edly in patients with CKD.[6-10] Cardiac failure is more common in patients with advanced CKD, showing a prevalence of ~40%.[11]

Several autopsy-based studies have shown a higher prevalence of arteriosclerotic lesions in individuals with CKD than in those without CKD.[12-14] Furthermore, patients with end-stage renal disease show more advanced atherosclerotic lesions with calcification in coronary arteries than the general population.[14] However, these studies were conducted in hospital-based populations, which are prone to underlying disease. Additionally, there are few studies investigating the histopathologic findings of coronary arteries in individuals with moderate stage of CKD.

The Hisayama Study is a prospective population-based study of cardiovascular disease risk factors in Japanese people[15] and is characterized by autopsy verification of the cause of death in ~80% of those who died.[16,17] The present study assessed the relationship between decreased kidney function and severity of coronary atherosclerosis in population-based autopsy samples.

METHODS

Study Population

The Hisayama Study was established in 1961 in the town of Hisayama, a suburban community adjacent to Fukuoka City in a metropolitan area of Kyushu Island in southern Japan. The population of Hisayama is ~7,000 and has been stable for 40 years. Full community surveys of residents have been repeated since 1961.[18] From January 1988 to November 2005, a total of 1,162 residents of Hisayama died; of these, 844 underwent autopsy examination. Individuals without health examination data within 3 years before death were excluded. The remaining 482 individuals were classified into 4 categories based on estimated glomerular filtration rate (eGFR): ≥60, 45-59, 30-44, and <30 mL/min/1.73 m^2 (data from the most recent health examination). Eighteen individuals had an eGFR < 30 mL/min/1.73 m^2. The individuals included in this study were randomly selected using a computer-generated random number from each category of eGFR level after matching for age at death and sex in a 1:2 ratio against individuals in the <30-mL/min/1.73 m^2 category. A final total of 126 individuals (49 men, 77 women) were enrolled in this study (Fig 1). The median period from the last health examination to death was 1.0 years (quartile [Q] 1 to 3, 0.0-2.0).

Risk Factors

At each health examination, study participants undertook a self-administered questionnaire covering medical history, antihypertensive treatment, smoking habits, and alcohol intake. The completed questionnaire was checked by trained interviewers. Blood pressures were measured 3 times using a standard mercury sphygmomanometer at each examination, with mean values used for the analysis. Hypertension was defined as systolic blood pressure ≥ 140 mm Hg and/or diastolic blood pressure ≥ 90 mm Hg or use of antihypertensive agents. Blood samples were collected after overnight fasting. Serum creatinine was measured using the Jaffé method. Hemoglobin A_{1c} was measured using high-performance liquid chromatography. Diabetes mellitus was diagnosed as hemoglobin A_{1c} level ≥ 6.0%. Total cholesterol, high-density lipoprotein cholesterol, and triglyceride levels were determined enzymatically. Dyslipidemia was defined as total cholesterol concentration ≥ 220 mg/dL, high-density lipoprotein cholesterol concentration ≤ 40 mg/dL, or triglyceride concentration ≥ 150 mg/dL.

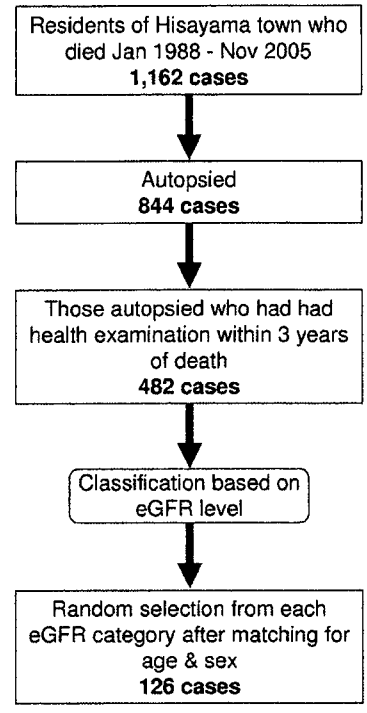

Figure 1. Flow diagram for study enrollment. Abbreviation: eGFR, estimated glomerular filtration rate.

Definition of CKD

eGFR was estimated using the 6-variable Modification of Diet in Renal Disease (MDRD) Study equation,[19] and is given by the following equation (only 5 variables are shown because the multiplier for black race was not applicable to this population):

$$\text{eGFR (mL/min/1.73 m}^2) = 170 \times [\text{serum creatinine (mg/dL)}]^{-0.999}$$

$$\times [\text{age (years)}]^{-0.176}$$
$$\times [\text{serum urea nitrogen (mg/dL)}]^{-0.170}$$
$$\times [\text{serum albumin (g/dL)}]^{0.318}$$
$$\times [0.762 \text{ if female}]$$

eGFR levels were classified into 4 categories: ≥60, 45-59, 30-44, and <30 mL/min/1.73 m^2, according to the National Kidney Foundation's Kidney Disease Outcomes Quality Initiative (KDOQI) guidelines.[20]

For sensitivity analyses, eGFR also was estimated using the 4-variable MDRD Study equation modified with the Japanese Society of Nephrology–Chronic Kidney Disease Initiative coefficient (ie, the JSN-CKDI equation)[21]:

$$\text{JSN-eGFR (mL/min/1.73 m}^2) = 0.808 \times 175$$
$$\times [\text{serum creatinine (enzymatic method [mg/dL])}]^{-1.154}$$
$$\times [\text{age (years)}]^{-0.203} \times [0.742 \text{ if female}]$$

where the value of serum creatinine measured using the Jaffé method was converted to values for the enzymatic method by subtracting 0.207 mg/dL.[22]

Coronary Artery Morphological Examination

Heart tissue obtained at autopsy was immersed in 10% buffered formaldehyde for at least 24 hours, making sure to include the 3 main coronary arteries. The right coronary artery (segment 1), left anterior descending coronary artery (segment 6), and left circumflex coronary artery (segment 11) were dissected free from the surface of the heart, cut perpendicular to the long axis at 3-mm intervals, and embedded in paraffin. The segment of the vessel showing the most severe stenosis was selected for histological examination, excluding areas near the branching site. Three blocks were excluded because the segments of the coronary arteries were not adequately defined. In total, 375 blocks were obtained and all blocks for each individual were cut into 3-μm-thick serial sections in 1 sequence (1 block provided insufficient sample to estimate the extent of arterial stenosis). Sections from each block were serially subjected to hematoxylin and eosin, elastica–van Gieson, and Masson trichrome staining. Histological examinations were made without reference to the associated clinical information by 2 independent pathologists (T. Nakano and S.S. in blinded assessments).

Estimation of Atherosclerotic Lesions

Atherosclerotic lesions found in each section were classified into 6 types in accordance with the definitions proposed by the Committee on Vascular Lesions of the Council on Atherosclerosis, American Heart Association (AHA)[23]: type I (initial lesion), intimal thickening with isolated foam cells; type II (fatty-streak lesion), intimal thickening with intracellular lipid accumulation; type III (intermediate lesion), type II changes and small extracellular lipid pools; type IV (atheroma), type II changes and core of extracellular lipid; type V (fibroatheroma), lipid core and fibrotic layer to lesions, or mainly calcified, or mainly fibrotic; and type VI (complicated lesion), disrupted lesion with hematoma or hemorrhage or thrombotic deposits. The AHA classification defines advanced atherosclerotic lesions as types IV-VI.[23] Lesion calcification was assessed on hematoxylin and eosin-stained paraffin sections from all specimens.

Morphometry of Luminal Stenosis in the Coronary Artery

All arteries were analyzed quantitatively for stenosis rate using computerized planimetry according to Taylor et al.[24] Morphometry was performed using National Institutes of Health (NIH) Image software (version 1.63; NIH, Bethesda, MD). Elastica-van Gieson–stained sections were magnified and digitized to measure the luminal internal and external elastic lamina perimeters. Arterial areas were calculated from diameter values derived from the measured arterial perimeter (area = πr^2) to avoid artifacts from vessel shape distortion during processing. Plaque areas were calculated as the differences between internal elastic lamina and luminal area measurements. Percentage luminal stenosis was calculated as plaque area/internal elastic lamina area × 100.[24]

Statistical Analysis

The SAS software package for Windows, version 9.1 (SAS Institute Inc, Cary, NC) was used to perform statistical analyses. Trends in mean values or frequencies of variables across subgroups of eGFR level were tested using linear regression analysis or logistic regression analysis, respectively. Mean stenosis rates according to eGFR levels were calculated using a linear mixed model to account for correlation between vessels within a patient. Stenosis rates between vessels correlated fairly, with a correlation coefficient range of 0.21-0.32. This analysis was carried out using the procedure "MIXED" in SAS. Odds ratios (ORs) and their 95% confidence intervals (CIs) were estimated using the generalized estimating equation methods to deal with modeling the correlation among repeated outcomes within a patient.[25] Correlation coefficients for the probabilities of advanced atherosclerosis and calcified lesion between vessels ranged from 0.08-0.34 and 0.25-0.37, respectively. These analyses were performed using procedure "GENMOD" in SAS. Trends in relationships between eGFR levels and risk of outcomes were tested by adding the median value of eGFR for each category to the relevant model. Two-tailed $P < 0.05$ was defined as statistically significant.

RESULTS

Baseline Characteristics

Table 1 lists baseline clinical and demographic characteristics of individuals included in the study according to eGFR levels. Individuals with lower eGFRs had higher systolic blood pressure and calcium-phosphorus product and lower hematocrit values. Frequency of hyper-

Table 1. Laboratory Variables and Risk Factors According to Kidney Function

	eGFR (mL/min/1.73 m²)				*P* for Trend
	≥60	45-59	30-44	<30	
eGFR (mL/min/1.73 m²)	72 (68-85)	55 (51-58)	40 (37-43)	21 (19-25)	
No. of patients	36	36	36	18	
Age (y)	84 ± 6	85 ± 6	85 ± 8	85 ± 7	0.8
Men (%)	39	39	39	39	0.9
Serum creatinine (mg/dL)	0.9 (0.8-1.0)	1.1 (1.0-1.3)	1.5 (1.3-1.7)	2.5 (2.0-3.2)	<0.001
Serum urea nitrogen (mg/dL)	16 (12-18)	19 (16-24)	24 (19-27)	39 (29-46)	<0.001
Serum albumin (g/dL)	4.0 ± 0.4	4.0 ± 0.5	3.9 ± 0.5	3.7 ± 0.4	0.1
Systolic blood pressure (mm Hg)	141 ± 23	142 ± 29	143 ± 29	165 ± 29	0.01
Diastolic blood pressure (mm Hg)	73 ± 12	74 ± 14	75 ± 10	77 ± 13	0.2
Use of antihypertensive agent (%)	28	36	56	50	0.03
Hypertension (%)	61	58	78	94	0.006
Hemoglobin A_{1c} (%)	5.2 ± 0.8	5.7 ± 1.5	5.4 ± 0.8	5.4 ± 0.9	0.6
Diabetes (%)	11	22	19	22	0.3
Total cholesterol (mg/dL)	184 ± 37	190 ± 43	195 ± 53	186 ± 45	0.6
High-density lipoprotein cholesterol (mg/dL)	60 ± 17	52 ± 13	56 ± 17	53 ± 15	0.3
Triglycerides (mg/dL)	76 (65-102)	91 (81-124)	88 (68-123)	113 (70-167)	0.1
Calcium-phosphorus product (mg^2/dL^2)	29 ± 6	31 ± 5	31 ± 4	33 ± 5	0.005
Hematocrit (%)	37 ± 5	37 ± 6	35 ± 5	30 ± 6	<0.001
Smoking habit (%)	19	28	6	17	0.3
Alcoholic intake (%)	17	11	11	6	0.3
Median time from last health examination (y)	1.0 (0.5-2.0)	2.0 (0.5-2.0)	1.5 (0.5-3.0)	1.0 (0-2.0)	0.7
Causes of death					
Malignant neoplasms (%)	28	31	28	0	0.2
Heart diseases (%)	17	17	11	11	0.1
Cerebrovascular diseases (%)	17	11	3	11	0.4
Other diseases of circulatory system (%)	0	6	6	6	0.2
Infectious diseases (%)	17	19	33	22	0.5
Other causes (%)	19	6	8	33	0.08

Note: Values expressed as mean ± SD, frequency, or median (Q1-Q3). Hypertension was defined as blood pressure ≥ 140/90 mm Hg or use of antihypertensive agent. Diabetes was defined as hemoglobin A_{1c} level ≥ 6.0%. Trends were tested using linear regression analysis for continuous variables or logistic regression analysis for categorical variables. Conversion factors for units: eGFR in mL/min/1.73 m² to mL/s/1.73 m², ×0.0167; serum creatinine in mg/dL to μmol/dL, ×76.26; serum albumin in g/dL to g/L, ×10; serum urea nitrogen in mg/dL to mmol/L, ×0.357; total and high-density lipoprotein cholesterol in mg/dL to mmol/L, ×0.02586; triglycerides in mg/dL to mmol/L, ×0.01129.

Abbreviation: eGFR, estimated glomerular filtration rate.

tension and use of antihypertensive agents increased significantly with decreased eGFR. Mean values or frequencies of other potential risk factors were not statistically different among eGFR levels.

Relationship Between Kidney Function and Severity of Atherosclerotic Lesions

Figure 2 shows a typical coronary artery for subgroups of eGFR levels. Age- and sex-ad-

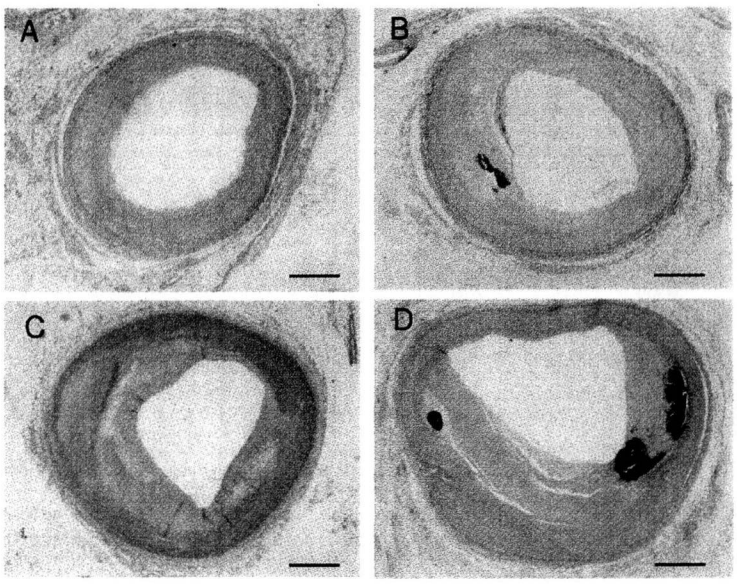

Figure 2. Typical arteries for each classification by glomerular filtration rate (GFR). (A-D) Typical light microscopic views of coronary arteries from respective cases with estimated GFRs (A) ≥60, (B) 45-59, (C) 30-44, and (D) <30 mL/min/1.73 m^2. Stenosis rates of respective arteries were (A) 36.8%, (B) 42.3%, (C) 54.2%, and (D) 58.9%. All sections were stained with hematoxylin and eosin. Scale bars = 1.0 mm.

justed mean values for coronary artery stenosis rate increased significantly with lower eGFRs (mean, 46.7% ± 1.9% [SE], 49.2% ± 1.9%, 51.9% ± 1.9%, and 53.7% ± 2.7% for eGFRs ≥60, 45-59, 30-44, and <30 mL/min/1.73 m^2, respectively; P for trend = 0.02).

Figure 3 shows proportions of atherosclerotic lesions using the AHA classification according to eGFR level. Prevalences of advanced atherosclerotic lesions defined as types IV-VI were 34.3% for eGFR ≥ 60 mL/min/1.73 m^2, 41.7% for eGFR of 45-59 mL/min/1.73 m^2, 52.3% for eGFR of

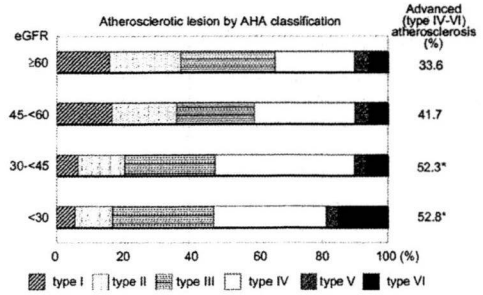

Figure 3. Proportions of atherosclerotic lesion types using American Heart Association (AHA) classification by level of kidney function. Percentages of advanced atherosclerosis (AHA types IV-VI) for each estimated glomerular filtration rate (eGFR) level is shown at the right side of the graphs. *P < 0.05 vs eGFR ≥ 60 mL/min/1.73 m^2.

30-44 mL/min/1.73 m^2, and 52.8% for eGFR < 30mL/min/1.73 m^2. Individuals in the latter 2 categories had a significantly higher proportion of advanced atherosclerotic lesions on autopsy than those with eGFR ≥ 60 mL/min/1.73 m^2. The risk of advanced atherosclerosis was doubled in individuals with eGFR < 45 mL/min/1.73 m^2 compared with those with eGFR ≥ 60 mL/min/1.73 m^2 after adjustment for potential confounding factors, including age, sex, hypertension, diabetes, total cholesterol level, high-density lipoprotein cholesterol level, triglyceride level, calcium-phosphorus product, hematocrit, smoking habit, and alcohol intake (Table 2).

Prevalence of Calcified Lesion in Coronary Artery According to Kidney Function

In a case of AHA type VI in the subgroup of eGFR < 30 mL/min/1.73 m^2, the arterial intima was thickened and associated with calcified plaque and hematoma (Fig 4).

Many coronary artery samples showed intimal calcified lesions, but there was no medial calcification in any specimen examined. Prevalences of calcified lesions were 36.5% for eGFR ≥ 60 mL/min/1.73 m^2, 37.0% for eGFR of 45-59 mL/min/1.73 m^2, 44.9% for eGFR of 30-44 mL/min/1.73

Table 2. Age- and Sex-Matched or Multivariate-Adjusted Odds Ratios for Advanced Coronary Atherosclerotic and Calcified Lesions According to Kidney Function

eGFR (mL/min/1.73 m²)	No. of Vessels Assessed	Age and Sex Adjusted[a] Matched Odds Ratio	95% Confidence Interval	P	P for Trend	Multivariate Adjusted[b] Matched Odds Ratio	95% Confidence Interval	P	P for Trend
Advanced Atherosclerosis (AHA type IV-VI)									
≥60	107	1.00	Reference		0.006	1.00	Reference		0.01
45-59	108	1.51	0.80-2.87	0.2		1.40	0.76-2.55	0.3	
30-44	107	2.22	1.11-4.43	0.02		2.02	0.99-4.15	0.05	
<30	53	2.38	1.18-4.81	0.02		3.02	1.22-7.49	0.02	
Calcified Lesion									
≥60	107	1.00	Reference		0.02	1.00	Reference		0.009
45-59	108	1.02	0.50-2.08	0.9		0.95	0.46-1.94	0.9	
30-44	107	1.43	0.71-2.89	0.3		1.43	0.69-2.95	0.3	
<30	53	2.75	1.19-6.34	0.02		4.71	1.78-12.50	0.002	

Abbreviations: AHA, American Heart Association; eGFR, estimated glomerular filtration rate.

[a]Odds ratios were adjusted for age and sex.

[b]Odds ratios were adjusted for age, sex, hypertension, diabetes, total cholesterol level, high-density lipoprotein cholesterol level, triglyceride level, calcium-phosphorus product, hematocrit, smoking habit, and alcohol intake.

m^2, and 60.4% for eGFR < 30 mL/min/1.73 m^2 (P for trend = 0.02). Lower eGFR was associated with a higher prevalence of calcified coronary artery lesions. The multivariate-adjusted OR of calcified lesions was 4.71 (95% CI, 1.78-12.50) in individuals with GFR < 30 mL/min/1.73 m^2 compared with those with GFR > 60 mL/min/1.73 m^2 (Table 2).

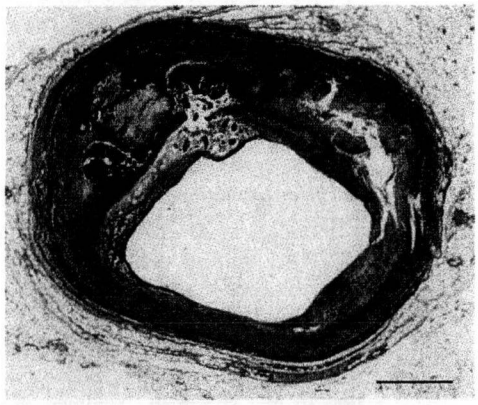

Figure 4. Typical artery of American Heart Association type VI lesion in the category glomerular filtration rate < 30 mL/min/1.73 m^2. (Masson trichrome stain; scale bar=1.0 mm.)

Association of Cardiovascular Risk Factors With Risk of Advanced Atherosclerotic Lesions and Calcified Lesions in Individuals With Decreased eGFR

Next, we assessed the relationship between the prevalence of advanced atherosclerotic lesions and cardiovascular risk factors, such as hypertension, diabetes, and dyslipidemia, in individuals with eGFR < 60 mL/min/1.73 m^2 (Table 3). The risk of advanced atherosclerotic lesions tended to be higher in individuals with hypertension than in those without hypertension (OR, 1.76; 95% CI, 0.93-3.35). Individuals with diabetes had a significantly higher risk of advanced atherosclerotic lesions (OR, 2.57; 95% CI, 1.26-5.24). Likewise, hypertension and diabetes were associated significantly with increased risk of calcified lesions in individuals with eGFR < 60 mL/min/1.73 m^2 (OR, 1.88; 95% CI, 1.04-3.39 for hypertension; OR, 2.91; 95% CI, 1.56-5.45 for diabetes).

Sensitivity Analyses Using the JSN-CKDI Equation to Estimate GFR

We also estimated GFRs using the JSN-CKDI equation.[21] The distribution of JSN-eGFR (median, 49 mL/min/1.73 m^2; Q1-Q3, 35-65) was similar to that of GFR estimated using the MDRD

Table 3. Association of Cardiovascular Risk Factors With Risk of Advanced Coronary Atherosclerotic and Calcified Lesions in Individuals With Decreased Kidney Function

	No. of Vessels Assessed	Frequency of Lesion (%)	Odds Ratio	95% Confidence Interval	*P*
Advanced Atherosclerosis (American Heart Association types IV-VI)					
Hypertension					0.08
No	71	38.0	1.00	Reference	
Yes	197	51.8	1.76	0.93-3.35	
Diabetes					0.01
No	212	43.4	1.00	Reference	
Yes	56	66.1	2.57	1.26-5.24	
Dyslipidemia					0.1
No	143	42.7	1.00	Reference	
Yes	125	54.4	1.61	0.91-2.86	
Calcified Lesion					
Hypertension					0.04
No	71	33.8	1.00	Reference	
Yes	197	48.7	1.88	1.04-3.39	
Diabetes					<0.001
No	212	40.1	1.00	Reference	
Yes	56	62.5	2.91	1.56-5.45	
Dyslipidemia					0.5
No	143	42.0	1.00	Reference	
Yes	125	48.0	1.25	0.71-2.20	

Note: Hypertension defined as blood pressure ≥ 140/90 mm Hg and/or use of antihypertensive agent. Diabetes defined as hemoglobin A_{1c} level ≥ 6.0%. Dyslipidemia defined as total cholesterol level ≥ 220 mg/dL, high-density lipoprotein cholesterol level < 40 mg/dL, and/or triglyceride level ≥ 150 mg/dL. Odds ratios adjusted for age and sex.

Study equation (median, 52 mL/min/1.73 m^2; Q1-Q3, 39-64), and these values correlated well (r = 0.98; $P < 0.0001$). Median (Q1-Q3) JSN-eGFR values for each category of GFR estimated using the MDRD Study equation were 77 (71-83), 54 (48-56), 36 (33-39), and 18 (15-21) mL/min/1.73 m^2 for eGFR categories ≥60, 45-59, 30-44, and <30 mL/min/1.73 m^2, respectively. Sensitivity analyses using the JSN-CKDI equation to estimate GFR made a little difference in the findings. Age- and sex-adjusted mean values for coronary artery stenosis rate increased gradually with lower JSN-eGFR levels (mean, 47.3% ± 1.9% [SE], 49.4% ± 2.1%, 51.7% ± 2.0%, and 52.3% ± 2.6% for JSN-eGFRs ≥60, 45-59, 30-44, and <30 mL/min/1.73 m^2, respectively; P for trend = 0.06). Lower JSN-eGFRs were associated significantly with higher risks of advanced atherosclerosis and calcified lesions after adjusting for age and sex (P for trend = 0.04 for both). Individuals with JSN-eGFRs < 30 mL/min/1.73 m^2 were likely to have greater risks of advanced atherosclerosis (OR, 1.80; 95% CI, 0.70-4.64) and calcified lesions (OR, 3.90; 95% CI, 1.45-10.49) than individuals with JSN-eGFR ≥ 60 mL/min/1.73 m^2 after adjusting for the mentioned confounding factors.

DISCUSSION

This study showed a clear relationship between lower kidney function and severity of coronary atherosclerosis in autopsy samples from a general population. To the best of our knowledge, this is the first histopathologic study showing the gradual progression of coronary atherosclerosis, even in individuals with moderate CKD. Additionally, cardiovascular risk factors, including hypertension, diabetes, and dyslipidemia, were associated with higher risk of advanced coronary atherosclerosis and calcified lesion in individuals with CKD. These findings imply the

importance of the management of cardiovascular risk factors before reaching an advanced stage of CKD to reduce the risk of coronary atherosclerosis.

Several authors have reported the relationship between kidney function and coronary atherosclerosis in people with advanced kidney failure. Lindner et al[26] showed that ~35% of all deaths in patients receiving hemodialysis were caused by coronary heart disease, partly confirmed by autopsy. Cross-sectional studies also showed that more than half the predialytic patients without signs and history of angina or myocardial infarction have had significant coronary artery stenosis, proved by coronary angiography.[27,28] Additionally, uremic patients are more likely to have coronary atherosclerotic lesions with plaque, medial thickness, and calcification than nonuremic patients in an autopsy-based study.[14] In the present study, the prevalence of advanced coronary atherosclerotic lesions increased gradually, even in individuals with moderate stages of CKD. These results emphasize the importance of considering kidney function status before patients reach advanced CKD in trying to reduce the burden of coronary atherosclerosis in the general population.

Several potential mechanisms can explain the association shown. Individuals with CKD often have a higher burden of traditional cardiovascular risk factors, such as aging, increased blood pressure, diabetes, and dyslipidemia.[29] Additionally, decreased eGFR may be associated with increased levels of novel cardiovascular disease risk factors, such as inflammation, oxidative stress, anemia, and abnormal calcium-phosphate metabolism.[29-31] Several experimental findings from uremic apolipoprotein E knockout mice support these results.[32-35] In the present study, the significant association between decreased GFR and severity of coronary arteriosclerosis was observed even after adjustment for all major traditional cardiovascular risk factors and some novel factors, including anemia and abnormal calcium-phosphate metabolism. However, we were unable to assess sufficiently how these other potential confounding factors influenced study findings. Further exploration clearly is needed to map risk factors for coronary atherosclerosis in individuals with CKD.

Several limitations of our study should be discussed. First, this was a cross-sectional study; therefore, it was difficult to infer causality between CKD and risk of progression of coronary atherosclerosis. However, the findings suggested strongly that individuals with CKD should be examined for progressive coronary atherosclerosis. Second, it has been well recognized that GFR estimated using the MDRD Study equation leads to a certain degree of misclassification of eGFR levels. However, this limitation is unlikely to change our conclusions because sensitivity analysis using the JSN-CKDI equation to estimate GFR did not make material differences in the findings. Third, no information was available regarding the severity or duration of hypertension and other cardiovascular disease risk factors. Furthermore, we also have no data available for medication use, such as lipid-lowering agents and phosphate binders. This limitation may reduce the experimental accuracy to some extent. Finally, this study is based on autopsy and the proportion of aged people is extremely high. Thus, these findings might not be applicable to the general living population. Nevertheless, information gained in this study contributes meaningfully toward better understanding the pathogenesis of coronary atherosclerosis in individuals with CKD.

In conclusion, decreased eGFR is associated significantly with severity of coronary atherosclerosis. The findings emphasize that individuals with CKD should be considered a high-risk population for coronary heart disease, and cardiovascular risk factors should be monitored substantially in this population to prevent the progression of coronary atherosclerosis. Further studies are needed to elucidate the precise mechanism mediating the deterioration of atherosclerotic lesions in individuals with CKD.

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Functional SNP of *ARHGEF10* confers risk of atherothrombotic stroke

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Although stroke is a common cause of death and a major cause of disability all over the world, genetic components of common forms of ischemic stroke are largely unknown. To identify susceptibility genes of atherothrombotic stroke, we performed a large case–control association study and a replication study in a total of 2775 cases with atherothrombotic stroke and 2839 controls. Through the analysis in 860 cases and 860 age- and sex-matched controls, we found that a single-nucleotide polymorphism (SNP), rs2280887, in the *ARHGEF10* gene was significantly associated with atherothrombotic stroke even after the adjustment of multiple testing by a permutation test [unadjusted $P = 1.2 \times 10^{-6}$, odds ratio = 1.80, 95% confidence interval (CI) = 1.42–2.28]. This association was replicated in independent 1915 cases and 1979 controls. Subsequent fine mapping found another three SNPs which showed similar association due to strong linkage disequilibrium to rs2280887 ($r^2 > 0.95$). In the functional analyses of these four highly associated SNPs, using luciferase assay and electrophoretic mobility shift assay we found that rs4376531 affected *ARHGEF10* transcriptional activity due to the different Sp1-binding affinity. In small GTPase activity assay, we found that a gene product of *ARHGEF10* specifically activated RhoA. A population-based cohort study revealed the subjects with rs4376531 CC or CG to increase the incidence of ischemic stroke ($P = 0.033$, hazard ratio = 1.79, 95% CI = 1.05–3.04). Our data suggest that the functional SNP of *ARHGEF10* confers the susceptibility to atherothrombotic stroke.

INTRODUCTION

Stroke is a common cause of death and a major cause of disability all over the world (1). Particularly, in the countries having a larger proportion of older people, the burden has been increasing more significantly. Twin and family studies have indicated that the risk for ischemic stroke is related to multiple genetic and environmental factors (2). Identification of susceptibility genes for ischemic stroke is expected to elucidate new pathophysiological mechanisms of the disease and to lead to the development of novel preventive measures. Although previous genome-wide association studies (GWASs) reported several susceptibility genes (3,4), genetic components of common forms of ischemic stroke are still largely undetermined.

Ischemic stroke is usually classified into several subtypes. From the aspects of pathophysiological mechanisms and preventive measures, ischemic stroke can be classified into atherothrombotic stroke and cardioembolic stroke (3,5,6). Atherothrombotic stroke is mainly caused by atherosclerosis in arteries of various sizes, and the main preventive measure is the control of cardiovascular risk factors such as hypertension, diabetes, dyslipidemia and smoking. In contrast, cardioembolic stroke is mainly caused by cardiac diseases such as atrial fibrillation and valvular heart

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disease, and the main preventive measure is the use of anticoagulants.

As for the genetics of these two subtypes, Jerrard-Dunne *et al.* showed that the genetic component of atherothrombotic stroke is stronger than that of cardioembolic subtype, and suggested that the genetic study might be more efficient by focusing on atherothrombotic stroke (7). Therefore, we performed a large-scale case–control association study in a Japanese population (8,9) by focusing on atherothrombotic stroke and identified a gene encoding guanine nucleotide exchange factor 10 (*ARHGEF10*) on chromosome 8p23 as a new susceptibility gene for atherothrombotic stroke. We found that a functional single-nucleotide polymorphism (SNP) of this gene affects its transcriptional activity by altering Sp1-binding affinity. Furthermore, small GTPase activity assay showed that a gene product of *ARHGEF10* specifically activates RhoA. Since RhoA–Rho kinase pathway has an important role for the pathogenesis of cardiovascular disease and atherosclerosis, the functional SNP of *ARHGEF10* may be involved in the susceptibility to the development of ischemic stroke.

RESULTS

Case–control association study

We previously performed a two-stage association analysis using 1112 cases with ischemic stroke and 1112 age- and sex-matched controls by examining 52 608 gene-based tag-SNPs selected from the JSNP database (8,9). To identify SNPs possibly associated with atherothrombotic stroke, we further analyzed candidate SNPs by focusing on 860 cases with atherothrombotic stroke and 860 age- and sex-matched controls (Set 1). We found that an SNP rs2280887 in intron 17 of *ARHGEF10* on chromosome 8p23 was found to be strongly associated with atherothrombotic stroke ($P = 1.2 \times 10^{-6}$ for dominant model; Table 1). This association remained significant after a permutation test for the adjustment of multiple testing ($P = 0.0006$). Although this SNP revealed a weak association even when we analyzed all ischemic stroke cases, no association was observed in the case of cardioembolic stroke (Supplementary Material, Table S1).

We subsequently selected and genotyped 93 tag-SNPs across the *ARHGEF10* gene selected from phase II of HapMap JPT data. We found an SNP rs4480162 in intron 17 of *ARHGEF10* to be in absolute linkage disequilibrium (LD) with rs2280887 ($D' = 1.0$ and $r^2 = 1.0$) and be significantly associated with atherothrombotic stroke ($P = 6.9 \times 10^{-7}$ for dominant model; Table 1). LD analysis showed that these two SNPs, rs4480162 and rs2280887, were located in a small LD block (block A) spanning 15.7 kb region, which corresponds to a region from intron 15 to intron 18 of *ARHGEF10* (Supplementary Material, Fig. S1). None of the remaining 92 tag-SNPs showed significant association with the disease. We subsequently searched for variants in this 15.7 kb region by direct sequencing using 48 affected individuals. This resequencing identified a total of 81 variants, of which 50 variants were already registered in dbSNP database, and 31 variants were new. After the exclusion of the variants genotyped or with minor allele frequency (MAF) of <0.05, we genotyped 43 additional SNPs. Figure 1 shows the result of fine mapping around the candidate region of *ARHGEF10*. The association of the SNPs with the disease was limited to the block A region of *ARHGEF10*. In block A, additional two SNPs, rs35234164 and rs4376531, were found to have significant associations similar to rs2280887 (Fig. 1C and Table 1). rs4376531 was only two bases apart from rs4480162, and these SNPs were absolutely linked with rs2280887. rs35234164 was a one-base insertion/deletion (T/del) polymorphism located at intron 16 and strongly linked with other three SNPs (each pairwise $D' = 1.0$ and $r^2 = 0.95$). No other SNP in block A was associated with atherothrombotic stroke. These four SNPs were found to be associated with atherothrombotic stroke in another case–control set of 1915 cases and 1979 controls (Set 2, $P = 0.010$ for dominant model; Table 1). The results of association analyses of those SNPs under allele and recessive models are shown in Supplementary Material, Table S2.

Susceptible allele of rs4376531 affects *ARHGEF10* transcriptional activity through the difference in Sp1-binding affinity

The four SNPs were located at intron 16 or 17 and mapped ~80 kb apart from 5′-untranslated region (UTR) and 50 kb apart from 3′-UTR. None of the four SNPs was located in splice donor, acceptor or branch sites of intron 16 or 17. Furthermore, the UCSC Genome Browser database indicated no additional annotated gene or non-coding RNA in the block A region. We hypothesized that some of these SNPs might exert some effect on transcription and prepared 5′-end biotin-labeled oligonucleotide probes that were derived from the genomic sequences corresponding to these SNPs. Although rs4480162 and rs4376531 were absolutely linked and only haplotypes of C-G and G-C were existed in our population, we also synthesized rs4480162_C/rs4376531_C and rs4480162_G/rs4376531_G probes to elucidate the function of each SNP. Electrophoretic mobility shift assay (EMSA) experiments using these oligonucleotides with nuclear extract of ·LoVo cells, in which the expression of *ARHGEF10* gene transcript is high, found a shifted band of a DNA–protein complex with a strong intensity in lanes corresponding to the susceptible allele of rs4376531 (C-G and G-G, Fig. 2A). This shifted band was weak in lanes corresponding to the non-susceptible allele (C-C and G-C). Although we also observed shifted bands for other oligonucleotides, no difference in the intensity between susceptible and non-susceptible alleles was observed. The competition assay with the unlabeled oligonucleotides demonstrated that the oligonucleotides containing the susceptibility allele of rs4376531 (C-G and G-G) inhibited the formation of DNA–protein complex in a dose-dependent manner but the other oligonucleotides (C-C and G-C) did not (Fig. 2B), suggesting that some nuclear proteins specifically bound to the DNA fragment corresponding to the susceptible allele of rs4376531. To identify which transcriptional factor binds to this susceptible allele, we added excess amount of unlabeled oligonucleotides corresponding to consensus sequences of various transcriptional factors as competitor and found that the unlabeled Sp1-binding consensus oligonucleotide effectively inhibited the formation of the DNA–protein complex (Fig. 2C).

Table 1. Association results among the four SNPs in *ARHGEF10* for atherothrombotic stroke

SNP allele (1/2)	Set	Case				Control				Dominant model			Additive model		
		11	12	22	Total	11	12	22	Total	*P*-value	OR	95% CI	*P*-value	OR	95% CI
rs2280887 (G/C)	Set 1	9	211	638	858	15	123	719	857	1.2×10^{-6}	1.80	1.42–2.28	5.2×10^{-5}	1.56	1.26–1.94
	Set 2	24	389	1501	1914	23	341	1615	1979	0.013	1.22	1.04–1.43	0.020	1.19	1.03–1.37
	Combined									2.1×10^{-6}	1.38	1.21–1.57	2.8×10^{-5}	1.29	1.15–1.46
rs35234164 (-/T)	Set 1	8	206	642	856	14	116	729	859	3.4×10^{-7}	1.87	1.47–2.38	1.9×10^{-5}	1.62	1.30–2.02
	Set 2	22	377	1507	1906	23	326	1627	1976	0.010	1.23	1.05–1.45	0.019	1.19	1.03–1.38
	Combined									7.0×10^{-7}	1.40	1.23–1.60	1.5×10^{-5}	1.31	1.16–1.48
rs4480162 (C/G)	Set 1	9	212	636	857	15	123	722	860	6.9×10^{-7}	1.82	1.43–2.31	3.4×10^{-5}	1.58	1.27–1.96
	Set 2	23	390	1502	1915	23	342	1613	1978	0.015	1.22	1.04–1.42	0.024	1.18	1.02–1.36
	Combined									2.0×10^{-6}	1.38	1.21–1.57	3.1×10^{-5}	1.29	1.14–1.46
rs4376531 (G/C)	Set 1	9	212	636	857	15	123	722	860	6.9×10^{-7}	1.82	1.43–2.31	3.4×10^{-5}	1.58	1.27–1.96
	Set 2	23	390	1499	1912	23	344	1610	1977	0.018	1.21	1.03–1.41	0.028	1.18	1.02–1.36
	Combined									2.6×10^{-6}	1.37	1.20–1.56	3.8×10^{-5}	1.29	1.14–1.45

For additive genetic model, we used logistic regression analysis coding genotypes 11, 12 and 22 as 2, 1 and 0, respectively. OR, odds ratio; CI, confidence interval.

Moreover, when we added anti-Sp1 antibody to the mixture, the band was further shifted to a higher molecular position, indicating the specific binding of the Sp1 protein to the susceptible allele of rs4376531. Similar shifted band was observed when we used rs4480162_G/rs4376531_G probe (data not shown).

To test whether rs4376531 affects the *ARHGEF10* transcriptional activity, we performed a luciferase assay using LoVo cells. We subcloned the sequences corresponding C-G and G-C EMSA probes into the pGL3-promoter vector. Luciferase activity was enhanced in the cells transfected with the reporter vector containing the susceptible allele of rs4376531 but the enhancement was low in the cells transfected with the vector containing the non-susceptible allele (Fig. 2D). These findings indicated that rs4376531 might affect *ARHGEF10* transcriptional activity through the difference in the binding affinity of Sp1 transcriptional factor.

Although the function of ARHGEF10 is not well understood, ARHGEF10 is a member of the family of guanine nucleotide exchange factors (GEFs), which regulate the activity of small Rho GTPases by catalyzing the exchange of bound GDP by GTP. To elucidate the role of ARHGEF10 in the pathogenesis of atherothrombotic stroke, we examined the effect of ARHGEF10 on the activation of RhoA, Rac1 and Cdc42 by small GTPase activity assay. As shown in Figure 3, overexpression of ARHGEF10 led to an increase in the GTP-bounded RhoA, indicating that ARHGEF10 might activate RhoA. In contrast, overexpression of ARHGEF10 had no effect on the GTP-bounded Rac1 or Cdc42. Since Sp1 is abundantly expressed in multiple tissues, the subjects with the disease-susceptible allele of rs4376531 are expected to have higher expression of *ARHGEF10* transcripts and might result in the higher activity of RhoA–Rho kinase pathway.

rs4376531 increases the incidence of ischemic stroke

Finally, we examined the effect of rs4376531 on the incidence of ischemic stroke using a population-based cohort study. During a 14-year follow-up of the cohort, 67 events of first-ever ischemic stroke were observed among 1656 subjects without a history of stroke at baseline examination. Figure 4 shows Kaplan–Meier estimates of the incidence of ischemic stroke by rs4376531. The cumulative incidence was 6.1% in the subjects who had at least one susceptible allele and 3.6% in the subjects with the homozygous of non-susceptible allele ($P = 0.042$ for log-rank test). Age- and sex-adjusted risk of atherothrombotic stroke was significantly higher in the subjects with susceptible allele of rs4376531 (adjusted $P = 0.033$, hazard ratio = 1.79, 95% confidence interval = 1.05–3.04).

DISCUSSION

In this study, we analyzed the data of a large-scale case–control association study by focusing on atherothrombotic stroke. We found a new candidate locus, rs2280887, located in intron 17 of *ARHGEF10*. This SNP was significantly associated with atherothrombotic stroke even after the adjustment of multiple testing, and the association was replicated in other case–control samples. Fine mapping of the *ARHGFE10* gene identified four highly linked SNPs (rs2280887, rs35234164, rs4480162 and rs4376531) as candidates with functional significance. Functional analysis of these four SNPs demonstrated that an SNP, rs4376531, altered the binding affinity of the Sp1 transcriptional factor and might enhance the *ARHGEF10* transcriptional activity in individuals with the susceptible allele. We also found that ARHGEF10 specifically activated RhoA, which has an important role in various process of atherosclerosis. From these findings, we suggest that the subjects with the susceptible allele of rs4376531 in *ARHGEF10* will have higher expression of transcript and might have higher RhoA activity. Since RhoA–Rho kinase pathway is involved in the pathogenesis of atherosclerosis, the functional SNP of *ARHGEF10* might confer the development of atherothrombotic stroke. A population-based cohort study supported this hypothesis.

Although several GWASs for ischemic stroke have been reported (3,4), they did not detect the association of SNPs in *ARHGEF10* with ischemic stroke. Among the four highly associated SNPs in this study, rs4480162 and rs4376531 were registered in the HapMap database. From the database,

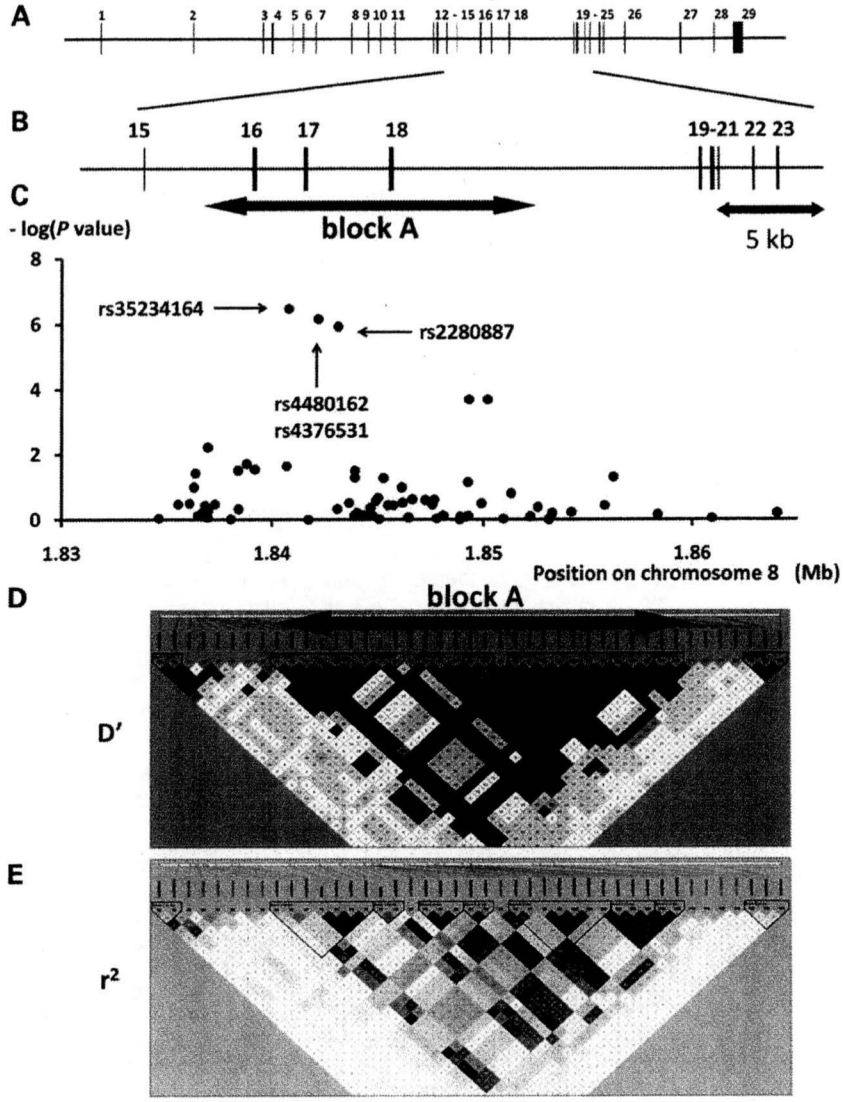

Figure 1. Exon–intron structure, case–control results and LD map in *ARHGEF10*. (**A**) All of the exon–intron structure of *ARHGEF10*. (**B**) Exon–intron structure around the marker SNP, rs2280887. (**C**) Case–control association study results. The $-\log_{10}$-transformed *P*-values for a dominant model are plotted on the *y*-axis. (**D**) Pairwise LD map between SNPs, as measured by *D'*. Black represents regions of high pairwise *D'*, and white represents regions of low pairwise *D'*. (**E**) Pairwise LD map between SNPs, as measured by r^2. Black represents regions of high pairwise r^2, and white represents regions of low pairwise r^2.

MAF of rs4480162 was 0.167 for CEU, 0.475 for YRI, 0.078 for CHB and 0.091 for JPT. Our sequence data showed that rs4376531 is absolutely linked with rs4480162 in the Japanese population; however, MAF of rs4376531 was 0 for CEU, 0.021 for YRI, 0 for CHB and 0 for JPT. These data speculate that the LD between causative variant (rs4376531) and other three SNPs might be different among different populations if the genotype data of CEU and YRI are correct. Moreover, our large-scale association study included rs2280887 as one of the 52 608 gene-based tag-SNPs selected from JSNP database. However, the current GWAS platforms do not contain all of the four SNPs even in Affymetrix Genome-Wide Human SNP Array 6.0 or Illumina Human1M-Duo BeadChip. Therefore, current GWASs using commercial chips cannot detect the association of SNPs in *ARHGEF10* and ischemic stroke.

GEFs activate small GTPases in response to diverse extracellular stimuli and ultimately regulate numerous cellular responses (10). Small GTPases, which were identified as the master regulators of the actin cytoskeleton, control a

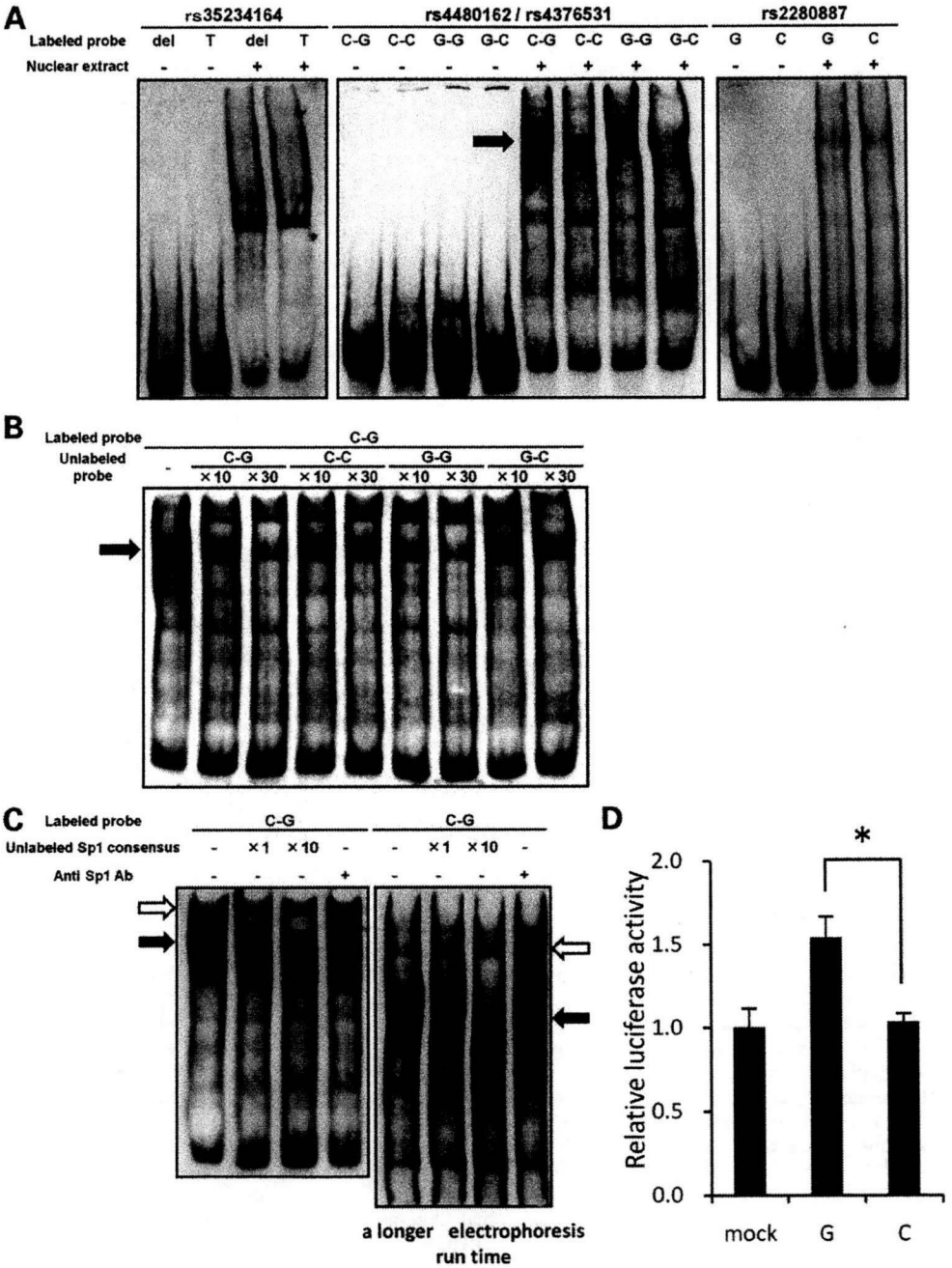

Figure 2. rs4376531 alters the binding affinity of Sp1 and affects *ARHGEF10* transcriptional activity. (**A**) EMSA using 5′ end-labeled 50 bp probes around each allele of SNPs in *ARHGEF10*. A black arrow indicates the shifted band that shows tighter binding of a nuclear protein to the susceptible allele of rs4376531(C-G and G-G) than the non-susceptible allele (G-C and C-C). (**B**) Competition assay with unlabeled self- or non-self-oligonucleotides. DNA–protein complex (black arrow) was more effectively competed by unlabeled oligonucleotides with G-allele of rs4376531 than those with C-allele. (**C**) Competition assay with unlabeled Sp1-binding consensus oligonucleotide and supershift assay using anti-Sp1 antibody. We could observe additional shifted band more clearly (white arrow) by a longer electrophoresis run time. (**D**) Luciferase assay. Fifty base pair fragments around each allele of rs4376531 were inserted into pGL3-prmoter vector. Each sample was studied in triplicate and data were shown as mean ± SD. Asterisk indicates $P < 0.05$ by Student's *t*-test.

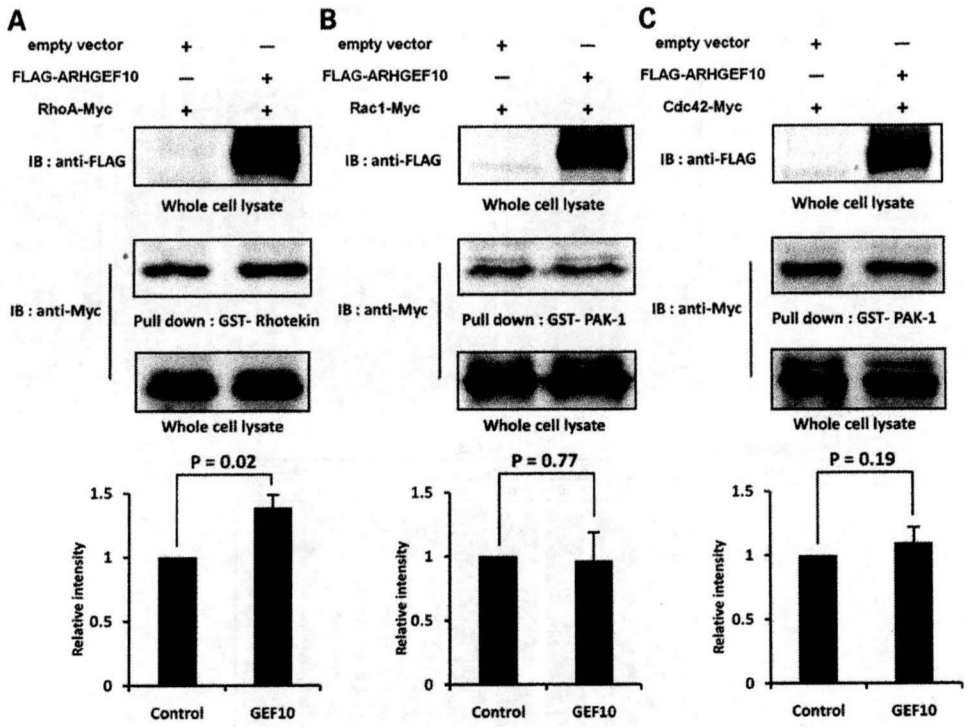

Figure 3. ARHGEF10 activates RhoA specifically. (**A**) RhoA activity assay. 293FT cells were co-transfected with the plasmids as indicated at the top. Cell lysates were pulled down by GST-Rhotekin and subjected to immunoblot with anti-Myc antibody. Whole-cell lysates were analyzed by immunoblot with anti-Myc and anti-FLAG antibody to detect total RhoA and ARHGEF10, respectively. GTP-bound RhoA and total RhoA were quantified by the intensity of the bands using Multi Gauge software of an LAS-3000 system (bottom). RhoA activity was calculated as the relative ratio of the intensity of GTP-bound RhoA against that of total RhoA. Relative intensity in the control (transfected empty vector) was expressed as 1 arbitrary unit. (**B**) Rac1 activity assay. (**C**) Cdc42 activity assay. (B) and (C) were analyzed as in (A) by pull-down assay using GST-PAK-1 instead of GST-Rhotekin. The experiments were repeated at least three times.

remarkable diversity of cell functions including contraction, motility, proliferation and apoptosis. Small GTPases act as molecular switches, which cycle between an inactive GDP-bound form and an active GTP-bound form (11). The Rho GEFs mediate the activation of small GTPases by promoting the release of GDP in exchange for GTP (12). ARHGEF10, a member of Rho GEFs, was identified by the sequencing of cDNA clones from the human brain (13) and found to be expressed not only in the brain but also in various tissues including the heart (14). A point mutation (T109I) of ARHGEF10 was reported to co-segregate in the family with slowed motor and sensory nerve conduction velocities of peripheral nerves with autosomal dominant inheritance (15). However, the function of ARHGEF10 is largely unknown. We found that ARHGEF10 specifically activated RhoA and might contribute to the development of atherothrombotic stroke through the regulation of RhoA–Rho kinase activity. Recent linkage studies have demonstrated that several Rho GEFs might be involved in the pathogenesis of atherosclerosis. A linkage scan for type 2 diabetes has identified that non-synonymous SNPs in LARG and PDZ-Rho GEF are associated with insulin sensitivity or insulin resistance (16,17). Another linkage study has found kalirin gene as a candidate gene for early-onset coronary artery disease (18). These results suggest that polymorphisms of Rho GEFs affect small GTPase signaling pathway and result in the pathogenesis of human atherosclerosis.

RhoA, which is the most characterized small GTPases (19), and one of its effectors, Rho-kinase, were reported to play an important role in the various processes of atherosclerosis including endothelial dysfunction, inflammation and vascular smooth muscle cell proliferation (20–24). From these aspects, drugs that inhibit RhoA–Rho kinase pathway such as Rho-kinase inhibitor (20) or statin (24) are already available in clinical setting. If our findings are confirmed in further studies, we could expect to use RhoA–Rho kinase pathway inhibitors in the subjects with susceptible allele of *ARHGEF10* SNP for the more effective prevention of atherothrombotic stroke.

In conclusion, rs4376531 located in intron 17 of *ARHGEF10* was significantly associated with atherothrombotic stroke. Individuals with the susceptible allele of the SNP might have a higher level of *ARHGEF10* transcript due to the higher binding-affinity of Sp1 and might have higher RhoA–Rho kinase activity. This higher activity will finally result in the increased incidence of atherothrombotic stroke

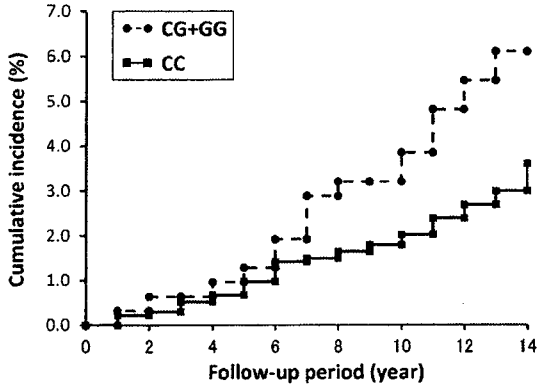

Figure 4. Kaplan–Meier estimates of the incidence of ischemic stroke by rs4376531 during 14-year follow-up period in the Hisayama study.

in the general population. Our findings might shed light on the elucidation of new atherosclerotic pathogenesis and to the development of preventive therapy for ischemic stroke.

MATERIALS AND METHODS

The flow chart of this study is shown in Supplementary Material, Figure S2.

Study populations

For the large-scale case–control association study, cases with ischemic stroke were registered from seven medical centers in and around Fukuoka City, Japan, in 2004. Details of registration were described previously (8). Briefly, all case subjects were diagnosed by stroke neurologists on the basis of detailed clinical features and ancillary laboratory examinations [such as brain imaging including computed tomography (CT) and magnetic resonance imaging (MRI), cerebral angiography, echocardiography and carotid duplex imaging]. Ischemic stroke was defined as a sudden onset of non-convulsive and focal neurological deficit persisting for >24 h without evidence of hemorrhagic stroke on brain imaging (CT or MRI). Ischemic stroke was further subdivided into atherothrombotic stroke, cardioembolic stroke and undetermined subtype. Subtypes of ischemic stroke were determined on the basis of the Classification of Cerebrovascular Disease III proposed by the National Institute of Neurological Disorders and Stroke (25), as well as on the basis of the diagnostic criteria of the Trial of Org 10172 in Acute Stroke Treatment (TOAST) study (26) and Cerebral Embolism Task Force (27). Details of the diagnostic criteria of ischemic stroke subtypes have been described previously (8). Briefly, small-artery occlusion (lacunar stroke) was diagnosed as the presence of a relevant brain stem or subcortical hemispheric lesion with a diameter of <1.5 cm demonstrated on brain imaging and no evidence of cerebral cortical or cerebellar impairment. Large artery atherosclerotic stroke was diagnosed when the subjects had significant stenosis (>50%) or occlusion of a major cerebral artery with infarct size ≥1.5 cm on brain imaging. The diagnosis of cardioembolic stroke was made on the basis of primary and secondary clinical features suggestive of cardioembolic stroke as reported by the Cerebral Embolism Task Force (27). The category of undetermined subtype included all ischemic stroke cases for which the subtype could not be determined because of insufficient clinical or morphological information. Both small-artery occlusion and large artery atherosclerotic stroke were included in the phenotype of atherothrombotic stroke. Subtypes of ischemic stroke were 860 in atherothrombotic, 136 in cardioembolic and 116 in undetermined subtype. Age- (within 5 years) and sex-matched control subjects were selected from the 3328 participants of the Hisayama screening survey between 2002 and 2003.

For the replication study, case samples were selected from the BioBank Japan project (28). Among the subjects with ischemic stroke in the BioBank Japan, we selected 1915 cases that were diagnosed as atherothrombotic stroke by brain imaging, same as the initial study. The remaining 1979 Hisayama participants who were not enrolled in the initial study were used as controls. Clinical characteristics of the study population in the two case–control sets were shown in Supplementary Material, Table S3.

For the prospective cohort study, we used a cohort population of the Hisayama study established in 1988 (8). In this cohort, 2637 Hisayama residents aged ≥40 years without a history of stroke or coronary heart disease were enrolled in 1988 and continuously followed up for 14 years until the occurrence of cardiovascular diseases or death. Among them, 1656 subjects participated in the examination between 2002 and 2003 were used in the present study.

Genomic DNA was extracted from peripheral blood leukocytes by a standard method in both populations. Written informed consent was obtained from all study subjects in both populations, and this study was approved by the ethics committees of the Graduate School of Medical Sciences, Kyushu University, and Yokohama Institute, RIKEN.

SNP selection and genotyping

In the previous large-scale case–control association study, we used a two-stage approach to identify susceptibility genes of ischemic stroke. We first genotyped 52 608 gene-based tag-SNPs selected from JSNP database using 188 cases and 188 age- and sex-matched controls. In the second stage, 1098 SNPs that showed $P < 0.01$ in the first stage were genotyped in the remaining samples. Details of this large-scale association study were described previously (8). For this study, we combined the data of the first and the second stage and re-analyzed by focusing on atherothrombotic stroke using the matched case–control samples. All SNPs in the large-scale association study were genotyped using the multiplex PCR-based Invader assay (Third Wave Technologies) described previously (29). All genotypes were called by visual inspection, and we determined genotyped success as less than 10 undetermined samples in a 384-well-plate.

For fine mapping across *ARHGEF10*, we selected tag-SNPs from phase II of the HapMap JPT data by pairwise tagging method with the following criteria: $r^2 > 0.8$, MAF > 5% and call rate >75%. We genotyped SNPs using the multiplex PCR-based Invader assay or by direct sequencing of PCR

products using ABI3700 capillary sequencers (Applied Biosystems) according to standard protocols.

Cell culture

Human colon cancer LoVo cells were grown in F12-HAM (Invitrogen) with 10% fetal bovine serum (FBS). Human embryonic kidney fibroblasts 293FT cells were grown in Dulbecco's modified Eagle's medium (Invitrogen) with 10% FBS. These cells were incubated in a humidified atmosphere with 5% CO_2 at 37°C.

Electrophoretic mobility shift assay

5′-biotin-labeled single-strand oligonucleotides were obtained from Invitrogen and annealed. The sequence of EMSA probe rs35234164_del is 5′-CAGTGAAGTAAAATATGGCCTAC C_TTAAGAAGTTAAGATAGTCATTTAA-3′; rs35234164_T: 5′-CAGTGAAGTAAAATATGGCCTACCTTTAAGAAGTT AAGATAGTCATTTAA-3′; rs4480162_C/rs4376531_G: 5′-AGTCGGACTCCTTAGTGTGAACTCCAGATCCACCTTC TCTGAACTCTGAA-3′; rs4480162_C/rs4376531_C: 5′-AGT CGGACTCCTTAGTGTGAACTCCACATCCACCTTCTCT GAACTCTGAA-3′; rs4480162_G/rs4376531_C: 5′-AGTCG GACTCCTTAGTGTGAACTGCACATCCACCTTCTCTGA ACTCTGAA-3′; rs4480162_G/rs4376531_G: 5′-AGTCGGA CTCCTTAGTGTGAACTGCAGATCCACCTTCTCTGAAC TCTGAA-3′; rs2280887_G: 5′-CTTGACTCTTGGGCAGTT TTAAGTAGGTTTAAAATTCTCCCGCTGCCAGA-3′; rs22 80887_C: 5′-CTTGACTCTTGGGCAGTTTTAAGTACGTT TAAAATTCTCCCGCTGCCAGA-3′ and Sp1 consensus is 5′-ATTCGATCGGGGCGGGGCGAGC-3′. EMSA probe of 20 fmol was incubated with 10 μg nuclear proteins for 30 min at room temperature in binding buffer (5 mM HEPES, pH 7.9, 0.05 mM EDTA, 0.5 μg poly(dI/dC), 50 mM KCl, 1 mM dithiothreitol and 10% glycerol). For the competition assay, each 1–30-fold molar excess of unlabeled probes was added and incubated for another 15 min at room temperature. For the supershift assay, 2 μg of rabbit polyclonal anti-human Sp1 antibody (sc-59X, Santa Cruz) was added and incubated for another 60 min on ice. The mixture was subjected to electrophoresis on a 4% polyacrylamide gel in 0.5× Tris–Borate–EDTA buffer at 4°C. Nucleic acids were transferred to a nylon membrane (Hybond-*N*+; Amersham Biosciences) at 100 V for 60 min. Biotin-labeled probes were detected using Chemiluminescent Nucleic Acid Detection Module (PIERCE, 89880) and analyzed with an LAS-3000 system (Fuji Film, Tokyo, Japan).

Luciferase reporter assay

The same DNA sequences around rs4480162 and rs4376531 as the EMSA probes were subcloned into pGL3-promoter luciferase vector (Promega). We transfected LoVo cells with 500 ng of each reporter construct and 50 ng of pRL-CMV vector (Promega) using FuGENE 6 Transfection Reagent (Roche). After 48 h, we collected the cells and measured luciferase activities using Dual Luciferase Assay System (Toyo B-Net).

Small GTPase activity assay

A plasmid designed to express full-length ARHGEF10 was obtained by cloning full-length human *ARHGEF10* cDNA into p3XFLAG-CMV-10 expression vector (SIGMA). We constructed three small GTPases overexpression plasmids by cloning full-length human RhoA, Rac1 or Cdc42 cDNA into pcDNA3.1/myc-His expression vector (Invitrogen). The cellular levels of GTP-loaded RhoA, Rac1 and Cdc42 were determined using GST fusion proteins containing the RhoGTPase-binding domain of Rhotekin (GST-RBD) (14-383, Upstate) or PAK-1 (GST-PBD) (14-325, Upstate) as described previously (30,31). In brief, pcDNA3.1-RhoA-Myc or pcDNA3.1-Rac1-Myc or pcDNA3.1-Cdc42-Myc was co-transfected into 293FT cells seeded in 10 cm dishes with p3XFLAG-CMV-10-ARHGEF10 or the corresponding empty vector using FuGENE 6 Transfection Reagent (Roche). After being cultured for 48 h, the cells were lysed in a buffer containing 25 mM HEPES, pH7.5, 150 mM NaCl, 1% Igepal CA-630, 10 mM $MgCl_2$, 1 mM EDTA, 10% glycerol and protease inhibitors, and the particular fraction was pelleted by centrifugation. The GTPase-containing supernatant was then incubated for 45–60 min at 4°C with GST fusion proteins bound to glutathione–Sepharose beads. After three times washing of the beads, bound proteins were eluted with sample buffer and separated by SDS–PAGE. ARHGEF10 and the small GTPases were then detected by immunoblotting with commercially available specific anti-FLAG antibody (F3165, SIGMA, 1 μg/ml) and anti-Myc antibody (562, MBL, 1 μl/ml), respectively. Proteins reacting with primary antibodies were visualized by an enhanced chemiluminescence system (GE Healthcare UK Ltd, Amersham) for detecting species-matched secondary antibodies and analyzed with an LAS-3000 system. Quantitative analyses of immunoblots were performed using Multi Gauge version 2.02 software included in an LAS-3000 system.

Statistical analysis

We assessed case–control association analysis by χ^2 test and Fisher's exact test, as appropriate. The shift of Hardy–Weinberg equilibrium was also tested by χ^2 test or Fisher's exact test. In the association analyses, we used allele, dominant and recessive models. All statistical analyses were performed without the adjustment of age and sex. Meta-analyses of the two case–control sample sets were performed using Mantel–Haenszel method (fixed effect analysis). Heterogeneities across the population were assessed using Cochran's Q test. For the adjustment of multiple testing in the discovery phase, we performed a random permutation test with 10 000 replications using MULTTEST procedure of SAS software version 9.12 (SAS Institute). LD were calculated as D' or r^2, and haplotype blocks were defined by Gabriel's criteria (32) using Haploview version 4.0 (Broad Institute). Luciferase assay data and small GTPase activity assay data were analyzed by Student's t-test.

SUPPLEMENTARY MATERIAL

Supplementary Material is available at *HMG* online.

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Conflict of Interest statement. None declared.

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分担研究報告書

メタボリックシンドロームと糖尿病発症リスク-舟形研究(Funagata study)- -

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研究要旨

メタボリックシンドローム(MetS)が、将来の糖尿病発症を予測する因子として、空腹時血糖値(FPG)に付加的な価値を持つかどうかは、少なくとも、日本人では、定説がない。そこで、舟形コホートを用いて、MetS、及び、FPG と糖尿病発症との関連を調べた。第2コホート(1995-1997 年受検者)の正常耐糖能(NGT)を対象に5年後の糖尿病発症をエンドポイントとした(n=779)。MetS の診断は IDF の基準を用いた(3種類のウエスト(Wc)基準を用いた(IDF の日本人基準(IDFWc: 男／女, 90/80 cm): 現在の日本人の基準(現行日本人 Wc: 85/90 cm);既報の日本人の代替基準(代替日本人 Wc: 85/80 cm)))。FPG が 100 mg/dl 以上を FPG 高値とした。人年法を用いて解析。MetS は有意な糖尿病発症のリスクであったが、FPG 高値に比して、どの Wc 基準を用いても、得意度は高いが、感度は低く、偽陽性率は高かった。FPG 高値は、将来の糖尿病発症を予測する因子としては、MetS の診断より有用と思われた。

A. 研究目的

メタボリックシンドローム(MetS)は大血管障害発症のリスクとして、提唱されてきた概念である。本邦の MetS の診断基準は、内蔵脂肪の蓄積(ウエスト周囲径(Wc)を基準)が必須項目であり、その他の集積が問題となる(非必須項目)メタボリック因子として、血圧、脂質異常(中性脂肪、あるいは、HDLc)、及び、空腹時血糖値(FPG)が含まれる。それでは、MetS の診断は、糖尿病発症リスクとして確立している空腹時血糖値と比較して、何らかの付加的価値を持つのであろうか？私達は、この疑問に答えるため、私達のコホート(舟形コホート)を用いて、MetS、及び、FPG と糖尿病発症との関連を調べた。

B. 研究方法

1979 年より山形県舟形町の35歳以上の住民を対象に行っている舟形研究では、1990 年から参加者全員に糖負荷試験を行い、確実な耐糖能の評価を行いコホートとして追跡調査を行っている。第2コホート(1995-1997 年受検者)の大血管障害の既往の無い正常耐糖能(NGT)を対象に5年後の糖尿病発症をエンドポイントとして追跡調査を行った(n=779)。

正常耐の評価は 1999 年の WHO 基準を用い、FPG が 100 mg/dl 以上を FPG 高値とした。MetS の診断は IDF の基準を用いたが、Wc については、3種類(IDF の日本人基準(IDFWc: 男／女, 90/80 cm): 本邦の現在の日本人の基準(現行日本人 Wc: 85/90 cm);既報の日本人の代替基準(代替日本人 Wc: 85/80 cm))を用いた。各群の糖尿病発症状況の違いについては、人年法を用い比較した。

C. 研究結果

有病率:ベースラインでの、MetS の有病率は、Wc 基準、IDF Wc、現行日本人 Wc、代替日本人 Wc、別に、5.1, 3.5, 6.4% であった。FPG 高値