[Standard Method of Provocation Test[16–19,128,129]]

(1) Insertion of a temporary pacing electrode in the right ventricle: Administration of acetylcholine, especially in the right coronary artery, may cause transient episodes of severe bradycardia. Perform backup pacing (40 to 50 bpm).
(2) Control angiography of left and right coronary arteries: Perform angiography in an appropriate projection that ensures the best separation of the branches of each coronary artery. After injection of acetylcholine, perform angiography in the same projection again.
(3) Injection of acetylcholine into the left coronary artery: Inject 20, 50, or 100 μg of acetylcholine in solution in 37°C physiological saline (concentration adjusted to obtain 5 ml solution volume for each quantity of acetylcholine) into the left coronary artery over a period of 20 seconds. Perform coronary angiography 1 minute after the start of each injection. In the event of an ischemic change on the ECG or chest pain, perform angiography at that time. Doses of acetylcholine should be given at 5-minute intervals.
(4) Injection of acetylcholine into right coronary artery: Inject 20 or 50 μg of acetylcholine (each in 5 ml solution) into the right coronary artery over a period of 20 seconds. The timing of angiography is the same as for the left coronary artery.
(5) Left and right coronary angiography after administration of nitrate: Administer a nitrate into each coronary artery, and perform angiography while the coronary artery is maximally dilated.

(2) Ergonovine Provocation Test[21,46,47,129–134]

Class I

Ergonovine provocation test during coronary angiography performed in patients in whom vasospastic angina is suspected based on symptoms, but in whom coronary spasm has not been diagnosed by non-invasive evaluation

Class IIa

Ergonovine provocation test during coronary angiography performed in patients who have been diagnosed with coronary spasm by non-invasive evaluation, and in whom drug treatment is ineffective or insufficiently effective

Class IIb

Ergonovine provocation test during coronary angiography performed in patients who have been diagnosed with coronary spasm by non-invasive or invasive evaluation, and in whom drug treatment has been proven to be effective

Class III

1. Ergonovine provocation test during coronary angiography performed in patients without symptoms suggestive of vasospastic angina
2. Ergonovine provocation test during coronary angiography performed in patients considered at high risk of suffering a life-threatening complication of induced coronary spasm (eg, patients with left main coronary trunk lesions; those with multivessel coronary lesions, including obstructive lesions; those with severe cardiac dysfunction; and those with untreated congestive heart failure) (however, in cases in which the onset of severe cardiac dysfunction or congestive heart failure may be a consequence of coronary spasm, the criteria for Class IIb apply)
3. Ergonovine provocation test during emergent coronary angiography performed in patients with acute coronary syndrome

As with the acetylcholine provocation test, coronary spasm during the ergonovine provocation test is defined as "transient, total, or sub-total occlusion (>90% stenosis) of a coronary artery with signs of myocardial ischemia (anginal pain and ischemic ST changes)." In the present guidelines, it is recommended for reasons of safety that the ergonovine provocation test be conducted with intracoronary rather than intravenous administration.

[Standard Method of Provocation Test[46,47,130–133]]
Intracoronary Administration

(1) Control angiography of left and right coronary arteries: Perform angiography an appropriate projection that ensures the best separation of the branches of each coronary artery. After injection of ergonovine, perform angiography in the same projection again.
(2) Injection of ergonovine into the left coronary artery: Inject 20 to 60 μg of ergonovine in solution in physiological saline into the left coronary artery over a period of several minutes (about 2 to 5 minutes). Perform coronary angiography 1 to 2 minutes after completion of the injection. In the event of an ischemic change on the ECG or chest symptom, perform angiography at the time of its onset. In case of a negative result in the provocation test, proceed to the right coronary provocation test 5 minutes later.
(3) Injection of ergonovine into the right coronary artery: Inject 20 to 60 μg of ergonovine in solution in physiological saline into the right coronary artery over a period of several minutes (about 2 to 5 minutes). The timing of angiography is the same as for the left coronary artery.
(4) Left and right coronary angiography after administration of nitrate: Administer a sufficient dose of nitrate into each coronary artery, and perform angiography while the coronary artery is maximally dilated.

(3) Measurement of Coronary Blood Flow[135]

Class I

None

Class IIa

None

Class IIb

Used for supplementary diagnosis in the drug-induced coronary spasm provocation test in patients suspected to have vasospastic angina

Class III

Used for supplementary diagnosis in the drug-induced coronary spasm provocation test in patients with severe organic stenosis

(4) Measurement of Coronary Sinus Lactate Levels[136–138]

Class I

None

Class IIa

None

Class IIb

Measurement of coronary sinus lactate levels during a drug-induced coronary spasm provocation test

Class III

None

A catheter is placed in the coronary sinus, and coronary spasm is induced with acetylcholine or a similar agent. Coronary venous blood and blood from the base of the aorta or coronary arterial blood is drawn before and after the induction, and lactate metabolism in the myocardium is

examined. Upon the development of ischemia, myocardial lactate consumption decreases; as the ischemia increases in severity, a shift to lactate production occurs.[136,137] Although lactate consumption decreases during coronary spasm, whether the shift to lactate production occurs depends on the severity of ischemia, the site where the ischemia occurs, and other factors. This parameter is also considered useful as a marker of the onset of myocardial ischemia in the diagnosis of coronary microvascular spasm.[138]

(5) Coronary Angioscopy[139–141]

Coronary angioscopy in patients with vasospastic angina is usually performed for the purpose of investigating the pathological condition or mechanism of onset of vasospastic angina, rather than for diagnostic purposes.

(6) Intravascular Ultrasound (IVUS)[5,142–146]

The major role of IVUS in the diagnosis of vasospastic angina is to elucidate its pathological condition and etiology based on its morphological (and sometimes functional) features.

III Treatment

1. Management of Daily Life (Correction of Risk Factors)[22,82,127,147–161]

Class I
1. Smoking cessation
2. Blood pressure control
3. Maintenance of ideal body weight
4. Correction of impaired glucose tolerance
5. Correction of lipid abnormalities
6. Avoidance of excessive fatigue and mental stress
7. No or moderate drinking

Class IIa
None

Class IIb
None

Class III
None

2. Drug Therapies

1 Nitrates[33,58,162–164]

Class I
Sublingual administration, spraying in the oral cavity, or intravenous administration during an attack

Class IIa
Administration of long-acting nitrates for prevention of coronary spasm

Class IIb
None

Class III
Administration of nitrates within 24 hours after taking an agent to treat erectile dysfunction

Nitrates are metabolized to NO in the body, which in turn activates guanylate cyclase to increase cGMP, resulting in relaxation of vascular smooth muscle.[33,58,162,163] Nitrates also suppress the activity of Rho-kinase via NO and thereby relax smooth muscle.[164] Nitrates exert effects in the treatment of coronary spasm by a mechanism of action different from that of calcium channel blockers; it is therefore desirable that patients be treated with the combination of a calcium channel blocker and nitrate or monotherapy with either drug alone based on the condition of individual patients.

2 Calcium Channel Blockers[165–172]

Class I
Administration of calcium channel blockers for vasospastic angina

Class IIa
None

Class IIb
None

Class III
None

Calcium channel blockers that suppress Ca^{2+} inflow into vascular smooth muscle cells are highly effective in preventing coronary spasm, and are deemed drugs of first choice for the treatment of vasospastic angina.[165,166] They can be used safely, without adverse reactions, at usual doses.[167–171]

3 Nicorandil[173–180]

Class I
None

Class IIa
Administration of nicorandil for vasospastic angina

Class IIb
None

Class III
Administration of nicorandil within 24 hours after taking an agent to treat erectile dysfunction

4 β-Blockers[1,171]

Class I
None

Class IIa
Concomitant use of β-blockers for vasospastic angina with significant stenosis of coronary artery

Class IIb
Concomitant use of β-blockers for vasospastic angina without significant stenosis of coronary artery

Class III
Monotherapy for vasospastic angina without significant stenosis of coronary artery

5 Other Drugs Possibly Effective in Suppressing Coronary Spasm

(1) Vitamins and Antioxidants[92,181–184]

Class I
None

Class IIa
None

Class IIb
Administration of vitamin E preparations for vasospastic angina

Class III
None

(2) Estrogens[69,98,185–193]
Class I
None
Class IIa
None
Class IIb
Administration of estrogens for vasospastic angina in postmenopausal women
Class III
None

(3) Steroids[194–198]
Class I
None
Class IIa
None
Class IIb
Administration of steroids for vasospastic angina
Class III
None

(4) Fasudil[138,199–203]
Class I
None
Class IIa
None
Class IIb
Administration of fasudil for vasospastic angina
Class III
None

3. Concomitant Percutaneous Coronary Intervention[18,204–209]

Class I
None
Class IIa
Percutaneous coronary intervention performed in combination with adequate administration of coronary dilators for vasospastic angina with severe organic stenosis
Class IIb
None
Class III
Coronary intervention performed for vasospastic angina without severe organic stenosis

IV Issues Related to Coronary Spasm

1. Intractable Vasospastic Angina

Although attacks of vasospastic angina can usually be relieved or suppressed with coronary vasodilators such as nitrates and calcium channel blockers, in some patients vasospastic angina is intractable and resists these drugs, and attacks cannot be relieved or suppressed. A Ministry of Health, Labour and Welfare-commissioned study was undertaken by a research task force to determine the incidence of intractable vasospastic angina. In that study, intractable vasospastic angina was defined as angina that cannot be controlled even with the administration of two types of coronary vasodilators. According to the report, vasospastic angina was found in 921 (40.9%) of 2,251 patients with angina reported from 15 institutions nationwide in Japan; 126 of these patients (13.7%) were intractable.[210] The patients with intractable vasospastic angina were characterized by younger age at the time of onset, and included high proportions of tobacco smokers and normotensive patients than the group of patients with treatable vasospastic angina.

For patients in whom control of coronary spasm with calcium channel blockers or nitrates is not possible, oral drugs that can control the other mechanism of action are required. It is strongly hoped that further advances will be made in research into the mechanisms of coronary spasm and the development of prophylactic medications. Reported non-drug treatments include the use of an implantable cardioverter defibrillator (ICD) for ventricular tachycardia and ventricular fibrillation during coronary spasm attacks in intractable vasospastic angina,[211,212] but no agreement exists concerning the validity of this treatment. If ischemic attacks can be prevented with drug therapy, use of an ICD is not considered indicated. If the patient's condition is intractable and attacks cannot be prevented, ICD may be considered. Further investigation is needed to develop appropriate treatment strategies for patients with intractable coronary spasm.

2. Coronary Microvascular Spasm

Some possibilities have been suggested regarding the mechanism of onset of myocardial ischemia based on abnormalities of the coronary microcirculation. They include (1) steal phenomenon resulting from reduction in coronary microvessel diastolic function or uneven vasodilation in the left ventricular wall, and (2) coronary microvascular spasm. In patients with microvascular angina, the decreased blood flow and ischemia in some regions of the myocardium or subendocardium are observed by the pacing stress test, handgrip stress test, or adenosine stress test. These types of impairment of metabolic vasodilation in the coronary microvessels can cause myocardial ischemia during exercise (effort angina). It is thought that if coronary microvascular hypercontraction (spasm) occurs, angina not accompanied by an increased myocardial oxygen demand, ie, rest angina, develops.

Because coronary microvascular spasm cannot be detected on angiography, its occurrence must be indirectly detected from the results of a provocation test.[138,213] If symptoms of angina are induced despite the absence of spasm in the major coronary arteries during a coronary spasm provocation test with administration of acetylcholine or ergonovine into the coronary arteries, and at the same time direct or indirect findings of myocardial ischemia, such as clear reduction of coronary blood flow rate, emergence of ischemic changes on the ECG, and myocardial lactate production, appear, then coronary microvascular spasm is diagnosed.

3. Coronary Spasm After Coronary Artery Bypass Grafting

During and after coronary artery bypass grafting, coronary spasm is likely to develop because endogenous vasopressor substances are produced as a result of anesthesia, surgical invasion, and cardiopulmonary bypass, and also because exogenous catecholamine and vasoconstrictors are administered. Furthermore, because hemodynamics are unstable in the perioperative period, coronary spasm can have serious, even life-threatening consequences in some cases. Perioperative coronary spasm develops suddenly, causing a broad range of signs of myocardial ischemia. Intraoperative and postoperative coronary spasm tends to be repetitive, and are sometimes accompanied by elevated pulmonary arterial pressure; careful monitoring is therefore essential with variety of devices. Because myocardial damage due to inadequate cardioplegia and graft blood flow insufficiency also lead to signs of myocardial ischemia during surgery, it is necessary to distinguish between these pathological conditions and coronary spasm.

In addition to coronary spasm, spasm of the graft itself is a potential problem following coronary artery bypass grafting. The ergonovine provocation test significantly alters the diameters of great saphenous vein grafts, but does not alter those of internal thoracic artery grafts.[214] In addition, it has been reported that radial artery and gastroepiploic artery grafts are more likely to exhibit spasm than internal thoracic artery grafts.[215]

4. Involvement of Coronary Spasm in Takotsubo Cardiomyopathy

Takotsubo cardiomyopathy is a transient myocardial damage of acute onset nature resembling acute coronary syndrome. It is characterized by sudden chest pain and chest symptoms as well as ECG changes such as ST elevation, abnormal Q waves, and negative T waves, is often triggered with physical or mental pain and stress, and occurs at relatively high incidence in elderly women. Its pathological features include slightly elevated levels of myocardial enzymes that are inconsistent with the severity of left ventricular wall motion abnormalities, and typical wall motion abnormalities unrelated to significant coronary stenotic lesions (ie, left ventricular apical ballooning, abnormal dilation of the left ventricular wall at the papillary muscle attachments, and excessive contraction at the cardiac base), that are observed during the acute phase but improved in the chronic phase.

Details of the etiology of Takotsubo cardiomyopathy remain unclear. In early reports from Japan[216,217] and several retrospective studies[218–221] of cases subsequently compiled, coronary spasm was observed in spontaneous attacks and drug provocation tests in the chronic phase of this disease. Although the incidence of coronary spasm in patients with Takotsubo cardiomyopathy varied between 0 to 43% in different reports,[218,220–224] it is believed that coronary spasm may play an important part in the development of myocardial damage in this population. However, Takotsubo cardiomyopathy differs from the common types of cardiomyopathy due to coronary spasm in pathological characteristics, patient characteristics, and causal factors. Reports from Western countries have suggested that whether coronary spasm is involved in the development of Takotsubo cardiomyopathy is unclear.[222–227] Coronary spasm cannot be considered the cause of all cases of Takotsubo cardiomyopathy.

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Appendix

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(The affiliations of the members are as of March 2010)

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Brief Communications

Plasma MicroRNA 499 as a Biomarker of Acute Myocardial Infarction

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BACKGROUND: MicroRNAs (miRNAs) are endogenous small RNAs 21–25 nucleotides in length. Recently, we reported that miRNA 208 (miR-208) is produced exclusively in the rat myocardium and that plasma miR-208 is a biomarker of myocardial injury in rats. In the present study, we assessed the hypothesis that plasma concentrations of myocardial-specific miRNAs can be used to diagnose myocardial injury in humans.

METHODS: We used array analysis of miRNA production in various human tissues to identify heart-specific miRNAs. We assessed the plasma concentrations of miR-499 in 14 individuals with acute coronary syndromes, 15 individuals with congestive heart failure, and 10 individuals without cardiovascular diseases. Plasma miR-499 concentrations were measured with a real-time reverse-transcription PCR method that used an artificial small RNA as an internal calibrator.

RESULTS: The miRNA array analysis of various human tissues indicated that miR-499 was produced almost exclusively in the heart. Plasma miR-499 concentrations were measurably increased in all individuals with acute myocardial infarction but were below the limit of detection for all individuals in the other patient groups.

CONCLUSIONS: The plasma concentration of miR-499 may be a useful biomarker of myocardial infarction in humans.

MicroRNAs (miRNAs),[3] endogenous small RNAs 21–25 nucleotides in length, can pair with the 3′ untranslated region sites in mRNAs of protein-coding genes to downregulate their expression *(1)*, and they play important roles in various physiological and pathologic processes *(2, 3)*. More than 500 human miRNAs have been identified *(4)*, and most human protein-coding genes appear to be targeted by these miRNAs *(5, 6)*. miRNAs appear to function as rheostats to fine-tune adjustments in the protein output *(7, 8)*.

The presence of miRNAs in various body fluids has recently been reported *(9–11)*, and we recently reported that the plasma concentration of miRNA 208 (miR-208), a myocardial-specific miRNA in rats, is a useful biomarker of myocardial injury *(12)*. Other groups have also reported that plasma miRNAs are sensitive and specific biomarkers of various tissue injuries *(13, 14)*. In the present study, we examined which human tissues produced miR-499 and assessed whether the plasma concentration of miR-499 is a useful biomarker of myocardial injury in humans.

We collected blood samples from 29 inpatients and 10 healthy asymptomatic outpatients at the National Cardiovascular Center Hospital after obtaining their written informed consent. This study was approved by the Ethics Committee of the National Cardiovascular Center.

The acute coronary syndromes group consisted of 9 patients with acute myocardial infarction (AMI) and 5 patients with unstable angina pectoris. All acute coronary syndrome patients underwent coronary angiography and percutaneous coronary intervention. The blood samples from the acute coronary syndrome patients were obtained within 48 h of the last onset of chest pain. We also obtained blood samples from AMI patients before their final discharge when their clinical status was stable. The congestive heart failure (CHF) group consisted of 8 patients with old myocardial infarction [New York Heart Association (NYHA) class III], 4 patients with dilated cardiomyopathy (NYHA class II), and 3 patients with valvular diseases (1 patient in NYHA class III and 2 in NYHA class II). The blood samples of patients in the CHF group were obtained while they were in NYHA functional class II or III. The control individuals consisted of asymptomatic healthy and/or borderline hypertensive outpatients who were visiting the hospital for regular health checkups. Creatine kinase MB was increased in the patients with AMI and not in the patients with unstable angina pectoris (Table 1).

We isolated total plasma RNA with the mirVana™ PARIS Kit (Ambion) according to the manufacturer's protocol. Before purification, we added a fixed amount of a small synthetic RNA to the plasma samples for a dual assay to verify the RNA-purification procedures. Details of the procedure are described in the Supplemental Data file available in the Data Supplement that accompanies the online version of this Brief Communication at http://www.clinchem.org/content/vol56/issue7.

[3] Nonstandard abbreviations: miRNA, microRNA; miR-208, miRNA 208; AMI, acute myocardial infarction; CHF, congestive heart failure; NYHA, New York Heart Association.

Table 1. Patient characteristics.[a]

	AMI[b] (n = 9)	UAP (n = 5)	CHF_III (n = 9)	CHF_II (n = 6)	Normal (n = 10)
F/M sex, n	3/6	2/3	2/7	2/4	5/5
Age, years	66.8 (9.28)	70.2 (16.2)	71.6 (6.6)	61.5 (16.4)	41.5 (8.0)
CKMB, U/L[c]	122.2 (124.9)	18.9 (6.6)	ND	ND	ND
BNP, ng/L[c]	ND	ND	674 (341)	175 (142)	ND
Log miR-499 copies/100 μL	4.19 (0.24)	<2.38	<2.38	<2.38	<2.38

[a] Data are expressed as the mean (SD) where indicated.
[b] AMI, acute myocardial infarction; UAP, unstable angina pectoris; CHF_III, congestive heart failure in NYHA class III; CHF_II, congestive heart failure in NYHA class II; Normal, healthy control individuals; CKMB, creatine kinase MB; BNP, brain natriuretic peptide; ND, not determined.
[c] CKMB (reference interval, 0–23 U/L) and BNP (reference interval, <18.4 ng/L) were measured in the AMI groups (AMI and UAP) and the CHF groups, respectively.

To identify myocardial-specific miRNAs, we used the ABI TaqMan MicroRNA Array kit (Applied Biosystems) according to the manufacturer's protocol for profiling the production of miRNAs in various human tissues and cultured cells.

To measure miR-499 concentrations, we used a TaqMan microRNA real-time RT-PCR kit (Applied Biosystems) *(15)* according to the manufacturer's protocol. We simultaneously assessed the concentration of the internal reference small RNA in a single tube. The limit of detection for miR-499 was 240 copies/100 μL. All assays were performed in duplicate. Calibration assays with various amounts of synthetic miR-499 were performed on each assay plate. Details of the statistical analyses are described in the Supplemental Data file in the online Data Supplement.

The miRNA array analyses of 671 species of miRNAs in various tissues and cells indicated that miR-499 is produced almost exclusively in the human heart (see Supplemental Table in the online Data Supplement). miR-208a and miR-208b concentrations appear to be very low in the human heart (see Supplemental Table in the online Data Supplement), and these 2 miRNAs appear not to be useful as plasma biomarkers.

Fig. 1 summarizes the data for plasma miR-499 concentrations in the study population. Plasma miR-499 concentrations were below the limit of detection in the control and CHF groups; however, plasma miR-499 concentrations were measurably increased in patients with AMI in the acute phase (within 48 h of the last onset of chest pain) and became undetectable before hospital discharge, whereas this miRNA was not detected in the plasma of patients with unstable angina pectoris. The large variation in the plasma miR-499 concentration in AMI patients was most likely related to variation in the time of blood collection. Our preliminary investigation indicated that the peak plasma miR-499 concentration occurred between 6 h and 12 h of the onset of myocardial infarction (data not shown). A positive correlation between creatine kinase MB activity and plasma miR-499 concentration was clearly

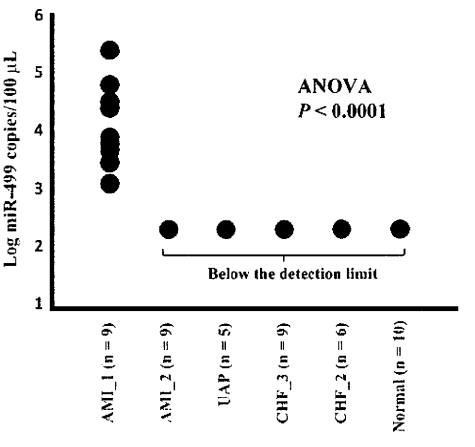

Fig. 1. Plasma concentrations of miR-499 in the study population.

Plasma miR-499 concentrations were assessed by real-time reverse-transcription PCR with a synthetic miRNA included as an internal calibrator. Values are expressed as log miR-499 copies/100 μL. Concentrations were measured in patients with AMI [repeatedly measured in samples obtained within 48 h (AMI_1) and at just before hospital discharge (AMI_2)], in patients with unstable angina pectoris (UAP), in CHF patients in NYHA class III (CHF_3), in CHF patients in NYHA class II (CHF_2), and in healthy control individuals (Normal). An ANOVA indicated that the mean miR-499 values were significantly different among the groups ($P < 0.0001$). The subsequent Dunnett test indicated that values in the AMI_1 group were significantly higher than those of the other groups ($P < 0.0001$ for all comparisons).

observed in individuals with AMI (see Supplemental Data in the online Data Supplement).

The present study is the first to confirm that a cardiac-specific miRNA, miR-499, can be a biomarker of myocardial infarction in humans. The next question is whether this assessment of the plasma miR-499 concentration has any clinical significance. We expected the PCR-based assay of plasma miR-499 to detect possible myocardial micronecrosis in CHF. In fact, our study showed that this method could not detect plasma miR-499 concentrations reliably in CHF patients. A more sensitive assay to detect plasma miR-499 can be developed, however, and it might establish miR-499 as a new biomarker of cardiovascular diseases in the same way that the recently developed high-sensitivity assays for troponins have become very useful for evaluating patients with cardiovascular diseases *(16)*.

Accumulating evidence suggests the usefulness of circulating miRNAs as stable blood-based biomarkers for various diseases *(9–11)*. The present study has confirmed, for the first time, that the plasma miR-499 concentration may be a biomarker of myocardial infarction in humans. Our array data indicate other intriguing candidates for clinical applications, including miR-124a for the central nervous system, miR-122 for the liver, and miR-133a for skeletal muscle. These observations await further clinical investigations.

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EDITORIAL

Exercise Training in Post-CABG Patients at Low Prognostic Risk

– Beyond Recovery From Surgery –

Yoichi Goto, MD, PhD

Cardiac rehabilitation with exercise training has been shown to improve exercise capacity, coronary risk factors, and health-related quality of life (QOL), to retard the progression of atherosclerosis, and to decrease morbidity and mortality in patients with coronary artery disease (CAD).[1] Based on these lines of evidence, the American College of Cardiology/American Heart Association (ACC/AHA) guidelines recommend cardiac rehabilitation for all eligible patients with CAD, including those after coronary artery bypass grafting (CABG).[2] However, among studies focusing exclusively on a CABG population, the existing evidence of the efficacy of exercise training is limited to improvements in exercise tolerance and psychological sense of well-being.[2,3] In this issue of the Journal, Bilinska et al report the effects of exercise training on hemodynamic and neurohumoral responses to static (handgrip) exercise and on inflammatory markers in patients after CABG.[4] Their study is unique in the following 3 aspects.

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Sympathetic and Metabolic Control of Cardiovascular Response to Exercise

The first of these is that the authors assessed the effects of dynamic exercise training on the response to static exercise. Static exercise is known to elicit a greater increase in systolic blood pressure (BP) than dynamic exercise, but the effects of exercise training on the hemodynamic and neurohumoral responses to static exercise have not been well understood. The finding that the increases in heart rate, systolic BP, total peripheral resistance and plasma norepinephrine concentration during handgrip exercise were attenuated after 6-week exercise training were anticipated, but the finding of the greater increase in the nitric oxide (NO) level in response to handgrip exercise after exercise training is intriguing. Recent studies suggest that not only the sympathetic nerve system but also NO-mediated metabolic regulation significantly contribute to the control of the cardiovascular response to acute exercise or mental stress.[5–7] Therefore, hemodynamic changes, such as increases in BP and vascular resistance during static exercise, are the composite result of interaction between 2 regulatory systems, that is, the sympathoexcitatory α-adrenergic and sympathoinhibitory NO systems.

Sugawara et al reported that, after exercise training, increased NO-mediated vasodilatation is counterbalanced by enhanced α-adrenergic vasoconstriction, resulting in an unchanged basal limb blood flow.[8] Additionally, the Bilinska study demonstrated that both attenuated norepinephrine release and enhanced NO release may be involved in the attenuated increases in systolic BP and peripheral vascular resistance during handgrip exercise after exercise training.[4] These findings may be important for explaining the mechanism of the beneficial cardiovascular effects of exercise training, because there is a view that high levels of baseline sympathetic outflow are not dangerous per se, but that high levels of sympathetic outflow in conjunction with endothelial dysfunction may have synergistic and detrimental effect in terms of cardiovascular risk.[9] If so, a plausible scenario is that the vicious cycle of autonomic dysfunction and endothelial dysfunction can be prevented or ameliorated by regular exercise training.

Effect of Exercise Training on Systemic Inflammation

Secondly, Bilinska et al demonstrate that exercise training results in a significant reduction in inflammatory markers in post-CABG patients. Although previous studies have reported a reduction in inflammatory markers after exercise training in CAD patients,[10,11] this is the first report in post-CABG patients. It is conceivable that, in post-CABG patients, even after active myocardial ischemia is extinguished, the remaining atherosclerotic plaques at the original sites may continue to be a source of chronic inflammation.

The precise mechanisms by which exercise training ameliorates systemic inflammation is unclear, but Handschin and Spiegelman proposed peroxisome proliferative-activated receptor γ (PPARγ) coactivator 1α (PGC-1α) as a key factor in the beneficial effect of exercise.[12] PGC-1α is a critical coordinator of the activation of metabolic genes controlling substrate use and mitochondrial biogenesis, and according to Handschin and Spiegelman, regular exercise induces PGC-1α in skeletal muscles, which in turn suppresses the production of proinflammatory cytokines such as interleukin-6 or tumor-necrosis factor-α in muscles.[12] Conversely, a sedentary lifestyle would decrease PGC-1α expression in skeletal muscles, resulting in elevation of proinflammatory cytokines and hence, chronic systemic inflammation.

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Interval vs Endurance Mode of Exercise Training

Thirdly, the study being discussed is unique because the investigators used interval training rather than endurance (continuous) training. Recent studies have demonstrated that high-intensity interval training is more effective than continuous moderate exercise training in enhancing exercise capacity, PGC-1α level, and endothelial function in patients with metabolic syndrome or chronic heart failure.[13,14] If interval training proves to be more effective than endurance training in gaining cardiovascular benefits, the mode of exercise training, and hence, the style of contemporary cardiac rehabilitation, will be greatly changed.

Remaining Issues

The study population was highly selected, young male patients after off-pump CABG with preserved left ventricular function and without myocardial ischemia, uncontrolled coronary risk factors, or comorbidities; that is, the patients were at very low prognostic risk, which means it is not easy to confirm that the observed beneficial effects will translate into meaningful clinical outcome, because the long-term event rate in this population should be very low. In addition, it remains unknown whether the presented findings obtained in a highly selected population can be generalized to real-world patients with multiple risk factors and comorbidities.

Lastly, despite the established and additional potential benefits, the use of outpatient exercise training/cardiac rehabilitation remains very low in Japan.[15] Considering the significant impact of exercise training on both the NO and PGC-1α systems that regulate fundamental cardiovascular pathophysiology, this important therapeutic modality warrants more widespread application.

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ORIGINAL ARTICLE
Molecular Cardiology

Association of the Functional Variant in the 3-Hydroxy-3-Methylglutaryl-Coenzyme A Reductase Gene With Low-Density Lipoprotein-Cholesterol in Japanese

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Background: The association between single nucleotide polymorphisms (SNPs) at 3-hydroxy-3-methylglutaryl-coenzyme A reductase (*HMGCR*) and low-density lipoprotein-cholesterol (LDL-C) levels has been well replicated in genome-wide association studies (GWAS) of white populations. Recently, the common intronic SNP of *HMGCR* (rs3846662) has been reported to be a functional variant, influencing the alternative splicing of exon 13. The aim of this study was to examine the association between rs3846662 of *HMGCR* and the level of LDL-C in Japanese.

Methods and Results: Significant differences in LDL-C levels were observed among the genotypes of rs3846662 (P=0.0002 (n=2,686) and P=0.004 (n=2,110)) for the Suita and Ehime samples, respectively. The G allele of rs3846662 was associated with higher LDL-C levels (β, 3.56; $P=4.91\times10^{-5}$). Consistent with this observation, the risk G allele at rs3846662 was more prevalent in subjects with myocardial infarction (MI) (n=701) than in subjects without MI (n=3,118); 0.559 and 0.511 in MI cases and controls, respectively (nominal P=0.0038). The odds ratio adjusted for age, sex, diabetes, hypertension, and drinking and smoking habits was 1.15 (95% confidence interval 1.04–1.28; P=0.0075).

Conclusions: The previously reported association of rs3846662 with LDL-C levels was replicated in the present Suita and Ehime samples. The LDL-associated SNP, rs3846662, appears to confer susceptibility to MI in Japanese. (*Circ J* 2010; **74:** 518–522)

Key Words: Genetics; Lipids; Myocardial infarction; Polymorphism

As outlined in the 2007 edition of the Japan Atherosclerosis Society (JAS) guideline for diagnosis and prevention of atherosclerotic cardiovascular diseases for Japanese,[1] elevated levels of low-density lipoprotein-cholesterol (LDL-C) are an important risk factor. LDL-C is known to be determined by both genetic and environmental factors. Substantial progress has been made toward detecting genes influencing circulating levels of LDL-C. In a recently published genome-wide association study (GWAS, n=19,840) with subsequent replication in 20,623 individuals,[2] 7 previously reported loci (*APOE/C1/C4/C2*, *APOB*, *HMGCR*, *LDLR*, *PCSK9*, *CELSR2/PSRC1/SORT1*, *CILP2/PBX4*),[3–8] as well as 4 novel loci (*ABCG8*, *TIMD4/HAVCR1*, *MAFB*, *HNF1A*) have shown genome-wide significant association with LDL-C levels. Although GWAS of lipid and lipoprotein levels have been predominantly conducted in populations of European ancestry, there have been only a few replication studies conducted in non-European populations.[4,9–11]

The association between single nucleotide polymorphisms (SNPs) of 3-hydroxy-3-methylglutaryl-coenzyme A reductase (*HMGCR*) and LDL-C levels has been well replicated in GWAS of white populations.[3,4,12] HMGCR is the rate-limiting enzyme in cholesterol synthesis, and inhibitors of HMGCR have been widely used as cholesterol-lowering drugs.[13] Recently, the common SNP in intron 13 of *HMGCR* (rs3846662) has been reported to be a functional variant, influencing the alternative splicing of exon 13.[14] In that study, lymphoblastoid cells from subjects homozygous for the major A allele showed higher levels of an alternatively spliced isoform missing exon 13 compared with those from

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Table 1. Clinical Characteristics of the Study Populations

	Suita sample		Ehime sample	
	Men	Women	Men	Women
No. of subjects	1,468	1,760	1,062	1,319
Age (years)	66.0±10.7	63.8±10.5	58.6±15.3	62.1±13.2
BMI (kg/m^2)	23.4±2.9	22.4±3.2	23.5±3.0	23.3±3.3
Total cholesterol (mg/dl)*	198.7±31.6	217.4±32.5	190.6±34.6	208.1±33.5
HDL-C (mg/dl)*	54.8±14.3	64.6±15.0	58.1±14.8	64.0±15.6
LDL-C (mg/dl)*	121.2±28.5	134.3±30.4	130.4±102.1	123.2±30.2
Triglycerides (mg/dl)*	119.0±84.8	93.0±55.6	59.8±7.3	103.7±55.5
% Medication for dyslipidemia	11.0	18.5	3.7	6.7
% Smokers	28.8	5.3	38.2	2.1
% Drinkers	67.2	27.3	85.0	33.9

Continuous variables are mean±standard deviation.
*Subjects with lipid-lowering medication were excluded.
BMI, body mass index; HDL-C, high-density lipoprotein-cholesterol; LDL-C, low-density lipoprotein-cholesterol.

homozygotes for the minor G allele. The alternatively spliced isoform of HMGCR has been detected in various human tissues, including kidney, liver, heart, spleen, lung, placenta, skeletal muscle, ovary, peripheral blood leukocytes, small intestine, bone marrow, brain, spinal cord, testes, thyroid gland, and uterus.[14,15] The proportion of the alternative splicing variant to the total HMGCR mRNA has been suggested to be tissue-specific.[14] In a recent pharmacogenetic study, in vitro upregulation of alternative splicing induced by statin treatment was inversely associated with the in vivo statin response and was partly determined by the genotypes of rs3846662.[16] Given the difference in allele frequencies and linkage disequilibrium (LD) patterns across the populations, it remains to be determined whether the previously reported functional variant, rs3846662, in *HMGCR* is associated with LDL-C levels in a Japanese population.

Methods

Study Populations

Suita Sample The study design of the Suita Study has been described previously.[17–24] In brief, the sample consisted of 14,200 men and women (30–79 years of age at enrollment), stratified by sex and 10-year age groups (10 groups and 1,420 subjects in each group) who had been randomly selected from the municipal population registry. They were all invited by letter to attend regular cycles of follow-up examination (every 2 years). Subjects were asked to estimate the amount and frequency of their alcohol intake per week, expressed as ethanol (g) per day.

To investigate the association of a genetic variation determining the LDL-C level with the risk of myocardial infarction (MI), genotyping of rs3846662 was carried out in 701 patients with MI randomly selected from in- and outpatients with documented MI and who were enrolled in the Division of Cardiology at the National Cardiovascular Center between May 2001 and April 2003. Those who were free from MI (n=3,118) served as controls.

Only those who gave written informed consent were included for the study. The study protocol was approved by the Institutional Ethics Committee and the Committee on Genetic Analysis and Gene Therapy of the National Cardiovascular Center.

Ehime Sample Ehime sample comprised subjects from the Nomura study of Ehime University, which is a longitudinal epidemiological study based on the Nomura Town residents.[25] Subjects were recruited through a community-based annual medical check-up process. Anthropometric and biochemical parameters were obtained from personal health records evaluated during the annual medical check-up. Information on smoking and drinking habits was obtained by interview. Subjects were asked to estimate average alcohol consumption per occasion expressed as 'gou', equivalent to 22.5 g of ethanol. All the study procedures were approved by the Ethics Committee of the Ehime University Graduate School of Medicine. Informed consent was given by each participating subject.

Genotyping Assays

Genotyping was performed by TaqMan allelic discrimination assays (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's instructions. Deviation from Hardy-Weinberg equilibrium and the degree of LD were analyzed using HaploView 4.0 (http://www.broad.mit.edu/mpg/haploview/).[26]

The exons 1–20 of *HMGCR* were sequenced in 48 subjects with low or high LDL-C levels using a 3730 DNA analyzer (Applied Biosystems) according to the manufacturer's instructions.

Statistical Analysis

Data are expressed as mean±standard deviation. Continuous variables were tested for normality of distribution, and logarithmic transformation was applied to those with skewed distributions. Residuals, defined as the observed minus predicted values on the basis of confounding factors, were used for the genotype–phenotype association analysis by 1-way analysis of variance (ANOVA) tests. Covariates included in the model were derived from multiple logistic regression analysis and used to calculate a residual value for each variable. Genotype frequencies between control and MI cases were compared by chi-square test. Odds ratio (OR) and 95% confidence interval (CI) for the risk allele were estimated by logistic regression analysis with adjustment for covariates. Statistical analysis was performed using a JMP statistical package 7.0 (SAS Institute, Cary, NC, USA).

Results

Clinical characteristics of the study populations are shown in

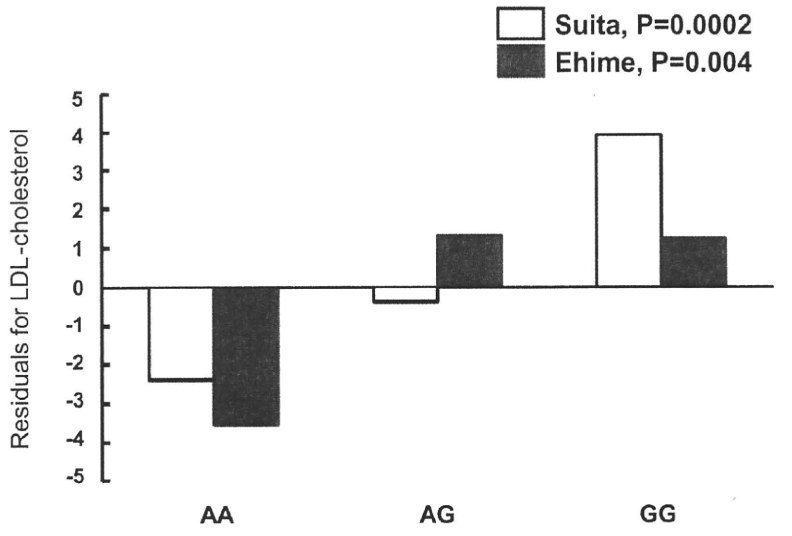

Figure. Association of rs3846662 with low-density lipoprotein (LDL)-cholesterol levels in the Suita and Ehime samples. Covariates used for the calculation of residual values for the Suita sample were sex, body mass index (BMI) and daily ethanol consumption. Covariates for the Ehime sample included age, sex, BMI and ethanol consumption per occasion.

Table 2. Logistic Regression Analysis of MI

rs3846662	Risk allele frequency	Genotype frequency			P value*	HWE†	OR‡ (95%CI)	P value
		AA	AG	GG				
Control (n=3,118)	0.511	0.232	0.514	0.254	0.0038	0.119	1.15 (1.04–1.28)	0.0075
MI cases (n=701)	0.559	0.193	0.496	0.311		0.905		

*Genotype frequencies between control and MI cases were compared by chi-square test.
†Deviation from HWE was analyzed by an exact test and P values are presented.
‡OR and 95%CI for the risk allele were estimated by logistic regression analysis with adjustment for age, sex, diabetes, hypertension, and drinking and smoking habits. BMI and the presence of hyperlipidemia were not significant predictors for MI and not included in the model.
MI, myocardial infarction; HWE, Hardy-Weinberg equilibrium; OR, odds ratio; CI, confidence interval; BMI, body mass index.

Table 1 and Figure summarizes the association of rs3846662 genotypes with LDL-C levels in the Suita and Ehime samples. Significant differences in residual values of LDL-C were observed among the genotypes of rs3846662 (P=0.0002 (n=2,686) and P=0.004 (n=2,110)) for the Suita and Ehime samples, respectively. In accordance with the previous report,[12] the G allele of rs3846662 was associated with higher LDL-C levels in the Suita sample (β, 3.56, $P=4.91\times10^{-5}$ with adjustment for sex, body mass index (BMI) and ethanol consumption). Although the association was in the same direction in both the Ehime and Suita samples, the frequency of the risk G allele was more common in the Suita than in the Ehime sample (0.511 among the Suita sample, 0.495 among the Ehime sample). In the Ehime sample, homozygotes for the A allele had significantly lower levels of LDL-C (β, −3.22, P=0.001 with adjustment for age, sex, BMI and ethanol consumption).

To examine the association between rs3846662 and the risk of MI, genotype frequencies were compared between patients with MI (n=701) and those free from MI (Table 2). The risk G allele of rs3846662 was more prevalent in subjects with MI than in subjects without MI (0.559 and 0.511 in MI cases and controls, respectively; nominal P=0.0038). The OR adjusted for age, sex, diabetes, hypertension, and smoking and drinking habits was 1.15 (95%CI 1.04–1.28; P=0.0075).

In order to assess whether a functional rare variant of *HMGCR* with a large effect is involved in influencing the variation in LDL-C levels in Japanese, we sequenced the exon regions of *HMGCR* in 48 subjects with low (n=18; residual LDL-C adjusted for sex, BMI and daily ethanol consumption: −71.76 to −4.25 mg/dl) or high (n=30; residual LDL-C adjusted for sex, BMI and daily ethanol consumption: 54.05–135.87 mg/dl) LDL-C levels. The sequencing analysis revealed 1 synonymous mutation on exon 17 (Thr758Thr) and 2 non-synonymous mutations on exon 9 (Tyr311Ser) and 19 (Gln824Lys). The minor allele frequency (MAF) for Thr758Thr, Tyr311Ser and Gln824Lys were 0.01, 0.03 and 0.01, respectively. Exons 11–20 are known to encode a catalytic domain.[27] Because only 1 subject with low LDL-C (uncorrected LDL-C: 46 mg/dl; residual LDL-C adjusted for sex, BMI and daily ethanol consumption: −71.8 mg/dl) had Gln824Lys, further genotyping of Gln824Lys on exon 19 was carried out in 192 subjects. However, we did not find any other subject with this mutation. Overall MAF (n=240) for Gln824Lys was 0.002.

Discussion

We have replicated the previously reported association of rs3846662 within intron 13 of *HMGCR* with LDL-C level in 2 independent Japanese populations: the Suita and Ehime samples. Furthermore, rs3846662 was found to be associated with the risk of MI. The risk allele frequency for rs3846662 was more common in patients with MI than in those without MI. The OR adjusted for age, sex, diabetes, hypertension,

and smoking and drinking habits was 1.15 (95%CI 1.04–1.28, P=0.0075).

Results of our GWAS[24] conducted in 900 Japanese men and women using the Illumina Sentrix HumanHap550 BeadChip (Illumina Inc, San Diego, CA, USA) are also in line with our current observation (see Supplement for more details). Among the 368,274 SNPs with a call rate >90% and MAF >0.1, rs3846662 of *HMGCR* was 1 of the top 38 SNPs exceeding the arbitrary threshold of $-\log_{10}P > 4.0$. Of 38 top-ranked SNPs, 20 were genotyped in the remaining Suita sample (n=1,000–1,500) for validation of the associations detected in the initial subpopulation (n=900). Although the strength of the association for the 20 SNPs genotyped in the additional Suita sample was weakened by increasing the sample size, the strongest association for LDL-C was observed for rs3846662, indicating this SNP as a good candidate for replication. Although it is possible that unrecognized genes or loci influencing LDL-C levels could be newly identified by increasing the sample size of the initial screening, the observation that none of the markers (n=368,274) achieved genome-wide significance after Bonferroni correction suggests that there is no master gene involved in determining LDL-C levels.

Because it can be speculated that multiple rare alleles with a much greater effect may contribute to variations in LDL-C levels in Japanese, we sequenced the 20 exons of *HMGCR* in 48 subjects with high or low LDL-C levels. Despite our anticipation, we failed to identify any unrecognized SNP with a larger effect.

One of the limitations of the current study is the use of the Friedewald formula to estimate LDL-C levels.[28] However, a recent study conducted in 27,331 women[29] demonstrated a significant correlation between the fasting LDL-C concentration by Friedewald equation and the direct method. Nearly identical results were obtained for fasting LDL-C levels by the 2 methods in terms of the ability to predict cardiovascular disease, questioning the advantage of the direct method over the Friedewald formula. Therefore, it is unlikely that the use of the Friedewald formula altered the outcome of the results significantly.

We have replicated the association of rs3846662 with LDL-C in 2 independent Japanese populations. In contrast to the remarkable effect of HMGCR inhibitors as a cholesterol-lowering drug, the effect of rs3846662 on LDL-C is rather small, explaining only a fraction. The physiological and functional importance of a gene may not necessarily be reflected by an effect size of a commonly occurring genetic mutation. There have been many reports investigating the effect of genetic polymorphisms on MI using a candidate gene approach.[30,31] Although our findings need to be tested in a larger sample, the LDL-associated functional SNP, rs3846662, identified through GWAS appears to confer susceptibility to MI in Japanese. The GWAS approach is a powerful tool for identifying genes involved in pathogenic pathways and will provide new clues to fundamental strategies for disease prevention and therapy. The possible candidate for future validation may be found in the GWAS data included in the Supplement.

In conclusion, the previously reported association of rs3846662 with LDL-C levels was replicated in Japanese populations.

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