Biochemical and Biophysical Research Communications 427 (2012) 280–284

Contents lists available at SciVerse ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

A subset of circulating microRNAs are predictive for cardiac death after discharge for acute myocardial infarction ☆

Sen Matsumoto [a,1], Yasuhiko Sakata [a,b,c,1,*], Daisaku Nakatani [a,1], Shinichiro Suna [a,1], Hiroya Mizuno [a,b,1], Masahiko Shimizu [a,1], Masaya Usami [a,1], Tatsuya Sasaki [d,1], Hiroshi Sato [e,1], Yukio Kawahara [f,1], Toshimitsu Hamasaki [g,1], Shinsuke Nanto [a,b,1], Masatsugu Hori [h,1], Issei Komuro [a,1]

[a] Department of Cardiovascular Medicine, Osaka University Graduate School of Medicine, Suita, Japan
[b] Department of Advanced Cardiovascular Therapeutics, Osaka University Graduate School of Medicine, Suita, Japan
[c] Department of Evidenced-Based Cardiovascular Medicine, Tohoku University Graduate School of Medicine, Sendai, Japan
[d] Cardiovascular Division, Osaka Minami Medical Center, National Hospital Organization, Kawachinagano, Japan
[e] School of Human Wilfare Studies Health Care Center and Clinic, Kwansei Gakuin University, Nishinomiya, Japan
[f] Laboratory of RNA Function, Osaka University Graduate School of Medicine, Suita, Japan
[g] Department of Biomedical Statistics, Osaka University Graduate School of Medicine, Suita, Japan
[h] Osaka Medical Center for Cancer and Cardiovascular Diseases, Osaka, Japan

ARTICLE INFO

Article history:
Received 24 August 2012
Available online 17 September 2012

Keywords:
Acute myocardial infarction
Circulating microRNAs
Secondary prevention

ABSTRACT

To investigate the prognostic impact of circulating microRNAs (miRs) in patients who survived acute myocardial infarction (AMI), we compared the circulating miR signature at the time of survival discharge among samples in the serum bank of the Osaka Acute Coronary Insufficiency Study. Using a high-throughput array consisting of 667 miRs, 11 miRs were found to be differentially expressed in the serum among patients at high-risk for cardiac death. Real-time RT-PCR confirmed that the serum levels of miR-155 and miR-380* were approximately 4- and 3-fold higher, respectively, in patients who experienced cardiac death within 1 year after discharge. Accordingly, a subset of circulating miRs might be predictive for cardiac death in post-AMI patients.

1. Introduction

MicroRNAs (miRs) are small endogenous noncoding RNAs that regulate gene expression by targeting the degradation or translational repression of mRNA. Recently, it has been demonstrated that circulating miRs in the blood are useful biomarkers for cardiovascular disease [1] as well as certain forms of cancer [2]. For example, Wang et al. [3] reported that miR-208a is an excellent diagnostic marker for AMI, as demonstrated by its sensitive detection in AMI patients within 4 h of the onset of symptoms. The authors also revealed that miR-208a had high sensitivity and specificity for diagnosing AMI by receiver operating characteristic curve analysis [4]. Kuwabara et al. [5] recently reported that circulating miR-133a serves as a useful marker for cardiomyocyte death and thus, can be used for the detection of several cardiovascular diseases, including acute myocardial infarction (AMI), and unstable angina, and takotsubo cardiomyopathy.

In patients with malignancy, the usefulness of circulating serum miRs as markers for prognosis and diagnosis has been established for several types of cancers. Few reports, however, have examined the predictive value of serum miRs in the field of cardiovascular medicine, particularly in the setting of secondary prevention after AMI. Here, we therefore investigated whether circulating miRs collected during the convalescent stage of AMI could predict cardiac death in post-AMI patients registered in the Osaka Acute Coronary Insufficiency Study (OACIS) database.

2. Materials and methods

2.1. OACIS registry

The OACIS is a prospective, multicenter observational study enrolling consecutive AMI patients in 25 collaborating hospitals from the Osaka region of Japan, and is registered with the

Abbreviations: ACEI, angiotensin-converting enzyme inhibitor; AMI, acute myocardial infarction; ARB, angiotensin receptor blocker; CI, confidence interval; HR, hazard ratio; miR, microRNA.

☆ This work was supported by Grants-in-Aid for University and Society Collaboration (#19590816 and #19390215) from the Japanese Ministry of Education, Culture, Sports, Science and Technology, Tokyo, Japan. The authors declare no relationships with industry.

* Corresponding author. Address: Department of Cardiovascular Medicine, Osaka University Graduate School of Medicine, 2-2 Yamadaoka, Suita 565-0871, Japan. Fax: +81 6 6879 6613.
E-mail address: sakatayk@cardiology.med.osaka-u.ac.jp (Y. Sakata).

[1] On behalf of The Osaka Acute Coronary Insufficiency Study (OACIS) Group (listed in the Appendix A).

0006-291X/$ - see front matter © 2012 Elsevier Inc. All rights reserved.
http://dx.doi.org/10.1016/j.bbrc.2012.09.039

University Hospital Medical Information Network Clinical Trials Registry, Japan (ID: UMIN000004575). A detailed description of the OACIS has been published elsewhere [6]. The present study protocol was approved by the ethics committee of each participating hospital.

2.2. Patients

Among 8603 patients with AMI who were registered in the OACIS between 1998 and 2009, we firstly selected 4160 consecutive patients fulfilling the following criteria: (1) discharged alive and (2) provided written informed consent for serum analysis at the time of registration. Among the selected patients, 60 cardiac deaths occurred after discharge. In the discovery phase, we randomly selected 7 patients who died of cardiac cause within a year after discharge and another 7 patients who did not experience any cardiovascular events during a 3-year follow-up period using propensity score-based matching of age, gender, classical coronary risk factors, infarction size, reperfusion therapy, and medical treatment at discharge. In the validation phase, we increased the number of patients in the cardiac death and survival groups to 19 and 21, respectively.

2.3. Serum collection

At each hospital, fasting blood samples were collected into serum separator tubes, which were then centrifuged at 1430g for 15 min at 4 °C to separate the clots. Serum was removed from the tubes and stored at −80 °C until the time of the assay.

2.4. RNA isolation and miR analysis

Total RNA was isolated from 1 ml of serum using a mirVana Paris kit (Life Technologies Co., Carlsbad, CA). Reverse transcription and preamplification steps were performed with a TaqMan MicroRNA RT kit (Life Technologies Co.) and Megaplex Primers (Life Technologies Co.). To identify miRs that could serve as predictive markers of cardiac death at 1 year, the expression levels of 667 miRs were compared between groups using TaqMan Human MicroRNA A and B Arrays, version 2.0 (Life Technologies Co.) (discovery phase). To confirm the results from the discovery phase, the expression levels of candidate miRs were examined by real-time PCR using a 7900HT Fast Real-Time PCR system (validation phase).

2.5. Data collection

Research cardiologists and trained research nurses or coordinators recorded data concerning sociodemographic variables, medical history, therapeutic procedures, and clinical events during patients' hospital stays. Clinical data after discharge were obtained at 3 and 12 months after the onset of AMI, and annually thereafter. The incidence of cardiac death was the clinical endpoint of the study.

2.6. Statistical analysis

To adjust for potential confounding factors, we selected two groups for the discovery and validation phases using a propensity score-based method. Briefly, a propensity score for cardiac death within 1 year after discharge was calculated using logistic regression analysis that included age, gender, diabetes mellitus (DM), hypertension (HT), dyslipidemia, smoking, previous MI, Killip class $\geqslant$ II at admission, infarction size, reperfusion therapy, and medication at discharge (ACEI or ARB, and statin) as variables. For the analysis, we first selected seven patients who died of cardiac cause within 1 year after discharge and another seven patients who did not experience any cardiovascular events during a 3-year follow-up period. We then selected 19 patients who died of cardiac cause after discharge and 21 patients who did not experience any cardiovascular events during a 2-year follow-up period. For the two sets of groups, patient backgrounds were compared using the χ^2 test. Expression levels of miRs between the two groups were analyzed by the Mann–Whitney U test. Associations were considered significant if the p value was <0.05. All statistical analyses were performed using SPSS software (SPSS Japan, Inc., Tokyo, Japan).

3. Results

3.1. Discovery phase

To investigate whether serum miRs could predict prognosis in the convalescent stage of AMI, we compared circulating miR signatures at the time of survival discharge using the OACIS serum bank. As shown in Table 1, patient backgrounds were well matched between patients who died of cardiac cause within 1 year after discharge ($N = 7$) and those who did not experience any cardiovascular events during the 3-year follow-up period ($N = 7$) in the discovery phase. High-throughput array analysis revealed

Table 1
Baseline characteristics in the discovery phase.

Variable	Cardiac death ($N = 7$)	Event free ($N = 7$)	p Value
Age (years)	68 ± 8	67 ± 7	0.810
Men (%)	86	71	1.000
Diabetes mellitus (%)	57	29	0.592
Hypertension (%)	83	57	0.559
Dyslipidemia (%)	71	57	1.000
Smoking (%)	57	86	0.559
Previous MI (%)	14	0	1.000
Peak CK $\geqslant$ 3000 IU/L (%)	14	43	0.559
Killip class $\geqslant$ II on admission (%)	43	43	1.000
Reperfusion therapy (%)	100	100	–
ACEI or ARB (%)	71	67	1.000
Statin (%)	57	67	1.000

ACEI: angiotensin-converting enzyme inhibitor, ARB: angiotensin receptor blocker, CPK: creatinine phosphokinase, MI: myocardial infarction.

Upregulated miRs	**hsa-miR-134, hsa-miR-155, hsa-miR-18a, hsa-miR-192, hsa-miR-380***
Downregulated miRs	**hsa-miR-125a-5p, hsa-miR-212, hsa-miR-331-3p, hsa-miR-223*, hsa-miR-190b, hsa-miR-93***

Fig. 1. High-throughput array analysis revealed that the levels of 5 miRs were increased and those of 6 miRs were decreased in the cardiac death group.

Table 2
Baseline characteristics in the validation phase.

Variable	Cardiac death ($N = 19$)	Event free ($N = 21$)	p Value
Age (years)	72 ± 12	69 ± 10	0.467
Men (%)	74	76	1.000
Body mass index (kg/m^2)	24.4 ± 3.51	23.0 ± 3.50	0.227
Diabetes mellitus (%)	53	62	0.750
Hypertension (%)	83	76	0.702
Dyslipidemia (%)	47	48	1.000
Smoking (%)	53	71	0.328
Previous MI (%)	21	20	1.000
Onset to admission time <24 h (%)	72	75	1.000
Peak CK ⩾ 3000 IU/L (%)	42	44	1.000
Killip class ⩾ II on admission (%)	41	29	0.502
Reperfusion therapy (%)	95	100	0.475
Multivessel disease (%)	63	52	0.538
ACEI or ARB (%)	74	60	0.501
Beta blocker (%)	68	60	0.741
Statin (%)	42	60	0.343
Antiplatelet therapy (%)	95	100	0.487

ACEI: angiotensin-converting enzyme inhibitor, ARB: angiotensin receptor blocker, CPK: creatinine phosphokinase, MI: myocardial infarction.

that 11 miRs were differently expressed between the two patient groups. The identified miRs were selected as initial candidates for the validation study (Fig. 1).

3.2. Validation phase

In the validation phase (cardiac death group, $N = 19$; and survival group, $N = 21$), real-time RT-PCR confirmed that 2 out of 11 miRs identified in the discovery phase were increased in the cardiac death group ($N = 19$) as compared with the survival group ($N = 19$). The serum levels of miR-155 and miR-380* were approximately 4- and 3-fold higher, respectively, in the cardiac death group, whereas the serum levels of the other 9 miRs differentially expressed in the discovery phase analysis were comparable between the two groups (Table 2, Fig. 2).

4. Discussion

To our knowledge, this is the first study to investigate whether circulating miRs are associated with prognosis in the field of cardiovascular medicine. Specifically, we examined the association between serum levels of 667 miRs and future cardiac events in post-AMI patients and found that serum levels of miR-155 and miR-380* in the convalescent stage of AMI were higher in patients who subsequently experienced cardiac death in 1 year. Although further investigation is required to confirm the predictive value of these miRs, our findings suggest the intriguing possibility that circulating miRs can serve as prognostic biomarkers for cardiovascular diseases.

MiRs are small endogenous RNAs that play important roles in animals and plants by targeting mRNAs for degradation or translational repression [7]. Dysregulation and tissue-specific patterns of intracellular miR expression have been reported in various diseases, particularly for several types of cancers [8]. In addition, miRs appear to circulate in the blood in a relatively stable form [9], suggesting that miRs may have biological functions outside the cell and thus, can potentially serve as diagnostic or prognostic biomarkers for cancer, as well as therapeutic targets. With regard to cardiovascular diseases, however, the potential of miRs as diagnostic markers has only recently been proposed [1], and few prognostic features or therapeutic potentials of circulating miRs have been reported.

In the present study, we found that the serum levels of miR-155 and miR-380* at the time of discharge after AMI were approximately 4- and 3-fold higher in patients who subsequently died of cardiac cause within 1 year of discharge than those in patients who did not experience cardiovascular events during the 3-year follow-up period.

This observation is of clinical significance, because it indicates serum miRs have the potential to predict prognosis in patients with cardiovascular disease, and also suggests that these miRs have the potential to be directly involved in future treatment approaches. Unlike studies investigating patients with malignancy [10], previous studies failed to identify miRs detected in ACS patients as therapeutic targets, possibly because such miRs were likely released into the circulation as a result of myocardial necrosis.

Although the underlying mechanism for the association between elevated serum levels of miR-155 and the increased risk for cardiac death after survival discharge of AMI is unclear, several explanations are possible. For example, Martin et al. [11] recently demonstrated that miR-155 directly interacts with the 3′-untranslated region of angiotensin II type 1 receptor (AT1R) mRNA, thereby modulating expression of AT1R and angiotensin II-induced extracellular signal-related kinase 1/2 (ERK1/2) activation. In addition, the expression levels of miR-155 are increased by angiotensin II in atherosclerotic cells *in vitro* (data not shown). This finding suggests that serum miR-155 levels may be increased through activation of the renin angiotensin system and thus, be associated with prognosis in post-AMI patients. Another possibility for elevated miR-155 in serum is as a result of inflammation. Yao et al. [12] reported that miR-155 is processed from BIC, a non-coding transcript that is highly expressed in both activated B and T cells, and monocytes/macrophages. Therefore, serum miR-155 levels might be increased following activation of monocytes/macrophages, which could lead to cardiovascular events. Similarly, elevated levels of serum miR-380* might reflect activation of p53 in failed myocardium, because miR-380-5p is reported to repress p53 expression via a conserved sequence in the p53 3′-untranslated region [13]. As up-regulation of the p53 pathway is one of the major causes for the development of heart failure in mouse models of pressure-overload and AMI [14], miR-380* might be secreted into the circulation from p53 up-regulated myocardium as a negative feedback loop of the p53 pathway, and thus be associated with prognosis after AMI.

Several limitations of this study warrant mention. First, this was a retrospective analysis using a small sample size of AMI patients selected from a prospective observational study. Second, our

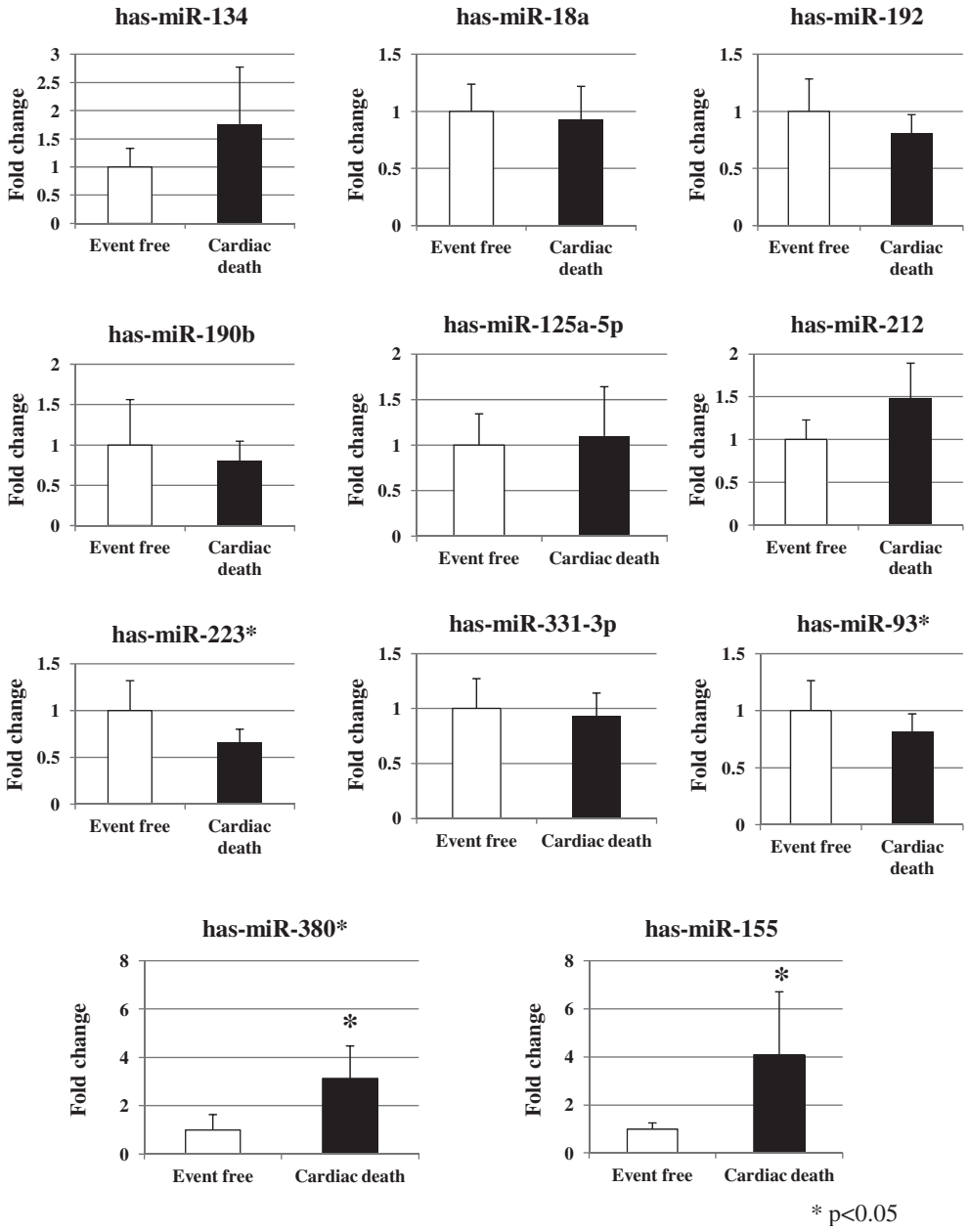

Fig. 2. Serum levels of miR-155 and miR-380* were approximately 4- and 3-fold higher, respectively, in the cardiac death group, whereas the serum levels of the 9 other examined miRs were between the two groups.

analysis was unable to detect a direct cause-effect relationship between the elevation of serum miR levels and cardiac death in post-AMI patients. Due to these limitations, further studies are warranted to confirm the present results.

Acknowledgments

We express our sincere appreciation to Mariko Kishida, Rie Nagai, Nanase Muraoka, Hiroko Takemori, Akiko Yamagishi, Kumiko Miyoshi, Chizuru Hamaguchi, Hiroko Machida, Mariko Yoneda, Nagisa Yoshioka, Mayuko Tomatsu, Kyoko Tatsumi, Tomoko Mizuoka, Shigemi Kohara, Junko Tsugawa, Junko Isotani, and all other OACIS research coordinators and nurses for their excellent assistance with data collection.

Appendix A

A.1. The OACIS Head Office

Chair: Issei Komuro, Department of Cardiovascular Medicine, Osaka University Graduate School of Medicine, 2-2 Yamada-oka, Suita 565-0871, Japan.

Secretariats: Yasuhiko Sakata (Chief), Daisaku Nakatani, Shinichiro Suna, Masaya Usami, Sen Matsumoto, Masahiko Hara, Mariko Kishida, Rie Nagai: Department of Cardiovascular Medicine, Osaka University Graduate School of Medicine, Suita, Japan.

Statisticians: Toshimitsu Hamasaki, Kenichi Hayashi, Toshifumi Sugitani: Department of Biomedical Statistics, Osaka University Graduate School of Medicine, Suita, Japan.

A.2. The OACIS Investigators (listed in alphabetical order for institutions)

Yoshiyuki Kijima, Yusuke Nakagawa, Minoru Ichikawa, Higashi-Osaka City General Hospital, Higashi-Osaka, Japan; Young-Jae Lim, Shigeo Kawano, Hideyuki Nanmori, Kawachi General Hospital, Higashi-Osaka, Japan; Hiroshi Sato, Kwasnsei Gakuin University, Nishinomiya, Japan; Takashi Shimazu, Hisakazu Fuji, Kazuhiro Aoki, Kobe Ekisaikai Hospital, Kobe, Japan; Masaaki Uematsu, Yoshio Ishida, Tetsuya Watanabe, Masashi Fujita, Masaki Awata, Kansai Rosai Hospital, Amagasaki, Japan; Michio Sugii, Meiwa Hospital, Nishinomiya, Japan; Masatake Fukunami, Takahisa Yamada, Takashi Morita, Osaka General Medical Center, Osaka, Japan; Shinji Hasegawa, Nobuyuki Ogasawara, Osaka Kosei Nenkin Hospital, Osaka, Japan; Tatsuya Sasaki, Yoshinori Yasuoka, Kiyoshi Kume, Osaka Minami Medical Center, National Hospital Organization, Kawachinagano, Japan; Hideo Kusuoka, Yukihiro Koretsune, Yoshio Yasumura, Keiji Hirooka, Osaka Medical Center, National Hospital Organization, Osaka, Japan; Masatsugu Hori (previous Chair), Osaka Prefectural Hospital Organization Osaka Medical Center for Cancer and Cardiovascular Diseases; Kazuhisa Kodama, Yasunori Ueda, Kazunori Kashiwase, Mayu Nishio, Osaka Police Hospital, Osaka, Japan; Yoshio Yamada, Jun Tanouchi, Masami Nishino, Hiroyasu Kato, Ryu Shutta, Osaka Rosai Hospital, Sakai, Japan; Shintaro Beppu, Hiroyoshi Yamamoto, Osaka Seamens Insurance Hospital, Osaka, Japan; Issei Komuro, Shinsuke Nanto, Yasushi Matsumura, Satoru Sumitsuji, Yasuhiko Sakata, Shungo Hikoso, Daisaku Nakatani, Masahiro Kumada, Michihiro Takeda, Shinichiro Suna, Osaka University Graduate School of Medicine, Suita, Japan; Toru Hayashi, Yasuji Doi, Ken-ichiro Okada, Noritoshi Ito, Saiseikai Senri Hospital, Suita, Japan; Kenshi Fujii, Katsuomi Iwakura, Atsushi Okamura, Motoo Date, Yoshiharu Higuchi, Koji Inoue, Sakurabashi Watanabe Hospital, Osaka, Japan; Noriyuki Akehi, Settsu Iseikai Hospital, Settsu, Japan; Eiji Hishida,Teramoto Memorial Hospital, Kawachinagano, Japan; and Shiro Hoshida, Kazuhiko Hashimura, Takayoshi Adachi, Yukinori Shinoda, Yao Municipal Hospital, Yao, Japan.

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