Association of CYP2C19 variants and epoxyeicosatrienoic acids on patients with microvascular angina

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MICROVASCULAR ANGINA (MVA) is caused by coronary microvascular dysfunction, which can arise from chronic inflammation (5, 22, 29). Epoxyeicosatrienoic acids (EETs) have been suggested to be anti-inflammatory (25) and potent vasodilators in the canine coronary microcirculation (27), raising the possibility that low levels of EETs may cause microvascular dysfunction in humans. EETs are endothelium-derived hyperpolarizing factors (EDHFs) generated from metabolism of arachidonic acid by cytochrome P450 (CYP) 2C19 epoxygenases (4, 37).

ENDOTHELIUM HYPERPOLARIZATION mediated by the opening of the potassium and calcium (KCa) channels is originally recognized as the important initiating step for EDH-mediated vasodilation (8). Node et al. (25) reported that EETs prevented leukocyte adhesion to the vascular wall by a mechanism involving inhibition of transcription factor, nuclear factor-κB, and IkB kinase. The inhibitory effects of EETs were independent of their membrane-hyperpolarizing effects, suggesting that these molecules play an important nonvasodilatory role in vascular inflammation. Thus EETs have been suggested to be anti-inflammatory properties (25). Moreover, EETs act as potent vasodilators in canine coronary microcirculation (27).

Therefore, low levels of EETs may cause microvascular dysfunction via inflammation in humans. CYP2C19 is expressed in human endothelial cells, and enzymatic activity varies according to the number of CYP2C19 loss-of-function (LOF) alleles (*2, *3). CYP2C19 poor metabolizers (PM) have two LOF alleles (*2/2, *2/3, or *3/*3), and non-PM have none or one LOF allele (*1/*1, *1/*2, or *1/*3) (8, 31). CYP2C19 plays an important role in metabolism of clopidogrel, and therefore previous studies that have shown a link between CYP2C19 PM and cardiovascular outcomes have been on patients treated with clopidogrel due to the inability of such patients to convert clopidogrel to its active form, especially in acute coronary syndrome (ACS) (2, 18). However, recent data suggested that CYP2C19 variants are an independent risk factor for diabetic retinopathy (9) and coronary artery disease (CAD) (12) regardless of clopidogrel therapy. Therefore, CYP2C19 PM may be a new candidate risk factor for MVA via EET.

In results, the CYP2C19 poor metabolizer genotype may be a new candidate risk factor for MVA via EET.
Thus we examined the incidence and impact of CYP2C19 variants in MVA by measuring serum hs-CRP and dihydroxyeicosatrienoic acid (DHET) as representative EET metabolite in patients with MVA.

METHODS

Study population. From April 2009 to January 2014, 1,336 consecutive, hemodynamically, and symptomatically stable patients with suspected angina were registered and underwent angiography in Kumamoto University Hospital. We excluded patients with possible heart failure (left ventricular ejection fraction <50%), previous diagnoses of CAD, left ventricular hypertrophy (defined as >12-mm left ventricular wall thickness in echocardiography), severe hypertension (HT; >160/110 mmHg), valvular heart disease, and malignant diseases. In addition, patients with elevated white blood cell counts (>9,000) and/or serum hs-CRP (>0.5 mg/dl) were excluded to avoid the potentially confounding effects of occult infection or other systemic inflammatory diseases on hs-CRP levels.

After filtering, 1,198 patients were enrolled in this study. Vasoreactive drugs, including calcium-antagonists, angiotensin-converting enzyme inhibitors, angiotensin receptor blockers, and nitrates were withdrawn at least 5 days before patients entered this study; sublingual nitroglycerin was withdrawn within 24 h before entering the study. Patients with HT were defined as >140/90 mmHg. Patients with diabetes mellitus (DM) were defined as those with a 2-h glucose tolerance test ≥200 mg/dl, a fasting glucose level ≥126 mg/dl, an HbA1c score ≥6.5%, physician-diagnosed diabetes and/or the use of diabetic medication, and chronic kidney disease (CKD) testing with an estimated glomerular filtration rate <60 ml/min/1.73 m². Dyslipidemia (DLP) was defined as low-density lipoprotein >140 mg/dl, high-density lipoprotein <40 mg/dl, or triglyceride >150 mg/dl.

Patients with no history of chest pain and no cardiovascular disease who underwent medical examinations at the Kumamoto Health Care Center in 2,010 were used as healthy control subjects (n = 471). Control data were adjusted by age and gender by a propensity score matching method (SPSS software, Version 21.0, IBM Institute) to control data were adjusted by age and gender by a propensity score matching method (SPSS software, Version 21.0, IBM Institute) to

Methods. Subjects were divided into two groups, obstructive CAD and nonobstructive CAD, according to the results of coronary angiography. In patients with obstructive CAD, epicardial angiography was performed using the CAG. In patients with nonobstructive CAD, transcardiac lactate production ratio and measurement of the ATP-CFR ratio were conducted according to the Japanese Circulation Society guidelines (15a). Measuring ATP-CFR scores was also used to diagnose microvascular coronary dysfunction in nonobstructive CAD (19, 24).

Intracoronary acetylcholine-provocation test. The method of intracoronary ACh-provocation test was described in detail previously (1, 14, 16). In brief, incremental doses (20, 50, and 100 µg) of ACh chloride were injected into the right coronary artery over a period of 30 s each, and CAG was performed 1 min after the start of each provocation. The doses of ACh were administered at 5-min intervals. Subsequently, 50 µg of ACh were injected into the right coronary artery without the administration of intracoronary isosorbide mononitrate (ISDN) and CAG at an interval of 10 min, adenosine triphosphate (ATP; 150 µg·kg⁻¹·min⁻¹) was administered via the central vein until maximal hyperemia was achieved for the calculation of ATP-CFR. ATP-CFR was calculated with the following formula: hyperemia average peak velocity (APV)/Post-ISDN APV (36).

Angiographical identification of epicardial spasm. A positive finding for coronary spasm on CAG in the ACh test is defined as “transient, total, or subtotal occlusion (>90% stenosis) of a coronary artery.” In addition, a definite diagnosis of vasospastic angina requires simultaneous signs or symptoms of myocardial ischemia (angina pain and ischemic ECG change).

Lactate measurement in ACh-provocation test. To assess myocardial ischemia on the basis of the detection of increased lactate production in the coronary circulation, paired blood samples were collected simultaneously from the aortic root (LAR) and the coronary sinus (LCS) by using a coronary sinus catheter at three time points: baseline, 1 min after delivery of 100 µg of ACh via the left coronary artery, and after administration of ISDN. The lactate production ratio

Fig. 1. Diagnostic flowchart. The definition of the patients with microvascular angina (MVA) is shown. ATP-CFR, adenosine triphosphate-induced coronary flow reserve; VSA, vasospastic angina; IHD, ischemic heart disease.
was calculated with the following formula: (LCS-LAR)/LAR × 100 (%). Normally, the ratio is negative, and a positive value definitively indicates the occurrence of myocardial ischemia.

**Genotyping.** Genomic DNA was extracted from whole blood using the DNA Extractor WB kit (Wako Pure Chemical Industries), using a modified version of the protocol described by Richards et al. (30). The protocol described by Richards et al. (30) is a modified version of the protocol described by Richards et al. (30).

**RESULTS**

**Characteristics of subjects.** A total of 1,198 patients were enrolled in this study, and 303 patients were defined as non- obstructive coronary artery disease after initial coronary angiography (CAG). Finally, 71 patients were defined as having MVA after an ACh-provocation test, the measurement of lactate in the coronary circulation, and ATP-provocation (Figs. 1 and 2).

In the clinical characteristics of the MVA and control groups, the prevalence of the characteristics of current smoker and HT were significantly higher in the MVA group than that of control group (12.7 vs. 2.8% and 59.2 vs. 40.8%, P < 0.05 was regarded as statistically significant. SPSS software, Version 21.0 (IBM Institute) was used for all statistical analyses.

**Table 1. Clinical characteristics of control and MVA groups**

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Control (n = 71)</th>
<th>MVA (n = 71)</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, yr</td>
<td>59.9 ± 13.58</td>
<td>62.4 ± 11.92</td>
<td>0.941</td>
</tr>
<tr>
<td>Male, %</td>
<td>58.5 (35.5)</td>
<td>33 (46.5)</td>
<td>0.401</td>
</tr>
<tr>
<td>BMI, kg/m²</td>
<td>23.4 ± 3.30</td>
<td>23.7 ± 4.04</td>
<td>0.551</td>
</tr>
<tr>
<td>Body weight, kg</td>
<td>62.4 ± 11.92</td>
<td>59.9 ± 13.58</td>
<td>0.293</td>
</tr>
<tr>
<td>Current smoker, %</td>
<td>2 (2.8)</td>
<td>9 (12.7)</td>
<td>0.028</td>
</tr>
<tr>
<td>Hypertension, %</td>
<td>29 (40.8)</td>
<td>42 (59.2)</td>
<td>0.029</td>
</tr>
<tr>
<td>Dyslipidemia, %</td>
<td>47 (66.2)</td>
<td>38 (53.5)</td>
<td>0.123</td>
</tr>
<tr>
<td>Diabetes mellitus, %</td>
<td>7 (9.2)</td>
<td>8 (15.0)</td>
<td>0.347</td>
</tr>
<tr>
<td>CKD, %</td>
<td>15 (21.1)</td>
<td>16 (22.5)</td>
<td>0.839</td>
</tr>
</tbody>
</table>

Data are means ± SD. MVA, microvascular angina; BMI, body mass index; CKD, chronic kidney disease.
Figure 3 shows that the incidence rates of CYP2C19 genotypes (PM and non-PM) were 35.2 and 64.8%, respectively, in the MVA group and 15.5 and 84.5%, respectively, in the control group. The incidence of CYP2C19 PM is significantly higher in the MVA group than that of the control group (P = 0.007).

The levels of hs-CRP were significantly higher in the MVA group than those of the control group (0.127 ± 0.142 vs. 0.086 ± 0.097 mg/dl, P = 0.043; Fig. 4A), especially with CYP2C19 PM (0.180 ± 0.107 vs. 0.086 ± 0.078 mg/dl, P = 0.016). Moreover, in the MVA group, the level of hs-CRP with CYP2C19 PM was significantly higher than that of non-PM (0.180 ± 0.107 vs. 0.106 ± 0.149 mg/dl, P = 0.045; Fig. 4B).

The levels of serum 11,12- and 14,15-DHET in the MVA group were significantly higher than those of the control group [13.8 ± 3.30 vs. 8.24 ± 3.17 ng/ml, P < 0.001 (Fig. 5A), 18.5 ± 4.97 vs. 10.8 ± 1.20 ng/ml, P < 0.001 (Fig. 5C)]. Moreover, in the MVA group, the levels of serum 11,12 and 14,15-DHET with CYP2C19 PM were significantly lower than that of non-PM [14.2 ± 5.39 vs. 10.9 ± 1.64 ng/ml, P = 0.019 (Fig. 5B); 18.9 ± 4.73 vs. 15.2 ± 4.39 ng/ml, P = 0.025 (Fig. 5D)].

In multiple regression analysis for MVA, current smoker, HT, high levels of hs-CRP, and CYP2C19 PM have been shown to be associated with MVA development (OR 5.53, 95% CI 1.08–28.2, P = 0.040; OR 2.15, 95% CI 1.06–4.40, P = 0.032; OR 2.07, 95% CI 1.00–4.25, P = 0.048; OR 2.49, 95% CI 1.06–5.85, P = 0.036; Table 2).

DISCUSSION

Our results show that MVA is related to CYP2C19 PM and chronic inflammation, suggesting that insufficient upregulation of EET, a vasodilator in microcirculation, is one possible cause of MVA.

The ratio of CYP2C19 PM and the hs-CRP level were significantly higher in the MVA group than those in the control group. The decreased enzyme activity of CYP2C19 PM, which decreases the level of EETs, appears to lead to increased inflammation and impaired microcirculation. Measurement of 11,12- and 14,15-DHET levels revealed that EETs were significantly higher in the MVA group than compared with the control group, suggesting that the MVA group had impaired microcirculation that activated the EET defense mechanisms and led to higher EET levels. However, the low rate of increase in the MVA PM group suggests dysfunctional EET upregulation. These results are consistent with a link between the low EET increase associated with decreased enzyme activity and the expansion of inflammation and impaired microcirculation in the CYP2C19 PM group. The previous report of comparison between CAD and control (non-CAD) group suggested that EETs levels increase in the patients with CAD relative to control subjects (34). On the other hand, the report in CAD group suggested that EETs levels decrease by stenosis progress (28). Moreover, Spiecker et al. (32) reported low EET levels in a subgroup with decreased enzymatic activity by CYP2J2 genetic mutations compared with another subgroup with normal enzymatic activity by wild CYP2J2 genotype among the CAD patients. CAD is defined as the presence of epicardial organic stenosis in above studies, and the definition of MVA was different from the definition of MVA in this study.

Fig. 3. Prevalence of cytochrome P450 (CYP) 2C19 genotypes. The ratio of CYP2C19 poor metabolizer (PM) in the MVA group is significantly higher than that of the control group.

Fig. 4. Comparison of high-sense C-reactive protein (hs-CRP) levels. A: comparison of the control and MVA groups. The mean hs-CRP level is significantly higher in the MVA group than that of the control group. B: comparison of CYP2C19 PM and non-PM in the control and MVA groups. The mean hs-CRP level in the MVA group was significantly higher than that of control group, especially for CYP2C19 PM. Moreover, in the MVA group, the mean hs-CRP level for CYP2C19 PM is significantly higher than that for non-PM.
however, the lower EET levels in groups with LOF alleles (CYP2C19*2 and *3, CYP2J2*7) for patients with MVA or CAD suggest that EET has some effect in cases of epicardial or microcirculation.

Although there are several DHET isomers (8,9-DHET, 11,12-DHET, and 14,15-DHET) from EETs (7), we measured the levels of serum 11,12 and 14,15-DHET in this study. 11,12-DHET constitutes 24% of DHETs and 14,15-DHET are 39% (largest percentage) (17). The same results were shown in not only 11,12-DHET but also 14,15-DHET, as reported previously (32, 33).

In multivariate analysis (Table 2), the risk factors for MVA were not only high levels of hs-CRP and CYP2C19 PM, but also current smoker and HT. It was reported that HT, diabetes mellitus, smoking status, dyslipidemia, insulin resistance, and obesity were reported as causes of microvascular dysfunction (5). Moreover, Itoh et al. (15) reported that the spasm group has high ratio of smoker and HT. Spasm is defined by the presence of epicardial vasodilation; herein, as the definition of MVA was completely different, it is possible that the strong factors of endothelial dysfunction as HT and smoking are predicted as incidence of MVA.

Microvascular dysfunction was reportedly observed in ~20% of patients who complained of chest pain but who had no significant organic stenosis in the epicardial coronary artery (22). In the present study, to define MVA, not only did we perform catheterization examinations in all the subjects but we also performed ACh-provocation testing, measurement of lactate acid, and ATP stress tests. Based on these results, we rigorously diagnosed the condition of the patients and obtained accurate results with no contradictions. From the group of patients in the present study who complained of chest pain and

Table 2. Multivariate analysis for MVA

<table>
<thead>
<tr>
<th>Factor</th>
<th>Univariate Analysis</th>
<th>Multivariate Analysis</th>
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<tbody>
<tr>
<td></td>
<td>OR</td>
<td>95% CI</td>
</tr>
<tr>
<td>Age, yr</td>
<td>0.999</td>
<td>0.945–1.040</td>
</tr>
<tr>
<td>Male</td>
<td>0.754</td>
<td>0.390–1.454</td>
</tr>
<tr>
<td>BMI, kg/m²</td>
<td>1.028</td>
<td>0.939–1.25</td>
</tr>
<tr>
<td>Current smoker</td>
<td>5.008</td>
<td>1.042–24.07</td>
</tr>
<tr>
<td>Body weight, kg</td>
<td>0.985</td>
<td>0.956–1.013</td>
</tr>
<tr>
<td>Dyslipidemia</td>
<td>0.588</td>
<td>0.299–1.158</td>
</tr>
<tr>
<td>Diabetes Mellitus</td>
<td>1.735</td>
<td>0.519–5.806</td>
</tr>
<tr>
<td>Hypertension</td>
<td>2.360</td>
<td>1.204–4.628</td>
</tr>
<tr>
<td>CKD</td>
<td>1.086</td>
<td>0.490–2.409</td>
</tr>
<tr>
<td>hs-CRP &gt; median, 0.07 mg/dl</td>
<td>2.503</td>
<td>1.274–4.917</td>
</tr>
<tr>
<td>CYP2C19 PM</td>
<td>2.964</td>
<td>1.323–6.640</td>
</tr>
</tbody>
</table>

Factors found to be significant (P < 0.05) in univariate analysis were subsequently entered into multivariate analysis. hs-CRP, high sense C-reactive protein; PM, poor metabolizer; OR, indicated odds ratio; CI, confidence interval.
did not present with epicardial stenosis, MVA was diagnosed in \( \sim 23.4\% \) of the patients, which was consistent with the rate reported by previous studies (22, 25).

Limitations. This study has a small sample size. Because we performed both cardiac catheterization and ACH and ATP provocation tests for all enrolled subjects to define MVA, the definition of MVA in this study is more accurate than that in studies in which subjects are not subjected to ACH and ATP provocation tests. Second, we cannot be sure that CAD and microvascular dysfunction were absent in the control subjects because the control subjects did not undergo cardiac catheterization or ACH and ATP provocation tests. However, we chose individuals with no medical history of chest pain or cardiovascular disease as control subjects. Third, we have no information about medical therapy, especially concerning clopidogrel. We have no data about medication in the control group, so we cannot compare with the MVA group medication. However, there were no patients with clopidogrel therapy in MVA group and we chose individuals with no medical history of chest pain or cardiovascular disease as control subjects. Therefore, there is little influence on microvascular dysfunction of clopidogrel therapy in this study.

Fourth, we consider that it is desirable to measure EET, EET + DHET, and EET/DHET ratio. In the present study, we used an ELISA kit, and it was difficult to measure EET concentration levels because preserved blood was hydrolyzed. We consider and discuss this study under the consideration that DHET is equal to EET; this consideration was also a study limitation. Finally, we identified reduced DHET, which reflects EET levels and hence the results of this study may not be necessarily applicable to other populations.

Clinical implications. The variant of CYP2C19 genotype was associated with MVA in the Japanese. This study thus identified reduced DHET, which reflects EET levels and hence an insufficient defensive mechanism as a risk factor to be targeted and intervened for the treatment and prevention of MVA. Recently, Iming et al. (13) showed that a novel molecule inhibitor of epoxide hydrolase (EH) inhibited the activity of EH and effectively restored the EET concentration in animal models. Accordingly, it is expected that this class of drug may serve as a new therapeutic for MVA in the future.

Conclusions. Our results indicate that CYP2C19 variants are associated with MVA. The decline of EET-based defensive mechanisms owing to CYP2C19 variants may affect coronary microvascular dysfunction.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

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