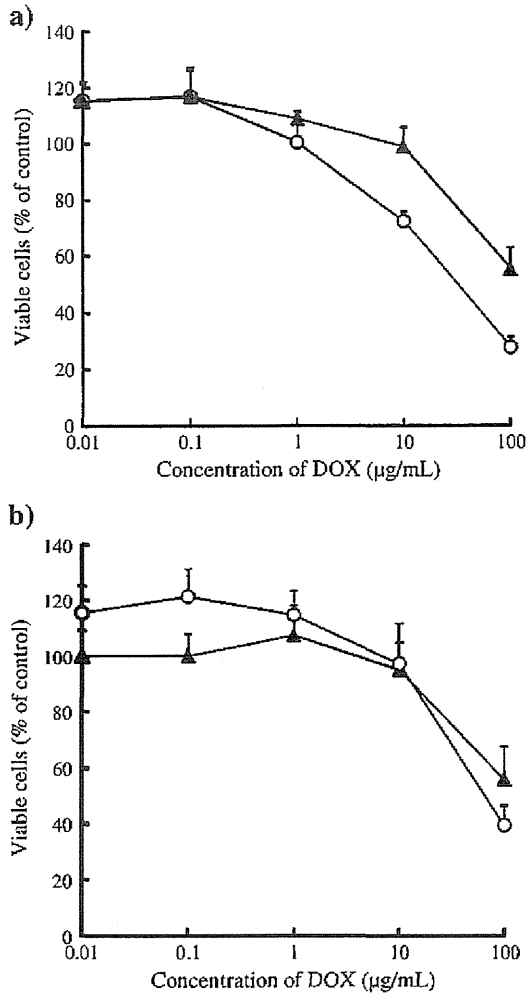

Fig. 4. Anti-proliferative effect of Ab-PEG-LipDOX on Vero-H and MDA-MB-231 cells. Vero-H (a) or MDA-MB-231(b) cells (2.5×10^3 cells/well) were seeded into a 96-well plate. Ab-PEG-LipDOX (▲) or free DOX (○) was added at the indicated concentrations (0.01, 0.1, 1, 10, and 100 μg/mL as DOX), and the cells were then incubated for 4 h at 37 °C. After having been washed with PBS, the cells were cultured in fresh medium for an additional 48 h at 37 °C. TetraColor ONE™ was then added to each well. After a 3-h incubation, the absorbance at 450 nm was measured. Data (n = 4) are presented as the percentage (mean and S.D.) of viable cells relative to the control (taken as 100%) at the indicated DOX dosages.

dose-dependent manner. Although free DOX suppressed the growth of both types of cells a little stronger than liposomal DOX, Ab-PEG-LipDOX might be expected to be effective *in vivo*. Since PEG-Lip did not show comparable association with the cells, we did not examine the effect of DOX encapsulated in PEG-Lip.

3.4. Therapeutic efficacy of DOX encapsulated in anti-HB-EGF immunoliposomes on MDA-MB-231 tumor-bearing mice

Finally, the therapeutic effect of DOX-encapsulated immunoliposomes on MDA-MB-231 solid tumors implanted subcutaneously into mice was examined. As shown in Fig. 5, both PEG-LipDOX and Ab-PEG-LipDOX strongly suppressed the tumor growth when give as 3 doses of 10 mg/kg DOX. Free DOX of this amount could not be injected due to the severe side effects. Between liposomal DOX-treated groups, PEG-LipDOX-treated group showed only a little tumor growth and the Ab-PEG-LipDOS-treated group showed tumor regression. The change in body weight was monitored as an indicator of side effects, and a decrease in body weight was observed in both liposomal DOX-treated

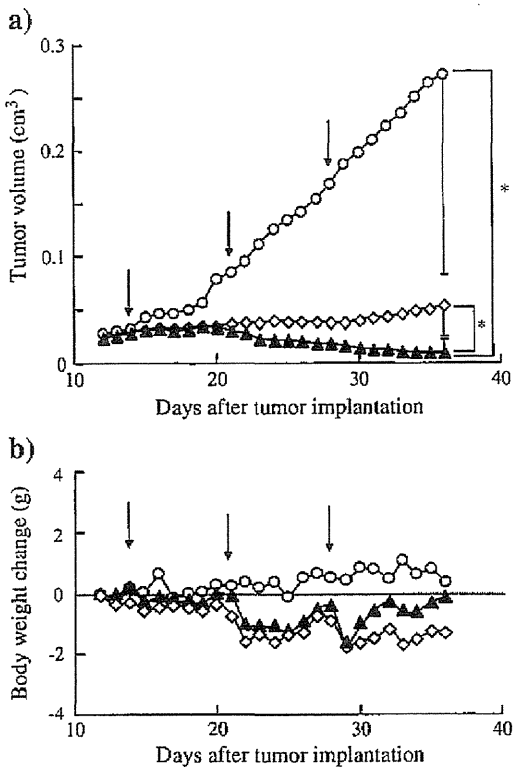

Fig. 5. Suppression of tumor growth in MDA-MB-231 carcinoma-bearing mice treated with Ab-PEG-LipDOX. BALB/C nu/nu female mice (n = 5) were implanted subcutaneously with MDA-MB-231 carcinoma into the left posterior flank. At 14, 21, and 28 days after tumor implantation, they were injected intravenously with PEG-LipDOX (◇), Ab-PEG-LipDOX (▲) or saline (○). The injected dose of liposomal DOX was 10 mg/kg as DOX for each administration. Tumor volume (a) and change in body weight (b) of the tumor-bearing mice were monitored daily after day 12. Data in "a" are presented as the mean tumor volume and S.D., where the S.D. bars are shown only for the last points for the sake of graphic clarity. Arrows show the day of treatment. Asterisks indicate a significant difference: * $p < 0.05$, as indicated by the brackets.

groups. This decrease, however, was not so much; and the body weight recovered at least by a week after the last treatment (Fig. 5b).

4. Discussion

In spite of diagnostic and therapeutic advances, cancer is still the leading cause of death in many countries. The present study focused on the treatment of human cancers by use of a HB-EGF-targeted liposomal drug delivery system, since various cancerous cells are known to frequently express this protein. For this purpose, we developed anti-HB-EGF antibody-decorated PEG liposomes encapsulating DOX. In this study, we used Fab' antibody instead of IgG, since removal of the Fc region endows antibody-decorated liposomes with a relatively long circulation time in the bloodstream by avoiding RES trapping [24]. In the present protocol, the efficiency of Fab' conjugation to the liposomal surface was about 70%, which amount was calculated to represent about 120 μg protein/μmol lipids. Since about 30 μg Fab'/μmol lipids is reported to be necessary for the function of immunoliposomes [25,26], the Ab-PEG-Lip prepared presently displayed a sufficient amount of Fab'. The size of liposomes is another important factor for deciding the pharmacokinetics of the liposomes, and about 140-nm liposomes are considered desirable for their accumulation in tumor tissue by the EPR effect [27].

Firstly the expression of HB-EGF in Vero, Vero-H, and MDA-MB-231 cells was examined at mRNA and protein levels. Extremely high expression of HB-EGF was observed in Vero-H cells that had been

constructed for overexpressing human HB-EGF. Vero cells, which are non-cancerous normal cells originally isolated from an African green monkey, also expressed HB-EGF; although the mRNA expression level was about 100-fold less than that of Vero-H cells, and 10-fold less than that of MDA-MB-231 cells. Since the anti-human HB-EGF antibody used for Western blotting is known to cross react with monkey HB-EGF, an HB-EGF band was detected in Vero cells.

By use of these cells, we determined the binding to and uptake of Ab-EGF-Lip into the cells. The cell-associated liposomes detected after incubation at 4 °C might have been mainly due to liposomes bound on the surface of the cells, whereas those detected after incubation at 37 °C would have included both bound and internalized liposomes. The binding of Ab-EGF-Lip to Vero H and MDA-MB-231 cells was very obvious compared with that to Vero cells, although Ab-EGF-Lip also bound to Vero cells to some extent. This finding suggests that the expression of endogenous HB-EGF in Vero cells was adequate for binding of the liposomes to some extent. Alternatively, part of the binding might be explained by non-specific binding of Ab-EGF-Lip via the Fab' despite its specificity. Since PEG-Lip showed little association with those cells, the anti-HB-EGF Fab'-decoration could have been responsible for the increased association of Ab-EGF-Lip with them.

DOX encapsulation into Ab-PEG-Lip was performed by the remote loading method using ammonium sulfate, since this method enables stable entrapment of DOX in the internal aqueous phase of PEG-liposomes [28] and immunoliposomes [29]. When the antiproliferative effect of Ab-PEG-LipDOX was examined, the cytotoxicity of Ab-PEG-LipDOX against Vero-H and MDA-MB-231 cells was found to be comparable to that of free DOX.

Finally, we performed a therapeutic experiment by use of MDA-MB-231 breast cancer cell-bearing mice. Both PEG-LipDOX and Ab-PEG-LipDOX strongly suppressed tumor growth. PEG-liposomes are known to accumulate in tumor tissues due to the EPR effect. Since this effect in tumor tissues is based on the leaky angiogenic vessels, hyper vascular tumors such as breast and ovarian tumors are desired targets. Therefore, PEG-liposomes encapsulating DOX were originally used for the treatment of ovarian and breast cancers and of HIV-associated Kaposi's sarcoma. The strong *in vivo* therapeutic effect of PEG-LipDOX against MDA-MB-231 tumors observed in this study is thus reasonable. Moreover, Ab-PEG-LipDOX, having both passive and active targeting characteristics, showed a stronger therapeutic effect against MDA-MB-231 tumors than PEG-LipDOX. PEG-liposomes that accumulate in tumor tissues after an intravenous injection are thought to reside mainly in the interstitial spaces in the tumor. On the other hand, decoration of them with some specific probes may alter the intratumoral distribution of the liposomes, and increase the uptake of liposomal drugs into the target cells, as observed in the present study.

As shown in Fig. 5, tumor growth inhibition was similar in both PEG-LipDOX- and Ab-PEG-LipDOX-treated groups until day 20, and differential therapeutic effect between targeted and non-targeted liposomes became obvious after second and third injection of them. We do not know the reason why the advantage of immunoliposomes was not obvious until day 20 at present. One possible explanation is as follows: The growth of MDA-MB-231 cells in vivo was not so fast, and the tumor mass was quite small at the first injection time, namely day 14. Therefore, the angiogenesis that produced leaky endothelium did not hardly occur. Moreover immune system would be still quite active that eliminate even PEGylated liposomes at this stage. In fact, although both PEG-LipDOX and Ab-PEG-LipDOX suppressed tumor growth to some extent compared to control, body weight change was not so obvious compared to that after second and third injection. At the time of second and third injection, immune systems were weakened because of tumor residing that helps the accumulation of liposomes in the tumor by EPR effect through neovessels. Since extravasation by EPR effect is prerequisite for the active targeting of immunoliposomes to the tumor cells, Ab-PEG-LipDOX thus accumulated in the interstitial space of the tumor interacted with tumor cells and produced higher therapeutic effect than PEG-LipDOX. Actually, tumor was hardly palpated in two mice out of five after the third treatment with Ab-PEG-LipDOX.

5. Conclusions

For the purpose of active targeting of anticancer drugs to cancer cells, anti-HB-EGF antibody-decorated liposomes were prepared. These immunoliposomes bound to and were taken up into not only Vero-H cells highly expressing HB-EGF but also MDA-MB-231 human breast cancer cells. Moreover, DOX-encapsulated, anti-HB-EGF antibody-decorated liposomes caused strong suppression and regression of MDA-MB-231 tumors in mice. These results indicate that anti-HB-EGF antibody-decorated liposomes could be a useful DDS carrier for the treatment of HB-EGF-expressing cancers.

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

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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.

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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 ⩾ 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 ⩾ 3000 IU/L (%)	14	43	0.559
Killip class ⩾ 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

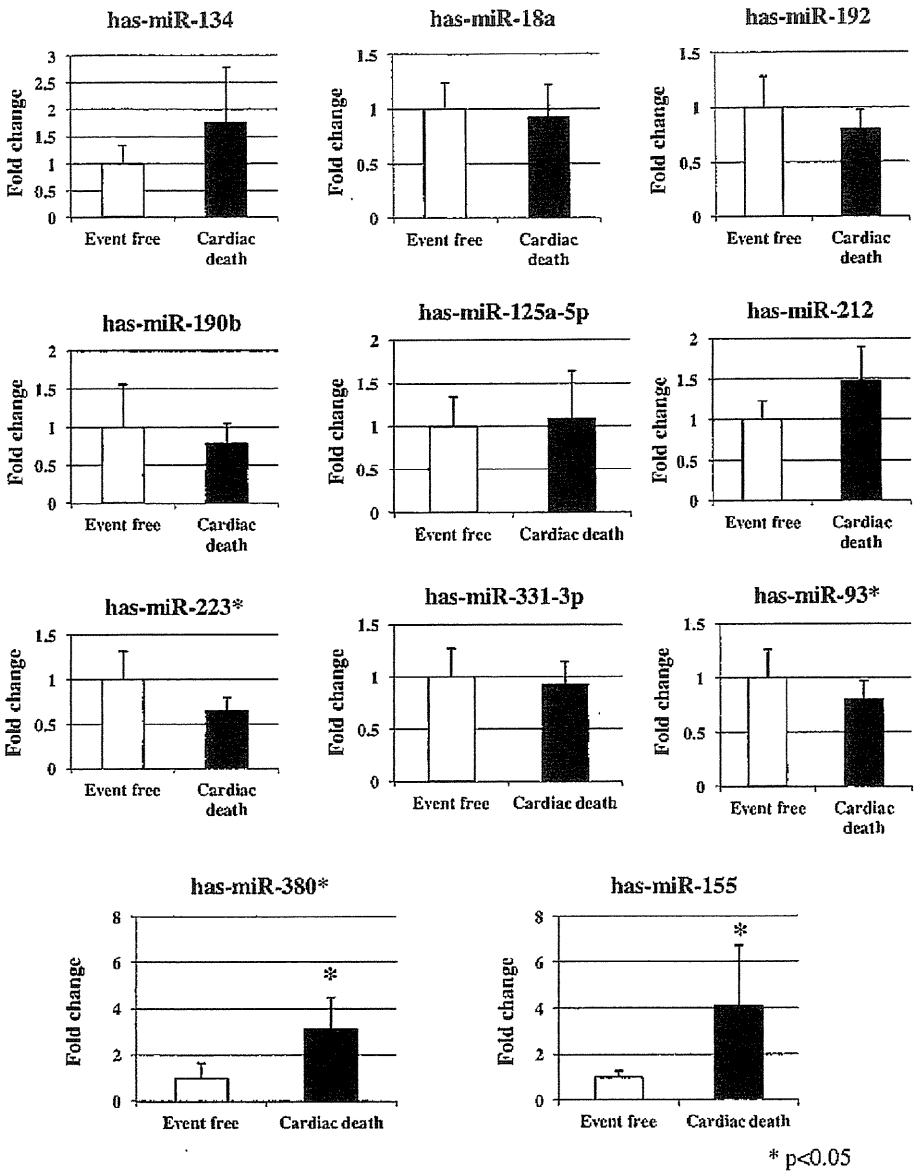

Fig. 2. Serum levels of miR-155 and miR-380* were approximately 4- and 3-fold higher, respectively, in the cardiac death group, whereas the serum levels of the 9 other examined miRs were between the two groups.

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.

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Appendix A

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Original article

Oral treatment with nicorandil at discharge is associated with reduced mortality after acute myocardial infarction

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KEYWORDS
Nicorandil;
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Mortality;
Secondary prevention

Summary
Background: Previous studies showed that nicorandil can reduce coronary events in patients with coronary artery disease. However, it is unclear whether oral nicorandil treatment may reduce mortality following acute myocardial infarction (AMI).
Methods and Results: We examined the impact of oral nicorandil treatment on cardiovascular events in 1846 AMI patients who were hospitalized within 24 h after AMI onset, treated with emergency percutaneous coronary intervention (PCI), and discharged alive. Patients

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were divided into those with (Group N, $n = 535$) and without (Group C, $n = 1311$) oral nicorandil treatment at discharge. No significant differences in age, gender, body mass index, prevalence of coronary risk factors, or history of myocardial infarction existed between the two groups; however, higher incidences of multi-vessel disease, and a lower rate of successful PCI were observed in Group N. During the median follow-up of 709 (340–1088) days, all-cause mortality rate was 43% lower in Group N compared with Group C (2.4% vs. 4.2%, stratified log-rank test: $p = 0.0358$). Multivariate Cox regression analysis revealed that nicorandil treatment was associated with all-cause death after discharge (Hazard ratio 0.495, 95% CI: 0.254–0.966, $p = 0.0393$), but not for other cardiovascular events such as re-infarction, admission for heart failure, stroke and arrhythmia.
Conclusions: The results suggest that oral administration of nicorandil is associated with reduced incidence of death in the setting of secondary prevention after AMI.

Introduction

Although recent progress in the management of acute myocardial infarction (AMI) has decreased mortality [1–3], long-term mortality remains high in post-AMI patients [4]. To further decrease mortality rates in the clinical setting after AMI, numerous efforts have been directed towards the pharmacological modification of left ventricular (LV) performance and remodeling, as well as stabilization of atherosclerotic coronary plaques, as it has been shown to be associated with prognosis [5]. In this context, the anti-anginal drug nicorandil is one of the promising candidates for improving outcomes of post-AMI patients due to its cardioprotective properties [6–19].

Nicorandil is a nicotinamide ester that possesses K-ATP channel-activating and nitrate-like properties and is being increasingly used to treat coronary artery disease (CAD). Nicorandil relieves symptoms of ischemia and also has numerous cardioprotective properties, such as pharmacological preconditioning [6–8], restoration of cardiac blood flow to ischemic and no-reflow myocardium [9–13], prevention of Ca^{2+} overload [14,15], and attenuation of cardiac sympathetic nerve injury [16–18], and so on. However, although the effects of nicorandil in protection of the myocardium during acute ischemic injury have been extensively reported in the clinical setting [9–13,17,20–25], little is known about the long-term impacts of nicorandil on mortality and secondary complications after AMI [18,26].

In the present study, we examined the mortality impact of oral nicorandil at discharge using a relatively large patient cohort in the setting of secondary prevention after AMI.

Methods

The Osaka acute coronary insufficiency study (OACIS)

The Osaka acute coronary insufficiency study (OACIS) is a prospective, multi-center observational study designed to collect and analyze demographic, procedural, outcome data, and blood samples in patients with AMI at 25 collaborating hospitals in the Osaka region of Japan [3,27–30]. As part of the OACIS, research cardiologists and specialized research nurses recorded data on socio-demographic variables, medical histories, therapeutic procedures, and clinical events during patient hospitalization, and also obtained follow-up clinical data at 3, 6, and 12 months after the occurrence of AMI, and annually thereafter. Information was obtained from hospital medical records and by direct interviews with patients, their family members, and their treating physicians. All data were transmitted to the data collection center at the Department of Cardiovascular Medicine, Osaka University Graduate School of Medicine, Suita, Japan for processing and analysis. The diagnosis of AMI required the presence of two of the following three criteria: (1) history of central chest pressure, pain, or tightness lasting more than 30 min, (2) ST-segment elevation 0.1 mV in 1 limb lead or 2 precordial leads, and (3) an increase in serum creatine kinase (CK) concentration of two times the upper limit of normal.

Patients

Among the patients registered with the OACIS registry, 1846 consecutive patients fulfilling the following criteria: (1) admission within 24 h after the onset of AMI between January 2005 and March 2009, (2) treatment with emergency percutaneous coronary intervention (PCI) on admission, and (3) survival discharge, were enrolled in the study. Of these patients, 535 were treated with oral nicorandil at discharge (Group N), while the remaining 1311 patients did not receive nicorandil at discharge (Group C).

Clinical endpoints

The demographic and clinical data and the primary endpoints during the five-year period following discharge, all-cause mortality, non-fatal re-infarction, re-admission for heart failure, and coronary revascularization, including PCI and coronary artery bypass grafting, were compared between patients in Groups N and C.

Statistical analysis

Results are expressed as medians (25th and 75th percentiles) or mean ± SD for continuous variables, and qualitative data are presented as numbers or percentages. Differences of continuous variables between groups were assessed using the Student's *t*-test, whereas categorical variables were compared using the chi-square test. Factors influencing mortality were analyzed using a multivariate Cox proportional hazard regression model with 14 variables from major patient backgrounds and treatments to minimize

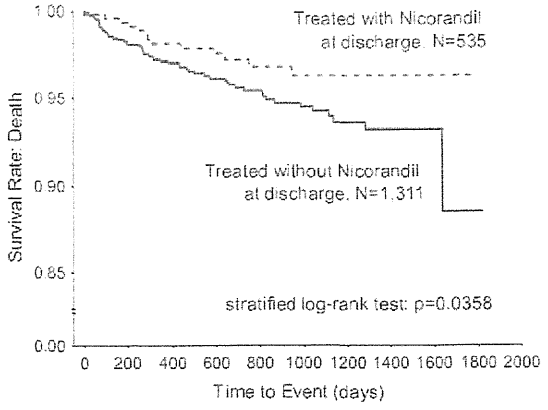

Figure 1 Kaplan–Meier plots for mortality during the five-year follow-up period following discharge for AMI. *p* value after adjustment with variables that had *p* values of <0.1 in the multivariate cox proportional hazard model is <0.05.

the effect of co-founders. Variables included in the model were age, gender, obesity, diabetes, hypertension, dyslipidemia, smoking, multi-vessel disease, PCI success, and prescription of statins, renin angiotensin system inhibitors, or beta-blockers. Survival curves were constructed using the Kaplan–Meier method, and the significance of differences in survival was assessed using the stratified log-rank test with variables as strata suggested by Cox regression. Analysis was performed using SAS version 9.1.3 for Windows (SAS Inc., Cary, NC) and PASW Statistics, version 18.0 (SPSS Inc., Chicago, IL). For all analyses, statistical significance was set at $p < 0.05$.

Results

The study population consisted of 535 patients who received nicorandil at discharge (Group N) and 1311 patients who did not (Group C). The patient baseline characteristics, including the cardiovascular medications being taken before and during the study, are summarized in Table 1. No significant differences in age, gender, body mass index, prevalence of coronary risk factors of diabetes, hypertension, dyslipidemia, obesity and smoking, or history of myocardial infarction were found between the two groups. However, a higher incidence of multi-vessel disease and treatment with intra-aortic balloon pumping, and a lower rate of successful PCI, defined as presence of TIMI3 flow grade after the procedures, were noted in Group N, suggesting that Group N included patients with more severe clinical conditions.

The median follow-up period was 709 (340–1088) days. During the follow-up period, the all-cause mortality rate was 43% lower in Group N compared with Group C, although this difference was not significant (2.4% vs. 4.2%, log-rank test: $p = 0.0849$). However, multivariate Cox proportional hazard analysis revealed that several variables were correlated with mortality following discharge of the AMI patients. After adjustment with the variables, Kaplan–Meier curves for mortality showed a significant difference between Groups N and C (stratified log-rank test: $p = 0.0358$, Fig. 1).

Despite the fact that patients of both groups were also administered other secondary prevention drugs, nicorandil was the only drug to have an association with decreased mortality (Table 2). Multivariate Cox proportional hazard analysis revealed that nicorandil treatment was a predictor for all-cause death after discharge (Hazard ratio (HR) 0.495, 95% CI: 0.254–0.966, $p = 0.039$), but not for re-infarction, admission for heart failure, arrhythmia and stroke (Table 3). Subgroup analysis revealed that no significant interaction were detected between the impact of nicorandil and variables of age, gender, diabetes, hypertension, dyslipidemia, multi-vessel disease, PCI success, and peak CK levels (Table 4), suggesting that nicorandil treatment displayed a significant reduction in mortality regardless of the subgrouping. In addition, subgroup analysis also suggested that nicorandil treatment was particularly associated with reduced mortality for patients with ages of <75 y.o., with hypertension, or of male gender (Table 4).

Discussion

This is the first study suggesting a mortality benefit of nicorandil for post-AMI patients in the clinical setting. In this retrospective analysis with a relatively large-scale AMI cohort, we have demonstrated that AMI patients who received oral nicorandil treatment displayed a reduction in all-cause mortality during a five-year follow-up period. Our results suggest that nicorandil may have the potential to improve survival outcomes in the setting of secondary prevention after AMI.

The most important finding of the present study is that nicorandil treatment was associated with a nearly 50% reduction in all-cause mortality following discharge for AMI (HR 0.495, 95% CI: 0.254–0.966, $p = 0.0393$). This finding also supports the results of a recent large-scale randomized control study, the Impact of Nicorandil in Angina [IONA] study [31], and a retrospective sub-analysis of the Japanese Coronary Artery Disease (JCAD) study [32], which suggested that nicorandil had a beneficial impact on mortality and morbidity in CAD patients. In the IONA study, a 20 mg twice-daily oral nicorandil treatment group ($N = 2565$) displayed a significant reduction in all cardiovascular events over a placebo group ($N = 2561$) [31]. In addition, the nicorandil group had a trend of reduced mortality compared to the placebo group (4.3% vs. 5.0%, respectively; HR 0.85, $p = 0.222$), although the difference was not statistically significant, likely due to the relatively short follow-up period (mean: 1.6 years). The JCAD study is a large multicenter collaborative prospective observational study designed to investigate risk factors, medication use, and outcomes of CAD patients in Japan ($N = 13,812$) [33]. In a retrospective analysis of the JCAD study, Horinaka et al. [32] compared the incidence of cardiovascular events between 2558 nicorandil-treated and 2558 control patients (mean follow-up: 2.7 years) in the JCAD cohort and revealed that death from all causes (primary endpoint) was 35% lower (HR 0.65, $p = 0.0008$) in the nicorandil group. Further, marked reductions in several secondary endpoints, including cardiac death (56%), fatal myocardial infarction (56%), cerebral or vascular death (71%), and congestive heart failure (33%), were noted in the nicorandil group [32]. Taken together, these lines of evidence suggest

Table 1 Patient characteristics of the nicorandil (Group N) and non-nicorandil (Group C) groups.

Variable	Group C (n = 1311)	Group N (n = 535)	p Value
Age (years)	65.7 ± 12.1	66.2 ± 11.8	0.388
Male	990 (75.5%)	411 (76.8%)	0.589
Symptom to admission time (h)	5.2 ± 5.5	4.9 ± 5.5	0.255
STEMI	1129 (86.8%)	467 (87.5%)	0.76
Killip class >1	1067 (85.3%)	429 (82.7%)	0.192
Cardiac pulmonary arrest	38 (2.9%)	12 (2.2%)	0.528
Peak creatine kinase (U/L)	2971 ± 2645	3144 ± 2634	0.223
Serum creatinine (mg/dL)	1.04 ± 1.16	1.06 ± 1.19	0.752
Common comorbidities			
Obesity	405 (32.6%)	174 (33.5%)	0.739
Diabetes mellitus	416 (31.7%)	192 (35.9%)	0.091
Hypertension	838 (65.5%)	342 (65.9%)	0.913
Dyslipidemia	557 (44.3%)	250 (48.3%)	0.142
Smoking history	786 (60.9%)	328 (62.4%)	0.595
Previous MI	149 (11.5%)	58 (11.0%)	0.807
Angiographic information			
Initial TIMI grade			0.484
0	754 (57.8%)	304 (57.3%)	
1	142 (10.9%)	54 (10.2%)	
2	246 (18.9%)	93 (17.5%)	
3	163 (12.5%)	80 (15.1%)	
Multiple vessel disease	534 (41.0%)	269 (50.5%)	
Collateral vessels	402 (31.2%)	164 (31.5%)	
Infarct related artery: LAD	552 (41.8%)	242 (46.0%)	
Reperfusion therapy			
PCI	1331 (100%)	535 (100%)	1
Stent	1225 (93.5%)	507 (94.9%)	0.282
Thrombectomy	921 (70.3%)	354 (66.2%)	0.086
Drug-eluting stent	89 (6.8%)	35 (6.8%)	0.918
PCPS	29 (2.2%)	6 (1.1%)	0.135
IABP	184 (14.0%)	97 (18.1%)	0.032
Emergent CABG	9 (0.7%)	5 (0.9%)	0.563
Temporary pacing	275 (21.3%)	101 (19.3%)	0.371
Successful reperfusion	1217 (93.6%)	481 (91.1%)	0.07
No-reflow phenomenon	36 (5.5%)	16 (6.8%)	0.517
Medications at discharge			
Anti-platelets	1289 (98.3%)	529 (98.9%)	0.529
ACEIs	419 (32.0%)	279 (52.1%)	<0.001
ARBs	647 (49.4%)	175 (32.7%)	<0.001
ACEI and/or ARB	1038 (79.2%)	435 (81.3%)	0.038
β-Blockers	813 (62.0%)	303 (56.6%)	0.036
Statin	689 (52.6%)	298 (55.7%)	0.237
Ca channel blockers	205 (15.6%)	70 (13.1%)	0.171
Nitrates	255 (19.5%)	91 (17.0%)	0.237
Diuretics	336 (25.6%)	143 (26.7%)	0.64

Results are expressed as mean ± SD for continuous variables. ACEI, angiotensin converting enzyme inhibitor; ARB, angiotensin II receptor blocker; CABG, coronary artery bypass graft; IABP, intraaortic balloon pumping; MI, myocardial infarction; PCI, percutaneous coronary intervention; PCPS, percutaneous cardio-pulmonary support; STEMI, ST elevation myocardial infarction; TIMI, thrombolysis in myocardial infarction.

that nicorandil has a beneficial effect for reducing mortality in patients with CAD, including post-AMI patients.

It is noteworthy that nicorandil treatment at discharge was associated with mortality reduction (Table 1), although nicorandil had been likely used as an adjunct to other cardioprotective drugs, rather than as an alternative, which is supported by the comparable prevalence of secondary prevention drugs between Groups N and C. Accordingly, nicorandil is potentially a good candidate to be given in an additive manner with popular cardiovascular secondary

Table 2 Predictors for death after discharge.

Variable	Multivariate cox proportional hazard model			
	HR	Lower limit	Upper limit	p Value
Statins	0.904	0.516	1.583	0.7247
RAS inhibitors	0.894	0.488	1.638	0.7166
Beta blockers	1.408	0.802	2.470	0.2332
Ca channel blockers	0.493	0.221	1.102	0.0849
Nicorandil	0.495	0.254	0.966	0.0393

Variables: age, gender, diabetes mellitus, hypertension, dyslipidemia, smoking, multi-vessel disease, successful percutaneous coronary, intervention, peak creatine kinase level, beta-blockers, renin-, angiotensin inhibitors, statins, calcium channel blockers, and nicorandil.

Table 3 Impact of oral nicorandil on cardiac events.

Cardiovascular event	HR	95% CI	p Value
Death	0.495	(0.254–0.966)	0.039
Myocardial infarction	0.873	(0.469–1.624)	0.667
Admission for heart failure	0.741	(0.410–1.338)	0.319
Arrhythmia	0.737	(0.360–1.509)	0.366
Stroke	0.363	(0.107–1.229)	0.103

Variables: age, gender, diabetes mellitus, hypertension, dyslipidemia, smoking, multi-vessel disease, successful percutaneous coronary, intervention, peak creatine kinase level, beta-blockers, renin-, angiotensin inhibitors, statins, calcium channel blockers, and nicorandil.

Table 4 Predictors for death after discharge.

Subgroup		N	HR	95% CI	p Value	p for Interaction
Age (years)	<75	1242	0.294	(0.088–0.984)	0.0471	0.2012
	≥75	435	0.817	(0.371–1.797)	0.6145	
Gender	Female	431	0.792	(0.219–2.866)	0.7224	0.4888
	Male	1394	0.474	(0.227–0.991)	0.0472	
Diabetes mellitus	No	1210	0.481	(0.182–1.269)	0.1391	0.6086
	Yes	608	0.610	(0.259–1.438)	0.2587	
Hypertension	No	618	0.802	(0.215–2.990)	0.7425	0.1672
	Yes	1180	0.429	(0.200–0.921)	0.0298	
Dyslipidemia	No	954	0.542	(0.250–1.173)	0.1199	0.6556
	Yes	805	0.308	(0.082–1.152)	0.0802	
Multi-vessel disease	No	1006	0.596	(0.205–1.733)	0.3423	0.8772
	Yes	803	0.483	(0.213–1.096)	0.0815	
PCI success	No	130	0.365	(0.041–3.291)	0.3692	0.9888
	Yes	1647	0.544	(0.277–1.069)	0.0775	
Peak CK level (U/L)	<3000	1203	0.532	(0.227–1.247)	0.1464	0.7414
	≥3000	621	0.530	(0.198–1.418)	0.2061	

CK, creatine kinase; PCI, percutaneous coronary intervention.

prevention drugs, such as beta-blockers and/or rennin-angiotensin system inhibitors. It is also noteworthy that the present study suggested that nicorandil was beneficial for all patients in the secondary prevention settings after AMI, because no significant interaction were detected after subgroup analysis (Table 4). In addition, the mortality benefit of nicorandil seemed particularly apparent for patients with ages <75 y.o., with hypertension, and of male gender (Table 4). Notably, a 71% reduction in mortality rate was found in nicorandil-treated patients with ages <75 y.o. (HR 0.294, 95% CI: 0.088–0.984, $p = 0.047$). Therefore, we suggest that nicorandil may represent a potent first-line drug for all patients after AMI, particularly for those with ages <75 y.o., male gender, or hypertension.

Although details of the mechanisms are unclear, the following pharmacologic and other properties of nicorandil may explain why this drug improves survival in patients with CAD or AMI. First, cardioprotective effects exerted by nicorandil during the acute stage of AMI and/or acute myocardial ischemia [9–13,17,20–25] may have also provided benefits in the convalescent or chronic stages of AMI. Second, as suggested in the J-WIND study [26], nicorandil treatment during the chronic phase of AMI may have improved left ventricular function, resulting in a reduction of mortality. Third, the positive effects of nicorandil on sympathetic nerve activity might have played a role in improving survival. Indeed, although it was a small sample-size study from a single center, Kasama et al. [18] reported that the long-term (six months) administration of 15 mg/dL nicorandil resulted in improved cardiac sympathetic nerve activity in AMI patients. Fourth, the anti-hypertensive properties of nicorandil might have reduced long-term mortality [34]. As discussed previously, nicorandil appeared to have been prescribed in an additive manner in the present cohort, rather than as an alternative to the other administered cardioprotective or antihypertensive drugs. Accordingly, the adjunctive use of nicorandil may have lowered blood pressure more effectively than in the control group, resulting in a reduction in long-term mortality. Finally, better long-term compliance and lack of tolerance to nicorandil [35] might be associated with improved mortality.

In the present study, we did not observe significant reductions in other cardiac events in Group N patients, unlike the results from the IONA study [31] and JCAD sub-study [32]. This discrepancy may have been due to differences in the backgrounds of the study populations; we only included patients who survived AMI in the present study, whereas the other two studies included individuals with stable angina or CAD [31,32]. Accordingly, it appears that the study cohort might have been more strictly treated with secondary prevention medications in the present study, as the prevalence of co-administered cardioprotective drugs such as antiplatelets, ACE inhibitors, angiotensin II receptor blockers (ARBs), β-blockers, and statin was higher than or at least equal to those in the IONA and JCAD studies [31,32]. In addition, the cohort subjects in the present study had all received emergent PCI for infarct related arteries and were likely to have undergone subsequent PCI for other diseased coronary arteries in the acute or convalescent stage of AMI, possibly resulting in a reduction of residual myocardial ischemia and prevention of ischemia-related cardiovascular complications. Therefore, the beneficial effects of nicorandil on cardiovascular events other than mortality may have been masked by the increased usage of co-administered cardioprotective drugs, as well as differences in the management of diseased coronary arteries.

A few limitations of the study warrant mention. First, as this was a retrospective observational study, precise information concerning the dose, duration and patient compliance for nicorandil treatment, was not available. In addition, information about cardiac function at discharge was not obtained in the present study. Second, as it was also observed that patients in Group N appeared to have more severe clinical CAD conditions, including a higher incidence of multi-vessel disease and a lower rate of successful PCI, the overall efficacy of nicorandil may have been underestimated. Third, the present study enrolled only the subjects who underwent emergent PCI in the acute stage of AMI. Accordingly, caution may be needed when interpreting the results for AMI patients who did not receive emergent PCI. Fourth, although the results suggested that nicorandil was effective to reduce mortality in those with age of <75 y.o. or those with male gender, the mechanisms were unclear and thus remain to be disclosed.

In conclusion, we have demonstrated that the oral administration of nicorandil following AMI was associated with reduced incidence of death for all patients, particularly in individuals with ages <75 y.o., male gender and hypertension. Although further randomized clinical investigations are needed, the promising clinical outcomes presented here suggest that nicorandil on oral administration may be effective for treating CAD and is expected to improve patient survival in the secondary prevention setting following AMI.

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ORIGINAL ARTICLE
Ischemic Heart Disease

Low Levels of Serum n-3 Polyunsaturated Fatty Acids Are Associated With Worse Heart Failure-Free Survival in Patients After Acute Myocardial Infarction

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Background: Intake of long-chain n-3 polyunsaturated fatty acids (n-3 PUFA), including docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA), is associated with a lower risk of atherosclerotic cardiovascular events, particularly acute myocardial infarction (AMI). However, limited data are available regarding the association between serum n-3 PUFA levels and heart failure (HF) events in survivors of AMI.

Methods and Results: We evaluated whether serum DHA and EPA levels were associated with HF-free survival and HF hospitalization rates after AMI. A total of 712 patients were divided into 3 groups according to their tertile serum levels of DHA and EPA (Low, Middle, and High). Propensity-score-stratified Cox regression analysis revealed that DHA- and EPA-Low groups presented statistically significant worse HF-free survival (hazard ratio (HR) 1.68, 95% confidence interval (CI) 1.03–2.72, P=0.0358, and HR 1.69, 95% CI 1.05–2.72, P=0.0280, respectively), with the EPA-Low group having a higher risk of HF hospitalization (HR 2.40, 95% CI 1.21–4.75, P=0.0097) than the DHA-Low group (HR 1.72, 95% CI 0.86–3.45, P=0.1224). The relationship between a low DHA or EPA level and decreased HF-free survival was almost common to all subgroups; however, the effect of low serum EPA on HF hospitalization was prominent in male patients, and those with low levels of high-density lipoprotein cholesterol or without statin therapy.

Conclusions: Low levels of circulating n-3 PUFA are associated with decreased HF-free survival in post-AMI patients. (*Circ J* 2013; 77: 153–162)

Key Words: Acute myocardial infarction; Docosahexaenoic acid; Eicosapentaenoic acid; Heart failure; n-3 polyunsaturated fatty acids

Docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA) are long-chain n-3 polyunsaturated fatty acids (n-3 PUFA) that are found in high levels in fish oil.[1,2] The n-3 PUFA are associated with a lower risk of cardiovascular events through a variety of mechanisms, such as favorable effects on lipid levels, platelets, endothelial function, blood pressure, cardiac excitability, and inflammatory cytokines.[1,2] Despite conflicting evidence,[3,4] many trials have demonstrated the beneficial effects of n-3 PUFA to reduce atherosclerotic cardiovascular events, including sudden cardiac death and acute myocardial infarction (AMI), in the primary and secondary prevention settings of cardiovascular diseases.[5–8] Limited data also

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Table 1. Clinical Background of Study Population

Parameter	All (n=712)
Follow-up duration, days	1,079 (721–1,442)
Age, years	65 (57–73)
Male, %	77.8
BMI, kg/m^2	23.9 (22.1–26.1)
STEMI, %	86.1
Coronary risk factors	
Diabetes, %	35.1
Hypertension, %	67.0
Dyslipidemia, %	50.8
Smoking, %	63.2
OMI, %	10.5
Laboratory data	
EPA, μg/ml	30.5 (21.6–44.2)
DHA, μg/ml	71.0 (54.6–91.9)
TC, mg/dl	191 (163–222)
LDL-C, mg/dl	122 (100–147)
HDL-C, mg/dl	44 (38–52)
TG, mg/dl	98 (60–153)
HbA_{1c}, %	6.1 (5.7–6.9)
eGFR, ml·min^{-1}·1.73m^{-2}	69.2 (55.1–85.1)
Peak CK, IU/L	2,207 (1,035–3,889)
Reperfusion, %	94.5
PCI, %	93.3
Medications at discharge	
Statin, %	60.5
ACEI or ARB, %	82.3
β-blocker, %	67.0
Antiplatelet, %	99.0
Ethyl icosapentate, %	2.5

ACEI, angiotensin-converting enzyme inhibitor; ARB, angiotensin 2 receptor blocker; BMI, body mass index; CK, creatine kinase; DHA, docosahexaenoic acid; eGFR, estimated glomerular filtration rate; EPA, eicosapentaenoic acid; HbA_{1c}, hemoglobin A_{1c}; HDL-C, high-density lipoprotein cholesterol; LDL-C, low-density lipoprotein cholesterol; OMI, old myocardial infarction; PCI, percutaneous coronary intervention; STEMI, ST-elevation myocardial infarction; TC, total cholesterol; TG, triglyceride.

suggest that n-3 PUFA are associated with a lower incidence of heart failure (HF), although data after AMI are lacking.[9–11] The aim of the present study was to evaluate whether serum DHA and EPA levels are associated with HF-free survival and HF hospitalization rates in AMI survivors enrolled in a multicenter prospective AMI registry in Japan.

Methods

Study Patients and Blood Sampling

The Osaka Acute Coronary Insufficiency Study (OACIS) is a prospective, multicenter observational study that enrolls consecutive patients with AMI in 25 collaborating hospitals from the Osaka region of Japan, and is registered to the University Hospital Medical Information Network Clinical Trials Registry (UMIN-CTR) in Japan (ID: UMIN000004575). Details of OACIS are reported elsewhere.[12,13] Among 2,579 patients with AMI who were registered in the OACIS between January 2006 and December 2009, we enrolled consecutive 712 patients who were discharged alive and whose blood samples were collected at least 10 days after the onset of AMI and within 14 days before and after discharge. We set the blood sampling period in order to avoid the acute phase impact of AMI (within 10 days after the onset of AMI), and tried to focus on the state of survival discharge (within 14 days before and after discharge). Patient selection flow is shown in Figure S1; we excluded 197 cases of in-hospital death, 804 cases without agreement of blood samples, and 866 cases in which samples were not obtained greater than 10 days after the onset of AMI and within 2 weeks before and after discharge from the present study in that order.

The diagnosis of AMI was based on the World Health Organization criteria using a combination of patient symptoms, electrocardiographic findings, and serum cardiac enzyme elevations. All study candidates were informed about data collection and blood sampling, and provided written informed consent. Follow-up clinical data were obtained at 3, 6, and 12 months after the onset of AMI and annually thereafter for 5 years. Fasting blood samples were collected at each local hospital. After centrifugation and prompt freezing at –80°C, serum samples were shipped to BML, Inc (Tokyo, Japan) for the measurement of DHA and EPA levels using a gas chromatography method. The study protocol complied with the Helsinki declaration and was approved by each participating institution's ethics committee.

Statistical Analysis

Categorical data are expressed as percentage and differences were analyzed by chi-square statistics. Continuous data are presented as the median (25–75 percentiles) and differences were analyzed by Kruskal-Wallis test or Tukey's test.

We set the primary endpoint as the HF-free survival rate and the secondary endpoints as HF hospitalization and all-cause death after survival discharge. The rates of these events were compared by dividing the patients into 3 groups (Low, Middle, and High) based on DHA and EPA tertile levels. The Kaplan-Meier method was used to estimate event rates, and estimated differences were compared by the log-rank test. The effects of DHA and EPA levels on the primary and secondary endpoints were assessed using Cox regression analysis by calculating hazard ratios (HR) and 95% confidence intervals (CI) of the DHA- and EPA-Low groups as compared with the Middle and High groups. Furthermore, to reduce possible confounding factors regarding patient background in the comparison, the survival function of event rates for the DHA- and EPA-Low groups was compared with the other groups by stratified log-rank test, where the strata classification was based on propensity scores.[14] Propensity scores were calculated by logistic regression analysis including the DHA- and EPA-Low groups as the response variable, and age, sex, ST-elevation MI, diabetes, hypertension, dyslipidemia, smoking, old MI, estimated glomerular filtration rate, reperfusion therapy, statin, angiotensin-converting enzyme inhibitor or angiotensin 2 receptor blocker, β-blocker, and antiplatelet agents as explanatory variables. Thus, all the abovementioned variables, which were used in the propensity score calculation, were adjusted during propensity-score-stratified analysis. The statistical significance was set as $P<0.05$. All statistical analyses were performed using R software ver.2.13.1 (http://cran.r-project.org/).

Results

The patients' backgrounds are shown in Table 1 (median age, 65 years; 77.8% men; 86.1% ST-elevation MI; 93.3% underwent percutaneous coronary interventions). The prescription rates of statin, angiotensin-converting enzyme inhibitor and/or angiotensin 2 receptor blocker, β-blocker, antiplatelet agents,

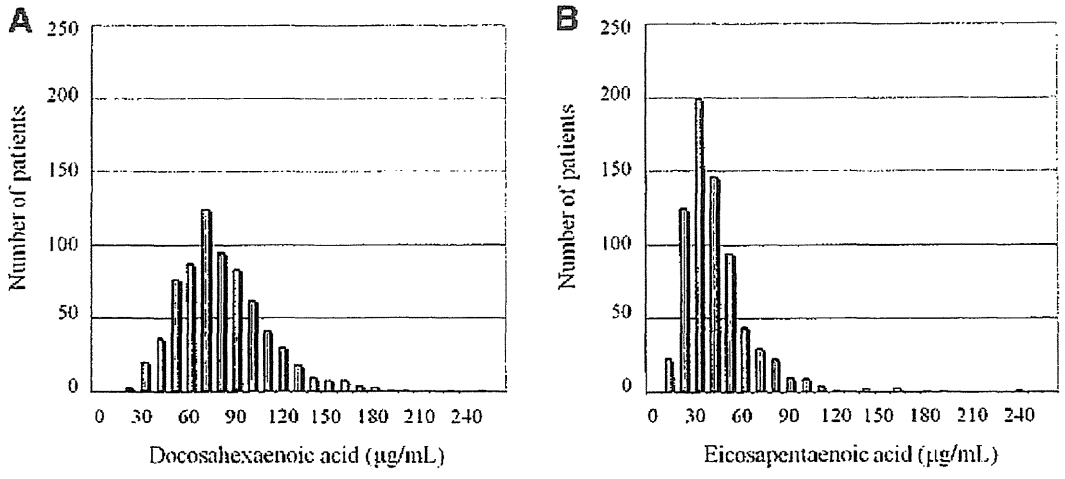

Figure 1. Distribution of (A) docosahexaenoic acid and (B) eicosapentaenoic acid.

Table 2. Patients' Backgrounds in the 3 Docosahexaenoic Acid Groups

Parameter	DHA			P value
	Low (n=239)	Middle (n=236)	High (n=237)	
Definition	DHA ≤61.4	61.4<DHA≤83.5	DHA >83.5	–
Age, years	66 (59–75)	66 (57–72)	64 (56–73)	0.0224
Male, %	81.2	80.5	71.7	0.0220
BMI, kg/m²	23.4 (21.8–25.2)	24.0 (22.3–26.7)	24.1 (22.3–26.4)	0.0181
STEMI, %	86.6	88.5	83.4	0.2775
Coronary risk factors				
Diabetes, %	37.7	36.4	31.2	0.2960
Hypertension, %	65.7	68.8	66.4	0.7520
Dyslipidemia, %	49.1	48.9	54.4	0.4155
Smoking, %	64.4	64.0	61.0	0.7042
OMI, %	11.6	7.6	12.3	0.1987
Laboratory data				
EPA, μg/ml	20.4 (15.1–28.2)	29.4 (23.8–39.1)	47.3 (36.0–65.6)	<0.0001
DHA, μg/ml	48.0 (40.3–54.7)	71.0 (66.6–76.6)	101.2 (92.0–117.2)	<0.0001
TC, mg/dl	184 (154–210)	194 (168–226)	198 (167–226)	0.0024
LDL-C, mg/dl	119 (95–144)	124 (104–153)	123 (101–149)	0.1591
HDL-C, mg/dl	42 (38–48)	45 (38–54)	44 (38–54)	0.0274
TG, mg/dl	88 (51–142)	100 (64–152)	104 (67–166)	0.0058
HbA_{1c}, %	6.1 (5.7–7.0)	6.1 (5.7–7.2)	6.0 (5.7–6.5)	0.1448
eGFR, $ml \cdot min^{-1} \cdot 1.73m^{-2}$	67.9 (52.5–84.0)	70.3 (56.6–85.4)	68.8 (55.6–84.7)	0.5054
Peak CK, IU/L	2,092 (997–4,169)	2,280 (1,063–3,723)	2,268 (1,066–3,940)	0.9868
Reperfusion, %	95.0	94.5	94.1	0.9134
PCI, %	92.9	94.5	92.4	0.6384
Medications at discharge				
Statin, %	68.6	60.6	52.3	0.0013
ACEI or ARB, %	80.8	83.5	82.7	0.7253
β-blocker, %	71.5	67.4	62.0	0.0862
Antiplatelet, %	98.7	99.2	99.2	0.8721
Ethyl icosapentate, %	3.8	0.8	3.0	0.1128

Abbreviations as in Table 1.

Table 3. Patients' Backgrounds in the 3 Eicosapentaenoic Acid Groups

Parameter	EPA			P value
	Low (n=237)	Middle (n=237)	High (n=238)	
Definition	EPA ≤24.6	24.6<EPA≤38.8	EPA >38.8	-
Age, years	66 (57–75)	64 (56–73)	66 (58–72)	0.2104
Male, %	77.6	81.0	74.8	0.2633
BMI, kg/m²	23.6 (22.1–25.6)	24.1 (22.2–26.7)	23.8 (22.1–25.8)	0.3196
STEMI, %	88.6	84.3	85.5	0.3901
Coronary risk factors				
Diabetes, %	38.4	36.3	30.7	0.1897
Hypertension, %	66.5	69.4	65.0	0.5895
Dyslipidemia, %	46.1	50.9	55.4	0.1370
Smoking, %	59.5	65.7	64.3	0.3427
OMI, %	9.8	10.3	11.4	0.8424
Laboratory data				
EPA, μg/ml	18.1 (14.8–21.5)	30.4 (27.6–33.9)	51.4 (44.2–68.6)	<0.0001
DHA, μg/ml	52.1 (41.1–67.0)	70.8 (59.6–83.9)	96.4 (78.3–115.5)	<0.0001
TC, mg/dl	186 (158–213)	194 (165–226)	195 (170–226)	0.0317
LDL-C, mg/dl	119 (95–142)	125 (102–153)	123 (103–149)	0.1320
HDL-C, mg/dl	42 (37–48)	44 (39–53)	45 (38–55)	0.0040
TG, mg/dl	99 (60–159)	93 (57–148)	97 (65–151)	0.3856
HbA_{1c}, %	6.0 (5.6–6.9)	6.1 (5.7–7.1)	6.0 (5.7–6.5)	0.5872
eGFR, $ml \cdot min^{-1} \cdot 1.73m^{-2}$	66.9 (51.3–83.0)	73.8 (59.1–88.4)	66.8 (55.3–83.3)	0.0026
Peak CK, IU/L	2,319 (1,100–4,227)	2,124 (1,023–3,819)	2,244 (1,003–3,667)	0.3117
Reperfusion, %	95.4	95.4	92.9	0.3839
PCI, %	94.5	94.1	91.2	0.2867
Medications at discharge				
Statin, %	61.2	61.2	59.2	0.8829
ACEI or ARB, %	82.3	83.1	81.5	0.8997
β-blocker, %	71.3	67.1	62.6	0.1307
Antiplatelet, %	98.3	99.6	99.2	0.3632
Ethyl icosapentate, %	3.0	1.3	3.4	0.3047

Abbreviations as in Table 1.

and ethyl icosapentate were 60.5%, 82.3%, 67.0%, 99.0%, and 2.5%, respectively. Ethyl icosapentate is the only available EPA formulation, and there is no available DHA formulation with medical care insurance in Japan. In the present study, there were no available data regarding over-the-counter DHA or EPA supplement capsule usage, and no available data regarding the amount of daily fish intake.

Possible selection bias was evaluated by comparing the patients' backgrounds among the enrolled patients (Subject group: n=712) and those not enrolled because of a lack of eligible serum samples (No-sample group: n=866) or a lack of the agreement of blood samples (No-agreement group: n=804). As shown in **Table S1**, patients' backgrounds differed significantly between the Subject and No-agreement groups, but were almost comparable between the Subject and No-sample groups, except for the duration of follow-up period and prevalences of dyslipidemia, smoking and ST-elevation MI.

Blood samples were obtained from patients at a median of 17 (quartile: 13–24) days after the onset of AMI. The distribution of serum DHA and EPA levels is shown in **Figure 1**. Based on the DHA tertile values, we grouped patients with DHA ≤61.4 μg/ml into a DHA-Low group (n=239), those with DHA >83.5 μg/ml formed the DHA-High group (n=237), and those with intermediate serum levels comprised the DHA-Middle group (n=236; Table 2). We also defined patients with serum EPA levels of ≤24.6 μg/ml as the EPA-Low group (n=237), those with EPA levels of >38.8 μg/ml as the EPA-High group (n=238), and all others as the EPA-Middle group (n=237) according to EPA tertile values (Table 3). A strong correlation was detected between the DHA and EPA values based on the Spearman's rank correlation coefficient of r=0.708 (P<0.0001).

Event rates were compared among the DHA and EPA groups by Kaplan-Meier analysis (**Figure 2**). There were a total of 35 HF hospitalizations and 45 all-cause death events (17 HF and 23 deaths in the DHA-Low group, 10 HF and 12 deaths in the DHA-Middle group, and 8 HF and 10 deaths in the DHA-High group; 20 HF and 19 deaths in the EPA-Low group, 7 HF and 16 deaths in the EPA-Middle group, and 8 HF and 10 deaths in the EPA High group) at the median follow-up duration of 1,079 (quartile: 721–1,442) days. There were 9 cardiovascular, 19 non-cardiovascular, and 17 unknown-cause deaths in the present study.

Both the DHA-and EPA-Low groups showed statistically significant worse HF-free survival as compared with the other groups (P=0.0204 and P=0.0353, respectively; **Figures 2A,B**). However, when we focused on each event rate, the EPA-Low group displayed a statistically significant worse outcome only for HF hospitalizations (P=0.0086; **Figures 2C,D**), while the DHA-Low group exhibited an adverse outcome only for all-