pressure on the risks of cerebral infarction and cerebral haemorrhage [7,8]. However, few observational studies have examined the association between blood pressure and the risks of cerebral infarction subtypes [6,33]. Our study confirmed the results from previous observational studies and provided more detailed information about the strong association of blood pressure levels with the risks of stroke subtypes in a general population of Japanese. This finding is directly in line with beneficial effects of blood pressure-lowering treatment for most of the stroke subtypes observed in randomized controlled trials [34–37].

In our study, despite the significant associations between blood pressure categories and the incidence of most stroke subtypes, the magnitude and patterns of the impact of blood pressure categories were different among stroke subtypes. The incidence of lacunar infarction in men and women and that of cerebral haemorrhage in men continuously increased with rising blood pressure categories, and the differences were significant between optimal blood pressure and grades 1–3 hypertension, whereas the incidence of atherothrombotic infarction in both sexes and that of cardioembolic infarction and subarachnoid haemorrhage in women significantly increased in grade 3 hypertension. Cerebral haemorrhage and lacunar infarction occur primarily in conjunction with arteriolosclerosis of the cerebral penetrating arteries. These arteries are tiny and mostly arise from larger arteries as unbranching end arteries, and are considered to be directly influenced by blood pressure [38]. In contrast, atherosclerotic diseases of cervical or intracranial large arteries, including atherothrombotic infarction and possibly subarachnoid haemorrhage, generally progress as part of a slow pathoanatomic process that may take a long time to reach a clinical end stage [39], and therefore only severe hypertension may have been able to accelerate the atherosclerotic process in our patients. The weak association between blood pressure and cardioembolic infarction may be due to the fact that hypertension indirectly influences the onset of cardioembolic infarction through the development of embolic sources such as atrial fibrillation and myocardial infarction.

There are several potential limitations to the findings in our study. First, it is possible that our results are biased, because some patients did not return for the follow-up examinations. However, more than 80% of the total number of surviving stroke-free patients participated in each examination, suggesting that such a bias did not invalidate the present findings. Second, we were unable to ascertain all risk factors, TOD and cardiovascular disease for the risk stratification of patients; for example, a family history of premature cardiovascular disease, subclinical atherosclerosis and low estimated glomerular filtration rate were difficult to identify. This limitation was likely to contribute to an underestimation of the stroke risk associated with risk groups, and our estimates for the impact of risk groups on the risk of stroke are probably quite conservative. Finally, cardiovascular risk factors and the risks of stroke and its subtypes have changed in Japan during the long-term follow-up period. However, we used the pooling of repeated-observations method, in which risk factors were allowed to change in accordance with data from the follow-up examinations, and therefore this bias is not likely to invalidate the present findings.

In conclusion, the findings of the present study clearly indicate that the blood pressure classification and risk stratifications recommended by the JSH 2009 guidelines [3] are useful in predicting the risk of stroke among Japanese. Though the magnitude and pattern of the impact of blood pressure were different among stroke subtypes, blood pressure levels were associated with the incidence of most stroke subtypes, suggesting that blood pressure lowering is likely to provide protection against a variety of stroke subtypes.

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ORIGINAL ARTICLE

Development and validation of a cardiovascular risk prediction model for Japanese: the Hisayama study

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The objective of this paper is to develop a new risk prediction model of cardiovascular disease and to validate its performance in a general population of Japanese. The Hisayama study is a population-based prospective cohort study. A total of 2634 participants aged 40 years or older were followed up for 14 years for incident cardiovascular disease (stroke and coronary heart disease (myocardial infarction, coronary revascularization and sudden cardiac death)). We used data among a random two-thirds (the derivation cohort, n=1756) to develop a new risk prediction model that was then tested to compare observed and predicted outcomes in the remaining one-third (the validation cohort, n=878). A multivariable cardiovascular risk prediction model was developed that incorporated age, sex, systolic blood pressure, diabetes, low-density lipoprotein cholesterol, high-density lipoprotein cholesterol and smoking. We assessed the performance of the model for predicting individual cardiovascular event among the validation cohort. The risk prediction model demonstrated good discrimination (c-statistic=0.81; 95% confidence interval, 0.77 to 0.86) and calibration (Hosmer–Lemeshow χ^2-statistic=6.46; P=0.60). A simple risk score sheet based on the cardiovascular risk prediction model was also presented. We developed and validated a new cardiovascular risk prediction model in a general population of Japanese. The risk prediction model would provide a useful guide to estimate absolute risk of cardiovascular disease and to treat individual risk factors.
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Keywords: cardiovascular disease; epidemiology; risk factors; risk prediction model

INTRODUCTION

Cardiovascular disease is estimated to be one of the leading causes of death in Japan, as well as other countries around the world, placing a burden on the community.[1] Although the incidence and mortality of cardiovascular disease in Japan have declined over several decades, the risk of cardiovascular events remains high.[2] Additional protection will require an effective strategy for prevention of cardiovascular disease. Among a number of cardiovascular prevention strategies, high-risk approaches are likely to be one of the most effective strategies for prevention of cardiovascular disease.[3] To identify individuals at high risk of cardiovascular disease, a number of risk prediction tools have been developed.[4–15] However, currently available risk prediction tools of cardiovascular disease are derived mainly from studies carried out in Western populations and few risk prediction tools are developed for general Japanese populations. The objective of this paper is to develop a new cardiovascular risk prediction model and to validate its performance in a general population of Japanese.

METHODS

Study design and participants

Since 1961, we have been conducting a long-term prospective cohort study of cardiovascular disease in the town of Hisayama, a suburb of Fukuoka City in Southern Japan.[2,16,17] In 1988, a screening survey for this study was performed in the town. A total of 2742 residents aged 40 years or older (80.9% of the total population of this age group) consented to participate in the examination.[2,18–21] After the exclusion of 106 subjects with a history of cardiovascular disease and two subjects who died during the examination, the remaining 2634 individuals were enrolled in this study.

The ethics committee of Kyushu University approved this study, participants provided written informed consent, and the procedures followed were in accordance with national guidelines.

Follow-up survey

The subjects were followed up prospectively from December 1988 to November 2002 by repeated health examinations. A detailed description of the study methods has been published previously.[2,18–21] In brief, the health status of any subject who had not undergone a regular examination or who had moved out

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of town was checked yearly by mail or telephone. We also established a daily monitoring system among the study team and local physicians or members of the town's Health and Welfare Office. When a subject died, an autopsy was performed at the Departments of Pathology of Kyushu University. During the follow-up period, 577 subjects died, of whom 438 (75.9%) underwent autopsy. Only one participant was lost to follow-up.

Outcomes

The primary outcome of the present analysis was cardiovascular disease. Cardiovascular disease was defined as first-ever development of coronary heart disease or stroke. The criteria for a diagnosis of coronary heart disease included first-ever acute myocardial infarction, silent myocardial infarction, sudden cardiac death within 1 h after the onset of acute illness, or coronary artery disease followed by coronary artery bypass surgery or angioplasty.[2] Acute myocardial infarction was diagnosed when a subject met at least two of the following criteria: (1) typical symptoms, including prolonged severe anterior chest pain; (2) abnormal cardiac enzymes more than twice the upper limit of the normal range; (3) evolving diagnostic electrocardiographic changes; and (4) morphological changes, including local asynergy of cardiac wall motion on echocardiography, persistent perfusion defect on cardiac scintigraphy, or myocardial necrosis or scars >1 cm long accompanied by coronary atherosclerosis at autopsy. Silent myocardial infarction was defined as myocardial scarring without any historical indication of clinical symptoms or abnormal cardiac enzyme changes, and was detected by electrocardiography, echocardiography, cardiac scintigraphy or autopsy. Stroke was defined as a sudden onset of nonconvulsive and focal neurological deficit persisting for >24 h. The diagnosis of stroke and the determination of its pathological type were based on the clinical history, neurological examination and all available clinical data, including brain CT/MRI and autopsy findings.[2]

Risk factors

Sitting blood pressure was measured three times at the right upper arm using a sphygmomanometer after 5 min of rest; an average of three measurements was used for the analysis. Plasma glucose levels were determined by the glucose-oxidase method, and diabetes was defined by a 75 g oral glucose tolerance test and by fasting ($\geqslant 7.0\,mmol\,l^{-1}$) or postprandial ($\geqslant 11.1\,mmol\,l^{-1}$) blood glucose levels or by the use of hypoglycemic agents. Total cholesterol, high-density lipoprotein cholesterol and triglyceride levels were determined enzymatically. Low-density lipoprotein (LDL) cholesterol level was estimated using the Friedewald formula.[22] Information on smoking habits was obtained using a standard questionnaire and was classified as either current or not.

Statistical analysis

Two-thirds of the study participants (n=1756) were randomly assigned to a risk prediction model derivation cohort and the remaining one-third (n=878) were reserved as an independent validation cohort using random digits generated by the Mersenne Twister method.[23] Among subjects allocated to the derivation cohort, a new risk prediction model was developed using Cox's proportional hazards model. Covariates included in Cox's proportional hazards model were age, sex, systolic blood pressure, diabetes, LDL cholesterol, high-density lipoprotein cholesterol and smoking habits that were traditional risk factors for cardiovascular disease established in the Hisayama study.[16,17,20,21] The performance of the risk prediction model was then tested among subjects allocated to the validation cohort. Ability of the risk prediction model to discriminate persons who experience a cardiovascular disease from those who do not were evaluated using c-statistic,[24] and calibration of the risk prediction model was evaluated using a Hosmer–Lemeshow χ^2-statistic with 8 d.f. The cardiovascular risk prediction model was translated into a risk score sheet using methods developed in the Framingham Heart Study.[25] To facilitate easier understanding of the concept of risk, 'vascular age' was also included in the risk score sheet. An individual's vascular age was calculated as the age of a person with the same predicted risk but with all other risk factor levels in optimal ranges.[10] All analyses were performed using the SAS software package (SAS Institute, Cary, NC, USA).

Table 1 Baseline characteristics in the derivation and the validation cohorts

	Derivation cohort (n=1756)	*Validation cohort (n=878)*
Age, years	59 (12)	59 (12)
Men	43%	40%
Systolic blood pressure, mm Hg	134 (21)	133 (22)
Diastolic blood pressure, mm Hg	78 (12)	77 (11)
Diabetes	11%	13%
LDL cholesterol, mg per 100 ml	131 (43)	133 (41)
HDL cholesterol, mg per 100 ml	50 (12)	50 (12)
Current smoker	24%	27%

Abbreviations: HDL, high-density lipoprotein; LDL, low-density lipoprotein.
Values are means (s.d.) or frequencies.
SI conversion factors: to convert LDL and HDL cholesterol to millimoles per liter, multiply by 0.0259.

Table 2 Regression coefficients and hazard ratios for the cardiovascular risk prediction model in the derivation cohort

	β	*Hazard ratio*	*95% CI*
Age, years	0.05775	1.059	1.046–1.073
Men	0.55569	1.743	1.264–2.404
Systolic blood pressure, mm Hg	0.01701	1.017	1.011–1.023
Diabetes	0.51977	1.682	1.193–2.370
LDL cholesterol, mg per 100 ml	0.00257	1.003	0.999–1.006
HDL cholesterol, mg per 100 ml	−0.01182	0.988	0.977–1.000
Current smoker	0.35287	1.423	1.024–1.978

Abbreviations: 95% CI, 95% confidence interval; HDL, high-density lipoprotein; LDL, low-density lipoprotein.
SI conversion factors: to convert LDL and HDL cholesterol to millimoles per liter, multiply by 0.0259.

RESULTS

The baseline characteristics of the subjects allocated to the derivation cohort and those to the validation cohort are shown in Table 1. There were no clear differences in these baseline characteristics between two cohorts.

During 14 years of follow-up, 216 cardiovascular events were observed in the derivation cohort and 125 in the validation cohort. The cardiovascular risk prediction model including covariates of age, sex, systolic blood pressure, diabetes, LDL cholesterol, high-density lipoprotein cholesterol and smoking habits were developed in the derivation cohort. The multivariate-adjusted regression coefficients and hazard ratios for the risk prediction model are shown in Table 2.

The performance of the risk prediction model was then evaluated among the validation cohort. In terms of discrimination, the c-statistic was as high as 0.81 (95% confidence interval, 0.77 to 0.86). Figure 1 demonstrates the calibration plots comparing actual and predicted cardiovascular events by deciles of risk. The calibration χ^2-statistic for the risk prediction model was 6.46 (d.f.=8), indicating excellent goodness of fit (P=0.60). The top 30% of predicted risk identified 70% of subjects who experienced cardiovascular disease during follow-up (sensitivity). Proportion of subjects without cardiovascular events who were not in the top 30% of predicted risk was 79% (specificity).

Tables 3 and 4 provide risk score sheets that can be used for estimation of the multivariable risk of cardiovascular disease at 10

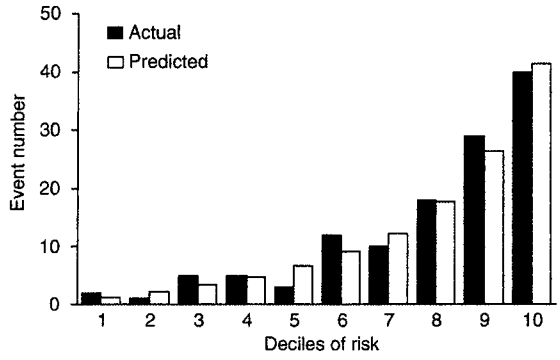

Figure 1 Actual and predicted cardiovascular events by deciles of risk in the validation cohort. Hosmer–Lemeshow χ^2-statistic=6.46, d.f.=8, P=0.60.

Table 3 Cardiovascular risk points

Points	*Age (years)*	*Sex*	*SBP (mm Hg)*	*Diabetic*	*LDL cholesterol (mg per 100 ml)*	*HDL cholesterol (mg per 100 ml)*	*Smoker*
0	40–44	Women	<119	No	<140	⩾40	No
1	45–49		120–139		⩾140	<40	Yes
2	50–54	Men	140–159	Yes			
3	55–59		160–179				
4	60–64		⩾180				
5	65–69						
6	70–74						
7	75–79						
8	⩾80						

Abbreviations: HDL, high-density lipoprotein; LDL, low-density lipoprotein; SBP, systolic blood pressure.
SI conversion factors: to convert LDL and HDL cholesterol to millimoles per liter, multiply by 0.0259.

years. Table 4 also provide a different quantification of the same risk in the form of vascular age.

DISCUSSION

In this paper, a new risk prediction model of cardiovascular disease has been developed using data obtained from a prospective cohort study of a general Japanese population. The risk prediction model demonstrated good performance in regard to both discrimination and calibration. A simple risk score sheet based on the cardiovascular risk prediction model was also presented. This simple risk prediction tool of cardiovascular disease for Japanese would provide a useful guide to estimate absolute risk of cardiovascular disease and to treat individual risk factors.

Large-scale cohort studies have developed a number of risk prediction tools of cardiovascular disease.[4–15] However, these risk prediction tools were mainly derived from studies carried out in Western populations and few risk prediction tools are developed among general Japanese populations. The NIPPON DATA 80 derived a cardiovascular risk prediction tool, in which age, sex, systolic blood pressure, glucose levels, total cholesterol and smoking habits were included as risk factors, using data obtained from a 19-year prospective cohort study of general Japanese populations, although the outcome of NIPPON DATA 80 risk charts was death from cardiovascular causes.[5] The Jichi Medical School (JMS) cohort study developed 10-year risk prediction tools for incidence of myocardial infarction[14] and stroke,[15] in which age, sex, systolic blood pressure, diabetes, total cholesterol and smoking habits were included as risk factors, using data obtained from a population-based prospective study of general Japanese populations. The present analysis from the Hisayama study developed a new risk prediction tool for incidence of cardiovascular disease in a general population of Japanese using similar risk factors used in the previous observational studies of Japanese. Cumulative incidence rates of cardiovascular events at 10 years estimated from the present risk prediction tool were almost similar to combined risks of myocardial infarction and stroke obtained from the JMS risk charts[14,15] and this finding supports the validity and the generalizability of the Hisayama risk prediction model.

Table 4 Estimated cardiovascular risk at 10 years and vascular age according to risk points

Points	*Risk[a] (%)*	*Vascular age for men[b] (years)*	*Vascular age for women[b] (years)*
0	1.4	—	40–44
1	1.8	—	45–49
2	2.4	40–44	50–54
3	3.2	45–49	55–59
4	4.2	50–54	60–64
5	5.6	55–59	65–69
6	7.4	60–64	70–74
7	9.8	65–69	75–79
8	12.8	70–74	80–84
9	16.7	75–79	85–89
10	21.7	80–84	90–94
11	27.8	85–89	95–99
⩾12	>30	⩾90	⩾100

[a]Estimated cardiovascular risk at 10 years.
[b]Age of a person with the same predicted risk but with all other risk factor levels in optimal ranges.

Several limitations of our study should be discussed. One limitation is a lack of external validation of the risk prediction model. However, split sample validation is an established method for internal validation of a risk prediction model and is widely used in other studies.[9,12] Similarity to the JMS risk chart[14,15] also supports the validity of the Hisayama risk prediction model. Another limitation is that LDL cholesterol, as a continuous variable, did not reach statistical significance in the derivation cohort. However, LDL cholesterol is an established risk factor for cardiovascular disease in the Hisayama study[21] and thus we included LDL cholesterol into the risk prediction model. A third limitation is that our findings are based on a one-time measurement of risk factors (for example, systolic blood pressure, plasma glucose levels, LDL cholesterol levels and high-density lipoprotein cholesterol levels), which may not accurately reflect the status of a study participant. A fourth limitation is that the value of LDL cholesterol was not directly assayed but was calculated by the Friedewald equation,[22] although the equation has been adopted in substantial epidemiologic and clinical studies of LDL cholesterol and cardiovascular disease. These limitations may have resulted in underestimation of the predicted risk among subjects at high risk of cardiovascular disease.

In conclusion, we developed and validated a new cardiovascular risk prediction model in a general population of Japanese. The risk prediction model would provide a useful guide to identify the individuals at high risk of cardiovascular disease in Japan. High-risk

approaches for the prevention of cardiovascular disease using the present risk prediction tool are likely to provide additional protection against the burden of cardiovascular disease in Japan.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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Lack of Association Between Variations of *PDE4D* and Ischemic Stroke in the Japanese Population

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Background and Purpose—After the first genomewide association study of ischemic stroke identified *PDE4D* as a susceptible gene, many replication studies have been conducted. However, the validity of the association has remained controversial because of the heterogeneity of both genetic markers and phenotypes.

Methods—We investigated the association between variations of *PDE4D* and ischemic stroke by 3 methods: single-marker, haplotype, and tag-single nucleotide polymorphism (SNP) analyses. In the single-marker analysis, we evaluated the association using 2 large case–control samples (1112 cases and 1112 control subjects in a sample obtained from Kyushu, Japan, and 1711 cases and 1786 control subjects in BioBank Japan) and a prospective cohort with 14 years of follow-up. These samples were analyzed both separately and pooled. Haplotype and tag-SNP analyses were performed using the 2 case–control samples together.

Results—In single-marker association tests, we found no significant association in the same direction among the 6 SNP reported in the initial study and ischemic stroke subtypes. Haplotype analysis revealed no significant association between the region around the 5′-end of the gene and combined atherothrombotic and cardioembolic infarction. Rs7730070, a SNP located around the 3′-end of *PDE4D*, showed the lowest nominal probability value by tag-SNP analysis but was not significant after adjustment for multiple testing (adjusted probability value =0.36).

Conclusions—These results suggest that variations in *PDE4D* are not associated with ischemic stroke risk in the Japanese population. (***Stroke*. 2009;40:1245-1251.**)

Key Words: cerebral infarct ■ genetics ■ PDE4D

Stroke is one of the most common causes of death and long-term disability around the world. Ischemic stroke is the most common form of stroke and is further subdivided into lacunar, atherothrombotic (ATI), and cardioembolic infarction (CEI). As for genetic contributions to the pathogenesis of ischemic stroke, twin and family studies[1,2] suggested that stroke risk was mediated by both environmental and genetic factors. The first genomewide association study of ischemic stroke reported the phosphodiesterase 4D gene (*PDE4D*) as a susceptible gene using 864 cases and 908 control subjects in an Icelandic population.[3] This study showed that the microsatellite marker AC008818–1 and 6 single nucleotide polymorphisms (SNPs) located in the 5′-end of the gene (SNP41, SNP45, SNP56, SNP87, SNP89, and SNP83) were significantly associated with ATI or with the combined ATI and CEI phenotype. Haplotype blocks B and C, which covered 260 kb around the 5′-end of the gene, were also associated, and the combination of the G allele of SNP45, the 0 allele of AC008818–1, and a common haplotype in block C led to the classification of individuals into at-risk, wild-type, and protective groups. Although the authors of the study showed that the affected individuals with the G0 haplotype had lower expression levels of some *PDE4D* isoforms, they could not find causative SNPs or haplotypes. Moreover, the biological role of *PDE4D* in ischemic stroke or the underlying atherosclerosis remained uncertain.

To our knowledge, 15 replication studies have been published on the association between SNPs in *PDE4D* and ischemic stroke.[4–18] However, the results are still controversial. Of the 4 studies that examined associations between the 2 markers (AC008818–1 and SNP45) and combined ATI and

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CEI, none replicated the original findings.[4–7] Among the 14 studies that examined at least SNP45,[4–17] only one found a nominal association with combined ATI and CEI.[6] Other groups reported significant associations between different phenotypes and different SNPs in the 1.5-Mb region of the gene.[6–13,19] There are thought to be several reasons for these inconsistencies among the results. The sample sizes in most studies were too small and had insufficient power to detect associations.[20] Sampling biases of cases and controls may have distorted true associations. Several positive findings in different SNPs might reflect associations among hidden causative variants linked to the SNPs or to the G0 haplotype. The association between variants in *PDE4D* and ischemic stroke risk might differ among ethnic groups.

According to the recent published criteria, replication studies should examine the same SNP or a SNP in perfect or very high linkage disequilibrium with the prior SNP on the same or a very similar phenotype. They also should show similar magnitude of effect and significance in the same direction.[21] Therefore, we performed single-marker association tests between the 6 SNPs and the same subtypes of ischemic stroke as in the initial study and used a sufficient sample size. We also performed haplotype analyses in blocks B and C using tag-SNPs selected from the same regions. To examine the possibility of hidden causative SNPs, we additionally genotyped 190 tag-SNPs that covered a 2.2-Mb region, including *PDE4D*, and performed association analyses.

Materials and Methods

Study Populations

We used 2 independent Japanese case–control samples and a prospective cohort for this study. One is a Kyushu sample consisting of 1112 cases of ischemic stroke and 1112 age- and sex-matched control subjects. Details on this population were described previously.[22] Briefly, patients with ischemic stroke were recruited from 7 medical centers in and around Fukuoka City, Japan, in 2004. These included 491 cases of lacunar infarction, 369 of ATI, 136 of CEI, and 116 of 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. All case subjects were diagnosed by stroke neurologists on the basis of detailed clinical features and ancillary laboratory examinations such as brain imaging. The 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 (NINDS-III).[23]

Another case–control sample was selected from the BioBank Japan project.[24] This project was started in 2003 to collect a total of 300 000 cases who have at least one of 47 diseases by a collaborative network of 66 hospitals located throughout Japan. The registration of cases was based on diagnoses made by physicians at the affiliated hospitals. From June 2003 to March 2006, 7974 cases with ischemic stroke were registered. We selected 1711 cases diagnosed with ischemic stroke subtypes by brain imaging, the same as with the Kyushu sample. The subtypes included 1143 with lacunar infarction, 355 with ATI, and 213 with CEI. Control subjects were randomly selected from the subjects who were registered with BioBank Japan for other diseases.

For the prospective cohort study, we used a cohort population of the Hisayama study established in 1988.[25] In this cohort, 2634 Hisayama residents aged ≥40 years and who had no history of stroke or coronary heart disease were enrolled in 1988 and continuously followed up for 14 years until the occurrence of cardiovascular disease or death. Among them, 1656 subjects participated in the examination between 2002 and 2003 and were used in the present study. During the 14-year follow-up, 67 events of first-ever ischemic stroke were observed.

Written informed consent was obtained from all study subjects. The study was approved by the ethics committees of the Graduate School of Medical Sciences at Kyushu University and the Institute of Physical and Chemical Research.

Clinical characteristics of 2 case–control samples are shown in Supplemental Table I, available online at http://stroke.ahajournals.org. In both samples, hypertension was defined as systolic blood pressure of ≥140 mm Hg or diastolic blood pressure of ≥90 mm Hg or current treatment with hypertensive medication.

SNP Selection and Genotyping

For the association study, we selected 6 SNPs that were significantly associated with ischemic stroke in the initial study: SNP41 (rs12153798), SNP45 (rs12188950), SNP56 (rs702553), SNP83 (rs966221), SNP87 (rs2910829), and SNP89 (rs1396476). In the haplotype analysis, we selected 16 additional tag-SNPs from the regions of blocks B and C defined in the initial study. For tag-SNP analysis, we selected 190 tag-SNPs from the 2.2-Mb region, including *PDE4D*. Tag-SNPs were selected from the Hapmap JPT data by the pairwise tagging method with the following criteria: $r^2>0.8$, minor allele frequency >5%, and call rate >75%.

Genomic DNA was extracted from peripheral blood leukocytes by a standard method. We genotyped SNPs using the multiplex polymerase chain reaction-based Invader assay[26] (Third Wave Technologies) or TaqMan assays (Applied Biosystems) in a blind fashion to the clinical information of study samples. All genotypes were called by visual inspection, and we determined genotype success as <10 undetermined samples in a 384-well plate. When we failed to genotype more than one 384-well plate in a total of 16 plates, we excluded the SNP from further analyses. To validate the genotyping data, we genotyped 10 SNPs in 48 subjects using direct sequencing, and the concordance rate was 99.6%.

Statistical Analysis

We examined the association both by each population and by meta-analysis. We assessed case–control association analysis and Hardy-Weinberg equilibrium by χ^2 test or Fisher exact test, as appropriate. In the association analysis, we mainly used an additive model and also referenced dominant and recessive models. For an easy understanding of the risk direction, we calculated the OR and 95% CI of each SNP according to the risk allele in the initial study. In a meta-analysis of the single-marker association test, pooled estimates of the ORs for 2 case–control studies and one prospective study were obtained using a fixed-effect model. Heterogeneities across the population were estimated formally using Cochran's Q test and the I^2 statistic. Haplotype analysis was performed using Haploview version 4.0 (Broad Institute). For the adjustment for multiple testing, we performed a random permutation test with 10 000 replications. linkage disequilibrium was calculated as D', and haplotype blocks were defined by Gabriel's criteria.[27]

Results

Single-Marker Association Test

We initially performed single-marker association tests between the 6 SNPs reported in the initial study and the same ischemic stroke subtypes (Table 1). SNP45 and SNP41, which showed the most significant association in

Table 1. Association Between SNPs Reported in the Initial Study and the Subtypes of Ischemic Stroke Among Japanese

Ischemic Stroke Subtype	SNP	Allele 1	Allele 2	Sample	Case AF	Case 11/12/22	Control AF	Control 11/12/22	*P* Value	OR (95% CI)	Meta-Analysis *P* Value	Meta-Analysis OR (95% CI)
ATI												
	SNP83	C	T	Kyushu	0.13	4/84/279	0.14	7/91/269	0.31	0.86 (0.64–1.16)		
				BioBank	0.13	7/79/269	0.15	42/436/1308	0.32	0.88 (0.70–1.12)	0.14	0.87 (0.73–1.05)
				Prospective	0.12	0/4/13	0.14	36/383/1157	0.66	0.79 (0.28–2.25)		
Combined ATI and CEI	SNP41	T	C	Kyushu	1.00	502/0/0	1.00	501/0/0				
				BioBank	1.00	568/0/0	1.00	1779/0/0				
				Prospective	1.00	24/0/0	1.00	1573/0/0				
	SNP45	C	T	Kyushu	1.00	502/0/0	1.00	501/0/0				
				BioBank	1.00	568/0/0	1.00	1779/0/0				
				Prospective	1.00	24/0/0	1.00	1573/0/0				
	SNP56	A	T	Kyushu	0.58	163/252/81	0.54	146/246/104	0.07	1.18 (0.99–1.41)		
				BioBank	0.56	169/290/102	0.56	554/860/352	0.88	1.01 (0.88–1.16)	0.11	1.09 (0.98–1.21)
				Prospective	0.73	14/7/3	0.55	485/766/315	0.02	2.17 (1.14–4.11)		
	SNP87	T	C	Kyushu	0.15	10/126/364	0.15	2/144/352	0.87	0.98 (0.76–1.25)		
				BioBank	0.11	7/116/445	0.13	32/412/1340	0.10	0.84 (0.68–1.03)	0.21	0.91 (0.78–1.06)
				Prospective	0.17	1/6/17	0.14	23/394/1148	0.60	1.22 (0.57–2.63)		
	SNP89	T	G	Kyushu	0.95	450/52/0	0.97	470/33/0	0.03	0.62 (0.40–0.97)		
				BioBank	0.95	516/50/2	0.95	1619/153/8	0.99	1.00 (0.73–1.37)	0.27	0.87 (0.67–1.12)
				Prospective	0.98	23/1/0	0.95	1430/143/3	0.39	2.33 (0.32–17.0)		

Allele 1 indicates the risk allele in the initial study; AF, allele frequency of allele 1; Meta-analysis was performed using a fixed-effect model.

the initial study, were monomorphic, and all individuals were homozygotes of the risk alleles in our population. In all samples, SNP83 showed no significant association with ATI. For the combined ATI and CEI subtypes, we found SNP56 to be significantly associated in the prospective cohort (P=0.02; OR, 2.17; 95% CI, 1.14 to 4.11), but it was not associated in the 2 case–control samples. In the meta-analysis, we could not find a significant association between SNP56 and the combined ATI and CEI phenotypes. SNP89 showed a significant association in the Kyushu sample, but its risk was in the opposite direction of the effect (P=0.03; OR, 0.62; 95% CI, 0.40 to 0.97). SNP89 was not significantly associated in the BioBank Japan sample and the prospective cohort, and we found no

Table 2. Association Between SNPs Reported in the Initial Study and Subtypes of Ischemic Stroke Among Combined Samples After Stratification by Hypertension

			Hypertension				Without Hypertension			
			Frequency, %				Frequency, %			
Ischemic Stroke Subtype	SNP	RA	Case (n=572)	Control (n=942)	*P* Value	OR (95% CI)	Case (n=130)	Control (n=842)	*P* Value	OR (95% CI)
ATI	SNP83	C	12.9	13.8	0.50	0.93 (0.75–1.15)	11.9	15.6	0.13	0.73 (0.49–1.09)
			Hypertension				Without Hypertension			
			Frequency, %				Frequency, %			
Ischemic Stroke Subtype	SNP	RA	Case (n=822)	Control (n=1017)	*P* Value	OR (95% CI)	Case (n=219)	Control (n=903)	*P* Value	OR (95% CI)
Combined ATI and CEI	SNP41	T	100	100			100	100		
	SNP45	C	100	100			100	100		
	SNP56	A	56.5	55.1	0.38	1.06 (0.93–1.21)	57.4	55.4	0.45	1.08 (0.88–1.34)
	SNP87	T	12.7	13.6	0.45	0.93 (0.77–1.13)	13.1	14.0	0.62	0.93 (0.68–1.26)
	SNP89	T	95.0	95.8	0.23	0.82 (0.60–1.12)	94.7	95.0	0.83	0.95 (0.59–1.52)

RA indicates risk allele in the initial study; Frequency, risk allele frequency; Due to the lack of hypertension status data, 22 ATI cases, 10 CEI cases, and 371 control subjects were excluded in the stratified analysis.

Table 3. Haplotype Analysis of SNPs Selected From the Region of Blocks B and C Among Combined Samples

Haplotype in Block B													
rs4502776	rs13172481	rs6869495	rs1423246	rs1345782	rs6860887	rs10514896	SNP56	rs27222	rs7712662	rs1423473	SNP45	rs153031	SNP41
A	G	A	A	C	C	A	A	C	T	C	C	A	T
G	C	G	G	C	T	G	T	T	T	C	C	G	T
A	G	A	A	C	C	A	A	T	C	T	C	G	T
A	G	A	G	A	T	A	T	T	C	T	C	G	T
G	C	A	G	C	T	G	T	T	T	C	C	G	T
A	G	A	A	C	T	G	T	T	T	C	C	G	T
G	C	A	G	A	T	A	T	T	C	T	C	G	T
G	C	A	A	C	C	A	A	C	T	C	C	A	T
G	C	A	G	C	T	G	T	C	T	C	C	A	T
A	G	A	G	C	C	A	A	C	T	C	C	A	T
A	G	A	A	C	C	G	T	T	T	C	C	G	T

Haplotypes with frequency >2% are shown.

significant association with SNP89 in the meta-analysis. SNP87 was not associated with the combined ATI and CEI phenotypes in any of the samples. We also examined the associations of these SNPs with ischemic stroke or other subtypes in the 2 case–control samples (Supplemental Table II, available online at http://stroke.ahajournals.org). SNP56 showed nominal association with ATI in the Kyushu sample (P=0.02; OR, 1.27; 95% CI, 1.03 to 1.57) but was not associated in the BioBank Japan sample. The meta-analysis showed no significant association between ATI and SNP56. No other SNPs showed a significant association with any phenotype in the same direction as the initial study.

Stratified Analysis by Hypertension Status

Some replication studies showed significant associations between the SNPs in *PDE4D* and ischemic stroke in subjects without hypertension.[11,17] Thus, we evaluated the association between the 6 SNPs and the subtypes of ischemic stroke among the combined samples stratified by hypertension status (Table 2). However, none of the SNPs were associated with ATI or the combined ATI and CEI phenotypes even in the subjects without hypertension.

Haplotype Analysis

Because SNP45 and SNP41, which are key SNPs for haplotype construction in block B, were monomorphic in our population, we constructed haplotypes using SNP56 and 16 additional tag SNPs selected from the regions of blocks B and C (Table 3). In block B, none of the haplotypes were significantly associated with the combined ATI and CEI phenotypes. In block C, the most common haplotype, G-C-C-A-G, showed the lowest probability value, but the association was not significant after adjustment for multiple testing (adjusted P=0.33). There was no significant haplotype in the combined region of blocks B and C (data not shown).

Tag-SNP Analysis

To determine the possibility of a hidden causative SNP, we attempted to examine the associations between tag-SNPs in *PDE4D* and ischemic stroke. We selected 190 additional tag-SNPs from the 2.2-Mb region that included *PDE4D* and genotyped in combined samples of 2823 cases and 2898 control subjects. Because 14 SNPs did not pass our criteria, we finally analyzed 198 SNPs (the 6 reported in the initial study and 192 tag-SNPs). The genomic structure, case–control results, and linkage disequilibrium map of the 2.2-Mb region are shown in the Figure. Although the initial study showed a strong association around the region of blocks A to C, none of the SNPs in this region showed any association. The rs7730070 SNP, located around the 3′-end of *PDE4D*, showed the lowest probability value (OR, 1.21; 95% CI, 1.06 to 1.37; P=0.0037). However, this SNP was not linked to the 5′-end of the gene that was the causative region in the initial study (Figure, C). Moreover, this association was not significant after adjustment for multiple testing (adjusted P=0.36).

Discussion

We examined the association between variations of *PDE4D* and ischemic stroke using 2 independent large case–control samples and a population-based cohort. Using these samples, we tried to replicate the previous reports in 3 ways: a single-marker association test, haplotype analysis in blocks B and C, and tag-SNP analysis, which covered the entire *PDE4D* gene region. Using 2 case–control samples consisting of 2823 cases and 2898 control subjects and a prospective cohort consisting of 1656 subjects, we found no significant association between the same SNPs and the same ischemic stroke subtypes in the single-marker tests. Similarly, no haplotypes in blocks B and C were found to be associated with the combined ATI and CEI phenotypes. Tag-SNP analysis could not find the hidden causative SNP in *PDE4D*. From these results, we suggest that the common variants of *PDE4D* did not confer risk for ischemic stroke, at least in the Japanese population.

Among the replication studies that examined variations of *PDE4D* and ischemic stroke, the most probable reason for the inconsistent findings is that the small sample sizes

Table 3. Continued

Frequency, %			Haplotype in Block C					Frequency, %		
Case	Control	*P* Value	rs35387	rs40512	rs26954	rs26950	rs26948	Case	Control	*P* Value
41.5	40.9	0.63	G	C	C	A	G	34.4	31.7	0.03
14.5	16.0	0.11	C	T	T	G	G	26.0	27.3	0.26
8.1	7.2	0.17	G	C	C	A	A	23.1	24.6	0.16
4.7	5.7	0.10	G	T	C	A	A	7.5	7.0	0.51
4.1	4.7	0.33	G	C	T	G	G	2.7	2.5	0.56
4.1	3.6	0.41								
3.1	3.1	0.87								
3.5	2.7	0.10								
2.9	2.2	0.08								
1.9	2.6	0.07								
2.1	2.1	0.82								

missed true associations of modest effect. Assuming our sample size, the allele frequencies of the SNPs in our control subjects, and the relative risks of the SNPs in the initial study, the power to detect associations at a significance level of 0.05 would be greater than 99% for SNP83 and SNP56, 98.3% for SNP87, and 69.7% for SNP89 in the case–control samples. In contrast, the statistical power of the prospective cohort was <30% for the 6 SNPs. However, a meta-analysis of these samples should increase the statistical power to detect the association. Therefore, if a true association exists, our study could detect the association between SNPs or haplotypes in *PDE4D* and ischemic stroke with high probability. A recent meta-analysis of 5216 cases and 6615 control subjects also showed that allele 0 of AC008818 and haplotype G0 carriers were associated with increased risk of ischemic stroke, but these associations become nonsignificant after exclusion of the initial study.[28] These results indicate that the effect size of *PDE4D* variants on ischemic stroke, if it exists, may be small.

Because the initial study could not determine a causative SNP or haplotype in *PDE4D*, many replication studies have reported positive associations between different SNPs in *PDE4D* and various ischemic stroke subtypes.[19] This indicates the possibility that hidden causative SNPs for ischemic stroke might exist in *PDE4D*. We analyzed a total of 198 tag-SNPs that covered the 2.2-Mb region, including *PDE4D*, but none of the SNPs were significant after adjustment for multiple testing. Because we selected tag-SNPs according to strict criteria, this analysis was able to capture the most common SNPs in *PDE4D*. Therefore, the previous positive findings of different SNPs may be attributable to chance.

One possible reason for the lack of association between *PDE4D* and ischemic stroke in our study was the difference in the ethnic background. Indeed, SNP45 and SNP41, which showed the most significant association with the combined ATI and CEI phenotypes in an Icelandic population, were monomorphic and all of the Japanese populations studied were homozygotes of the risk alleles in both SNPs. If SNP45 or SNP41 or absolutely linked variations are causative, we cannot estimate the effects of these variations on ischemic stroke, because all causative variations are homozygotes of risk alleles in both cases and control subjects.

Several limitations of this study should be discussed. First, we did not genotype the microsatellite marker, AC008818-1, in this study. However, we genotyped 16 tag-SNPs selected

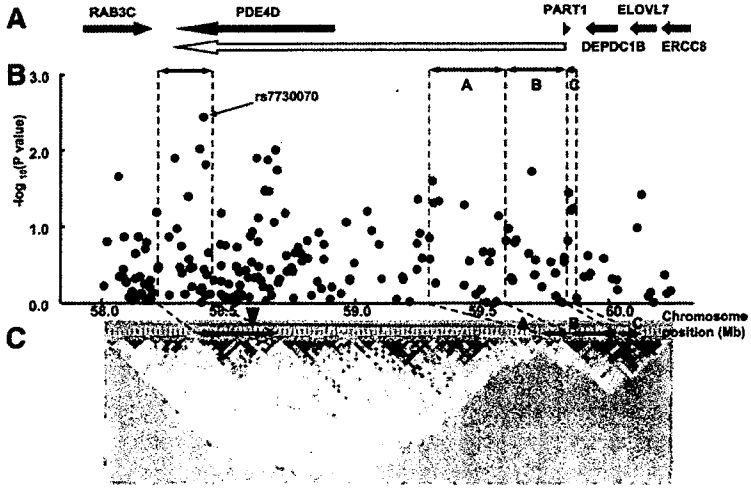

Figure. Genomic structure, case–control results, and linkage disequilibrium map of the 2.2-Mb region, including *PDE4D*. A, Genomic structure around *PDE4D*. The white arrow indicates *PDE4D* reported by the initial study. B, Case–control association results for ischemic stroke among Japanese. The log 10-transformed probability values calculated by the Cochran-Armitage trend test are plotted on the y axis. "A" indicates block A; "B," block B; "C," block C in the initial study. C, Pairwise linkage disequilibrium map between SNPs. The strength of the linkage disequilibrium increases from white to black. A black inverse triangle indicates the location of rs7730070 in the map.

from the regions of blocks B and C according to strict criteria. Therefore, we believe that the effect of AC008818-1 could be sufficiently covered by haplotype analysis using tag-SNPs. Second, we could use only 1656 of 2634 subjects in the prospective cohort. Subjects who developed ischemic stroke would have a higher mortality rate than subjects who did not, and this may have resulted in the lower participation rate in this study. There is a possibility that the results of the prospective cohort might have been distorted by a survivorship bias. Third, the criteria used for classifying ischemic stroke were different between the initial study and ours. For classification of ischemic stroke, the initial study used the Trial of Org 10172 in Acute Stroke Treatment research criteria[29] and we used NINDS-III.[23] However, these 2 classifications are similar to each other, and we diagnosed the subtypes of ischemic stroke by adequate laboratory examinations. We believe that there is no large difference in the phenotype definition.

In conclusion, although we performed a replication study between the variations of *PDE4D* and ischemic stroke risk using 2 independent large case–control samples and a population-based prospective cohort, we failed to replicate the associations. We suggest that variations of *PDE4D* do not confer risk for ischemic stroke in the Japanese population.

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Disclosures

None.

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ORIGINAL ARTICLE

Risk factors for coronary atherosclerosis in a general Japanese population: The Hisayama study

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Abstract

To investigate risk factors for coronary atherosclerosis in men and women in the recent general Japanese population, we examined coronary arteries obtained from subjects autopsied in the Hisayama cohort study (autopsy rate: 78.7%). The subjects were over 40 years of age and consisted of 125 men and 108 women. They underwent an antemortem medical examination in 1988 and were subject to autopsy at death during an 8-year follow-up period. Atherosclerosis was globally assessed by examining 14 specimens taken from wide areas of epicardial coronary arteries and classified into 6 grades. The frequency of more severe grades of coronary atherosclerosis increased with age in both genders and was greater in men than in women of the same age. Multiple regression analysis revealed that age, systolic blood pressure, serum total cholesterol, and hemoglobin A_{1C} were significant risk factors for men. Age, systolic blood pressure, and waist to hip ratio were risk factors for women. Smoking was not significantly correlated with the grade of coronary atherosclerosis in either gender. Thus, aging, hypertension, hypercholesterolemia, obesity, and glucose intolerance are risk factors for coronary atherosclerosis in recent Japanese populations, and the significance of the metabolic risk factors is different between men and women.

Keywords: Risk factors; Coronary atherosclerosis; Epidemiology; Autopsy; Japanese

Introduction

The mortality and morbidity of cardiovascular diseases have changed over the last several decades in Japan. The most striking change is the decrease in the incidence of stroke, and declining blood pressure in the general population has contributed greatly to this trend [15,37,39]. Although a reduced risk of hypertension was

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expected to diminish the morbidity and mortality of coronary heart disease (CHD), this change is minimal [15,39]. This is thought to be the result of mixed effects of other risk factors, such as hyperlipidemia, obesity and glucose intolerance, because the prevalences of these risk factors have rapidly increased in Japanese society during the same time period through Westernization of the Japanese lifestyle [7,15,39]. However, it is not well understood how changes in the risk factors actually affect the development of CHD events and coronary atherosclerosis. From 1961 to 1984, we conducted investigations on risk factors for CHD [14] and coronary atherosclerosis [24] in the Hisayama cohort study, using CHD patients and autopsy results, respectively. In the present study, we examined coronary arteries obtained from more recently autopsied subjects, aiming to clarify the risk factors for coronary atherosclerosis in men and women in the recent general Japanese population and to compare the results with those of the previous studies. The risk factors for coronary atherosclerosis are sometimes regarded as the same as those for CHD. However, they should be distinguished, because coronary atherosclerosis is not the only causative factor for CHD, and CHD is not necessarily developed in all patients with severe coronary atherosclerosis but developed often in patients with moderate atherosclerosis [16].

The method most frequently used in assessing coronary atherosclerosis is to measure surface involvement (SI), in which the proximal portion of the artery is longitudinally opened, and the area affected by atherosclerosis is measured. While this method certainly works well for young and middle-aged subjects [1,8,9,17,34,35], it is ineffective for older subjects like those in the present study. First, calcification is often present in the proximal portion of coronary arteries and prevents longitudinal opening. Second, older subjects tend to have higher SI scores for the proximal portion, so that the discriminatory value of the method is reduced. Therefore, we employed a novel method in the present study in which atherosclerosis was globally assessed by examining histologic specimens taken from wide areas of epicardial coronary arteries.

Material and methods

Hisayama study

A prospective population survey of cardiovascular disease and its risk factors has been conducted in Hisayama, a suburban community adjacent to Fukuoka, since 1961. The population over 40 years of age on January 1, 1988, included 3558 subjects. Full details of the sampling procedures, the methods of baseline examination and subsequent follow-up have been previously described [15,37,38]. From June 29 to November 10, 1988, 2742 men and women over 40 years of age underwent a medical examination (participation rate, 80.9%). Six subjects who died before the follow-up period and 12 subjects who had already had a history of myocardial infarction were excluded from the study, leaving 2724 subjects.

Subjects of the present study

During the follow-up period from December 1, 1988, to July 31, 1996, 310 subjects died, of whom 244 were autopsied (autopsy rate: 78.7%). Eleven subjects were excluded because whole lengths of coronary arteries were not available for pathologic examinations. Thus, 233 subjects (125 men and 108 women) were investigated in the present study. The study was approved by the Ethics Committee of the Department of Pathology, Kyushu University, and informed consent was given by the families of the autopsied subjects. The intervals between the antemortem examination and the autopsy ranged from 1 month to 8 years. The mean ages at the antemortem examination and death are shown in Table 1. Eighteen subjects were affected by CHD during the follow-up period, i.e., 14 subjects experienced myocardial infarction, and 4 subjects were victims of sudden death with severe chest pain or precordial oppression. Sixty six and 18 subjects were treated with anti-hypertensive and anti-diabetic drugs, respectively.

Risk factors

The variables that were selected as possible predictors for coronary atherosclerosis included age at the antemortem examination, systolic blood pressure (SBP), diastolic blood pressure (DBP), body mass index (BMI), waist to hip ratio (WHR), total serum cholesterol (TC), high-density lipoprotein cholesterol (HDL-C), hemoglobin A_{1C} (HbA_{1C}), smoking habits, and daily alcohol intake. The mean values of the factors are shown in Table 1. Blood pressures were measured 3 times in a sitting position and averaged. The concentrations of TC and HDL-C were measured enzymatically. HbA_{1C} levels were measured by high-pressure liquid chromatography (TBA-80S, Toshiba Inc., Tokyo, Japan). Non-smokers at the time of the antemortem examination were classified as 0 and ex-smokers and current smokers as 1. Non-drinkers and ex-drinkers were classified as 0 and current drinkers as 1.

Coronary arteries

In all subjects, the heart was immersion-fixed with 10% formalin. As illustrated in Fig. 1, 14 specimens of

Table 1. Characteristics of the subjects.

Variable	Men			Women		
	n	Mean ± SD	Range	*n*	Mean ± SD	Range
Age at exam. (years)	125	72.56 ± 10.75	(44.00–94.00)	108	74.41 ± 11.01	(46.00–96.00)
Age at death (years)	125	76.95 ± 10.35	(49.00–94.00)	108	78.84 ± 11.09	(53.00–98.00)
SBP (mmHg)	125	143.96 ± 23.03	(103.00–237.00)	108	148.35 ± 25.85	(96.00–213.00)
DBP (mmHg)	125	77.55 ± 10.75	(41.00–104.00)	108	75.05 ± 11.76	(47.00–111.00)
BMI (kg/m^2)	125	21.15 ± 2.76	(14.67–27.46)	106	21.24 ± 3.66	(13.33–34.04)
WHR	122	0.92 ± 0.06	(0.80–1.08)	103	0.91 ± 0.09	(0.70–1.12)
TC (mg/dL)	125	187.04 ± 48.64	(75.00–354.00)	108	201.76 ± 38.84	(123.00–311.00)
HDL-C (mg/dL)	125	46.50 ± 12.48	(20.00–84.00)	108	47.06 ± 13.03	(25.00–82.00)
HbA_{1C} (%)	125	5.77 ± 0.96	(4.00–11.10)	108	5.68 ± 0.80	(4.20–8.90)

SBP indicates systolic blood pressure; DBP, diastolic blood pressure; BMI, body mass index; WHR, waist to hip ratio; TC, serum total cholesterol; HDL-C, high-density lipoprotein cholesterol; HbA_{1C}, hemoglobin A_{1C}.

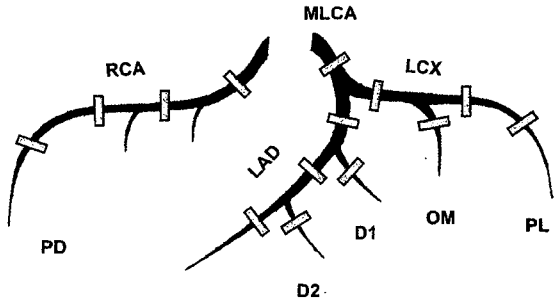

Fig. 1. Examination of 14 sites (gray bars) from epicardial coronary arteries. D1: first diagonal branch, D2: second diagonal branch, LAD: left anterior descending artery, LCX: left circumflex artery, MLCA: main left coronary artery, OM: obtuse marginal branch, PD: posterior descending branch, PL: posterolateral branch, RCA: right coronary artery.

epicardial coronary arteries were taken from each heart for histologic examinations; 4 specimens from the right coronary artery (RCA) system (proximal, mid and distal portions of RCA and posterior descending branch), 1 from the main left coronary artery (MLCA), 5 from the left anterior descending artery (LAD) system (proximal, mid and distal portions of LAD and first and second diagonal branches), and 4 from the left circumflex artery (LCX) system (proximal and distal portions of LCX and obtuse marginal and posterolateral branches). The arteries were cut *in situ* at 3–4 mm intervals, and the most narrowed sites were taken. In cases of anatomical variations, specimens were obtained from other arteries distributing blood to the corresponding cardiac areas such that the total number was 14. Specimens with severe calcification were decalcified.

Histologic classification

Histologic sections were stained with hematoxylin and eosin, elastica van Gieson, and Masson's trichrome stains. Atherosclerotic lesions were classified into two categories, i.e., preatheromas and advanced lesions, according to the classification of the American Heart Association [31]. Preatheromas included foam cell lesions (type II) and intermediate lesions (type III). Advanced lesions included atheromas (type IV) and fibroatheromas (type V).

Grading of coronary atherosclerosis

As shown in Table 2, the global state of coronary atherosclerosis in each subject was classified into 6 grades by assessing the histology of intimal lesions and luminal stenosis in 14 specimens. When there was neither preatheroma nor advanced lesion, the subjects were classified as grade 0. If the number of preatheromas was 1–4, and no advanced lesions were present, the subjects were classified as grade 1. In grade 2, subjects who fulfilled either of the following criteria were included. In criterion A, the number of preatheromas was 5 or more, and no advanced lesions were present. In criterion B, the number of advanced lesions was 1 or 2, and the total number of lesions (preatheromas + advanced lesions) was less than 5. For example, a subject with 12 preatheromas and no advanced lesions and a subject with no preatheromas and only 1 advanced lesion were classified in this grade. In grade 3, subjects who had 1–4 advanced lesions with variable numbers of preatheromas were included, and those who fulfilled criterion B in grade 2 were excluded. Subjects were classified as grade 4 when the number of advanced lesions was 5–8. For grade 5, subjects who fulfilled either of the following criteria were included. In criterion A, the number of advanced lesions was the same as that for grade 4 and, in addition, the luminal stenosis was over 80% in at least 2 of 3 (RCA, LAD, and LCX) arterial systems. In criterion B, the number of the advanced lesions was 9 or more. For example, a subject having 6 advanced lesions with severe stenosis in RCA and LAD

Table 2. Grading of coronary atherosclerosis.

Grade		Number of lesions in 14 sites		Luminal stenosis
		Preatheroma	Advanced lesion	
0		0	0	
1		$1 \leq < 5$	0	
2	A	$5 \leq$	0	
	B		$1 \leq < 3$	
		(preatheroma + advanced lesion: < 5)		
3		[a]	$1 \leq < 5$ (except subjects who fulfill the criterion B in grade 2)	
4		[a]	$5 \leq < 9$	
5	A	[a]	$5 \leq < 9$ and	Over 80% stenosis in at least 2 of 3 major arterial systems
	B	[a]	$9 \leq$	

[a]In grades 3–5, the number of preatheroma was not concerned in grading.

and a subject having 11 advanced lesions were classified in this grade. Luminal stenosis was measured as previously described [24] with a computer-assisted morphometric analyzer (MacScope, Fukui, Japan). To investigate the effect of SBP and DBP on LAD and RCA systems, atherosclerosis of LAD and RCA was classified into 6 grades, respectively, using a similar grading method as described above.

Statistical analysis

An SAS computer package (SAS Institute, Cary. N.C., USA) was used for regression analysis and stepwise multiple regression analysis. A Mann–Whitney non-parametric test was used to compare the grade of atherosclerosis in subjects with CHD with those with non-CHD. The age-adjusted SBP and DPB values were calculated by the covariance method to investigate their effects on atherosclerosis in LAD and RCA. A $p < 0.05$ value was considered to be statistically significant.

Results

Frequency of each grade of coronary atherosclerosis

The number of subjects who were classified in grades 0, 1, 2, 3, 4, and 5 were 20, 32, 47, 62, 45, and 27, respectively. The mean (m) of the grade for all subjects was 2.70. The grade of the subjects affected by CHD during the follow-up period ($m = 3.83$, $n = 18$) was significantly higher than that for non-CHD subjects ($m = 2.60$, $n = 215$, $p < 0.001$). The number of subjects treated with anti-hypertensive drugs was greater in the higher grades than in lower grades (3, 6, 6, 17, 20, and 14 in grades 0, 1, 2, 3, 4, and 5, respectively), and so was the number of subjects treated with anti-diabetic drugs (1, 0, 3, 4, 5 and 5 in grades 0, 1, 2, 3, 4, and 5, respectively).

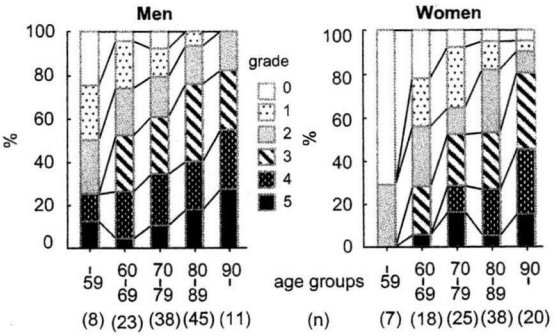

Fig. 2. Frequency (%) of coronary atherosclerosis graded as grades 0–5 in five age groups. n: number of subjects.

Correlation of coronary atherosclerosis with age and gender

Fig. 2 shows the correlation between the grade of coronary atherosclerosis and age at death. Subjects were divided into 5 groups according to their age (less than 60 years of age, 60–69, 70–79, 80–89, and 90 or more). In the youngest group, all subjects were over 50 years of age, except one 49-year-old man. The ordinate represents the frequency of grades 0–5 expressed as a percentage. As shown in this figure, the percentages of higher grades of coronary atherosclerosis increased with age in both genders, and were greater in men than in women of the same age. Generally, the proportion of

each grade in men was similar to that for women in the age group one decade older.

Age-adjusted regression analysis of risk factors

As shown in Table 3, age-adjusted regression analysis revealed significant correlations between the grade of coronary atherosclerosis and SBP, BMI, WHR, TC, and HbA_{1C} for men, and SBP, BMI, and WHR for women. In addition, a negative correlation was found for HDL-C for women.

Multivariate analysis

As shown in Table 4, stepwise multiple regression analysis revealed age, SBP, TC, and HbA_{1C} as risk factors for coronary atherosclerosis in men, and age, SBP, and WHR as risk factors in women.

Effect of blood pressure on atherosclerosis in LAD and RCA

SBP was a significant risk factor for atherosclerosis in LAD ($p = 0.0063$) and RCA ($p = 0.0391$) in men, and for atherosclerosis in RCA ($p = 0.0003$) but not in LAD ($p = 0.2790$) in women. DPB was not a significant risk factor for atherosclerosis either in LAD or in RCA in men and women.

Discussion

The Hisayama study

Although there are numerous reports examining risk factors for CHD, there are fewer studies on risk factors for coronary atherosclerosis, and the factors investigated are limited [1,2,8,9,10,19,24,28,30,32,34]. It is likely that the major reason for this paucity of information is the difficulty of obtaining sufficient data on risk factors and of precisely assessing the grade of coronary atherosclerosis in the same individuals. This is especially true when general populations are targeted because autopsy is required for the assessment of atherosclerosis. The Hisayama study is a prospective cohort study of the general Japanese population, having favorable prerequisite conditions. The autopsy ratio is high (about 80%), and data on risk factors were obtained from records of antemortem examinations performed by the same examiners in the same period.

Table 3. Age-adjusted regression analysis of risk factors for coronary atherosclerosis.

Risk factor	Men ($n = 125$)			Women ($n = 108$)		
	β^a	F	R^{2b}	β^a	F	R^{2b}
SBP	0.014	7.40*	0.0509	0.017	11.04**	0.0789
DBP	0.012	1.15	0.0083	0.014	1.51	0.0117
BMI	0.098	4.71*	0.0330	0.106	8.69**	0.0646
WHR	5.895	8.56**	0.0594	4.168	7.71**	0.0607
TC	0.287	10.11**	0.0680	0.188	2.06	0.0159
HDL-C	−0.327	0.81	0.0059	−0.779	4.09*	0.0311
HbA_{1C}	0.332	7.84*	0.0537	0.216	1.71	0.0133
Smoking	0.406	1.54	0.0111	0.125	0.14	0.0011
Drinking	−0.164	0.43	0.0031	−0.614	2.51	0.0193

[a] β: regression coefficient.
[b] R^2: coefficient of determination.
* $p<0.05$.
** $p<0.01$.

Table 4. Stepwise multiple regression analysis of risk factors for coronary atherosclerosis.

Risk factor	Men ($n = 125$)			Women ($n = 108$)		
	β^a	F	R^{2b}	β^a	F	R^{2b}
Age	0.041	14.90**	0.1151	0.042	12.48**	0.0827
SBP	0.013	6.97**	0.0444	0.015	8.54**	0.1586
WHR				3.193	4.59*	0.0339
TC	0.224	6.11**	0.0730			
HbA_{1C}	0.275	5.37*	0.0337			
Sum			0.2662			0.2752

[a] β: regression coefficient.
[b] R^2: coefficient of determination.
* $p<0.05$.
** $p<0.01$.

Global assessment of coronary atherosclerosis

In the present study, coronary atherosclerosis was globally assessed by examining wide areas of epicardial coronary arteries. Consequently, the number of subjects was largest in grade 3 and gradually decreased in both higher and lower grades. This result indicates that the grading method functioned well in the present study, and suggests that it can also be applied to other studies aiming at similar age groups. However, the grade of atherosclerosis can be variable among races and people who live in different geographic conditions [32,35]. Therefore, a small probing study will be necessary to see whether this method gives a proper distribution of subjects when applied to other situations.

Age and gender

Many studies have revealed that age is a significant risk factor for coronary atherosclerosis [9,10,19,34,35]. However, most of the data were obtained from young to middle-aged subjects, and subjects over 70 years of age have seldom been surveyed. The present study demonstrated that atherosclerosis continued to progress even in

the elderly in both genders. Examining coronary stenosis in similar age groups of Hisayama-autopsied subjects, Okumiya et al. [24] reached the same conclusion.

Coronary atherosclerosis in women is milder than in men [21,35] and this is particularly true in premenopausal women [19,34]. However, we are unaware of any detailed reports on coronary atherosclerosis in postmenopausal women. The results of the present study suggest that the progression of coronary atherosclerosis in women is about 10 years behind that found in men in middle-aged and elderly people. This is consistent with clinical observations that the incidence and mortality rate of CHD in women is lower than that for men at the same age even in the postmenopausal period [11,36].

Blood pressure

A positive association between blood pressure and coronary atherosclerosis has been reported by many authors [1,8–10,30,34]. Investigating Hisayama autopsy subjects who died between 1971 and 1981, Okumiya et al. [24] also reached the same conclusion. At the time of this study, blood pressure began to be managed rigorously in Hisayama, and the prevalence of hypertension was significantly decreased in the examination in 1988, compared to those in the 1960s and 1970s when the old WHO criteria were used [7]. Therefore, we expected some modification in the correlation between blood pressure and coronary atherosclerosis before the present study started. However, the analyses revealed that blood pressure remained a strong risk for coronary atherosclerosis in both genders even in the newer subjects. This result may be explained by the fact that the prevalence of hypertension in 1988 was not different from that of the 1960s and 1970s when new WHO criteria were used [15]. The average blood pressures of the present subjects, in particular those for SBP, were high in the light of the new WHO criteria, as shown in Table 1.

Hemodynamics is different between the left coronary artery (LCA) and RCA systems. A major difference is that blood flow in LCA is dominant in diastole, while RCA has a relatively systolic predominance [5]. This difference suggests that LCA is more affected by DBP than SBP and RCA by SBP. However, the present study showed that SBP, but not DBP, was a risk factor for atherosclerosis both in LCA and in RCA. The difference in the blood flow pattern between LCA and RCA may not be large enough to create the different effects of SBP and DBP on coronary atherosclerosis.

TC and HDL-C

TC has been demonstrated as a strong risk factor for coronary atherosclerosis in western countries [1,8,30]. As shown in the present study and other domestic studies performed in the past [24,34], TC is also a risk factor for Japanese people. Regrettably, the risk of TC may become more significant in the future, because the level of TC has rapidly increased in the Japanese population recently [39]. The present study also demonstrated a correlation of TC and coronary atherosclerosis in men but not in women. Although no data on gender differences are available in other pathologic studies, the same trend is demonstrated for CHD. Some epidemiologic studies, including Kiyohara's Hisayama study, demonstrated that the association between TC and CHD was significant for men but not for women [12,14,29], and men had higher rates of CHD than women with the same TC values [3]. Thus, TC has a stronger association with coronary atherosclerosis and CHD in men than in women.

A negative correlation between HDL-C and coronary atherosclerosis has been reported in the Oslo study and the PDAY (Pathobiological Determinants of Atherosclerosis in Youth) study [8,19]. In the present study, however, HDL-C showed a weak association with the grade of coronary atherosclerosis only in women in the age-adjusted regression analysis, and no significance was found in the multivariate analysis in either gender. This difference between the present study and other studies may be related to the difference in the age of the subjects and the grade of atherosclerosis. Compared to the present study, the age groups examined in the Oslo and PDAY studies are much younger [8,19]. In addition, a significant association between HDL-C and atherosclerosis was detected only in early preatheromatous lesions but not in advanced lesions in the PDAY study [19]. However, advanced lesions were observed in many subjects in the present study.

HbA_{1C} and glucose intolerance

A positive correlation between glucose intolerance and coronary atherosclerosis has been reported in only a few epidemiological studies [10,18]. On the other hand, a positive association with glucose intolerance and CHD has been reported in many epidemiologic studies. However, Kiyohara's Hisayama study, in which the subjects were followed from 1961 to 1984, did not reveal a significant correlation between glucose intolerance and the development of CHD events [14]. Interestingly, a subsequent study of Hisayama subjects, in which the same cohort as that used in the present study was followed from 1988 to 1993, demonstrated a positive association [6]. The difference between the two studies is probably explained by the difference in the morbidity of diabetes mellitus at the times of the two investigations. Along with the rapid increase in diabetic patients among Japanese populations, the prevalence of glucose