of miR-486 or miR-150 were quantified using quantitative real-time PCR (qRT-PCR) using TaqMan human microRNA assay kits (Applied Biosystems) according to the manufacturer’s instructions.”

3. In the first section of Result, Page 9, line 4 (line 136): “EF%” should be changed to “EF”.
Response: Thanks for your advice. We have changed “EF%” to “EF”.

4. In the title of fourth section of Results, Page 10, line 9 (line 163): “Pattern of expression pattern of miR-486 and miR-150”?
Response: We are very sorry for our negligence and we have corrected the mistake. “Expression pattern of miR-486 and miR-150 in STEMI and NSTEMI”

5. In the second paragraph of Discussion, Page 11, line 8-10 (line 184-186): miR-499, miR-1, and miR-208 are not cardiac-specific miRNAs but muscle-enriched miRNAs. Cardiac specific miRNA is miR-208a only.
Response: We are very sorry for our negligence and we have corrected the mistake. “These include cardiac-specific miRNAs (miR-208a) and non-cardiac-specific miRNAs (miR-126, miR-328, miR-134)”

6. In Table 1, the number of hyperlipidemia patients in all cohort is correct?
Response: Thank you for your comment. We checked the data again, and the number of hyperlipidemia patients in all cohort is indeed correct.
7. In Figure 4F, the ROC curves of miR-486 and miR-150 are correct? I assume that the colors are reversed.

Response: We are very sorry for our negligence and we have corrected it.

Referee 2:

Major Compulsory Revisions

Abstract

Results

-“...in discriminating AMI patients and NSTEMI...” NSTEMI are also AMI patients, please re-write the sentence.

Response: Thank you for your suggestion. We have revised the sentence:

“The combined ROC analysis revealed an AUC value of 0.771 in discriminating AMI patients from healthy controls and an AUC value of 0.845 in discriminating NSTEMI patients from healthy controls.”

Introduction

Last paragraph
-Please define, why miR-150 and miR-486.

Response: According to your comment, we have revised the manuscript, details as follows: “It have been reported that miR-486 is a potent modulator in cardiac/skeletal muscle and miR-150 is involved in many cardiovascular diseases [27]. We also performed a preliminary plasma miRNA microassay chip analysis of AMI patients and healthy controls and the results showed significant changes in the levels of miR-486 and miR-150 in AMI patients which was in accordance with a recent study about serum miR-486 and miR-150 [28].”

-Please include as the aim also discrimination of NSTEMI and STEMI.

Response: Thank you for your comment. We have supplemented the aim of discrimination of NSTEMI and STEMI. The details were in manuscript. “AMI is separated into two categories based on changes seen in the electrocardiography (ECG): ST-segment elevation myocardial infarction (STEMI) and non-ST-segment elevation myocardial infarction (NSTEMI). In STEMI, the infarct-related artery is usually totally occluded by fibrin-rich clots, and immediate reperfusion therapy is the initial approach. In contrast, the initial conservative strategy or the initial invasive strategy can be taken in patients with NSTEMI whose infarct-related artery is partially occluded by platelet-rich clots. Prompt diagnosis is critical to controlling the development of AMI and initiating appropriate therapy to reduce the mortality rate and improve prognosis. ECG is significant to differentiate the two AMI types. However, ECG has several limitations. For example, normal findings do not exclude the possibility of AMI. NSTEMI patients are often misdiagnosed because they frequently lack typical symptoms and obvious elevated ST-segment in their ECG.”

Methods

qRT-PCR

-TaqMan human microRNA assay kits are not from Qiagen.

Response: We are very sorry for our negligence and we have corrected it. “…using TaqMan human microRNA assay kits (Applied Biosystems) according to the
manufacturer’s instructions.”

-Please define the preparation of cDNA (detailed reaction with cycling/incubation conditions or according to manufacture)? What was the input of RNA (equal among 220 samples or different)?

Response: The cDNA was prepared using TaqMan MicroRNA Reverse Transcription Kit (Applied Biosystems) according to the manufacturer’s instructions. The 15µL RT reaction mix contains 0.3 µL of 100mM dNTPs, 3 µL of MultiScribe Reverse Transcriptase (50 U/µL), 1.5 µL of 10×RT buffer, 0.19 µL of RNase inhibitor (20 U/µL), 6 µL of RT primer, 3µL of RNA sample (180ng/ul) and 1.01 µL of Nuclease-free water. For RNA from plasma samples, the concentration was diluted to 180ng/µL. The reagent mixes were incubated at 16 °C for 30 min, 42 °C for 30 min and 85 °C for 5 min.

-How the reaction of qPCR was performed (detailed reaction with cycling/incubation conditions)? Was the cDNA diluted or un-diluted? Was the one-step or two-step real time PCR performed? Based on what did you choose U6 as an internal control? Did you perform efficiency analysis? Further, according to Livak et al, Methods, 2001, 2-δCt may be used to analyze relative gene expression data when only one gene is being studied. For an example, when you analyze differences before and after i.e. treatment for reference gene, to determine the effect of treatment on the expression of a candidate internal control gene, where the target gene and the endogenous reference gene are only in the same. It would be therefore useful to perform detailed description of equation or define the reference, where the above equation is described as possible to use also in case of target gene and reference gene. Another consideration is using this equation, in different samples you have used different amounts of total RNA, which can be affected by efficiency (when is not the same for target and reference gene) that was not performed.

Response: The 20 µL PCR reaction mix includes 1.0µL 20×TaqMan MicroRNA Assays, 0.16 µL RT product, 10 µL TaqMan Universal Master Mix Ⅱ, No AmpErase UNG (2×),
8.84 µL Nuclease-free water. The PCR cycles consisted of an initial denaturation at 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s and 60 °C for 60 s. The cDNA was un-diluted. We performed one-step real time PCR. Spike-in method using cel-miR-39 for evaluating the levels of circulating miRNAs that the referee’s recommend is really excellent. While U6 snRNA is also used widely as an internal control and we just had the internal control U6 snRNA during the experiments in our lab. We will follow your suggestion and adopt the spike-in method in the future studies. Both the $2^{-\Delta\Delta Ct}$ method and the $2^{-\Delta Ct}$ method may be used to analyze relative gene expression data, but we chose the $2^{-\Delta Ct}$ method.

Statistical analysis

-“... to asses distinguishing AMI.” From what?
  
  Response: We have revised the sentence. “... to assess distinguishing AMI from healthy controls.”

Results

Circulating miR-486 and miR-150 expression levels as predictors of AMI

-Last paragraph first sentence. It is not clear which value is for certain miRNA.
  
  Response: We have revised the sentence follow your comment. “The AUC of miR-486 or miR-150 in AMI patients was 0.731 (P<0.001), 0.678 (P<0.001).”

Pattern of expression pattern of miR-486 and miR-150 in STEMI and NSTEMI

-One word “pattern” should be removed from title. Last two sentences: again, it is not clear which value is for certain miRNA or condition.
  
  Response: We have revised the manuscript according to your comments.
  “Expression pattern of miR-486 and miR-150 in STEMI and NSTEMI” “These data suggested miR-486 or miR-150 were highly sensitive and specific for the discrimination of NSTEMI cases from controls, and the combination of the two miRNAs was shown to predict NSTEMI with even higher power.”

Discussion
Third paragraph, first sentence. You have obtained RNAs from plasma; however, they have obtained from serum. Please, include this. First and forth sentence should be part of introduction for better understanding of the aims of your study.

Response: We have revised the manuscript according to your comment. The details were in manuscript. “...ECG is significant to differentiate the two AMI types. However, ECG has several limitations. For example, normal findings do not exclude the possibility of AMI. NSTEMI patients are often misdiagnosed because they frequently lack typical symptoms and obvious elevated ST-segment in their ECG...” “It has been reported that miR-486 is a potent modulator in cardiac/skeletal muscle and miR-150 is involved in many cardiovascular diseases [27]...”

Last paragraph. Including some other expression patterns/function of miR-150 in other cardiac disease (I believe there are approx. 10 publications) would improve the discussion.

Response: Thank you for your comment. We have revised the discussion. “miR-150, an miRNA related to inflammation, was reported to be implicated in the pathogenesis of various cardiovascular diseases [36-38]. A microarray screen of plasma samples showed reduced levels of miR-150 in patients with pulmonary arterial hypertension compared to healthy controls. Moreover, plasma miR-150 level in these patients was a significant predictor of survival [39]. miR-150 was also dysregulated in serum of patients with unstable angina pectoris. The diagnostic accuracy for unstable angina pectoris was obviously improved by applying the miRNA panel including miR-132, miR-150, and miR-186 [40]. Furthermore, miR-150 may inhibit cardiac structural and functional remodeling during ischemic injury partly by direct repression of the pro-apoptotic gene egr2 and p2x7r (pro-inflammatory ATP receptor) in cardiomyocytes [41]. In addition, the transcription factor c-Myb, NOTCH3 receptor, and nonmetastatic melanoma protein B were the potential targets of miR-150.”

Conclusion
-Why therapeutic target from your results?

Response: Thank you for your comment. We have revised this sentence. “It is possible that miR-486 and miR-150 could be suitable biomarkers against AMI.”

Referee 3:

1. The abbreviation “PCI” must be explained when first mentioned in the text, despite being commonly used.

Response: We have corrected the mistake according to your comments.

“Patients with previous MI or percutaneous coronary intervention (PCI) ...”

2. Line 159-161: the sentence about microRNAs which “might be suitable for use as biomarkers in the diagnosis of AMI” does not fit in the “Results”. This is mentioned in the Discussion.

Response: Thank you for your comment. We have revised this sentence.

“These data suggested that the combination of circulating miR-486 and miR-150, which both had both high sensitivity and specificity, might be more suitable than miR-486 or miR-150 alone for diagnosing AMI.”

3. The conclusion that the analysed microRNAs could be suitable therapeutic targets is too strong. There is long way before a microRNA that has been found to be elevated in a certain disease, can become a therapeutic target.

Response: Thank you for your comment. We have revised this sentence. “It is possible that miR-486 and miR-150 could be suitable biomarkers against AMI.”