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the beneficial effects of atrial natriuretic peptide and nicorandil have only been tested in single-centre studies with small sample sizes.²⁴⁻²⁵ The Japan working group studies on acute myocardial infarction for the reduction of necrotic damage by human atrial natriuretic peptide or nicorandil (J-WIND-ANP and J-WIND-KATP, respectively) aimed to assess the value of these drugs as adjuncts to percutaneous coronary intervention for patients with acute myocardial infarction.

Methods
Patients

We have described the protocols for the two trials previously.^{26,27} In brief, we recruited patients to two independent, investigator-initiated, investigator-led, multicentre, prospective, randomised, single-blind, controlled trials at 65 hospitals. 27 hospitals participated in the atrial natriuretic peptide trial, and 38 separate hospitals in the nicorandil trial (table 1); the two studies were completely independent. We initially planned to include fewer hospitals, but we increased the number to promote enrolment of sufficient patients.

Eligibility criteria were age between 20 and 79 years; chest pain for more than 30 min; at least 0.1 mV of ST segment elevation in two adjacent ECG leads; admission to hospital within 12 h of the onset of symptoms; and one instance of acute myocardial infarction. Exclusion criteria were a history of myocardial infarction; left main trunk stenosis; severe liver or kidney dysfunction or both; suspected aortic dissection; previous coronary artery bypass grafting; and a history of drug allergy.

All patients gave written informed consent immediately after admission to hospital, and were asked to sign the same consent form again after 2 weeks when they had more time to decide. This system was applied on the recommendation of the institutional review boards. Only one patient, who was in the nicorandil group, withdrew their consent at their second opportunity. We enrolled patients from Oct 24, 2001, to Dec 13, 2005. The study protocol was approved by the institutional review boards and ethics committees of all participating hospitals, and was in accordance with the Declaration of Helsinki.

Procedures

An independent statistician generated our randomisation lists with a computer, by the permuted-block method. Within each centre, the block length was eight. Treatment allocations were concealed in opaque sealed envelopes until patients were enrolled. Physicians were not aware of the random assignments of patients until the follow-up stage; patients and those who analysed the data were unaware of the treatment assignment for the duration of the study. Both trials were designed as single-blind studies.

277 patients who were enrolled in the atrial natriuretic peptide trial were randomly assigned to receive an intra-

venous infusion of this drug after reperfusion treatment, at 0.025 µg/kg per min for 3 days, and 292 a placebo of 5% glucose solution by the same method. 276 patients in the other trial were randomly assigned to intravenous nicorandil, infused at 1.67 µg/kg per min for 24 h after bolus injection of nicorandil at a dose of 0.067 mg/kg, and 269 were assigned to 0.9% saline solution, by the same method. Previous studies have shown substantial cardiovascular protection with atrial natriuretic peptide and nicorandil at these doses.^{20,22} Of the 276 patients assigned to receive nicorandil, 61 were given nicorandil orally, at the discretion of individual investigators, during the follow-up period.

We planned to stop the administration of treatment drugs in case of severe hypotension, which was defined as systolic blood pressure of less than 90 mm Hg, because of the vasodilator effect of these drugs. The study protocol did not restrict or specify any other diagnostic or therapeutic methods in the acute phase (2–8 weeks after acute myocardial infarction) or chronic phase (6–12 months).

We obtained data on baseline characteristics, emergent catheterisation, and medication at discharge after 1 month; data on follow-up catheterisation and medication after 6 months; and data on medication after 24 months. We also followed up all patients for cardiovascular events (ie, cardiac death, readmission to hospital due to heart failure, new onset of acute coronary syndrome, or revascularisation of new lesions) until the end of August, 2006. We took blood samples to measure concentrations of creatine kinase at a central laboratory, before the procedure and at 1, 3, 6, 9, 12, 18, 24, 36, 48, and 72 h after the onset of reperfusion.¹⁴ We analysed total creatine kinase for all patients with at least six blood samples. We obtained right anterior oblique views with angiography of the left ventricle once in the acute phase (2–8 weeks), and once in the chronic phase (6–12 months).

Our primary endpoints were infarct size (which was estimated as the area under the concentration versus time curve for creatine kinase)¹⁴ and ventricular ejection fraction (which was assessed by angiography of the left ventricle at 6–12 months after hospital admission).¹⁴ The prespecified secondary endpoints were survival rate; cardiovascular events (such as cardiac death, readmission to hospital for heart failure, new onset of acute coronary syndrome, or revascularisation of new lesions); incidence of cardiac death or readmission to hospital for

	J-WIND-ANP study	J-WIND-KATP study
1-4 patients	7 hospitals	9 hospitals
5-9 patients	3 hospitals	13 hospitals
10-19 patients	7 hospitals	6 hospitals
More than 20 patients	10 hospitals	10 hospitals

Table 1: Distribution of patients between participating hospitals

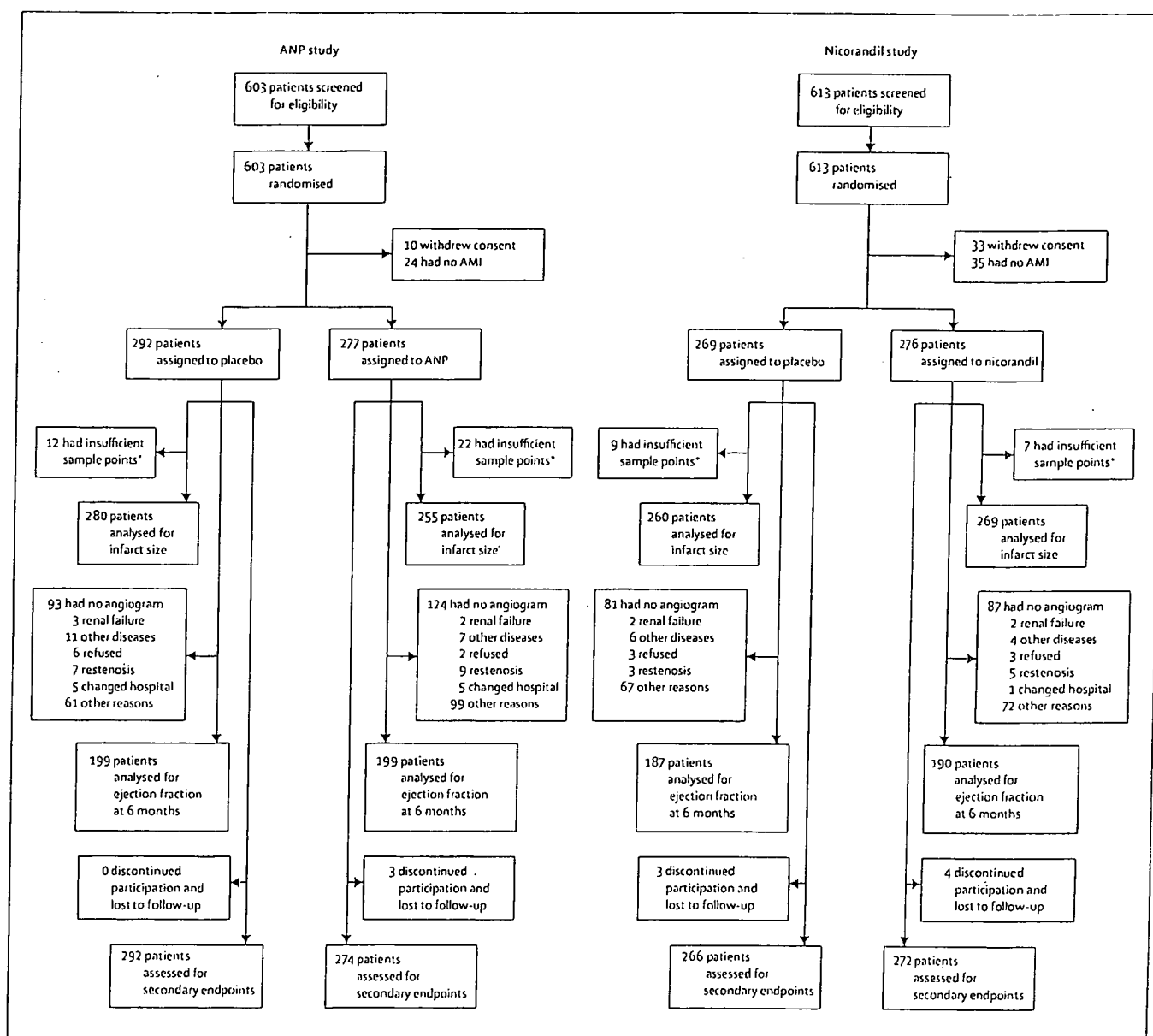


Figure 1: Trial profiles

ANP=atrial natriuretic peptide. AMI=acute myocardial infarction. *Fewer than six blood samples.

heart failure; or reperfusion injury before discharge from coronary care unit (such as malignant ventricular arrhythmia during reperfusion, recurrence of ST segment elevation, or worsening of chest pain). We also assessed infarct size, estimated by peak creatine kinase and troponin T;^{28,29} left ventricular ejection fraction at acute phase; and end-diastolic or end-systolic volume index (assessed by angiography of the left ventricle). We looked at the effects of each drug on the primary endpoints in prespecified subgroups (sex, age, body-mass index, pre-angina, elapsed time between acute

myocardial infarction and intervention, diabetes mellitus, hyperlipidaemia, smoking, and family history of acute myocardial infarction). We also did post-hoc analyses on the effect of chronic administration of nicorandil on the ejection fraction.

All data were collected by Koteisho-kyokai (Tokyo), an organisation established by the Japanese government in 2001–2003 and by NTT Data (Tokyo) in 2004–2006. Left ventricular ejection fraction and end-diastolic volume were measured by the area-length method, from angiography of the left ventricle. Two independent

interpreters, who were unaware of the treatment assigned to patients, measured left ventricular ejection fractions from the angiographs. We calculated the average value, unless the two investigators disagreed, in which case we referred to a third opinion.

Clinical findings and medications during the follow-up period were reported to a data and safety committee after registration. This committee, which consisted of three physicians and one statistician who did not participate in the trial, monitored all adverse events. Research nurses or doctors visited all participating hospitals to check that patients were registered, drugs were given, and data collected according to the protocol. Committee members did not provide any results to the steering committee, because discontinuation of the study was not recommended.

Statistical analysis

We calculated that a sample size of 300 patients would be needed in each group to detect a 20% reduction in the most important primary endpoint (total creatine kinase) with a statistical power of 80% at significance level of 0.05 (with a two-sided *t* test), accounting for dropout of some patients. We set equal sample sizes in both groups, because we expected to see almost the same reduction in infarct size with either treatment. Since creatine kinase and total creatine kinase are both log-normally distributed,¹⁰ total creatine kinase was log-transformed before analysis. The left ventricular ejection fraction was also log-transformed before the analysis since the distribution was skewed.

Statistical analysis was done according to a prespecified analytical plan. Efficacy analysis was based on intention to treat. The primary efficacy analyses for total creatine kinase and left ventricular ejection fraction were done simply by *t* test. The estimated mean and differences on the log scale were transformed back to the original scale and were expressed as geometric means and ratios of geometric mean. If the calculated

95% CI for the ratio of the geometric mean did not cross the point of no effect (ie, 1) the difference between groups was regarded as significant. Furthermore, analysis of covariance for the two endpoints was used to estimate adjusted mean comparison, with effect of covariates and the interactions. We imputed missing data for patients by the predicted mean imputation method, with nonlinear regression. We applied multiple imputation techniques (with group means, Markov Chain Monte Carlo, Bayesian bootstrap, and last-observation-carried-forward methods) to assess the robustness and sensitivity of our conclusions.

Proportions were examined by Fisher's exact test. We examined time-to-event by the Kaplan-Meier method to estimate the survival for each group and then the differences in survival between groups by the log-rank test. The Cox proportional hazards model was used to assess baseline risk factors and an adjusted hazard ratio. The proportional hazards assumption was investigated graphically, with a test based on Schoenfeld residuals.^{11,12}

All tests were two-sided, and a *p* value of less than 0.05 was regarded as significant. All analyses were done with SAS software (version 8.2). The trials are registered with Clinicaltrials.gov, numbers NCT00212056 and NCT00212030.

Role of the funding source

The sponsors of the study had no role in study design, data collection, data analysis, data interpretation, or writing of the report. The corresponding author had full access to all data at the end of the study, and had final responsibility for the decision to submit for publication.

Results

Figure 1 shows the trial profile. Table 2 shows baseline characteristics. Median follow-up was 2.7 (IQR 1.5–3.6) years in the atrial natriuretic peptide trial and 2.5 (1.5–3.7) years in the nicorandil trial. Table 3 shows

	Atrial natriuretic peptide study		<i>p</i>	Nicorandil study		<i>p</i>
	ANP (n=277)	Control (n=292)		Nicorandil (n=276)	Control (n=269)	
Age (years)	63.0 (10.4)	61.8 (10.7)	0.1652	61.1 (11.4)	63.7 (10.2)	0.0035
Sex (male)	211 (76.2%)	243 (83.2%)	0.0374	246 (89.1%)	220 (81.8%)	0.0153
Body-mass index	24.3 (3.5)	24.0 (2.9)	0.3733	24.2 (3.0)	23.4 (2.8)	0.0007
Killip classification (I, II, III, IV)	88.6%, 9.5%, 1.1%, 0.8%	90.3%, 7.5%, 1.4%, 0.7%	0.5274	91.1%, 8.2%, 0.4%, 0.4%	92.0%, 4.2%, 2.7%, 1.1%	0.7843
Pre-angina	105 (44.5%)	118 (46.1%)	0.7862	111 (44.6%)	111 (43.9%)	0.9284
Risk factors						
Hypertension	137 (56.1%)	162 (62.1%)	0.2046	127 (48.5%)	137 (53.9%)	0.2190
Diabetes mellitus	81 (33.8%)	86 (33.9%)	1.0000	104 (39.5%)	82 (32.9%)	0.1413
Hyperlipidaemia	127 (54.3%)	131 (50.6%)	0.4181	121 (46.7%)	114 (46.2%)	0.9291
Smoking	158 (63.7%)	175 (67.3%)	0.4022	178 (68.7%)	170 (66.1%)	0.5732

Data are number (%) or mean (SD), unless otherwise specified. ANP=atrial natriuretic peptide.

Table 2: Baseline characteristics on admission



	Atrial natriuretic peptide study		Nicorandil study	
	ANP (n=277)	Control (n=292)	Nicorandil (n=276)	Control (n=269)
Elapsed time (h)*	4.00 (3.00-6.00)	4.00 (2.50-6.00)	3.50 (2.50-5.00)	3.50 (2.50-5.00)
Infusion time (h)	1.00 (0.50-1.00)	1.00 (0.50-1.00)	0.70 (0.50-1.00)	0.75 (0.50-1.00)
IRA (LAD, LCx, RCA)	55.3%, 6.4%, 38.3%	52.3, 10.6, 37.1%	53.9, 7.4, 38.7%	44.5, 9.9, 45.6%
Stents	176 (63.5%)	193 (66.1%)	187 (67.8%)	183 (68.0%)
Rescue	64 (23.1%)	92 (31.5%)	94 (34.1%)	92 (34.2%)
Intra-aortic balloon pump	17 (6.1%)	14 (4.8%)	14 (5.1%)	15 (5.6%)
Final stenosis (<75%)	246 (93.5%)	266 (94.7%)	257 (96.6%)	255 (97.0%)
Final thrombolysis in myocardial infarction (0, 1, 2, 3)	3.9%, 1.9%, 5.0%, 89.1%	5.2%, 0.7%, 4.1%, 90.0%	3.7%, 0.7%, 5.2%, 90.3%	3.4%, 1.1%, 6.9%, 88.5%
Medications at 1 month				
ACE inhibitor	155 (57.8%)	173 (60.7%)	164 (61.0%)	163 (62.0%)
ARB	77 (28.7%)	99 (34.7%)	72 (26.8%)	69 (26.2%)
Spironolactone	28 (10.4%)	33 (11.6%)	17 (6.3%)	22 (8.4%)
β blocker	112 (41.8%)	128 (44.9%)	110 (40.9%)	121 (46.0%)
Aspirin	225 (84.0%)	252 (88.4%)	251 (93.3%)	250 (95.1%)
Nitrates	81 (30.2%)	86 (30.2%)	50 (18.6%)	63 (24.0%)
Statins	129 (48.1%)	156 (54.7%)	126 (46.8%)	115 (43.7%)
Nicorandil	62 (23.1%)	52 (18.2%)	79 (29.4%)	34 (12.9%)
Medications at 6 months				
ACE inhibitor	103 (48.1%)	117 (44.8%)	120 (50.6%)	131 (53.9%)
ARB	69 (32.2%)	110 (42.1%)	68 (28.7%)	75 (30.9%)
Spironolactone	26 (12.1%)	26 (10.0%)	11 (4.6%)	15 (6.2%)
β blocker	93 (43.5%)	118 (45.2%)	104 (43.9%)	113 (46.5%)
Aspirin	179 (83.6%)	233 (89.3%)	217 (91.6%)	229 (94.2%)
Nitrates	51 (23.8%)	63 (24.1%)	37 (15.6%)	49 (20.2%)
Statins	112 (52.3%)	150 (57.5%)	123 (51.9%)	118 (48.6%)
Nicorandil	46 (21.5%)	39 (14.9%)	55 (23.2%)	23 (9.5%)
Medications at 24 months				
ACE inhibitor	66 (47.5%)	63 (37.5%)	83 (52.5%)	75 (49.3%)
ARB	42 (30.2%)	72 (42.9%)	39 (24.7%)	43 (28.3%)
Spironolactone	13 (9.4%)	21 (12.5%)	9 (5.7%)	4 (2.6%)
β blocker	57 (41.0%)	61 (36.3%)	77 (48.7%)	71 (46.7%)
Aspirin	113 (81.3%)	133 (79.2%)	143 (90.5%)	137 (90.1%)
Nitrates	29 (20.9%)	45 (26.8%)	23 (14.6%)	25 (16.4%)
Statins	66 (47.5%)	78 (46.4%)	81 (51.3%)	71 (46.7%)
Nicorandil	26 (18.7%)	26 (15.5%)	28 (17.7%)	11 (7.2%)

Data are median (IQR), number (%) or mean (SD), unless otherwise specified. ANP=atrial natriuretic peptide. IRA=infarct-related artery. LAD=left anterior descending coronary artery. LCx=left circumflex artery. RCA=right coronary artery. ARB=angiotensin receptor blocker. ACE=angiotensin-converting enzyme. *Period between acute myocardial infarction and start of intervention.

Table 3: Treatments and prescribed drugs

treatments and drugs throughout the study. Drugs used in the chronic stage did not differ between groups in either study, except that some patients in the nicorandil trial were given oral nicorandil during follow-up.

Table 4 and figure 2 show infarct size and left ventricular function at 2–8 weeks and 6–12 months in both studies. The ratio of total creatine kinase between the atrial natriuretic peptide and placebo groups was 0.85 (95% CI 0.75–0.97, $p=0.0155$); which indicates that atrial natriuretic peptide was associated with a reduction of 14.7% in infarct size. Subanalyses identified no factors that enhanced or reduced the

influence of atrial natriuretic peptide on infarct size (figure 2). Nicorandil did not reduce infarct size compared with placebo, and no factors affected this finding. Treatment with atrial natriuretic peptide tended to increase the left ventricular ejection fraction (ratio 1.043, 95% CI 1.000–1.089, $p=0.0525$) at 2–8 weeks after the onset of acute myocardial infarction, and at 6–12 months (ratio 1.051, 95% CI 1.006–1.099, $p=0.0236$). By contrast, table 4 and figure 2 show that left ventricular ejection fraction did not differ in patients given nicorandil and controls at either 2–8 weeks or 6–12 months.

	J-WIND-ANP study		p	J-WIND-KATP study		p
	Atrial natriuretic peptide	Control		Nicorandil	Control	
Infarct size						
n	255	280		269	260	
Creatine kinase (area under curve) (IU/L h)	66459.9 (60258.2-73300.0)	77878.9 (71590.2-84720.1)	0.016	70520.5 (64309.8-77331.0)	70852.7 (65066.7-77153.2)	0.941
Peak creatine kinase (IU/L)	2487.5 (2217.6-2790.3)	2784.2 (2526.7-3067.9)	0.141	2557.1 (2306.1-2835.4)	2428.7 (2199.8-2681.5)	0.479
Troponin-T concentration (12-18 h) (ng/mL)	5.36 (4.76-6.03)	6.13 (5.55-6.79)	0.084	6.18 (5.51-6.93)	5.60 (4.97-6.32)	0.244
Troponin T (96 h) (ng/mL)	2.57 (2.25-2.94)	2.94 (2.64-3.27)	0.125	2.63 (2.36-2.94)	2.89 (2.61-3.19)	0.225
Left ventricle (2-8 weeks)						
n	187	207		168	170	
Median elapsed time (days)*	18.5 (IQR 15.0-27.0)	19.0 (IQR 16.0-25.0)		17.0 (IQR 14.0-23.0)	17.0 (IQR 14.0-24.0)	
Ejection fraction	43.0% (41.8-44.3)	41.3% (40.0-42.6)	0.053	42.0% (40.7-43.3)	41.6% (40.4-42.9)	0.680
End diastolic volume index (mL/m ²)	98.8 (94.4-103.4)	102.3 (98.1-106.6)	0.272	111.2 (106.4-116.3)	105.9 (100.9-111.3)	0.147
End systolic volume index (mL/m ²)	54.2 (51.2-57.4)	58.3 (55.5-61.4)	0.058	62.8 (59.2-66.6)	60.4 (57.0-64.1)	0.360
Left ventricle (6-12 months)						
n	155	199		190	187	
Median elapsed time (days)*	196.5 (IQR 180.5-230.5)	200.5 (IQR 183.0-226.0)		195.0 (IQR 180.0-231.0)	195.5 (IQR 183.0-232.0)	
Ejection fraction	44.7% (43.4-46.0)	42.5% (41.2-43.9)	0.024	42.5% (41.2-43.8)	43.2% (42.0-44.4)	0.460
End diastolic volume index (mL/m ²)	100.6 (95.2-106.2)	100.9 (96.8-105.1)	0.930	109.8 (105.4-114.4)	105.7 (100.8-110.8)	0.230
End systolic volume index (mL/m ²)	54.2 (50.6-58.0)	56.0 (53.1-58.9)	0.452	61.7 (58.4-65.2)	58.5 (55.1-62.1)	0.198

Data are mean (95% CI) or median (IQR). *Time between acute myocardial infarction and start of intervention.

Table 4: Primary endpoints and other outcomes obtained by angiography of left ventricles

Figure 3 shows reperfusion injuries, survival rates, and cardiovascular events. Reperfusion injuries were less common in the atrial natriuretic peptide group than in the placebo group (ratio 0.743, 95% CI 0.58-0.952, $p=0.019$). Although there were no differences between groups in either survival rates or the incidence of cardiovascular events, both cardiac death and readmission to hospital for heart failure were lower in patients given atrial natriuretic peptide than in controls (HR 0.267, 95% CI 0.089-0.799, $p=0.0112$). By contrast, cardiac death and readmission to hospital for heart failure were not significantly lower in patients given nicorandil than in controls (HR 0.799, 95% CI 0.307-1.973, $p=0.5972$). When nicorandil was given orally throughout the study after reperfusion treatment, the change of left ventricular ejection fraction increased substantially between the acute and chronic phase. The ejection fraction was 3.66% in the 61 patients who were given nicorandil orally, and 1.47% in the 241 patients who were not (difference 2.20, 95% CI 0.17-4.22, $p=0.0338$).

In the atrial natriuretic peptide trial, 29 patients given that drug had severe hypotension during the acute phase, compared with one control. In the other trial, three patients in the nicorandil group had severe hypotension, compared with no controls. No other severe adverse events were reported during the course of either study.

Discussion

We showed that adjunctive, acute-phase treatment with atrial natriuretic peptide after reperfusion therapy in patients with acute myocardial infarction reduced infarct

size by 14.7%, increased the left ventricular ejection fraction during the chronic phase, and decreased the incidence of cardiac death and readmission to hospital because of heart failure. Intravenous treatment with nicorandil did not affect the primary endpoints, although patients who were given nicorandil orally had better cardiac function outcomes.

Interest in the cardioprotective effects of adenosine has increased, because of its variety of cardioprotective mechanisms. Unfortunately, in trials of adenosine, it only marginally improved infarct size and showed no clinical benefits.²³ We hypothesised that treatment with atrial natriuretic peptide and nicorandil in the acute phase might prove more effective than chronic-phase treatment for limitation of infarct size. The first window of ischaemic preconditioning is mediated by opening of the KATP channel,²⁴ which is the mechanism of action of nicorandil; and the second window is mediated by nitric oxide and activation of G kinase, which is the mechanism of action of atrial natriuretic peptide.

Before this clinical trial, we had tested whether atrial natriuretic peptide could limit infarct size in a canine model in which the left anterior coronary artery was ligated for 90 min, followed by 6 h of reperfusion. Treatment with atrial natriuretic peptide reduced infarct size by about 40% after reperfusion (unpublished data). Our results are consistent with the finding of Hayashi and coworkers²⁵ that infusion of atrial natriuretic peptide immediately after reperfusion in patients with their first anterior acute myocardial infarction increased left ventricular ejection fraction.

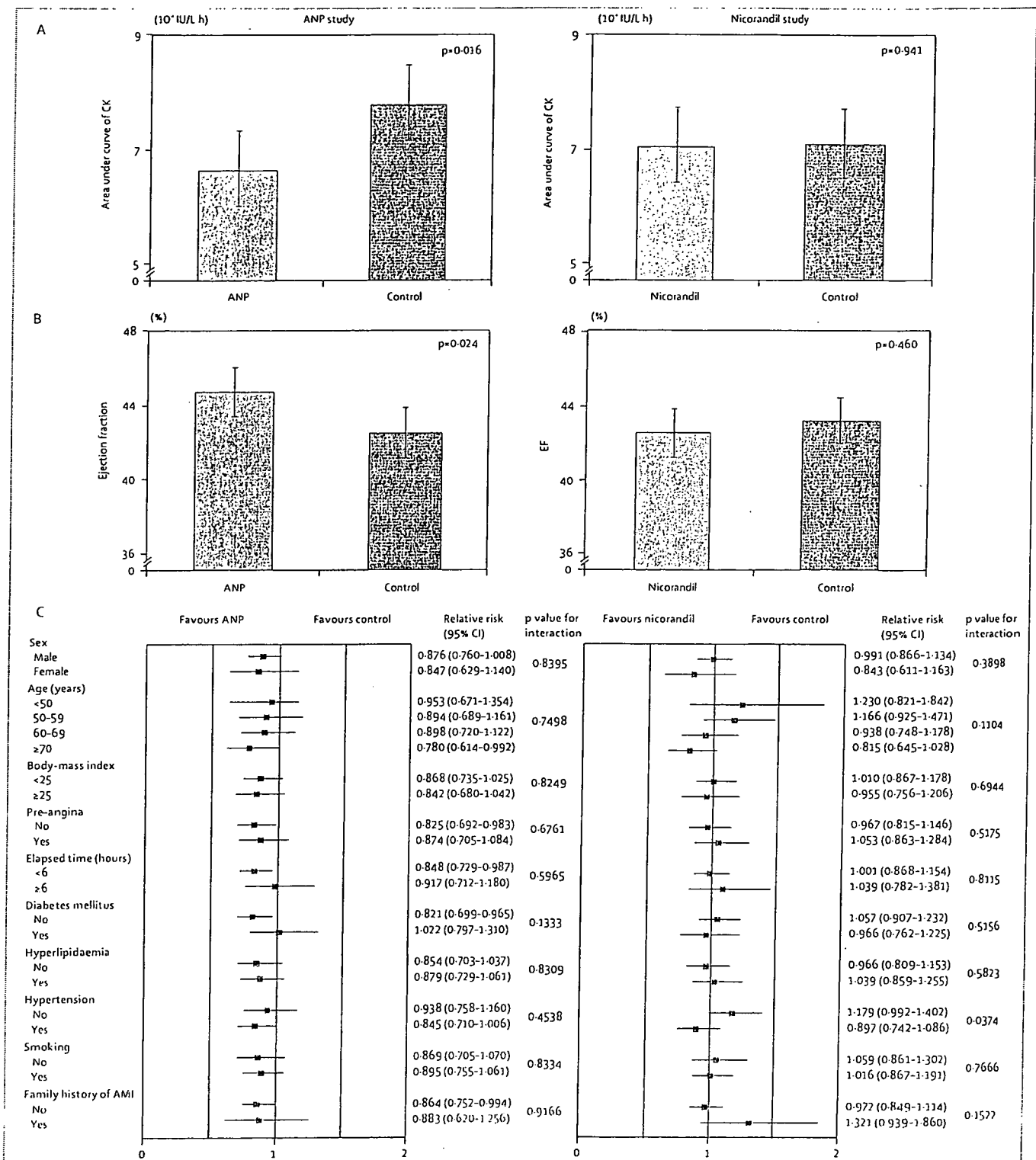


Figure 2: Primary endpoints and subgroup analyses. CK=creatinine kinase. AMI=acute myocardial infarction. ANP=atrial natriuretic peptide. Panel A shows area under curve of creatine kinase concentration versus time. Panel B represents left ventricular ejection fraction measured at 6-12 months.

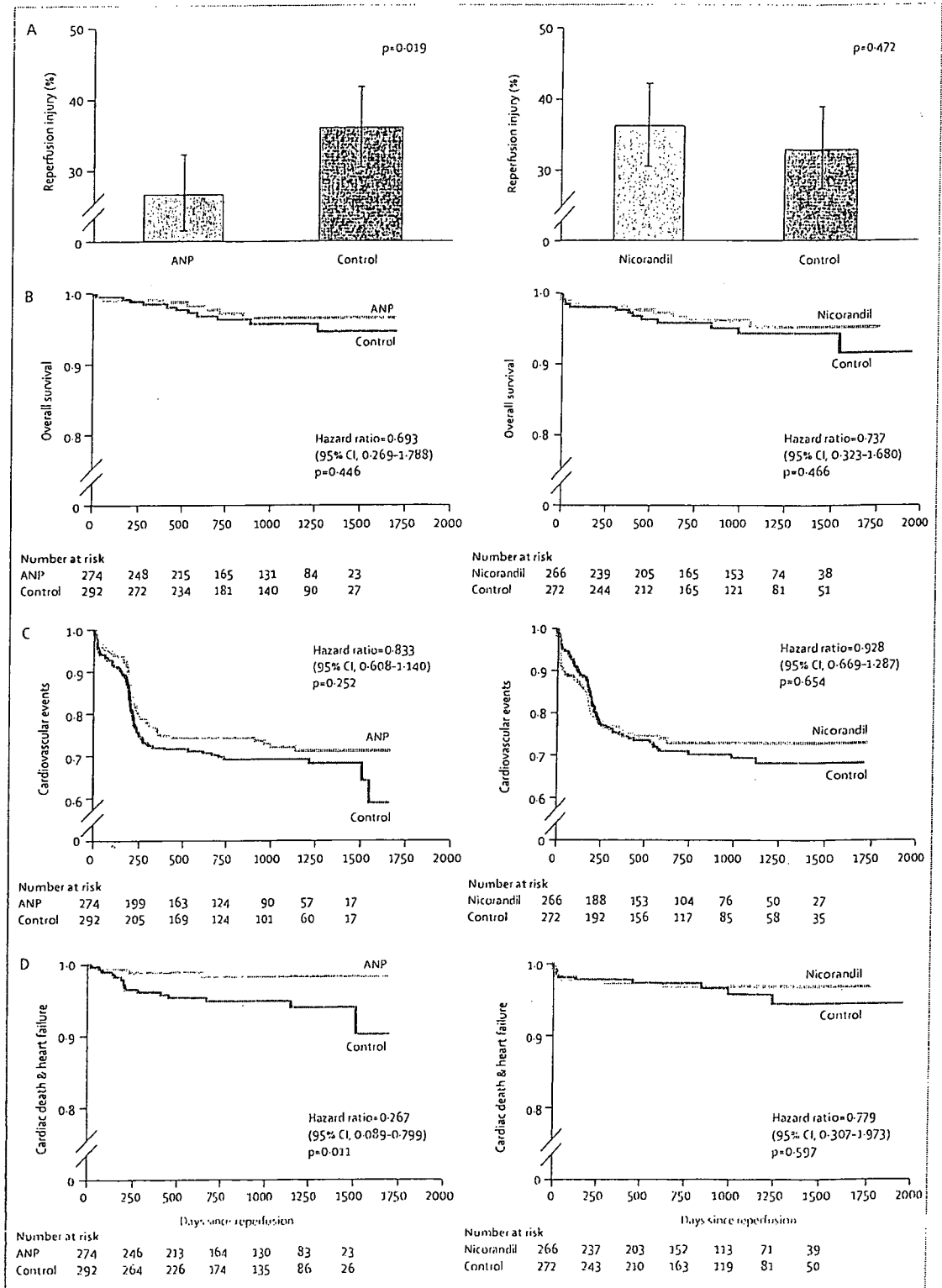


Figure 3: Secondary endpoints and other subanalyses
ANP=atrial natriuretic peptide.

The reduction of infarct size and the improvement of left ventricular ejection fraction might decrease mechanical stress on the non-infarcted myocardium, which might decrease hypertrophy and dilatation of the non-infarcted myocardium. Since cardiac hypertrophy and dilatation cause diastolic and systolic heart failure, a reduction of infarct size and an increase of left ventricular ejection fraction could mediate beneficial clinical outcomes. However, we need to do another large-scale clinical trial to target clinical outcomes such as cardiovascular death, because our primary aim here was to test the reduction of infarct size. Moreover, Hayashi and colleagues⁷⁰ showed that plasma concentrations of angiotensin II, aldosterone, and endothelin-1 were lower in patients given atrial natriuretic peptide than in controls. Sudden exposure to high concentrations of angiotensin II, aldosterone, and endothelin-1 for several days caused vascular or ventricular remodelling, and attenuation of these harmful effects by infusion of atrial natriuretic peptide could reduce the incidence of cardiac death and readmission to hospital for chronic heart failure.⁷⁰

One reason that nicorandil treatment did not limit infarct size in our study could be the size of the dose. Ishii and colleagues³⁵ have reported that one intravenous administration of a dose of nicorandil that was three times higher than that which we used decreased the infarct size and reduced the rate of cardiovascular death or readmission to hospital for chronic heart failure in 368 patients with acute myocardial infarction.

Patients in the nicorandil study who were given nicorandil orally in the chronic phase had greater increases in left ventricular ejection fraction, irrespective of whether nicorandil was given intravenously or orally. Since microvascular obstruction ten days after myocardial infarction was associated with left ventricular remodelling and poor prognosis, coronary perfusion might be improved by opening KATP channels in coronary blood vessels during the healing stage. The IONA study³⁵ showed that nicorandil could reduce the incidence of unstable angina in patients with stable angina.

Our finding that treatment with atrial natriuretic peptide in the acute phase reduced the incidence of readmission to hospital for chronic heart failure could help to reduce the physical, medical, and economic burdens on people around the world. Moreover, since intravenous nicorandil in the acute phase, followed by oral administration in the chronic phase, increased the left ventricular ejection fraction, chronic treatment with nicorandil could improve ventricular function for patients with myocardial infarction in the chronic phase.

Several limitations of our study should be discussed. First, physicians knew the random assignment of patients, and treatment for acute myocardial infarction in the chronic phase was not restricted accordingly; this

could have affected the difference in nicorandil treatment at the chronic phase. Second, although we planned to do angiography of the left ventricle when patients were admitted to hospital, some hospitals could not take angiographs, because of the additional medical cost. Therefore, baseline angiographs were absent for some patients. Third, the patterns of missing angiography data on left ventriculography differed between the two studies (which were done at different hospitals) and also between the atrial natriuretic peptide group and corresponding placebo group. We cannot explain this difference, but since we did not intervene in this procedure, we believe that it must be due to chance.

Contributors

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Conflict of interest statement

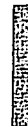
We declare that we have no conflict of interest.

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REGULAR ARTICLE

Haplotype of thrombomodulin gene associated with plasma thrombomodulin level and deep vein thrombosis in the Japanese population

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Abstract

Introduction: Thrombomodulin (TM) is an essential cofactor in protein C activation by thrombin. Here, we evaluated the contribution of genetic variations in the TM gene to soluble TM (sTM) level and deep vein thrombosis (DVT) in Japanese. **Patients and methods:** We sequenced the TM putative promoter, exon, and 3'-untranslated region in DVT patients (n=118). Among 17 genetic variations we identified, two missense mutations (R385K, D468Y) and three common single nucleotide polymorphisms (–202G>A, 2487A>T, 2729A>C) were genotyped in a general population of 2247 subjects (1032 men and 1215 women) whose sTM levels were measured. We then compared the frequency of these mutations in DVT patients

Abbreviations: DVT, deep vein thrombosis; TM, thrombomodulin; PC, protein C; APC, activated protein C; PS, protein S; EGF, epidermal growth factor; SNP, single-nucleotide polymorphism; sTM, soluble TM; 5'-UTR, 5'-untranslated region; 3'-UTR, 3'-untranslated region.

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with that in the age, body mass index-adjusted population-based controls.

Results: We identified one neutral mutation (H381) and three missense mutations (R385K; $n=2$, A455V; $n=53$ heterozygous, $n=14$ homozygous, D468Y; $n=2$) of TM in the DVT patients. Age-adjusted mean values of sTM were lower in C-allele carriers of 2729A>C than in noncarriers in the Japanese general population (women: 16.7 ± 0.3 U/ml vs. 17.9 ± 0.2 U/ml, $p < 0.01$, men: 19.4 ± 0.3 U/ml vs. 20.4 ± 0.3 U/ml, $p = 0.03$). Additionally, the CC genotype of this mutation was more common in the male DVT patients than in the male individuals of the general population (odds ratio = 2.76, 95% confidence interval = 1.14–6.67; $p = 0.02$). This mutation was in linkage disequilibrium (r -square > 0.9) with A455V mutation.

Conclusions: TM mutations, especially those with a haplotype consisting of 2729A>C and A455V missense mutation, affect sTM levels, and may be associated with DVT in Japanese.

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Introduction

Family-based studies have established that venous thromboembolism is, at least in part, an inherited disease with estimated heritabilities of approximately 60% [1,2]. The mode of inheritance of venous thromboembolism is probably complex [2]. Moreover, family-based and twin studies have established that over 25 plasma hemostasis-related analytes (traits) both correlate with thrombosis and are heritable [3–5]. In Caucasians, the factor V-Leiden mutation and prothrombin G20210A mutation are widely recognized as genetic risk factors for deep vein thrombosis (DVT) [6]. However these mutations are not present in the Japanese [7,8]. Recently, we and others found that the protein S (PS) K196E mutation, known as the PS Tokushima mutation, is a genetic risk for DVT in the Japanese population, indicating large differences in the genetics of DVT among ethnicities [9,10].

Thrombomodulin (TM) is a transmembrane protein that is constitutively expressed on the luminal surface of vascular endothelial cells [11]. The anticoagulant function of TM is mediated by interaction with thrombin and protein C (PC). Endothelial membrane-bound TM forms a high-affinity complex with thrombin via thrombin exosite 1, and inhibits thrombin interaction with fibrinogen and protease-activated receptor-1. In contrast, the thrombin–TM complex is a potent activator of PC, and TM enhances thrombin-dependent PC activation by more than two orders of magnitude. Due to the abundance of TM in the microvasculature, the vast majority of thrombin generated under ambient conditions is sequestered by TM. Constitutive inhibition of the procoagulant function of thrombin and tonic formation of activated PC (APC) comprise an essential anticoagulant mechanism that prevents the amplification of

thrombin generation, via proteolysis of activated coagulation factors Va and VIIIa by APC.

TM encoded by an intron-less gene consists of a large N-terminal extracellular region, a single transmembrane segment, and a short cytoplasmic tail [12]. The extracellular region is comprised of an N-terminal lectin-like domain followed by six tandem repeats of epidermal growth factor (EGF)-like domains, and a glycosylated (chondroitin sulfate) serine/threonine-rich domain. The thrombin-binding region has been localized to the fifth and sixth EGF-like domains, while the fourth EGF-like domain is required for PC binding to the thrombin–TM complex. The serine/threonine-rich spacer region is required for both thrombin binding and TM cofactor activity for membrane-associated TM. The chondroitin sulfate domain may stabilize thrombin binding to TM, possibly by interacting with the thrombin apolar region [13,14].

Animal model data suggest that TM dysfunction or deficiency is associated with a prothrombotic disorder. Knock-in mice with a TM mutant that has a mutation corresponding to human E387P exhibit a prothrombotic disorder [15]. This amino acid change is located between the interdomain loop of the fourth and fifth EGF-like domains and abolishes the ability of soluble TM (sTM) to catalyze in vitro thrombin activation of PC to APC. Mice with TM deficiency limited to the vascular endothelium die shortly after birth as a result of a consumptive coagulopathy that can be prevented by warfarin anticoagulation [16].

Based on the important antithrombotic role of TM, we hypothesized that genetic variations within the TM gene that alter TM expression and/or impair anticoagulant function could predispose to venous thromboembolism. To test this hypothesis, we screened the promoter, exon, and 3' untranslated regions (3'-UTR) of the TM gene in unrelated patients with idiopathic, objectively confirmed

DVT for genetic variation. By genotyping three polymorphisms (-202G>A, 2487A>T, 2729A>G) and two missense mutations (R385K, D468Y) in a Japanese general population, we assessed the prevalence of these genetic variations. We then evaluated the association of sTM levels with genetic variations. We finally compared the genotype prevalence of these genetic variations in DVT patients with those in population-based controls to test whether these mutations are associated with DVT in the Japanese.

Patients and methods

DVT patients

A total of 118 Japanese DVT patients (59 men and 59 women, mean age: 52.3 ± 16.1 years old) were recruited from Osaka University Hospital from 2000 to 2004 and the National Cardiovascular Center from 2002 to 2004. All patients examined in this study were unselected patients diagnosed with DVT. Clinical diagnosis of DVT was confirmed by imaging analysis including computerized tomography and ultrasonography.

Screening of genetic variations in TM gene

Blood samples were obtained from DVT patients and genomic DNA was isolated from peripheral blood leukocytes [17]. All the putative promoter, exon, and 3' -UTR regions in 118 Japanese DVT patients were directly sequenced with an ABI

PRISM3700DNA analyzer (Applied Biosystems, Foster City, CA) using seven sets of primers. Primer sequences are available upon request. The obtained sequences were examined for the presence of variations using Sequencher software (Gene Codes Corporation, Ann Arbor, MI), followed by visual inspection [18]. The A of ATG of the initiator Met codon is denoted nucleotide +1, and the initial Met residue is denoted amino acid +1 [19]. The nucleotide sequence (GenBank Accession ID: AF-495471) was used as a reference sequence.

General population (Suita Study)

The sample selection and study design of the Suita Study have been described previously [20–22]. Briefly, the subjects visited the National Cardiovascular Center every 2 years for general health checkups, underwent a routine blood examination that included lipid profiles and glucose levels, and underwent blood pressure measurements. The basic characteristics of the individuals have been reported previously [23,24]. sTM levels of 2247 population-based samples were measured by an enzyme-linked immunosorbent assay (Mitsubishi Gas Chemical Co., Inc., Tokyo, Japan).

Genotyping of mutations and single nucleotide polymorphisms (SNPs) in the general population

Two common SNPs with a minor allele frequency of greater than 5% and all of the missense mutations we detected were tried for genotyping by the

Table 1 Clinical profiles of 118 DVT patients

Clinical profiles		Clinical profiles	
Age, years \pm S.D.	52.3 \pm 16.1	Nephrotic syndrome, <i>n</i> (%)	0 (0.0)
Women, <i>n</i> (%)	59 (50.0)	Chronic heart failure, <i>n</i> (%)	17 (14.4)
BMI, kg/m ² , mean \pm S.D.	23.7 \pm 3.2	Diabetes Mellitus, <i>n</i> (%)	47 (39.8)
DVT family history, <i>n</i> (%)	8 (6.8)	Hypertlipidemia, <i>n</i> (%)	48 (40.7)
Previous DVT, <i>n</i> (%)	12 (10.2)	Autoimmune disease, <i>n</i> (%)	11 (9.3)
Pregnancy, <i>n</i> (%)	5 (4.2)	Inflammatory bowel disease, <i>n</i> (%)	2 (1.7)
Stroke, <i>n</i> (%)	1 (1.5)	Estrogen use, <i>n</i> (%)	3 (2.5)
Prolonged immobility, <i>n</i> (%)	14 (11.9)	Steroid use, <i>n</i> (%)	9 (7.6)
Malignancy, <i>n</i> (%)	16 (13.6)	Paralysis, <i>n</i> (%)	5 (4.2)
Major surgery (abd, hip, leg), <i>n</i> (%)	21 (17.8)	Myeloproliferative disease, <i>n</i> (%)	1 (0.8)
Trauma (pelvis, hip, leg), <i>n</i> (%)	3 (2.5)	Reduced plasminogen activity, <i>n</i> (%)	7 (5.9)
Stasis due to compression, <i>n</i> (%)	6 (5.1)	Reduced antithrombin activity, <i>n</i> (%)	7 (5.9)
Central venous catheter, <i>n</i> (%)	0 (0.0)	Reduced protein C activity, <i>n</i> (%)	8 (6.8)
		Reduced protein S antigen, <i>n</i> (%)	10 (8.5)
		Lupus anticoagulant (cardiolipin, ACLb2), <i>n</i> (%)	3 (11.0)

BMI, body mass index; DVT, deep vein thrombosis; Diabetes mellitus indicates fasting plasma glucose ≥ 126 mg/dl or non-fasting plasma glucose ≥ 200 mg/dl or HbA1c $\geq 6.5\%$ or use of antidiabetic medication; Hypertension, systolic blood pressure ≥ 140 mm Hg and/or diastolic blood pressure ≥ 90 mm Hg or use of antihypertensive medication; Hypertlipidemia, total cholesterol ≥ 220 mg/dl or use of antihypertlipidemia medication; Myeloproliferative disease, Plt. $>5 \times 10^5$ and Ht. $>55\%$; Reduced plasminogen activity, plasminogen activity $<70\%$; Reduced antithrombin activity, antithrombin activity $<80\%$; Reduced protein C activity, protein C activity $<70\%$; Reduced protein S antigen, protein S antigen $<60\%$.

TaqMan-PCR method [25]. Among three missense mutations, genotyping for 1418C>T (A455V) was failed. Additionally, another common SNP (2729A>C) which was in linkage disequilibrium (r -square>0.9) with A455V mutation was genotyped instead of A455V mutation. Thus, five genetic variations were successfully genotyped in 2247 subjects (1032 men and 1215 women). The sequences of PCR primers and probes for the TaqMan-PCR method are available upon request. All clinical data and sequencing and genotyping results were anonymous. The study protocol was approved by the Ethical Review Committee of Osaka University Hospital and National Cardiovascular Center. Gene analyses were performed after informed consent had been obtained in written.

Statistical analysis

Values are means \pm S.E. The distributions of basic characteristics in men and women in the Japanese general population were examined using the Student's t -test or X^2 analysis. The correlations of two missense mutations and three common SNPs with sTM levels were examined by logistic analysis, with adjustment for confounding factors, including age, body mass index (BMI), present illness (hyperlipidemia and diabetes mellitus), and lifestyle (smoking and drinking). Odds ratios for each mutation are presented both adjusted for age and age-BMI. All analyses were performed using SAS (release 8.2, SAS Institute Inc.). Statistical significance was estab-

lished at $p < 0.05$. Linkage disequilibrium was calculated using SNPalyze version 4.0 (DYNACOM Co., Ltd., Mobara, Japan).

Results

Characteristics of DVT patients

The clinical profiles of the 118 Japanese DVT patients (59 men, 59 women aged 52.3 ± 16.1) are summarized in Table 1. Eight patients (6.8%) had a DVT family history and 12 patients (10.2%) had previous DVT. Sixteen patients (13.6%) suffered from cancer and 21 (17.8%) had undergone major surgery of the abdomen, hip or leg. Seven patients (5.9%) had reduced plasminogen activity (<70%) and 7 (5.9%) had reduced antithrombin activity (<80%). Eight patients (6.8%) had reduced PC activity (<70%), and 10 patients (8.5%) had reduced PS antigen (<60%). To eliminate effects of warfarin on PS/PC activities, we did not count numbers of patients having reduced PC activity (PC<70%) and PS antigen (PS<60%) when they had taken warfarin.

Screening of TM gene for sequence variation in DVT patients

On sequencing the TM gene in 118 DVT patients, we identified 17 genetic variants (Table 2). Three of 17

Table 2 Genetic variations in TM gene identified in 118 Japanese DVT patients.

SNPs	LD	Region	Amino acid substitution	Allele 1 frequency (%)	Allele 2 frequency (%)	Flanking sequence	db SNP ID
*-832C>A		Promoter		99.6	0.4	gggcagaggcgc [c/a] tggtttaggcc	
*-754G>C		Promoter		99.1	0.9	caagcgcgctcc [g/c] ctggttcctga	
*-265C>A		Exon(5' UTR)		99.6	0.4	aatccgagtatg [c/a] ggcatcagccct	
-202G>A	A	Exon(5' UTR)		89.2	10.8	ggagggaggccc [g/a] ggcattataaa	
*-58G>C		Exon(5' UTR)		98.3	1.7	ctgctccggcac [g/c] gccctgtcgag	
*1197C>T		Exon(EGF4)	H38I	99.6	0.4	gccccattcccca [c/t] gagccgcacagg	
1208G>A		Exon(EGF4)	R385K	99.1	0.9	acgagccgcaca [g/a]gtgccagatgtt	
1418C>T	B	Exon(EGF6)	A455V	65.1	34.9	actcgcccttg [c/t] ccgccacattgg	rs1042579
1456G>T		Exon(Ser/Thr-rich)	D468Y	99.1	0.9	tccggcaaggtg [g/t] acggtggcgaca	
1754C>T		Exon(3' UTR)		98.7	1.3	aggagcctggct [c/t] cgctccaggagcc	rs13306852
2005G>A	A	Exon(3' UTR)		89.2	10.8	gtcctcactacc [g/a]ggcgcaggagg	rs3176134
*2230T>C		Exon(3' UTR)		99.6	0.4	tcttgggaatt [t/c] ttttttccctagc	
*2487A>T		Exon(3' UTR)		93.1	6.9	ttcccagagcaa [a/t] ataattttaaac	
2521A>G		Exon(3' UTR)		79.8	20.2	gatgtaaaaggt [a/g] ttaaattgatgt	rs1042580
2729A>C	B	Exon(3' UTR)		65.0	35.0	tgctctagattg [a/c] gagaagagacaa	rs3176123
*3521-3522insT		3' flanking		99.6	0.4	ctcggttgtgt [-/t] gtctgttacctt	
*3559T>A		3' flanking		99.6	0.4	gccctcatttta [t/a] gtcattaaaatgg	

LD, mutations in linkage disequilibrium (group A; r -square=0.84, group B r -square=0.93); allele 1, major allele; allele 2, minor allele; *, novel mutation; EGF, epidermal growth factor like domain; Ser/Thr-rich, serine/threonine-rich domain; UTR, untranslated region.

Table 3 Basic characteristics of subjects in general population

	Women (n=1215)	Men (n=1032)	p
Age, years \pm S.D.	64.6 \pm 10.7	67.1 \pm 10.9	<0.0001
Systolic blood pressure, mm Hg \pm S.D.	123.5 \pm 19.8	126.1 \pm 17.9	0.0008
Diastolic blood pressure, mm Hg \pm S.D.	74.3 \pm 10.4	77.2 \pm 10.4	<0.0001
Body mass index, kg/m ² \pm S.D.	22.4 \pm 3.2	23.4 \pm 3.0	<0.0001
Total cholesterol, mg/dl \pm S.D.	215.9 \pm 31.6	198.7 \pm 31.5	<0.0001
HDL-cholesterol, mg/dl \pm S.D.	64.4 \pm 15.1	55.2 \pm 14.0	<0.0001
Current smokers, %	4.4	27.2	<0.0001
Current drinkers, %	26.0	67.0	<0.0001
Present illness, %			
Hypertension	35.3	42.8	0.0003
Hyperlipidemia	55.7	34.3	<0.0001
Diabetes mellitus	6.1	13.2	<0.0001

Hypertension indicates systolic blood pressure \geq 140 mm Hg and/or diastolic blood pressure \geq 90 mm Hg or use of antihypertensive medication; Hyperlipidemia, total cholesterol \geq 220 mg/dl or use of antihyperlipidemia medication; Diabetes mellitus, fasting plasma glucose \geq 126 mg/dl or non-fasting plasma glucose \geq 200 mg/dl or HbA1c \geq 6.5% or use of antidiabetic medication. The distributions of basic characteristics in men and women in general population were analyzed using the Student's *t*-test or χ^2 analysis.

mutations were missense mutations (R385K; $n=2$, A455V; $n=53$ heterozygous, $n=14$ homozygous, D468Y; $n=2$). Four mutations within the TM promoter region and the 5' -untranslated region (5' -UTR) (-832C>A, -754G>C, -265C>A, -58G>C) were rare. Twenty-five patients were heterozygous carriers for the -202G>A mutation within the promoter region, which was reported as a -33G>A mutation. This mutation has been reported to decrease TM promoter activity in vitro [26]. It was in linkage disequilibrium (r -square>0.8) with 2005G>A in the 3' -UTR. No patients were carriers for previously reported mutations in the lectin-like

domain [A25A (847G>C), E61A (954G>C)] [27,28]. One patient was heterozygous for a novel neutral mutation within the fourth EGF-like domain [H381 (1197C>T)]. Two patients were heterozygous carriers for the previously described R385K mutation (1208G>A) in the fourth EGF-like domain [28]. The previously reported A455V mutation (1418C>T) was found within the sixth EGF-like domain ($n=53$ heterozygous, $n=14$ homozygous), an important region for thrombin binding and activation of PC [13]. This mutation was in linkage disequilibrium (r -square>0.9) with the 2729A>C mutation within the 3' -UTR. Within the serine/threonine-rich domain,

Table 4 Genotype distribution of two missense mutations and three common single nucleotide polymorphisms (SNPs) of TM gene in DVT patients and in individuals in general population

SNPs (amino acid change)	Genotypes	Individuals in general population			DVT patients		
		Women n (%)	Men n (%)	Total n (%)	Women n (%)	Men n (%)	Total n (%)
-202 G>A	GG	1009 (83.1)	855 (82.9)	1864 (83.0)	45 (76.3)	46 (80.7)	91 (78.5)
	GA	192 (15.8)	157 (15.2)	349 (15.5)	14 (23.7)	11 (19.3)	25 (21.6)
	AA	14 (1.2)	19 (1.8)	33 (1.5)	0 (0.0)	0 (0.0)	0 (0.0)
	Total	1215	1031	2246	59	57	116
1208 G>A (R385K)	GG	1207 (99.3)	1023 (99.1)	2230 (99.2)	57 (98.3)	56 (98.3)	113 (98.3)
	GA	8 (0.7)	9 (0.9)	17 (0.8)	1 (1.7)	1 (1.8)	2 (1.7)
	AA	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
	Total	1215	1032	2247	58	57	115
1456 G>T (D468Y)	GG	1181 (97.3)	1015 (98.5)	2196 (97.7)	57 (96.6)	57 (100.0)	114 (98.3)
	GT	33 (2.7)	16 (1.6)	49 (2.2)	2 (3.4)	0 (0.0)	2 (1.7)
	TT	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
	Total	1214	1031	2245	59	57	116
2487 A>T	AA	1001 (82.4)	873 (84.6)	1874 (83.4)	41 (83.7)	47 (87.0)	94 (86.2)
	AT	206 (17.0)	155 (15.0)	361 (16.1)	8 (16.3)	7 (13.0)	15 (13.8)
	TT	8 (0.7)	4 (0.4)	12 (0.5)	0 (0.0)	0 (0.0)	0 (0.0)
	Total	1215	1032	2247	49	54	109
2729 A>C	AA	707 (58.2)	570 (55.2)	1277 (56.8)	24 (43.6)	22 (40.0)	46 (41.8)
	AC	419 (34.5)	393 (38.1)	812 (36.1)	26 (47.3)	25 (45.5)	51 (46.4)
	CC	89 (7.3)	69 (6.7)	158 (7.0)	5 (9.1)	8 (14.6)	13 (11.8)
	Total	1215	1032	2247	55	55	110

Table 5 Comparison of sTM levels by genetic variations of TM gene in general population

SNPs (amino acid change)	Genotypes	Women				Men			
		Age-adjusted		Multi-adjusted		Age-adjusted		Multi-adjusted	
		Mean \pm SE U/ml	<i>p</i>	Mean \pm SE U/ml	<i>p</i>	Mean \pm SE U/ml	<i>p</i>	Mean \pm SE U/ml	<i>p</i>
-202 G>A	GG	16.9 \pm 1.6		17.0 \pm 1.6		19.2 \pm 1.9		19.6 \pm 1.9	
	GA+AA	17.4 \pm 0.2	0.73	17.4 \pm 0.2	0.77	19.9 \pm 0.2	0.68	19.9 \pm 0.2	0.87
1208 G>A (R385K)	GG	17.4 \pm 0.2		17.4 \pm 0.2		19.9 \pm 0.2		19.9 \pm 0.2	
	GA+AA	16.2 \pm 2.4	0.62	16.0 \pm 2.3	0.54	20.5 \pm 2.2	0.79	20.4 \pm 2.2	0.84
1456 G>T (D468Y)	GG	17.4 \pm 0.2		17.4 \pm 0.2		19.9 \pm 0.2		19.9 \pm 0.2	
	GT+TT	18.1 \pm 1.0	0.51	18.1 \pm 1.0	0.52	22.2 \pm 1.7	0.20	22.6 \pm 1.7	0.11
2487 A>T	AA	17.6 \pm 0.2		17.6 \pm 0.2		20.0 \pm 0.2		20.0 \pm 0.2	
	AT+TT	16.7 \pm 0.4	0.04	16.7 \pm 0.4	0.04	19.6 \pm 0.6	0.54	19.5 \pm 0.6	0.40
2729 A>C	AA	17.9 \pm 0.2		17.9 \pm 0.2		20.4 \pm 0.3		20.3 \pm 0.3	
	AC+CC	16.7 \pm 0.3	<0.01	16.8 \pm 0.3	<0.01	19.4 \pm 0.3	0.03	19.5 \pm 0.3	0.07

The correlations of five genetic variations with sTM level were examined by logistic analysis, adjusting for age and multiple factors, including age, BMI, present illness (hyperlipidemia and diabetes mellitus), and lifestyle (smoking and drinking).

two patients were heterozygous carriers for the previously described D468Y mutation (1456G>T) [29].

Characteristics of individuals in the general population

The characteristics of the 2247 subjects of the Japanese general population group (1032 men, 1215 women) are shown in Table 3. Age, systolic blood pressure, diastolic blood pressure, BMI, percentage current smokers, percentage current drinkers, and frequencies of hypertension and diabetes mellitus were significantly higher in men than in women, while total cholesterol, HDL-cholesterol, and percentage of subjects with hyperlipidemia were significantly higher in women than in men.

Genotyping of two missense mutations (R385K, D468Y) and three common SNPs (-202G>A, 2487A>T, 2729A>C) and association of sTM levels with TM genotypes in the general population

In the general population of 2247 subjects, five mutations were successfully genotyped (Table 4). Plasma levels of sTM were measured in all subjects.

As shown in Table 5, sTM levels were significantly lower in C-allele carriers of the 2729A>C mutation than in non-carriers in the general population (women: 16.7 \pm 0.3 U/ml vs. 17.9 \pm 0.2 U/ml, p <0.01, men: 19.4 \pm 0.3 U/ml vs. 20.4 \pm 0.3 U/ml, p =0.03), when adjusted for age. Additionally, in male patients, the CC genotype group was associated with significantly higher DVT risk than the combined AA/AC genotype after adjustment for age and age-BMI (odds ratio=2.76, 95% confidence interval=1.14–6.67; p =0.02 and odds ratio=2.98, 95% confidence interval=0.21–7.33; p =0.02, respectively) (Table 6). This mutation was in linkage disequilibrium (r -square>0.9) with the A455V mutation (Table 2).

Discussion

Several mutations within the TM gene have been reported in small numbers of patients with DVT [27,30–33]. However, it was reported that polymorphisms within the TM gene were not common risk factors for incidental DVT in a recent Caucasian population-based case-control study [34]. Because the factor V-Leiden mutation is not detected in Japanese DVT patients [7], while PS Tokushima mutation (K196E) is a risk factor for DVT in a

Table 6 Odds ratios and 95% confidence intervals for DVT in relation to 2729A>C in TM gene

Genotypes	Women				Men			
	Age-adjusted		Age, BMI-adjusted		Age-adjusted		Age, BMI-adjusted	
	Odds ratio (95% CI)	<i>p</i>	Odds ratio (95% CI)	<i>p</i>	Odds ratio (95% CI)	<i>p</i>	Odds ratio (95% CI)	<i>p</i>
AA+AC	1 (reference)		1 (reference)		1 (reference)		1 (reference)	
CC	0.97 (0.35–2.70)	0.95	0.96 (0.34–2.70)	0.93	2.76 (1.14–6.67)	0.02	2.98 (0.21–7.33)	0.02

CI, confidence interval.

Japanese population [9,10], we suspected that frequencies of the TM mutations in Japanese DVT patients might differ from those in Caucasians. We therefore performed a case-control study to test TM polymorphisms for associations with DVT in Japanese. In this study, we found that sTM levels were lower in those with 2729C and 2729C was more common in DVT patients than in the general population. It is a reasonable assumption that the low sTM levels in plasma reflect the decreased TM expression on endothelial cells. If so, the capacity of the PC anticoagulant system, which is comprised of TM, PC and PS, would be decreased to thrombosis-prone.

We first screened the TM putative promoter, exon, and 3' -UTR regions for sequence variations in a random sample ($n=118$) of DVT patients, and identified one novel neutral mutation (1197C>T; H381) and three previously described missense mutations (1208G>A; R385K, 1418C>T; A455V, 1456G>T; D468Y) (Table 2). As shown in previous report showing A455V mutation within the sixth EGF-like domain, an important region for thrombin binding and activation of PC, was a common missense mutation [13], the frequency of A455V mutation was also higher than the other mutation found in this study. The 1197C>T (H381, $n=1$) mutation and 1208G>A (R385K, $n=2$) mutation within the fourth EGF-like domain were rare. Although the fourth EGF-like domain serves as the binding site for PC, the functional consequences of the Arg-to-Lys substitution at position 385 are not known. D468Y mutation lies in the serine/threonine-rich domain. An in vitro study showed that this mutation did not cause any abnormality in levels of production or functional activity of TM [31]. In our study, patients carrying this mutation were rare ($n=2$).

We genotyped five genetic variants in the 2247 population-based controls (Table 4). We failed in genotyping for the A455V mutation, so the 2729A>C mutation in linkage disequilibrium with the A455V mutation was genotyped. In the Japanese general population, the frequency of 2729A>C mutation (36.1% heterozygous, 7.0% homozygous) was higher than that of A455V mutation in Caucasians (24.0% heterozygous, 4.3% homozygous) and African-Americans (15.9% heterozygous, 2.2% homozygous) [33]. Since the frequency of A455V mutation in the Chinese population has been reported to be 45% heterozygous and 9% homozygous [35], the frequency of the 2729A>C mutation in our study was similar to the result in the Chinese population. This difference in genotype frequency may be associated with differences in ethnical genetic background.

The extracellular region of endothelial TM is cleaved and the cleaved fragments are called sTM. sTM processes anticoagulant properties, and sTM levels reported to have a statistically significant correlation with sTM cofactor activity in healthy individuals [36,37]. The LITE Study reported that sTM levels tended to exhibit gene dosage effects, with AA-genotype of A455V mutation carriers exhibiting approximately 10% higher sTM levels than VV-genotype of A455V mutation carriers, and values for the AV-genotype carriers were intermediate, with no significant differences among these three groups [33]. In our study, particularly in women, sTM levels in individuals carrying 2729A>C mutation were lower than those in noncarriers (Table 5). Since the 2729A>C mutation and the A455V missense mutation are in linkage disequilibrium, our findings might support those of these previous reports. For the other mutations, there was no significant difference in sTM level among the genotypes. Despite much interest in sTM as a marker of endothelial injury, few studies have investigated the relationship between sTM and DVT. The findings of previous studies are conflicting or difficult to judge, partly because of small sample sizes or cross-sectional design [33,38–40]. However, systemic infusion of recombinant sTM has been shown to have antithrombotic potential and dose-dependent effects in the prevention of venous thrombosis after total hip replacement [41,42]. Moreover, the ARIC Study, performed in the United States, reported that high levels of sTM are associated with a lower risk of incidental coronary heart disease [43].

Finally, we compared the genotype frequencies in the population-based controls with those in the DVT patients. In male DVT patients, the frequency of 2729A>C mutation was higher than in the population-based controls (Table 6). The LITE Study reported no difference in the frequency of A455V mutation between DVT patients and controls among Caucasians and African-Americans [33]. This discrepancy might come from the difference of sample size, ethnical genetic background or study design. Especially, in our study, difference of mean ages between DVT patients (52.3 ± 16.1 years old) and general population (women: 64.6 ± 10.7 years old, men: 67.1 ± 10.9 years old) may affect the results, although all analysis has been done in age-adjusted manner.

Additionally, significant decrease of sTM levels in the C-allele carriers of 2729A>C mutation was found in women, whereas not much in men in our study (Table 5). However, the incidence of DVT was associated with only men, but not women (Table 6). The mechanisms by which 2729A>C mutation might

contribute to DVT in only men are unknown. This inconsistency might be derived from gender differences or a lack of statistical power due to the sample size. Regarding the gender differences, TM proteins are known to be modulated by estrogens [44]. 17 β -estradiol is known to reduce the anticoagulant properties of endothelial cells by decreasing thrombomodulin expression. This can well explain the gender difference of sTM levels, where men showed higher sTM levels than women. The anticoagulant activity of TM was destroyed by oxidation caused by chloramine T, H₂O₂, or hypochlorous acid generated from H₂O₂ by myeloperoxidase [45]. Activated neutrophil, the primary in vivo source of biological oxidants, also rapidly inactivate TM. Oxidation of Met388 in the sixth EGF-like domain was critical for inactivation. Men are supposed to have greater oxidative stress than women. If so, men might be exposed more for DVT risk. Thus, we suppose that the cause of gender difference in relationship between TM polymorphism and DVT may be via the influences of hormonal and environmental effects.

We observed that 2729A>C mutation and A455V mutation are in linkage disequilibrium and 2729A>C mutation is associated with sTM levels and DVT. At present, the causative genetic mutations for this association are not known. A455V mutation may directly affect the expression of TM molecule. 2729A>C mutation in the 3' -UTR may affect the mRNA stability. TM mRNA is known to be unstable [46], and C-allele may create more unstable mRNA. Two polymorphisms may be in linkage disequilibrium with another genetic variation in the region that was not examined by sequencing. Therefore, additional in vitro studies are required for the identification of the functional genetic variation. Since association studies are not consistently reproducible due to false-positives, false-negatives or true variability in association between different populations [47], the association of TM polymorphism to sTM levels and DVT must be reexamined in other populations.

In summary, TM mutations, especially those with a haplotype consisting of 2729A>C and A455V, affect sTM levels, and may be associated with DVT in Japanese.

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Original Article

Association of Genetic Polymorphisms of Endothelin-Converting Enzyme-1 Gene with Hypertension in a Japanese Population and Rare Missense Mutation in Preproendothelin-1 in Japanese Hypertensives

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Endothelin-1 (EDN1), a 21-amino acid peptide, is a potent vasoconstrictor with various pharmacological responses. EDN1 is synthesized from a 212-amino acid precursor protein, preproEDN1, through multiple proteolytic steps. Endothelin-converting enzyme (ECE) cleaves a Trp73-Val74 peptide bond in big-EDN1 to give rise to mature EDN1. In this study, we examined the possible association of genetic variations in *ECE1* with hypertension in a general Japanese population and searched for missense mutations in and around the EDN1 polypeptide. We genotyped 5 single nucleotide polymorphisms (SNPs) in the *ECE1* gene in 1,873 individuals from a general Japanese population and identified one SNP associated with hypertension in women (rs212528: TT vs. TC+CC: odds ratio=1.40; 95% confidence intervals: 1.04–1.89; $p=0.026$), after adjusting for confounding factors. The systolic blood pressure in women with the CC genotype was 6.44 mmHg higher than that in those with the TT genotype ($p=0.007$), after adjusting for the same factors. Next, to identify the missense mutations that may influence the biological activity of EDN1, we sequenced the genomic region that encodes EDN1 in 942 Japanese hypertensive patients. We identified a novel missense mutation, G36R, in one hypertensive patient, but no mutations were observed in EDN1. A gene polymorphism in *EDN1*, Lys198Asn, has been reported to be associated with hypertension in obese subjects. Taken together, these findings reveal that the EDN-ECE pathway is an important system involved in essential hypertension in Japanese. (*Hypertens Res* 2007; 30: 513–520)

Key Words: endothelin, endothelin-converting enzyme, gene variants, hypertension, general population

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