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Rho-Associated Kinase Activity Is a Predictor of Cardiovascular Outcomes

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Abstract

Cardiovascular diseases are associated with chronic activation of Rho-associated kinase. Rho-associated kinase activity is significantly correlated with endothelial function and Framingham risk score. However, there is no information on the prognostic value on Rho-associated kinase activity. We evaluated Rho-associated kinase activity in peripheral leukocytes by Western blot analysis in 633 subjects who underwent health-screening examination at Hiroshima University Hospital. We assessed the associations between Rho-associated kinase activity and first major cardiovascular events (death from cardiovascular causes, myocardial infarction, and stroke), death from cardiovascular causes, acute myocardial infarction, stroke, revascularization (percutaneous coronary intervention, coronary artery bypass grafting) and hospitalization for heart failure. During a median period of 42.0 months (interquartile range, 24.4 to 56.6 months) of follow-up, 29 subjects died (10 from cardiovascular causes), 2 myocardial infarction, 20 revascularization, 15 stroke, and 17 hospitalization for heart failure. After adjustment for age, sex, cardiovascular risk factors and other relevant variables, Rho-associated kinase activity remained a strong independent indicator of first major cardiovascular events (hazard ratio, 2.19; 95 percent confidence interval, 1.35 to 3.70; P=0.002), death from cardiovascular disease (hazard ratio, 2.57; 95 percent

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confidence interval, 1.18 to 6.60; P=0.002), stroke (hazard ratio, 2.14; 95 percent confidence interval, 1.24 to 3.86; P=0.006) and revascularization (hazard ratio, 2.68; 95 percent confidence interval, 1.60 to 4.66; P<0.001). Leukocyte Rho-associated kinase activity may be a new biomarker of cardiovascular events. These findings suggest that inhibition of Rho-associated kinase activity may be a therapeutic target for prevention of cardiovascular events.

Keywords

Rho-associated kinase activity; Biomarker; Atherosclerosis; Cardiovascular events

Introduction

Rho-associated kinase, one of the first downstream targets of the small GTP-binding protein RhoA, plays a pivotal role in the regulation of vascular smooth muscle contraction, endothelial function, and many cellular functions, including cell proliferation, migration, adhesion, and apoptosis.[1–6] It has been shown, *in vivo*, that Rho-associated kinase contributes to early atherosclerotic lesion formation, neointimal formation, vascular remodeling, and cardiac hypertrophy.[7–10] In addition, previous studies have shown that Rho-associated kinase activity is enhanced in patients with hypertension, angina pectoris, vasospastic angina, pulmonary hypertension, heart failure, and stroke, and even in smokers.[11–17] Recently, we reported that Rho-associated kinase activity was significantly correlated with endothelial function and Framingham risk score in healthy men and in men with cardiovascular risk factors but without established cardiovascular or cerebrovascular diseases.[18] Increased Rho-associated kinase activity may play a critical role in the pathogenesis and progression of cardiovascular diseases.

However, there is no information on the prognostic value on Rho-associated kinase activity for cardiovascular outcomes. In this study, we assessed the prognostic value of Rho-associated kinase activity in a large population.

Methods

Subjects

Between June 2007 and March 2012, Rho-associated kinase activity was assayed in 751 subjects who underwent health examinations and medical consultation at Hiroshima University Hospital and were enrolled in a database of the Rho-associated Kinase Study Registry. Exclusion criteria were hospitalization within one month (n=11), current pregnancy or breastfeeding (n=41), and no available information on morbidity and death (n=66). Finally, we enrolled a total of 633 subjects (398 men and 235 women; mean age, 53±18 yr). Hypertension was defined as systolic blood pressure of more than 140 mm Hg and/or diastolic blood pressure of more than 90 mm Hg, in a sitting position, on at least three different occasions. In order to reduce the potential bias, patients with secondary hypertension were excluded on the basis of complete history, physical examination, radiological and ultrasound examinations, urinalysis, plasma renin activity, plasma aldosterone and norepinephrine concentrations, serum creatinine, potassium, calcium, and

free thyroxine concentrations, and 24-hour urinary excretion of 17-hydroxycorticosteroids, 17-ketogenic steroids, and vanillymandelic acid. Normotension was defined as systolic blood pressure of less than 140 mm Hg and diastolic blood pressure of less than 90 mm Hg. Diabetes was defined according to the American Diabetes Association or a previous diagnosis of diabetes.[19] Dyslipidemia was defined according to the third report of the National Cholesterol Education Program.[20] The estimated glomerular filtration rate (eGFR) values were calculated by the following equation: $194 \times \text{serum creatinine}^{-1.094} \times \text{age}^{-0.287}$ ($\times 0.739$ if female).[21] The ethical committees of our institutions approved the study protocol. Written informed consent for participation in the study was obtained from all of the subjects.

Subjects fasted the previous night for at least 12 hours. After remaining in the supine position for 30 minutes, basal Rho-associated kinase activity and fasting serum concentrations of total cholesterol, high-density lipoprotein cholesterol, low-density lipoprotein cholesterol, triglycerides, blood urea nitrogen, creatinine, glucose, and electrolytes were measured.

From September 2012 to November 2012, we collected the information on potential outcomes or adverse events from medical records and/or one telephone survey. We first assessed the associations of tertiles of Rho-associated kinase activity with first major cardiovascular events (myocardial infarction, stroke, and death from cardiovascular causes) and then assessed the associations with death from cardiovascular causes, acute myocardial infarction, stroke, revascularization (percutaneous coronary intervention, coronary artery bypass grafting), and hospitalization for heart failure.

Measurement of Rho-associated Kinase Activity

Rho-associated kinase activity was assayed in peripheral blood leukocytes as the amount of phospho-Thr853 in the myosin-binding subunit of myosin light chain phosphatase (MLCPh), because myosin-binding subunit on MLCPh is one of the downstream targets of Rho-associated kinase. Blood was collected at room temperature in heparinized tubes (20 U/mL). After adding an equal volume of 2% dextran, each sample was kept at room temperature for 30 min. The supernatant was spun at 1450 rpm for 10 min. Red blood cells in the resulting cell pellet were lysed with the addition of water and spun at 1450 rpm for 10 min after the addition of Hank's balanced salt solution (Hyclone, Logan, UT, USA). The resulting leukocyte pellet was resuspended in medium 199 (Sigma Chemical Co., Saint Louis, Missouri, USA) and the number of cells was counted using a hematocytometer. Cells were fixed in 10% trichloroacetic acid and 10 mmol/L dichlorodiphenyltrichloroethane. After centrifugation, the cell pellets were stored at −80°C for Western blot analysis. Cells pellets were dissolved in 10 μL of 1 mol/L Tris base and then mixed with 100 μL of extraction buffer (8 mol/L urea, 2% sodium dodecyl sulfate, 5% sucrose, and 5% 2-mercaptoethanol). Equal amounts of cell extracts were subjected to 7.5% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes. NIH 3T3 cell lysates were used as a positive control and to standardize the results of Western blot analyses from several membranes. After serum starvation for 20 hours, confluent cells were stimulated with 10 μmol/L lysophosphatidic acid for 10 minutes and then subsequently fixed and harvested in 10% trichloroacetic acid and 10 mmol/L dichlorodiphenyltrichloroethane.

Following centrifugation at 1450 rpm for 10 minutes at 4°C, precipitates were dissolved in 10 μL of 1 mol/L Tris base and mixed with 100 μL of extraction buffer. An equal volume of positive control cell lysate was used for each gel. Membranes were incubated with rabbit anti–phospho-specific Thr853–myosin-binding subunit polyclonal antibody (Biosource Invitrogen, Carlsbad, California, USA), rabbit anti-myosin-binding subunit polyclonal antibody (Covance Laboratories, Evansville, Indiana, USA), or antiactin monoclonal antibody (Sigma). Bands were visualized using the ECL system (Amersham-Pharmacia Co., London, UK). Images were captured using Adobe Photoshop, and the band intensities were quantified using National Institutes of Health Image 1.61. Rho-associated kinase activity was expressed as the ratio of phospho myosin-binding subunit in each sample to phospho myosin-binding subunit in each positive control divided by total myosin-binding subunit in each sample per total myosin-binding subunit in each positive control.

Statistical Analysis

Results are presented as means±SD. All reported probability values were 2-sided, and a probability value of <0.05 was considered statistically significant. Continuous variables were compared by using ANOVA for multiple groups and the *t* test between 2 groups. Categorical variables were compared by means of the $\chi 2$ test. Time-to-event end-point analyses were performed by using the Kaplan-Meier method. A log-rank test was used to compare survival in the groups. Multivariable Cox proportional hazard regression analysis was performed to assess the association between Rho-associated kinase activity and cardiovascular events in addition to univariate analysis. The data were processed using the software package Stata, version 9 (Stata Co, College Station, TX).

Results

Baseline Clinical Characteristics

The baseline characteristics of the 633 subjects are summarized in Table 1. Of the 633 subjects, 398 (62.9%) were men and 235 (37.1%) were women. Three hundred thirty-eight (53.4%) had hypertension, 387 (61.1%) had dyslipidemia, 145 (22.9%) had diabetes mellitus, and 171 (27.0%) were current smokers. The mean value of Rho-associated kinase activity was 0.83±0.65. Of the 633 subjects who were evaluated, 95 (15.0%) had known coronary artery disease and 35 (5.5%) had known stroke.

Rho-associated Kinase Activity and Clinical Outcomes

We next categorized subjects into 3 tertiles based on Rho-associated kinase activity (Table 1). The low group had Rho-associated kinase activity of 0.521 or less (0.38±0.11), the intermediate group had Rho-associated kinase activity between 0.522 and 0.810 (0.66±0.08), and the high group had Rho-associated kinase activity of 0.811 or more (1.46±0.78) (Fig. 1). Rho-associated kinase activity was correlated with difference in gender (P=0.009), total cholesterol (P=0.004), high-density lipoprotein cholesterol (P=0.02) and glucose (P=0.01). In addition, univariate regression analysis revealed that leukocyte Rho-associated kinase activity inversely correlated with eGFR (r=−0.09, P=0.02) (Supplemental Fig. S1). Other parameters such as systolic blood pressure, diastolic blood pressure, high-sensitivity C-reactive protein, HbA1c and number of white blood cells did not correlate with Rho-

associated kinase activity (Supplemental Figs. S2–S5). There were not notable differences in baseline clinical characteristics, including age, risk factors (hypertension, diabetes, smoking), complications, and drugs used according to level of Rho-associated kinase activity.

During a median period of 42.0 months (interquartile range, 24.4 to 56.6 months) of follow-up, 29 subjects died (10 from cardiovascular causes), 2 myocardial infarction, 20 revascularization, 15 stroke, and 17 hospitalization for heart failure (Table 2). First major cardiovascular events were significantly more frequent among patients with high levels of Rho-associated kinase activity than among those with low levels (P=0.014) (Fig. 2). There were significant differences between the Kaplan-Meier curves for death from cardiovascular disease (P=0.049), stroke (P=0.013), and revascularization (P=0.025), but there were no significant differences between the Kaplan-Meier curves for acute myocardial infarction (P=0.61), hospitalization for congestive heart failure (P=0.99), and death from any cause (P=0.94) (Fig. 3). The causes of non-cardiac death included cancer (n=11), septic shock (n=3), renal failure (n=2), asthma (n=1), pneumonia (n=1), and unknown (n=1) (Table 3).

After adjustment for age, sex, cardiovascular risk factors, previous coronary heart disease, previous stroke and concomitant treatment with an antiplatelet, an angiotensin-converting enzyme, an angiotensin type 1 receptor blocker, a calcium channel blocker, a statin, or diabetes agents, the associations between increasing levels of Rho-associated kinase activity and increasing risk of first major cardiovascular events (hazard ratio, 2.19; 95 percent confidence interval, 1.35 to 3.70; P=0.002), death from cardiovascular disease (hazard ratio, 2.57; 95 percent confidence interval, 1.18 to 6.60; P=0.002), stroke (hazard ratio, 2.14; 95 percent confidence interval, 1.24 to 3.86; P=0.027), and revascularization (hazard ratio, 2.68; 95 percent confidence interval, 1.60 to 4.66; P<0.001) remained significant (Table 4).

Discussion

The results of this prospective study showed for the first time the prognostic impact of Rho-associated kinase activity for first major cardiovascular events. Previously, we have shown that cumulative cardiovascular risk factors increase Rho-associated kinase activity and that Rho-associated kinase activity is an independent predictor of flow-mediated vasodilation, an index of endothelial function, in healthy men.[18] These findings suggest that Rho-associated kinase activity may be a biomarker for predicting cardiovascular events and may be a therapeutic target for cardiovascular disease.

It is well known that cumulative cardiovascular risk contributes to onset of cardiovascular and cerebrovascular events. Hypertension, dyslipidemia, diabetes, aging, smoking, and obesity are contributing risk factors in cardiovascular and cerebrovascular diseases. In the present study, there were not notable differences in baseline clinical characteristics, including age, risk factors, complications, and drugs used according to level of Rho-associated kinase activity. After adjustment of cardiovascular risk factors, Rho-associated kinase activity remained an independent predictor of major cardiovascular events. Among the events, stroke was substantially associated with Rho-associated kinase activity. Indeed, previous studies have demonstrated a significant correlation of stroke with Rho-associated

kinase activity not only in an experimental setting but also a clinical setting.[16,22–25] Collectively, the increase in Rho-associated kinase activity may simultaneously or independently affect the maintenance and progression of arteriosclerosis and atherosclerosis.

We found that elevated Rho-associated kinase activity correlated with first major cardiovascular events. First major cardiovascular events include myocardial infarction, stroke, and death from cardiovascular causes, all of which may be important and essential events to consider morbidity and mortality from cardiovascular diseases. In experimental studies, several investigators have shown that inhibition of Rho-kinase activity prevents the maintenance and development of cardiovascular damage.[6,26,27] It is expected that appropriate inhibition of Rho-associated kinase activity will prevent the pathogenesis, maintenance and development of cardiovascular dysfunction, resulting in reduced cardiovascular events. In a clinical setting, several interventions, including treatment with statins and antihypertensive agents, have been shown to improve Rho-associated kinase activity in patients with dyslipidemia and essential hypertension.[28,29] These findings suggest that Rho-associated kinase activity is reversible, at least in part, in patients with cardiovascular diseases and/or risk factors. Assessment of Rho-associated kinase activity may be useful as a therapeutic target for cardiovascular disease as well as a biomarker for cardiovascular events. Rho-associated kinase activity may be a therapeutic target for prevention of cardiovascular events. However, unfortunately, there is no information concerning the long-term effects of interventions that inhibit Rho-associated kinase activity on cardiovascular morbidity and mortality.

Rho-associated kinase is known to be activated by several neurohormonal factors, such as angiotensin II, thrombin, and a high-glucose condition in endothelial cells or smooth muscle cells, and subsequently to modulate cell contraction, proliferation, apoptosis, and gene expression via several signaling pathways, including nuclear factor κB.[2,30] Several lines of evidence have shown that Rho-associated kinase play an important role in cardiovascular damage through modulation of the expression of various genes.[30–35] It is thought that several downstream mediators of Rho-associated kinase contribute to the processes of arteriosclerosis and atherosclerosis. RhoA is a downstream target of angiotensin II, endothelin-1 and phenylephrine in vessels and the heart via G protein-coupled receptors (GPCRs).[29,36–38] GPCR signaling mediated by these neurohumoral factors increases the expression of Rho families, including RhoA, Rac1 and Cdc42.[38] Furthemore, GPCR signaling activates RhoA not only through translocation of RhoA to the membrane from the cytosol but also through an increase of an active GTP-bound form of RhoA by Rho guanine nucleotide exchange factors (GEFs). Indeed, the cycle between active GTP-bound form and inactive GDP-bound form of RhoA is controlled by Rho GEFs as activators and Rho GTPase-activating proteins (GAPs) as inactivators. There is growing evidence that RhoA is also a key mediator of atherosclerotic responses in vascular smooth muscle cells and cardiomyocytes through increasing Rho-associated kinase activity.[30–38] Rho and Ras have been shown to play important roles in modulation of the expression of various genes, including beta-myosin heavy chain and atrial natriuretic factor, that is induced by signaling through $G_{12/13}$ and Gq.[30,38,39] These findings suggest that the Rho/Rho-associated kinase pathway is involved in cellular responses in the process of cardiovascular damage.

It is therefore clinically important to estimate the grade of Rho-associated kinase activity. Previously, vascular Rho-associated kinase has been clinically assessed by venous occlusion mercury-filled plethysmography using an intra-arterial infusion of fasudil, a specific Rho-associated kinase inhibitor. In previous studies, increased Rho-associated kinase activity has been shown to play a critical role in the pathogenesis and progression of hypertension, angina pectoris, vasospastic angina, pulmonary hypertension, heart failure, and stroke. Furthermore, Rho-associated kinase activity has been shown to be enhanced in smokers.[11–17] Although the evaluation of vascular Rho-associated kinase activity using plethysmography has been demonstrated to be specific due to the fact that the dose-response curve of forearm blood flow response to a Rho-associated kinase inhibitor could be evaluated, evaluation using plethysmography is invasive, burdensome, and time-consuming for subjects. In the present study, we measured leukocyte phospho-myosin-binding subunit/total-myosin-binding subunit as a noninvasive method for assessing Rho-associated kinase activity, since peripheral leukocytes are able to be simply obtained. Measurement of Rho-associated kinase activity in endothelial cells or vascular smooth muscle cells from human vessels would enable more specific conclusions concerning the role of Rho-associated kinase activity in cardiovascular events to be drawn. However, unfortunately, we cannot easily obtain samples of endothelial cells or vascular smooth muscle cells under a healthy condition. In addition, several investigators including us have previously reported that measurement of leukocyte Rho-associate kinase activity is a useful method as is measurement of vascular Rho-associated kinase activity.[18,33,40–42] We have also confirmed that leukocyte Rho-associated kinase activity substantially correlates with vascular Rho-associated kinase activity. Indeed, leukocyte Rho-associated kinase activity was related to Framingham risk score, which is a risk calculator and an index of cumulative cardiovascular risk commonly used for assessing heart attack or death from heart disease within 10 years.[18] However, technical complexity remains when measuring leukocyte Rho-associated kinase activity. Further studies are needed to standardize the method for measurement of leukocyte Rho-associated kinase activity.

Limitations

A small number of events, particularly acute myocardial infarction, were registered during the follow-up period, leading to reduction in statistical power. Also, there were subjects with various investigation periods, because the subjects were enrolled during a long period from June 2007 to March 2012. Nevertheless, Rho-associated kinase activity was an independent predictor of cardiovascular events after adjustment of cardiovascular risk factors. Future studies are needed to confirm the prognostic value of Rho-associated kinase activity in a larger population.

In the present study, we measured Rho-associated kinase activity just one time when entering subjects. Follow-up data on Rho-associated kinase activity are lacking. Repeated measurements of Rho-associated kinase activity and evaluation of the changes in Rho-associated kinase activity may be useful to confirm the therapeutic efficacy for prevention of cardiovascular events.

We enrolled subjects who underwent health examinations in a single medical center and found a critical correlation of Rho-associated kinase activity with future cardiovascular events in the present study. A multicenter study is needed to confirm the results of this study.

Measurements of combinations of Rho-associated kinase activity and markers of oxidative stress and inflammation-related circulating levels of cytokines/chemokines as well as growth factors would enable more specific conclusions concerning the role of Rho-associated kinase activity in the onset of cardiovascular events to be drawn.

Finally, several investigators have previously focused on leukocyte Rho-associated kinase activity.[18,28,29,40–42] However, the reproducibility of leukocyte Rho-associated kinase measurement has not been discussed due to the complexity of isolating leukocytes to measure Rho-associated kinase activity. Although leukocyte Rho-associated kinase is becoming established as a biomarker for cardiovascular morbidity and mortality, further studies concerning reproducibility of measurement are necessary.

Perspectives

Previously, several investigators including us reported relationships of Rho-associated kinase activity with cardiovascular risk factors but not with cardiovascular outcomes. In this study, we demonstrated that Rho-associated kinase activity in peripheral leukocytes measured by Western blot analysis was associated with future cardiovascular events in a general population. These findings suggest that leukocyte Rho-associated kinase may be a useful surrogate marker for cardiovascular outcomes and that inhibition of Rho-associated kinase activity may be a therapeutic target for prevention of cardiovascular events.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Novelty and Significance

1) What is new?

The present study is the first study showing the prognostic impact of Rho-associated kinase activity for major cardiovascular events.

Rho-associated kinase activity may be used as a surrogate marker of future cardiovascular events.

2) What is Relevant?

Inhibition of leukocyte Rho-associated kinase activity may be a therapeutic target for prevention of cardiovascular events.

3) Summary

The results of this prospective study showed for the first time the prognostic impact of Rho-associated kinase activity for first major cardiovascular events.

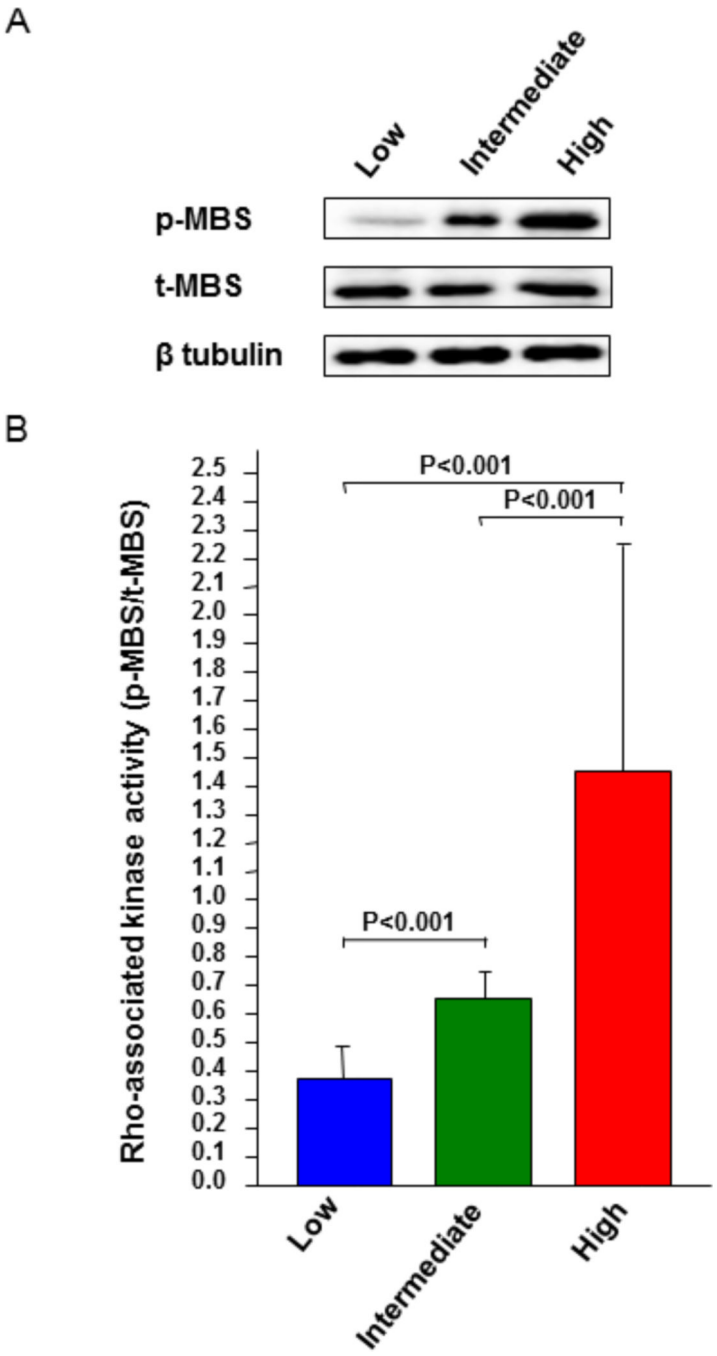

Figure 1.

(A) Representative western blot analysis for phospho myosin-binding subunit (p-MBS), total myosin-binding subunit (t-MBS), and β tublin in peripheral blood leukocytes of subjects with low, intermediate, and high Rho-associated kinase activity. (B) Expression levels of p-MBS, t-MBS, and β tubulin in subjects with low, intermediate and high Rho-associated kinase activity divided into three tertiles.

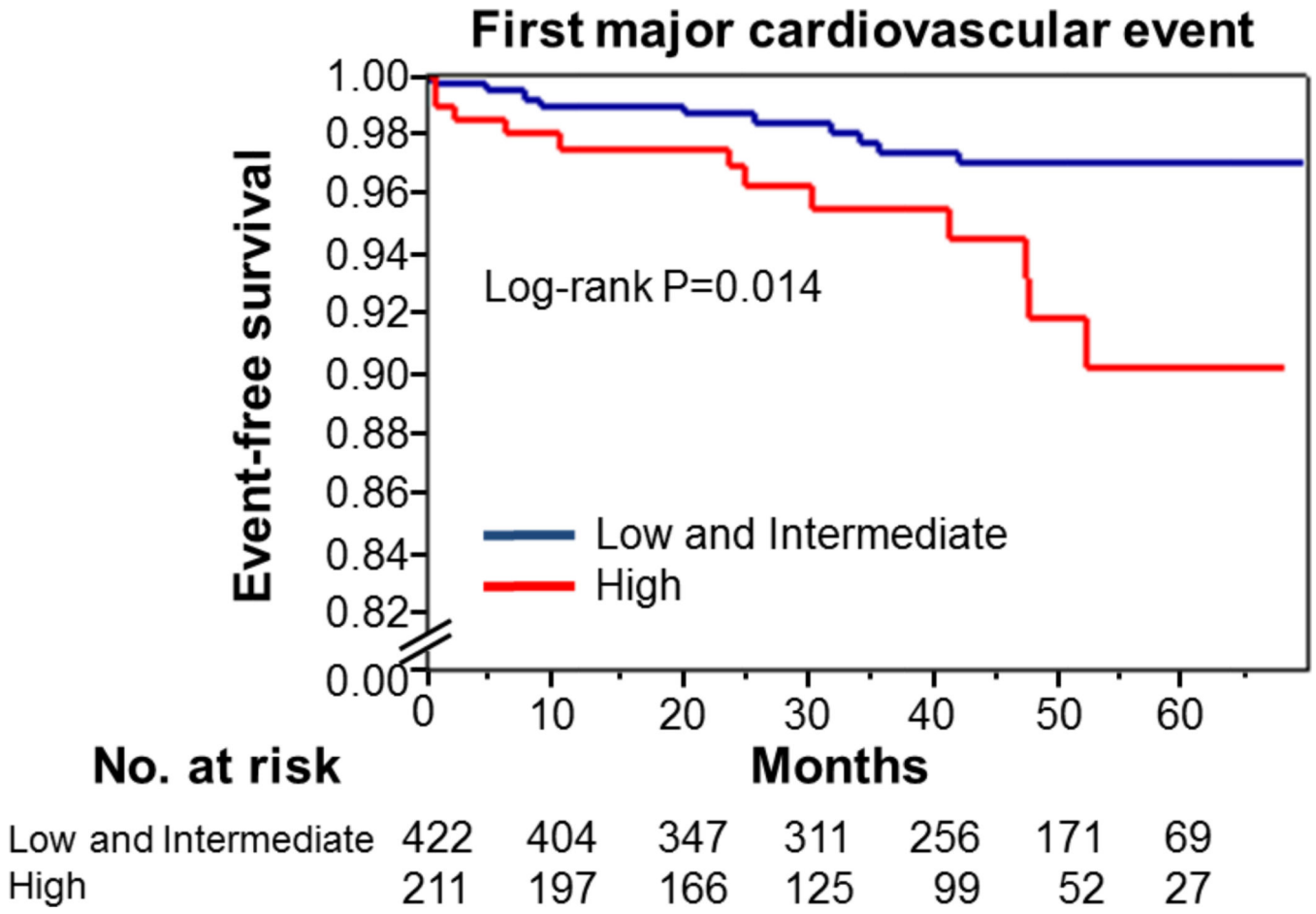

Figure 2.

Kaplan-Meier curves of cumulative event-free survival of major cardiovascular events (death from cardiovascular causes, myocardial infarction, and stroke), according to Rho-associated kinase activity. The P value was calculated from the log-rank test.

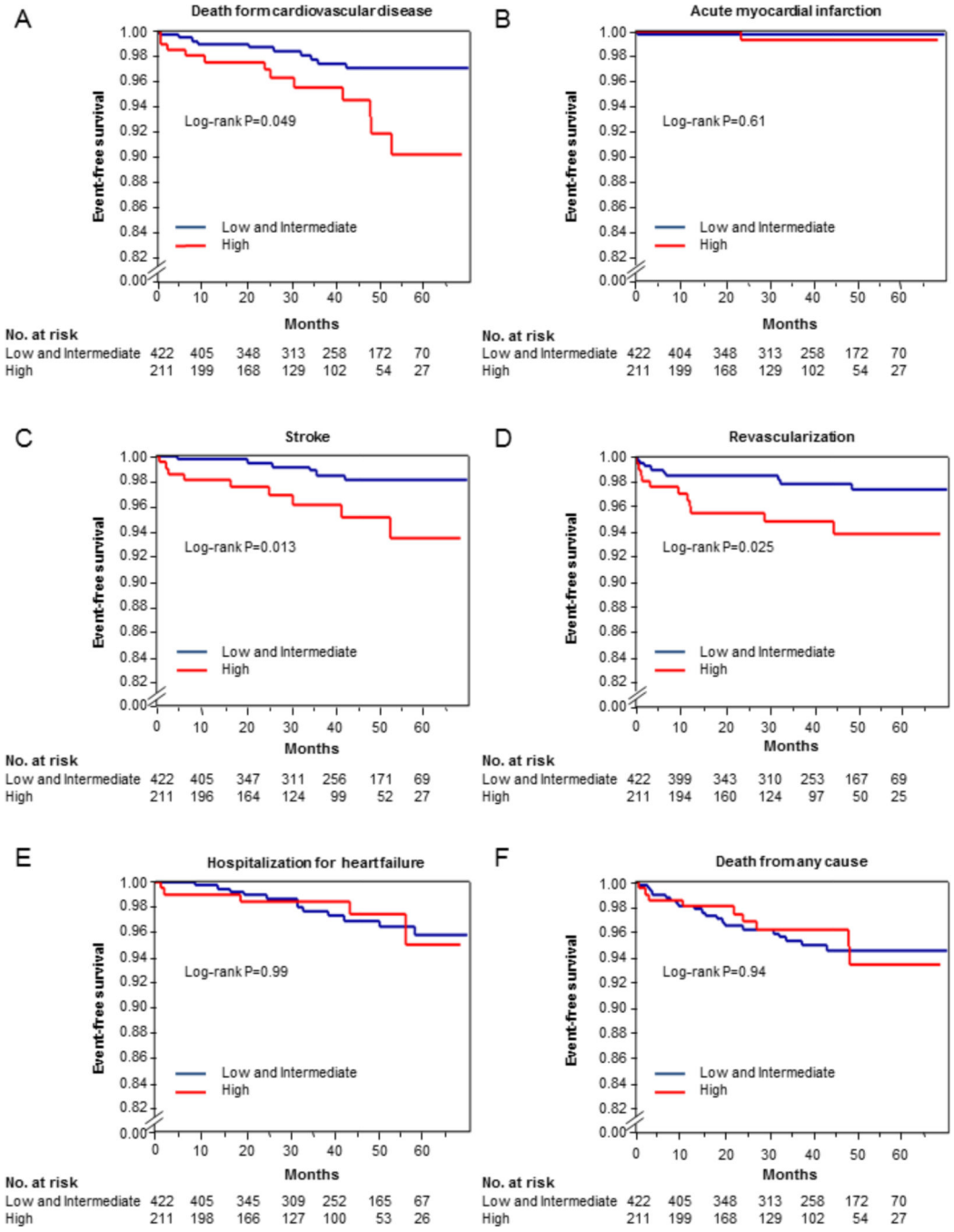

Figure 3.

Kaplan-Meier curves of cumulative event-free survival of death from cardiovascular causes (panel A), acute myocardial infraction (panel B), stroke (panel C), revascularization (percutaneous coronary intervention, coronary artery bypass grafting) (panel D), hospitalization for heart failure (panel E), and death from any cause (panel F), according to Rho-associated kinase activity. The P value was calculated from the log-rank test.

Table 1

Clinical Characteristics of the Subjects

Variable	Total (n=633)	Low Group (n=211)	Intermediate Group (n=211)	High Group (n=211)	P value for Trend
Age, yr	53±18	53±18	54±19	54±18	0.69
Gender, men/women	398/235	116/95	136/75	146/65	0.009
Body mass index, kg/m^2	23.2±3.7	23.3±4.0	23.1±3.5	23.1±3.7	0.74
Systolic blood pressure, mmHg	128±20	127±19	128±21	128±21	0.89
Diastolic blood pressure, mmHg	73±14	73±14	72±14	73±15	0.60
Heart rate, bpm	72±13	73±13	71±13	71±12	0.09
Medical history – no. (%)					
Hypertension	338 (53.4)	118 (55.9)	115 (54.5)	105 (49.8)	0.45
Dyslipidemia	387 (61.1)	140 (66.4)	118 (55.9)	129 (61.1)	0.09
Diabetes mellitus	145 (22.9)	50 (23.7)	52 (24.6)	43 (20.4)	0.56
Current smoker	171 (27.0)	51 (24.2)	57 (27.0)	63 (29.9)	0.39
Previous coronary artery disease	95 (15.0)	34 (16.1)	34 (16.1)	27 (12.8)	0.55
Myocardial infarction	38 (6.0)	11 (5.2)	13 (6.2)	14 (6.6)	0.81
Angina pectoris	74 (11.7)	29 (13.7)	25 (11.8)	20 (9.5)	0.40
Previous cerebrovascular disease	35 (5.5)	10 (4.7)	10 (4.7)	15 (7.1)	0.47
Cerebral infarction	30 (4.7)	10 (4.7)	7 (3.3)	13 (6.2)	0.38
Cerebral hemorrhage	8 (1.3)	1 (0.5)	3 (1.4)	4 (1.9)	0.36
Laboratory determinations					
Blood urea nitrogen, mmol/L	5.60±2.78	5.46±2.32	5.64±3.36	5.68±2.57	0.65
Creatinine, umol/L	84.9±82.2	79.6±67.2	77.8±53.9	96.4±113.2	0.06
estimated glomerular filtration rate, mL/min/1.73m^2	74.8±23.9	76.0±23.9	75.9±23.6	72.4±24.1	0.23
Total cholesterol, mmol/L	5.09±1.09	5.30±1.29	4.94±0.98	5.04±0.96	0.004
Triglycerides, mmol/L	1.65±1.13	1.73±1.16	1.58±1.05	1.61±1.16	0.42
High-density lipoprotein cholesterol, mmol/L	1.53±0.47	1.60±0.52	1.50±0.41	1.47±0.47	0.02
Low-density lipoprotein cholesterol, mmol/L	2.97±0.88	3.08±1.01	2.87±0.83	2.97±0.80	0.09
Glucose, mmol/L	6.22±2.61	5.88±2.22	6.66±3.39	6.11±2.00	0.01
Medications – no. (%)					

Variable	Total (n=633)	Low Group (n=211)	Intermediate Group (n=211)	High Group (n=211)	P value for Trend
Antiplatelets	168 (25.2)	62 (29.4)	53 (25.1)	53 (25.1)	0.55
Calcium-channel blockers	192 (30.3)	73 (34.6)	65 (30.8)	54 (25.6)	0.13
Statins	132 (20.9)	43 (20.4)	49 (23.2)	40 (19.0)	0.53
Renin angiotensin system inhibitors	145 (22.9)	53 (25.1)	46 (21.8)	46 (21.8)	0.68
Medically treated diabetes					
Any	91 (14.4)	31 (14.7)	29 (13.7)	31 (14.7)	0.95
Insulin-dependent	26 (4.1)	6 (2.8)	7 (3.3)	13 (6.2)	0.19

Low group indicates less than 0.521 Rho-associated kinase activity, intermediate group indicates 0.522 to 0.810, high group indicates more than 0.811.

All results are presented as mean ± SD.

Table 2

Clinical Outcomes of All Subjects on the Basis of Rho-associated Kinase Activity

Variable	Total (n=633)	Low Group (n=211)	Intermediate Group (n=211)	High Group (n=211)	P value
First major cardiovascular event, n (%)	22 (3.5)	4 (1.9)	6 (2.8)	12 (5.7)	0.03
Death from cardiovascular disease, n (%)	10 (1.6)	2 (1.0)	2 (1.0)	6 (2.8)	0.08
Acute myocardial infarction, n (%)	2 (0.3)	0 (0.0)	1 (0.5)	1 (0.5)	0.63
Stroke, n (%)	15 (2.4)	3 (1.4)	3 (1.4)	9 (4.3)	0.03
Revascularization (PCI or CABG), n (%)	20 (3.2)	6 (2.8)	3 (1.4)	11 (5.2)	0.04
Hospitalization for heart failure, n (%)	17 (2.7)	5 (2.4)	7 (3.3)	5 (2.4)	0.73

First major cardiovascular events include cardiovascular death, myocardial infarction, and stroke.

Low group indicates less than 0.521 Rho-associated kinase activity, intermediate group indicates 0.522 to 0.810, high group indicates more than 0.811. PCI indicates percutaneous coronary intervention; CABG, coronary artery bypass grafting.

P value was presented low and intermediate Rho-associated kinase activity group vs. high group.

Table 3

Cause of Death

Cause of death	Total (n=29/633)	Low Group (n=12/211)	Intermediate Group (n=8/211)	High Group (n=9/211)
Heart failure, n	4	1	1	2
Aortic dissection or upture, n	3	1	1	1
Systemic embolism, n	1	0	0	1
Acute myocardial infarction, n	1	0	0	1
Stent thrombosis, n	1	0	0	1
Malignancy, n	11	5	4	2
Septic shock, n	3	1	1	1
Renal failure, n	2	2	0	0
Asthma, n	1	1	0	0
Pneumonia, n	1	0	1	0
Unknown, n	1	1	0	0

Low group indicates less than 0.521 Rho-associated kinase activity, intermediate group indicates 0.522 to 0.810, high group indicates more than 0.811.

Table 4

Association Between Rho-associated Kinase Activity and Events During Follow-up

Variable	Unadjusted HR (95% CI) P value	Adjusted* HR (95% CI) P value	Adjusted† HR (95% CI) P value
First major cardiovascular event	1.66 (1.09–2.55) 0.01	1.82 (1.18–2.84) 0.008	2.19 (1.35–3.70) 0.002
Death from cardiovascular disease	1.83 (0.97–3.61) 0.06	1.87 (0.97–3.81) 0.06	2.57 (1.18–6.60) 0.002
Acute myocardial infarction	1.43 (0.28–7.19) 0.62	NA	NA
Stroke	1.85 (1.11–3.20) 0.01	1.98 (1.17–3.46) 0.01	2.14 (1.24–3.86) 0.006
Revascularization (PCI or CABG)	1.63 (1.05–2.56) 0.03	1.96 (1.25–3.13) 0.004	2.68 (1.60–4.66) <0.001
Hospitalization for heart failure	1.00 (0.56–1.64) 0.99	0.98 (0.55–1.65) 0.96	1.01 (0.55–1.74) 0.96

First major cardiovascular events include cardiovascular death, myocardial infarction, and stroke.

HR indicates hazard ratio; CI, confidence interval; NA, not applicable; PCI, percutaneous coronary intervention; CABG, coronary artery bypass grafting.

Hazard ratios are for high Rho-associated kinase activity group, using the low and intermediate Rho-associated kinase activity group as the reference.

*Adjusted for age; gender; the presence of hypertension, dyslipidemia, and diabetes; current smoking.

†Adjusted also for age; gender; the presence of hypertension, dyslipidemia, and diabetes; current smoking; previous coronary heart disease; previous stroke; concomitant treatment with antiplatelets, angiotensin-converting enzyme inhibitors, angiotensin type 1 receptor blockers, calcium channel blockers, statins, and diabetes agents.