Table 1 Case-control study showing association between nonsynonymous SNP 1425G/A in *PRKCH* and cerebral infarction

	Case				Control				Minor allele frequency		*P* value (adjusted *P*)		Odds ratio (95% c.i.)	
Samples	AA	AG	GG	Sum	AA	AG	GG	Sum	Case	Control	A vs. G	AA+AG vs. GG	A vs. G	AA+AG vs. GG
Screening														
Cerebral infarction	57	390	662	1,109	40	332	724	1,096	0.227	0.188	1.31×10^{-3}	1.98×10^{-3}	1.27 (1.10–1.47)	1.31 (1.11–1.56)
Lacunar	27	178	286	491	11	130	344	485	0.236	0.157	9.84×10^{-6} (0.0004)	3.47×10^{-5} (0.0009)	1.66 (1.33–2.09)	1.75 (1.34–2.28)
Atherothrombotic	14	132	220	366	16	108	238	362	0.218	0.194	0.234	0.115	1.17 (0.90–1.50)	1.27 (0.94–1.72)
Cardioembolic	7	39	90	136	9	48	77	134	0.195	0.246	0.150	0.141	0.74 (0.49–1.11)	0.69 (0.42–1.13)
Undetermined	9	41	66	116	4	46	65	115	0.254	0.235	0.625	0.952	1.11 (0.73–1.70)	0.98 (0.59–1.66)
BioBank Japan														
Lacunar	56	416	665	1,137	81	575	1,219	1,875	0.232	0.197	9.89×10^{-4}	3.34×10^{-4}	1.24 (1.09–1.40)	1.32 (1.13–1.54)

Adjusted *P* values were obtained from case-control samples with 10^4 permutation tests in lacunar infarction. c.i.: confidence interval.

role for genetic factors in cerebral infarction, although their contribution is not very large[4]. There have been several approaches to identifying genetic variants associated with susceptibility to common diseases[5]. With the availability of a large volume of SNP information and large-scale genotyping methods, genome-wide association studies have successfully identified genetic variants associated with susceptibility to common diseases such as myocardial infarction[6], rheumatoid arthritis[7,8] and Crohn disease[9].

In regard to the genetic risk for stroke, *PDE4D* has been reported as a candidate for cerebral infarction through a genome-wide linkage study[10]. Here we report identification of *PRKCH* as a candidate risk locus for cerebral infarction through a case-control study by means of large-scale gene-based SNP analysis. We replicated this association in independent samples from BioBank Japan and confirmed it by a 14-year population-based follow-up study.

To identify variants associated with susceptibility to cerebral infarction, we performed a genome-wide case-control study using 1,112 Japanese individuals with cerebral infarction and 1,112 age- and sex-matched controls. First, we genotyped 188 individuals with cerebral infarction and 188 age- and sex-matched controls using 52,608 gene-based tag SNPs selected from the JSNP database[11]. We compared allele frequencies of 48,083 successfully genotyped SNPs (overall success rate of 91.4%) between the two groups and identified 1,098 SNPs showing *P* values of < 0.01. In a second round of screening, we genotyped the remaining cases and controls for these SNPs. As the subjects included subtypes of cerebral infarction, we also analyzed data by subgroups. Through this analysis, we found that SNP_15 in *PRKCH* (IMS_JST140193) was strongly associated with lacunar infarction ($P = 4.73 \times 10^{-6}$ for allele frequency model). We did not find any significant association of this SNP with atherothrombotic infarction, probably because of the small number of subjects. SNP_15 retained a significant association with lacunar infarction ($P = 0.0036$) after a permutation test. We therefore concluded that the susceptibility locus for lacunar infarction was likely to be in a region including *PRKCH*.

We subsequently attempted to construct a fine linkage disequilibrium (LD) map of the *PRKCH* locus, and we defined the region showing a strong association with lacunar infarction. We genotyped 45 SNPs among 491 cases with lacunar infarction and age- and sex-matched controls (**Supplementary Table 1** online), and we constructed a high-resolution LD map consisting of 27 SNPs that had minor allele frequencies of >0.2 (**Fig. 1**). Of two LD blocks defined, SNP_15 was located within block 1, and the association with lacunar infarction peaked at SNPs located in block 1, suggesting that *PRKCH* is the most likely candidate for harboring variants associated with susceptibility to lacunar infarction. We further sequenced all exons in *PRKCH* using DNA from 48 affected individuals and 48 controls, and we identified four SNPs: 695A/G in exon 2 (rs3742633), 1425G/A in exon 9 (rs2230500), 1427A/C in exon 9 (rs2230501) and 1979C/T in exon 12 (rs1088680). Among them, the first and the last SNPs were not located within block 1 and showed no association. We considered the remaining two SNPs to be in absolute LD from the DNA sequence results. The 1425G/A SNP caused an amino acid substitution (V374I). We then genotyped the 1425G/A SNP for all cases and controls by direct sequencing, and we confirmed its significant association with lacunar infarction ($P = 9.84 \times 10^{-6}$, odds ratio (OR) = 1.66; 95% confidence interval (c.i.) = 1.33–2.09 for allele frequency model; **Table 1**). We replicated this association in an independent case-control

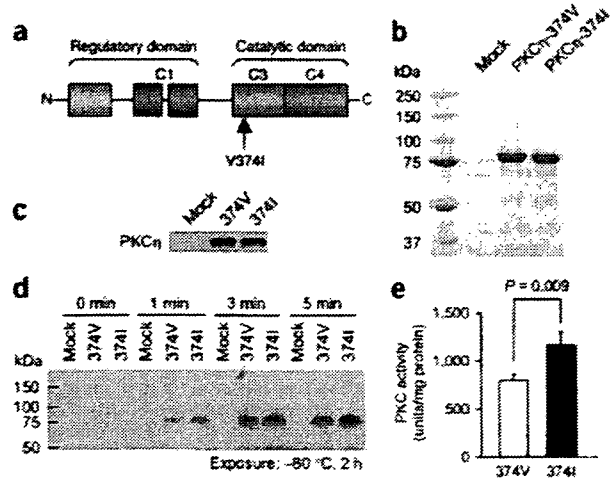

Figure 2 Comparison of the PKC activity of PKCη-374V and PKCη-374I. (a) Domain structure of *PRKCH*. Arrow indicates the position of 1425G/A. (b) Immunoprecipitates of mock, PKCη-374V and PKCη-374I by Coomassie brilliant blue staining. (c) Protein blotting using equal amounts of immunoprecipitates of mock, PKCη-374V and PKCη-374I. (d) Autophosphorylation assay of mock, PKCη-374V and PKCη-374I after stimulation with 10 μM phosphatidylserine and 100 nM PDBu. (e) PKC activity of PKCη-374V and PKCη-374I using myelin basic protein peptide as a substrate after 3-min stimulation with 10 μM phosphatidylserine and 100 nM PDBu.

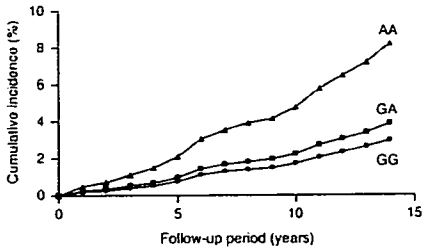

Figure 3 Age- and sex-adjusted cumulative incidence of cerebral infarction by nonsynonymous SNP 1425G/A in *PRKCH* (leading to amino acid substitution V374I) during a 14-year follow-up period in the Hisayama study.

group of 1,137 cases with lacunar infarction and 1,875 controls selected from BioBank Japan project ($P = 9.89 \times 10^{-4}$, OR of 1.24, with 95% c.i. of 1.09–1.40). Combined analysis of two case-control samples showed that the A allele was significantly associated with lacunar infarction under a dominant model, with an OR of 1.40 (95% c.i., 1.23–1.59, $P = 5.1 \times 10^{-7}$). Although the association was slightly stronger at SNP_15 than at 1425G/A SNP, these SNPs were in almost absolute LD ($D' = 0.99$, $\Delta^2 = 0.98$). Therefore, either one or a combination of these SNPs in block 1 might have functional significance by altering the quality or quantity of the gene product.

To further clarify an independent effect of these SNPs, we performed multivariate analysis with adjustment for clinical risk factors using a conditional logistic regression model in 491 individuals with lacunar infarction along with age- and sex-matched controls. The genotypic risk for lacunar infarction was substantially unchanged after adjustment for age, sex, hypertension, hyperlipidemia and diabetes (**Supplementary Table 2** online). We also evaluated the impact of population stratification on all 1,112 individuals with cerebral infarction, along with age- and sex-matched controls using STRUCTURE[12] and did not find any significant population stratification (**Supplementary Fig. 1** online).

Among the SNPs in block 1, a 1425G/A SNP (leading to V374I) is located within an ATP-binding site of PKCη (ref. 13). Therefore, we first examined the effect of V374I on PKCη kinase activity (**Fig. 2**). We constructed expression vectors of Flag-tagged PKCη corresponding to a valine-encoding allele (Flag-PKCη-374V) and to an isoleucine-encoding allele (Flag-PKCη-374I). We transfected these vectors to 293T cells and immunoprecipitated the Flag-tagged proteins. We subjected equal amounts of immunoprecipitates to SDS-PAGE and examined the purity of the immunoprecipitates by Coomassie brilliant blue staining. We also checked the amount of the two forms of Flag-PKCη by protein blot analysis (**Fig. 2b,c**). As PKCη was reported to be activated by autophosphorylation[14], we examined the kinase activity of these two proteins by autophosphorylation assay. After stimulation with 10 μM phosphatidylserine and 100 nM phorbol 12,13-dibutyrate, we observed autophosphorylation of PKCη 1 min later; the degree of autophosphorylation was higher for PKCη-374I than for PKCη-374V (**Fig. 2d**). To further confirm these results, we examined PKC activity using myelin basic protein as a substrate and found that PKCη-374I has 1.6 times the activity of PKCη-374V ($P = 0.009$, Student's *t*-test; **Fig. 2e**). These results suggest that the amino acid substitution of V374I in PKCη results in higher autophosphorylation and kinase activity after stimuli and activates its signaling pathway.

Replication of the association using different populations is critical. However, the minor allelic frequencies of SNP_15, which is in absolute LD with the candidate 1425G/A SNP, were reported in the HapMap database as 0.239 in Japanese in Tokyo, 0.178 in Han Chinese in Beijing, 0.008 in CEPH samples (Utah residents with ancestry from northern and western Europe) and 0.00 in Yoruba from Ibadan, Nigeria. As these data suggest that this candidate SNP is likely to be specific to Asian populations, we attempted to confirm the association of this SNP using a population-based prospective cohort[2] established in 1988. During a 14-year follow-up period, 67 individuals experienced their first cerebral infarction (42 cases of lacunar infarction, 18 cases of atherothrombotic infarction and 7 cases of cardioembolic infarction) among 1,642 subjects without a history of stroke at baseline examination. The age- and sex-adjusted cumulative incidence of cerebral infarction was 2.96% in the GG genotype, 3.86% in GA and 8.18% in the AA (**Fig. 3**), and we found a significant difference between the GG and AA genotypes ($P = 0.030$, age- and sex-adjusted hazard ratio of 2.83, with a 95% c.i. of 1.11–7.22; **Table 2**). This relationship remained significant even after adjustment for baseline clinical risk factors. We estimated the population attributable risk of the AA genotype to be 30 per 100,000 person-years in this cohort (population attributable risk percentage of 10.1%). We also examined the impact of the 1425G/A SNP on the development of lacunar infarction in particular. A Kaplan-Meier estimate showed similar results to those seen for cerebral infarction, but the differences among the genotypes were not significant, probably owing to the small number of events (**Supplementary Fig. 2** online). In addition, when we examined the effect of the 1425G/A SNP on the development of coronary heart disease among 1,661 subjects without a history of coronary heart disease, we found a similar result ($P = 0.024$, age- and sex-adjusted hazard ratio of the AA genotype was 3.31 (95% c.i., 1.17–9.36) compared with the GA and GG genotype combined; **Supplementary Fig. 3** online). These findings indicate that the 1425G/A SNP is a common genetic risk factor for the development of atherosclerotic diseases.

Table 2 Hazard ratios for the incidence of cerebral infarction

			Age- and sex-adjusted			Multivariate-adjusted		
Genotype of nonsynonymous SNP 1425G/A	Total number of subjects	Number of cerebral infarctions	Hazard ratio	95% c.i.	*P* value	Hazard ratio	95% c.i.	*P* value
GG	1,063	39	1.00			1.00		
AG	518	23	1.31	0.78–2.19	0.309	1.31	0.78–2.20	0.317
AA	61	5	2.83	1.11–7.22	0.030	2.91	1.14–7.47	0.026
GG+AG	1,581	62	1.00			1.00		
AA	61	5	2.58	1.03–6.44	0.043	2.66	1.06–6.68	0.038

Multivariate analysis was performed with adjustment for age, sex, hypertension, diabetes, cholesterol and smoking and drinking habits. c.i.: confidence interval.

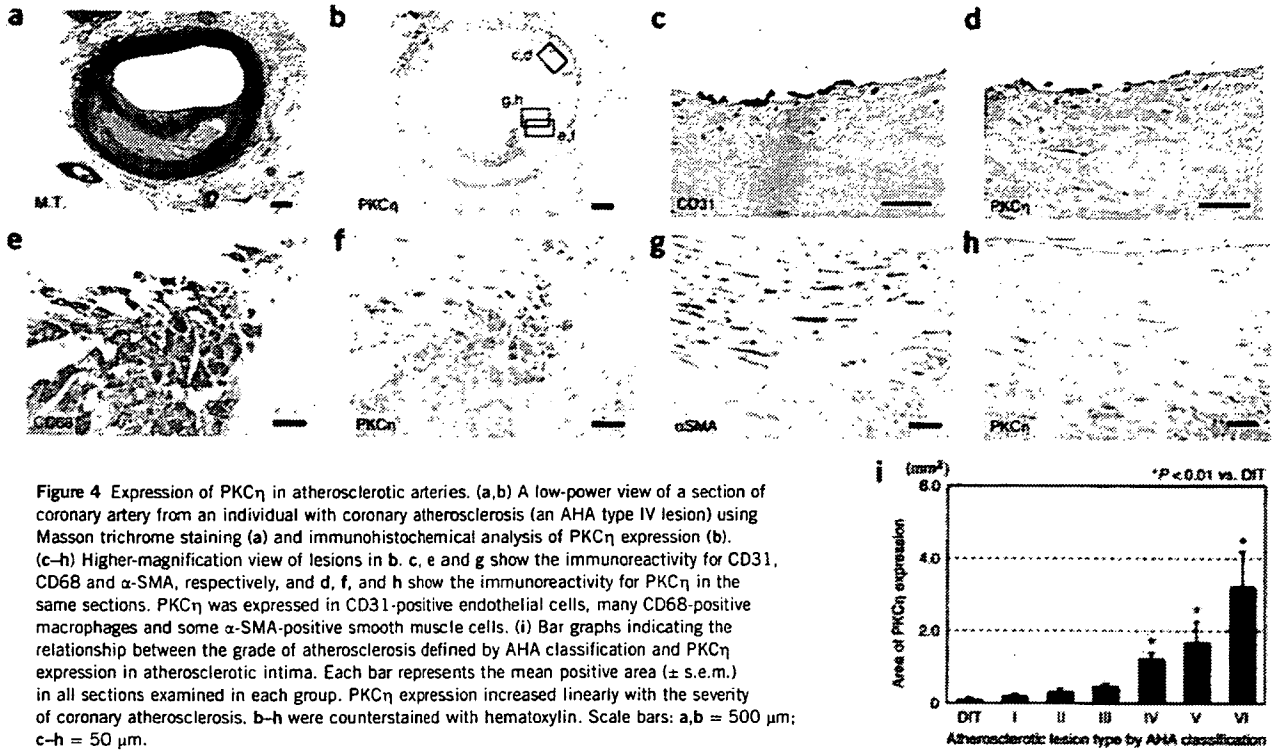

Figure 4 Expression of PKCη in atherosclerotic arteries. (a,b) A low-power view of a section of coronary artery from an individual with coronary atherosclerosis (an AHA type IV lesion) using Masson trichrome staining (**a**) and immunohistochemical analysis of PKCη expression (**b**). (c–h) Higher-magnification view of lesions in **b**. **c**, **e** and **g** show the immunoreactivity for CD31, CD68 and α-SMA, respectively, and **d**, **f**, and **h** show the immunoreactivity for PKCη in the same sections. PKCη was expressed in CD31-positive endothelial cells, many CD68-positive macrophages and some α-SMA-positive smooth muscle cells. (**i**) Bar graphs indicating the relationship between the grade of atherosclerosis defined by AHA classification and PKCη expression in atherosclerotic intima. Each bar represents the mean positive area (± s.e.m.) in all sections examined in each group. PKCη expression increased linearly with the severity of coronary atherosclerosis. **b–h** were counterstained with hematoxylin. Scale bars: **a,b** = 500 μm; **c–h** = 50 μm.

PKCη is known to be expressed predominantly in epithelial tissues in mouse[14], but its expression pattern in humans was unknown. Therefore, we carried out quantitative real-time PCR using mRNA obtained from various human tissues. PKCη was ubiquitously expressed and showed slightly higher expression in thymus and spleen, suggesting its physiological importance in white blood cells (**Supplementary Fig. 4** online). On the basis of this result and the association with atherosclerotic diseases, we subsequently investigated the expression of PKCη in human atherosclerotic lesions (**Fig. 4**). Immunohistochemical analysis of a coronary arterial specimen showed that PKCη was expressed in endothelial cells and some foamy macrophages in the intima and in a part of spindle-shaped smooth muscle cells in the intima and media (**Fig. 4b–h**). This immunohistochemical positivity was completely abolished by preabsorbing antibody with an excess of immunogenic peptide. To further investigate a role of PKCη in atherosclerosis, two independent pathologists carefully classified 60 coronary arterial specimens according to the type of atherosclerotic lesion[15]. The expression of PKCη increased in accordance with the progression of coronary atherosclerotic lesion type ($P < 0.0001$ for Spearman's rank correlation; **Fig. 4i**). These results suggest a role for PKCη in the development and progression of atherosclerosis in humans.

PKC is a serine-threonine kinase that regulates a wide variety of important cellular functions including proliferation, differentiation and apoptosis. PKCs are classified into three subfamilies based on their molecular structure and cofactor requirements. Classical PKC isoforms (α, βI, βII, and γ) are regulated by calcium, diacylglycerol and phospholipids. Novel PKC isoforms (δ, ε, η and θ) are regulated by diacylglycerol and phospholipids but are insensitive to calcium. Atypical PKC isoforms (ζ and λ or ι) are insensitive to either calcium or diacylglycerol[16]. Despite their high degree of sequence homology, different PKC isoforms mediate unique cellular functions and phosphorylate unique protein substrates[17]. Although PKCη was identified in 1990 (ref. 13), its specific substrates are not yet known. Therefore, it is unclear how PKCη is involved in the development and progression of atherosclerosis. Our pathological findings showed that PKCη was abundantly expressed in foamy macrophages that are essential in all phases of atherosclerosis. Macrophages contribute to the uptake of lipoproteins, release of reactive oxygen species and immune mediators that have important roles in the development of atherosclerosis[18]. Previous studies indicated that PKCη was involved in oxidative stress. Overexpression of PKCη in human monocytic cells resulted in the induction of inducible nitric oxide synthase and nitric oxide (NO) production after endotoxin exposure[19]. Antisense oligonucleotides for PKCη inhibited LPS-induced NO release in primary astrocytes[20]. The other possible downstream signal is the promotion of cell growth through suppression of cyclin E (ref. 21) and caspase-3 activity[22] as well as activation of the Akt pathway[23], which is involved in diverse cellular processes.

In conclusion, we have identified *PRKCH* as a new candidate gene for cerebral infarction using genome-wide SNP analysis and have replicated this association in an independent case-control sample. The nonsynonymous SNP (1425G/A) in *PRKCH* causes enhancement of PKC activity and is associated with a higher incidence of cerebral infarction in a Japanese population-based sample. Although the function and the signaling pathway of *PRKCH* are not fully elucidated, our findings may contribute new insights into the pathogenesis of atherosclerosis and suggest the possibility of new preventive measures for cerebral infarction.

METHODS

Study subjects. Details of the study subjects and the flow diagram of this study are shown in **Supplementary Figure 5** online. For the genome-wide case-control study, affected individuals with cerebral infarction were recruited from

seven affiliated hospitals of Kyushu University (Kyushu University Hospital, Hakujyuji Hospital, Fukuoka Red Cross Hospital, Kyushu Medical Center, Imazu Red Cross Hospital, Fukuoka Higashi Medical Center and Seiai Rehabilitation Hospital) in 2004. The diagnoses of cerebral infarction and its subtypes for all cases were made by stroke neurologists of the affiliated hospitals, referring to detailed clinical features and ancillary laboratory examinations: namely, cerebral angiography, brain imaging (including computed tomography and magnetic resonance imaging), echocardiography and carotid duplex imaging. Details of the diagnostic criteria for cerebral infarction and its subtypes have been described previously[24]. Briefly, subtypes of cerebral infarction were determined on the basis of the Classification of Cerebrovascular Disease III proposed by the National Institute of Neurological Disorders and Stroke[3]. Lacunar infarction was diagnosed as the presence, as observed by brain imaging, of a relevant brainstem or subcortical hemispheric lesion with a diameter of <1.5 cm and no evidence of cerebral cortical or cerebellar impairment. Atherothrombotic infarction was diagnosed when the subjects had significant stenosis or occlusion of a major cerebral artery with infarct size ≥ 1.5 cm on brain imaging. The diagnosis of cardioembolic infarction was made on the basis of primary and secondary clinical features suggestive of cardioembolism as reported by the Cerebral Embolism Task Force[25] with the TOAST classification of risk sources of cardioembolism[26]. The category of undetermined subtype included all cerebral infarction cases for which the subtype could not be determined because of insufficient clinical or morphological information.

Control subjects were enrolled from the participants in the Hisayama study, an ongoing prospective population-based epidemiological study of cardiovascular disease established in 1961. Details of this study have been described previously[1,2]. Between 2002 and 2003, a screening survey for the present study was performed. Briefly, a total of 3,328 individuals aged 40 years or older consented to participate in the screening survey and underwent a comprehensive assessment (participation rate of 78%). After excluding subjects with a history of stroke or coronary heart disease, we selected age-matched (within 5 years) and sex-matched control subjects from the participants by 1:1 matching using random numbers (**Supplementary Table 3** online).

The replication case-control samples were recruited from the BioBank Japan project. We selected lacunar infarction cases from subjects registered to have cerebral infarction in the BioBank. Cases with lacunar infarction were diagnosed on the basis of clinical findings, including neuroimaging results. Controls were randomly selected from the subjects registered in the BioBank Japan.

To investigate the impact of 1425G/A on the development of cerebral infarction, we used a cohort population of the Hisayama study established in 1988 (ref. 2). A total of 2,742 Hisayama residents aged 40 years or older participated in the health examination (participation rate of 81%) in 1988. After we excluded individuals with a history of stroke or coronary heart disease, we monitored 2,637 subjects for the occurrence of cardiovascular disease or death. Of these, 1,683 participated in the 2002–2003 examination.

We obtained written informed consent from all study subjects, and procedures were approved by the ethics committees of the Graduate School of Medical Sciences of Kyushu University and the Institute of Medical Science of the University of Tokyo.

SNP genotyping. We extracted genomic DNA from peripheral blood leukocytes using standard protocols. We genotyped SNPs using the multiplex PCR-based Invader assay (Third Wave Technologies) as previously described[27] or by direct sequencing of PCR products using ABI3700 capillary sequencers (Applied Biosystems) according to standard protocols.

Cell culture, transfection and immunoprecipitation. We maintained 293T cells in D-MEM supplemented with 10% (vol/vol) FBS and 1% (vol/vol) antibiotic/antimycotic solution (Sigma) at 37 °C under 5% CO_2. We constructed a plasmid designed to express full-length PKCη-374V by cloning human thymus cDNA into a p3xFLAG-CMV-14 expression vector (Sigma). A plasmid expressing full-length PKCη-374I was constructed from p3xFLAG-CMV-14-PKCη-374V vector using the QuikChange XL Site-Directed Mutagenesis Kit (Stratagene) following the manufacturer's instructions. For transient transfection, we plated 293T cells in 15-cm dishes and transfected p3xFLAG-CMV-14-PKCη-374V or p3xFLAG-CMV-14-PKCη-374I using FuGENE6 (Roche) according to the manufacturer's instructions. After 48 h, we collected cells and lysed them at 4 °C in lysis buffer containing 1% Nonidet P-40, 150 mM NaCl, 50 mM Tris-HCl (pH 8.0), 1 mM phenylmethyl sulfonyl fluoride, 1 mM dithiothreitol and 0.1% protease inhibitor cocktail set III (Calbiochem). After a 30-min incubation on ice, the lysates were centrifuged at 20,000*g* for 15 min at 4 °C. The supernatant was incubated with anti-Flag M2 affinity gel (Sigma) for 3–4 h at 4 °C after predearing with rec-Protein G–Sepharose 4B conjugate (Zymed) and mouse normal IgG for 30 min. After incubation, the gels were washed twice with lysis buffer and with 1× TBS buffer, and the Flag-tagged protein was eluted by addition of 15 μg of 3× Flag peptide (Sigma). We assessed the purity of immunoprecipitates by Coomassie brilliant blue staining. Equal amounts of immunoprecipitates were subjected to SDS-PAGE, and the amount of Flag-PKCη was assessed by protein blotting using antibody to PKCη (Santa Cruz) and the ECL protein blotting detection system (Amersham). The protein concentration was measured by the Bradford method.

PKC activity and autophosphorylation assay. PKC autophosphorylation activity and kinase activity were measured according to a previously described method[28]. For the PKC autophosphorylation assay, equal amounts of immunoprecipitates for mock (empty vector), PKCη-374V and PKCη-374I were incubated at 30 °C for the indicated times in a total volume of 50 μl of reaction mixture containing 20 mM Tris-HCl (pH 7.5), 5 mM $MgSO_4$, 1 mM EGTA and 5 μCi [γ-^{32}P]ATP with 10 μM phosphatidylserine (Sigma) and 100 nM phorbol-12,13-dibutyrate (PDBu, Sigma) and were then subjected to SDS-PAGE and autoradiography. Protein kinase activity was measured by incorporation of ^{32}P from [γ-^{32}P]ATP into myelin basic protein (MBP) peptide (Sigma). The incubation mixture contained 20 mM Tris-HCl (pH 7.5), 5 mM $MgSO_4$, 1 mM EGTA, 100 μM ATP, 1 μCi [γ-^{32}P]ATP, 10 μg MBP peptide and PKCη immunoprecipitant in a total volume of 50 μl, with 10 μM phosphatidylserine and 100 nM PDBu. After incubation for 3 min at 30 °C, the reaction was terminated by direct application to P81 phosphocellulose squares (Upstate) followed by washing with 75 mM phosphoric acid and measurement of radioactivity. One unit of the activity was defined by incorporation of 1 nmol/min of radioactive phosphate from ATP into MBP.

Quantification of *PRKCH* expression using real-time PCR. We carried out real-time quantitative PCR using an ABI 7700 (Applied Biosystems) with SYBR Premix ExTaq (TaKaRa) in accordance with the manufacturer's instructions. We purchased total RNA from various human tissues (Clontech) and synthesized first-strand cDNA from 1 μg of total RNA using oligo $d(T)_{12-18}$ primers and Superscript III Reverse Transcriptase (Invitrogen). The relative expression of *PRKCH* mRNA was normalized to the amount of β-actin expression in the same cDNA using the standard curve method described by the manufacturer.

Immunohistochemistry and morphometric analysis of coronary arteries. Hearts were obtained at autopsy at the Department of Pathology of Kyushu University from 16 deceased Hisayama residents (eight men and eight women), ranging in age from 68–91 years old (81.1 ± 6.2), within 16 h of death. The coronary arteries were cannulated, washed with 0.1 mol/l PBS (pH 7.4) and perfused with 1 l of 4% (wt/vol) paraformaldehyde in 0.1 mol/l sodium phosphate (pH 7.4) at 100 mm Hg. Then, the heart was immersed in 4% paraformaldehyde for at least 24 h at 4 °C. The right coronary artery and left anterior descending coronary artery were dissected free from the surface of the heart, cut perpendicular to the long axis at 3-mm intervals and then embedded in paraffin. Sixty blocks were obtained and cut into 3-μm-thick serial sections at once. Serial sections from each block were separately subjected to hematoxylin and eosin staining, elastica–van Gieson staining and Masson trichrome staining, as well as immunohistochemistry. In accordance with the definitions proposed by the Committee on Vascular Lesions of the Council on Arteriosclerosis of the American Heart Association (AHA)[15], the atherosclerotic lesion type of each section was carefully classified by two independent pathologists (T.N. and K.S.).

Immunohistochemical examinations were performed as described previously[29]. In brief, deparaffinized sections were incubated with 3% nonfat milk; with primary antibodies against human PKCη (Santa Cruz), endothelial cells (anti-human CD31, Dako), monocytes/macrophages (anti-human CD68, Dako) and smooth muscle cells (anti-human α-SMA, Sigma); and then with peroxidase-labeled secondary antibody (Dako). The slides were incubated with 3,3′-diaminobenzidine tetrahydrochloride (DAB) and counterstained with

hematoxylin. As a negative control, we substituted nonimmune rabbit IgG or nonimmune mouse IgG of each isotype instead of primary antibody. As a second negative control, we used antibody preabsorbed with an excess amount (10:1 molar ratio) of immunogenic peptide. As PKCη is expressed in normal epithelium of human mammary glands[30], we used the tissue blocks retrieved from human mammary glands as positive controls. A single observer, blind to the atherosclerotic lesion types, quantified PKCη-positive lesions by determining the positive area in atherosclerotic intima. All images were captured and analyzed by US National Institutes of Health Image software.

Statistical analysis. Data are presented as mean ± s.d. unless otherwise stated. We assessed association and Hardy-Weinberg equilibrium by χ^2 test and Fisher's exact test. We calculated linkage disequilibrium index and Δ index and created **Figure 1d** as described previously[7]. For adjustment of multiple testing, we performed a random permutation test with 10,000 replications using the MULTTEST procedure in SAS9.12 software. In the 14-year follow-up cohort study, we estimated hazard ratios and 95% confidence intervals for the development of cerebral infarction among the candidate genotypes using Cox proportional hazards model after adjustment for age and sex. In adjustment for clinical risk factors, we used a conditional logistic regression model for the case-control study and Cox proportional hazards regression model for the prospective cohort study using SAS software. We compared the PKCη-positive area among the grade of coronary atherosclerosis using Spearman's rank correlation with Bonferroni correction. The difference in PKC activity between 374V and 374I was tested by Student's *t*-test.

Note: Supplementary information is available on the Nature Genetics website.

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AUTHOR CONTRIBUTIONS

M.K., J.H., T.M. and K. Yamazaki performed SNP genotyping; T. Ninomiya and K. Yonemoto provided Hisayama samples and carried out statistical analyses; T. Nakano and K.S. performed immunohistochemical experiments; M.K., K. Yamazaki and K.M. performed protein blotting experiments; Y.O. and S.S. performed genotyping of genome-wide screening samples; T.K. and S.I. provided clinical information and samples of individuals with cerebral infarction; Y.N. provided BioBank Japan samples; M.K. performed all other experiments and wrote the manuscript with contributions from K.S., Y.N. and Y.K.; T. Ninomiya, K. Yonemoto, Y.K. helped with revisions and M.I., Y.N. and Y.K. jointly directed the project.

COMPETING INTERESTS STATEMENT

The authors declare that they have no competing financial interests.

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Impact of Kir6.2 E23K Polymorphism on the Development of Type 2 Diabetes in a General Japanese Population

The Hisayama Study

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OBJECTIVE—The association between the E23K polymorphism of ATP-sensitive K^+ channel subunit Kir6.2 and diabetes has been reported in Caucasians but not in Asians. We examined this issue in follow-up and cross-sectional studies in a general Japanese population.

RESEARCH DESIGN AND METHODS—In a 14-year follow-up study of 976 subjects aged 40–79 years with normal glucose tolerance (NGT), we investigated the impact of the E23K polymorphism on change of glucose tolerance status using a 75-g oral glucose tolerance test. Additionally, we confirmed this association in a cross-sectional survey of 2,862 subjects.

RESULTS—In the follow-up study, the frequencies of the K/K genotype or K-allele were significantly higher in subjects with conversion from NGT to diabetes than in those in whom NGT was maintained (genotypes, $P = 0.01$; alleles, $P = 0.008$). In multivariate analysis, the risk for progression to diabetes was significantly higher in subjects with the E/K (odds ratio 2.10 [95% CI 1.16–3.83]) and K/K (2.40 [1.01–5.70], P for trend = 0.01) genotypes than in those with the E/E genotype after adjustment for confounding factors, namely, age, sex, fasting plasma glucose, family history of diabetes, BMI, physical activity, current drinking, and current smoking. In the cross-sectional study, the frequencies of the K/K genotype or K-allele were also significantly higher in those with diabetes than in those with NGT (genotypes, $P = 0.006$; alleles, $P = 0.001$).

CONCLUSIONS—Our findings suggest that the Kir6.2 E23K polymorphism is an independent genetic risk factor for diabetes in the general Japanese population. ***Diabetes* 56:2829–2833, 2007**

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IFG, impaired fasting glycemia; IGT, impaired glucose tolerance; K_{ATP} channel, ATP-sensitive K^+ channel; NGT, normal glucose tolerance; OGTT, oral glucose tolerance test; PAR, population-attributable risk.

Variants in genes encoding key components of insulin secretion pathways may confer a susceptibility to type 2 diabetes. Among candidate genes for such variants, the ATP-sensitive K^+ channel (K_{ATP} channel) genes play an essential role in glucose-stimulated insulin secretion (1). The K_{ATP} channel in pancreatic β-cells is composed of an ATP-sensitive and pore-forming inwardly rectifying K^+ channel (Kir6.2) subunit (2) and a regulatory sulfonylurea receptor 1 subunit (3). A single nucleotide polymorphism at codon 23 of the *KCNJ11* gene (rs5219) results in a glutamic acid to lysine substitution (E23K) in the NH_2-terminal tail of Kir6.2 (4–6), and this polymorphism may cause modest reductions in ATP sensitivity and insulin secretion (7). Although there is general consensus that this polymorphism is a risk factor for type 2 diabetes in Caucasian populations (8–12), very few studies have examined its effect in Asian populations. Recently, one study reported that the E23K polymorphism was not significantly related to diabetes in Japanese (13). The aim of the present article is to assess the association of this polymorphism with diabetes in a defined Japanese population after accounting for comprehensive risk factors for diabetes.

RESEARCH DESIGN AND METHODS

Follow-up study group. In 1988, a screening survey for the present study was performed in the town of Hisayama, a suburb of Fukuoka, Japan (14). The age and occupational distributions for Hisayama were almost identical to those of Japan as a whole based on data from the national census. Of all residents aged 40–79 years in 1988, 2,587 participated in the baseline survey (participation rate 80.2%). After excluding 82 subjects who had already had breakfast, 15 who ceased taking a 75-g oral glucose tolerance test (OGTT) due to nausea or general fatigue during the ingestion of glucose, and 10 who were on insulin therapy, 2,480 subjects completed the OGTT. Of these, 1,561 subjects with normal glucose tolerance (NGT) were enrolled in the baseline examination. After the initial screening, glucose tolerance levels were measured again in 2002. The genotype data of the E23K polymorphism and glucose tolerance levels were successfully obtained for a total of 976 subjects (383 men and 593 women), and these were the subjects selected for the 14-year follow-up study.

Cross-sectional study group. A diabetes survey similar to that done in 1988 was performed in 2002. Of all residents aged 40 years or over, 3,328 underwent the examination (participation rate 77.6%). Among the participating residents, 3,196 subjects agreed to the genetic analysis. Of these, a total of 2,851 subjects completed the OGTT after excluding 234 who refused the OGTT, 76 who had already eaten breakfast, and 35 who were on insulin therapy. Among those who did not undergo the OGTT, the following 78 subjects were categorized as having diabetes and included in the cross-sectional study: 21 with fasting plasma glucose ≥7.0 mmol/l, 6 with plasma glucose ≥11.1 mmol/l after eating breakfast, 16 who were taking oral hypoglycemic agents, and 35 on insulin

TABLE 1
Clinical characteristics of subjects according to the Kir6.2 E23K polymorphism in NGT subjects in 1988 and total subjects in 2002

	NGT subjects at baseline from the follow-up study in 1988				Total subjects in the cross-sectional study in 2002			
	E/E	E/K	K/K	*P* for trend	E/E	E/K	K/K	*P* for trend
n	414	442	120		1,197	1,296	369	
Age (years)	53 ± 8	52 ± 8	52 ± 8	0.48	60 ± 12	60 ± 12	61 ± 12	0.56
Men (%)	38.7	39.4	40.8	0.67	44.6	44.4	43.1	0.26
Fasting plasma glucose (mmol/l)	5.3 ± 0.4	5.3 ± 0.4	5.3 ± 0.4	0.76	6.1 ± 1.4	6.2 ± 1.4	6.3 ± 1.4	0.03
2-h postload glucose (mmol/l)	5.9 ± 1.1	5.9 ± 1.1	6.1 ± 1.0	0.29	7.9 ± 3.8	8.3 ± 3.8	8.5 ± 3.8	0.002
Fasting insulin (pmol/l)	32.9 (14.2–76.2)	31.2 (13.6–71.1)	31.5 (14.5–68.6)	0.11	39.7 (12.8–123.5)	39.1 (12.6–121.5)	40.0 (12.9–124.5)	0.92
2-h postload insulin (pmol/l)	157.1 (42.0–587.9)	144.1 (37.1–559.9)	140.0 (40.6–482.7)	0.03	241.9 (57.7–1,013.6)	238.9 (57.0–1001.8)	230.2 (54.9–965.4)	0.30
HOMA-IR	1.3 (0.5–3.1)	1.2 (0.5–2.9)	1.2 (0.5–2.9)	0.11	1.8 (0.5–6.4)	1.7 (0.5–6.4)	1.8 (0.5–6.6)	0.72
Family history of diabetes (%)	6.1	9.6	8.4	0.14	14.9	15.8	15.3	0.61
BMI (kg/m^2)	23.0 ± 2.8	22.9 ± 2.8	22.5 ± 2.8	0.09	23.3 ± 3.3	23.2 ± 3.3	23.2 ± 3.3	0.65
Regular exercise (%)	9.0	10.9	15.8	0.04	10.9	10.2	11.6	0.90
Current drinking (%)	29.0	31.5	35.8	0.15	46.0	45.3	46.6	0.97
Current smoking (%)	19.6	21.3	33.3	0.006	22.5	22.1	24.5	0.35

Data are means ± SD, percentages, or geometric mean (range). Fasting insulin, 2-h postload insulin, and homeostasis model assessment of insulin resistance (HOMA-IR) values are geometric mean and value by 1 SD computed on the log-transformed variable and the mean and SD values converted to the original scale. Age and percent of men are not adjusted.

therapy. Consequently, 2,929 subjects whose glucose tolerance levels were determined underwent analysis of the E23K polymorphism. Of these, the genotype data were successfully obtained from a total of 2,862 subjects (1,268 men and 1,594 women), for a genotyping success rate of 97.7%.

Genotyping. Genotyping of the Kir6.2 E23K polymorphism was done by a TaqMan assay at HuBit Genomix (Tokyo, Japan). The TaqMan genotyping reaction was amplified on a GeneAmp PCR System 9700, and fluorescence was detected on an ABIPRISM 7900HT Sequence Detection System. We confirmed the genotyping results of 376 randomly selected subjects by the direct sequencing method at the RIKEN Institute (Yokohama, Japan). Consequently, the concordance rate was 100% in the 369 subjects who were successfully genotyped by both the TaqMan and direct sequencing methods. The distributions of the E23K polymorphism were in Hardy-Weinberg equilibrium.

Clinical evaluation. In both the 1988 and 2002 surveys, the study subjects underwent the OGTT between 8:00 and 10:30 A.M. after at least a 12-h overnight fast. The plasma glucose levels were determined by the glucose-oxidase method and serum insulin by a radioimmunoassay in 1988 and a chemiluminescent enzyme immunoassay in 2002. Plasma glucose levels were classified according to World Health Organization criteria (15). In the 2002 survey, the subjects who were taking antidiabetic medicine or had a glucose concentration of ≥11.1 mmol/l after eating breakfast were categorized as having diabetes. Homeostasis model assessment of insulin resistance was calculated from fasting plasma glucose and serum insulin (16). BMI was calculated from the height and weight. Diabetes in first- or second-degree relatives was taken to indicate a family history of diabetes. Alcohol intake and smoking habits were classified as either currently habitual or not. Subjects engaging in sports at least three times per week during their leisure time were classified into a regular exercise group.

Statistical analysis. The distributions of the E23K polymorphism were analyzed according to glucose tolerance levels by χ^2 test. Adjusted odds ratios (ORs) and 95% CIs were calculated by logistic regression model. The population-attributable risk (PAR) percentage and its 95% CI were estimated according to the method used in the previous report (17). If assuming a significance level of $P < 0.05$ and 80% power, it is estimated that the OR for the risk of diabetes between the E/E and K/K genotypes becomes significant at the difference of 1.37-fold or greater in our cross-sectional survey. In a meta-analysis of the cross-sectional studies (10), it has been reported that the OR for the risk of diabetes in the K/K genotype was 1.49, significantly higher than that for the E/E genotype. Thus, the sample size of our study seems to be enough for the detection of the significant association.

This study was conducted with the approval of the ethics committee of the Faculty of Medicine, Kyushu University, and written informed consent was obtained from all study subjects.

RESULTS

Table 1 shows the clinical characteristics of NGT subjects in 1988 and total subjects in 2002 according to Kir6.2 E23K genotypes.

The frequencies of the E23K polymorphism based on change of glucose tolerance status are shown in Table 2. The distributions of the E23K polymorphism in subjects with conversion from NGT to impaired fasting glycemia (IFG) or impaired glucose tolerance (IGT) were not significantly different from those in whom NGT was maintained over the 14-year follow-up. The frequencies of the E/K and K/K genotypes or K-allele, however, were significantly higher in individuals with progression to diabetes than in those in whom NGT was maintained (genotypes, $P = 0.01$; alleles, $P = 0.008$).

The age- and sex-adjusted or multivariate-adjusted ORs of the E23K polymorphism for the development of diabetes were analyzed between subjects with conversion from NGT to diabetes during the follow-up period and subjects with NGT maintained (Table 3). The risk for progression from NGT to diabetes was more than twofold higher in the E/K and K/K genotypes than in the E/E genotype after age and sex adjustment. This association remained significant even after adjustment for confounding factors at baseline, i.e., age, sex, fasting plasma glucose, family history of diabetes, BMI, physical activity, current drinking, and current smoking (E/K genotype, adjusted OR 2.10 [95% CI

TABLE 2
Genotype and allele frequencies of the Kir6.2 E23K polymorphism according to change of glucose tolerance status in the follow-up study, 1988–2002

	Change of glucose tolerance status			
	NGT → NGT	NGT → IFG	NGT → IGT	NGT → diabetes
E23K genotype				
E/E	259 (0.44)	41 (0.46)	95 (0.42)	19 (0.26)
E/K	260 (0.44)	35 (0.39)	104 (0.46)	43 (0.59)
K/K	66 (0.11)	14 (0.16)	29 (0.13)	11 (0.15)
P vs. NGT		0.41	0.74	0.01
E23K allele				
E	778 (0.66)	117 (0.65)	294 (0.64)	81 (0.55)
K	392 (0.34)	63 (0.35)	162 (0.36)	65 (0.45)
P vs. NGT		0.69	0.43	0.008

Data are *n* (frequency).

1.16–3.83]; K/K genotype, 2.40, [1.01–5.70]; *P* for trend = 0.01). A similar result was revealed in the allele frequency model; the risk for conversion to diabetes was higher in subjects with the minor K-allele than in those with the major E-allele (1.58 [1.09–2.30], $P = 0.01$). The likelihood ratio in the multivariate-adjusted model that included the above-mentioned confounding risk factors and the E23K polymorphism was significantly higher than that in the model with only the confounding factors (genotypes, $P = 0.02$; alleles, $P = 0.01$). Furthermore, the multivariate-adjusted OR of the dominant model in which the E/K and K/K genotypes were combined was 2.16 (95% CI 1.21–3.85) compared with the E/E genotype. When estimating the PAR percentage, 40.1% (10.8–62.2) of the progression to diabetes among NGT subjects was attributable to these dominant genotypes.

Furthermore, we investigated the association between the E23K polymorphism and the risk of diabetes in the total subject group in 2002 (Table 4). The genotype and allele frequencies of the E23K polymorphism in subjects with IFG and IGT were not significantly different from those in the subjects with NGT, while the frequencies of the K/K genotype or K-allele were significantly higher in those with diabetes than in those with NGT (genotypes, $P = 0.006$; alleles, $P = 0.001$).

DISCUSSION

In our follow-up and large cross-sectional studies, we showed a positive association between the Kir6.2 E23K polymorphism and type 2 diabetes. These associations remained significant even after adjusting for other confounding factors. The likelihood ratio was significantly higher in the multivariate-adjusted model with the polymorphism than in that without. These findings suggest that the E23K polymorphism is a significant predictor of future diabetes in the general Japanese population.

Several case-control studies indicated that the E23K polymorphism is a risk factor for type 2 diabetes (8–12), but this association was not observed in cohort studies—two Finnish prospective studies showed that the E23K polymorphism had no effect on the development of diabetes (18,19). The present study is the first to indicate an association between the E23K polymorphism and diabetes using a follow-up design. Our study group consisted of exclusively Japanese subjects, with no population stratifications (20), and their glucose tolerance levels were determined in principle using an OGTT. This study design provided us an opportunity to precisely examine the ability of the E23K polymorphism to predict diabetes.

In our follow-up study, the PAR percentage for progression to diabetes was 40.1% in the NGT subjects, which was higher than that reported in previous studies of this gene (8,12). This difference seems to be attributable to the fact that we used control subjects in whom NGT was maintained, as confirmed by the OGTT, over a 14-year interval. However, since our result was based on a small number of subjects, it is better to confirm this value in other large populations.

The present study is the first to show a significant association between the E23K polymorphism and diabetes in Japanese. Recently, it was reported that this polymorphism was not associated with diabetes in Japanese indi-

TABLE 3
Age- and sex-adjusted or multivariate-adjusted ORs and 95% CIs for the progression to diabetes from NGT by the Kir6.2 E23K genotype and allele in the follow-up study, 1988–2002

	E23K genotype				E23K allele		
	E/E	E/K	K/K	*P* value for trend	E	K	*P*
Subjects at risk (*n*)	278	303	77		859	457	
Cases of diabetes (*n*)	19	43	11		81	65	
Age- and sex-adjusted OR (95% CI)	1 (referent)	2.25 (1.28–3.97)	2.27 (1.03–5.02)	0.008	1 (referent)	1.59 (1.12–2.26)	0.009
Multivariate-adjusted OR (95% CI)	1 (referent)	2.10 (1.16–3.83)	2.40 (1.01–5.70)	0.01	1 (referent)	1.58 (1.09–2.30)	0.01

Multivariate adjustment was made for age, sex, fasting plasma glucose, family history of diabetes, BMI, physical activity, current drinking, and current smoking.

TABLE 4
Genotype and allele frequencies of the Kir6.2 E23K polymorphism according to glucose tolerance status in the cross-sectional study, 2002

	Glucose tolerance status			
	NGT	IFG	IGT	Diabetes
E23K genotype				
E/E	617 (0.43)	124 (0.44)	254 (0.42)	202 (0.37)
E/K	655 (0.46)	117 (0.42)	261 (0.44)	263 (0.48)
K/K	161 (0.11)	39 (0.14)	84 (0.14)	85 (0.15)
P vs. NGT		0.31	0.20	0.006
E23K allele				
E	1,889 (0.66)	365 (0.65)	769 (0.64)	667 (0.60)
K	977 (0.34)	195 (0.35)	429 (0.37)	433 (0.39)
P vs. NGT		0.73	0.29	0.001

Data are *n* (frequency).

viduals (13). In that study, subjects were recruited from university hospitals and the control subjects defined as subjects with A1C levels <5.6%. However, a group of subjects with A1C <5.6% could include individuals with diabetes, as well as IFG and IGT (21). Actually, the frequency of the minor K-allele in the control subjects of that report was significantly higher than that in our NGT subjects, although the K-allele frequencies in the respective groups of diabetic subjects were similar. The different criteria used to define the phenotype are partly responsible for these different outcomes.

Some limitations of our study must be mentioned. First, there is an overlap of subjects used in both the cross-sectional and follow-up analyses—34% of the subjects in the cross-sectional analysis were also enrolled in the follow-up analysis, and thus our results are not really replicated. Further investigations will be needed to confirm our results in other Asian populations. Second, a 75-g OGTT has low reproducibility (22). Some of the participants might have been categorized into different glucose tolerance levels after repeat testing. Nonetheless, any misclassification would be expected to weaken rather than strengthen the association found in this study. Thus, the true association may be stronger than that shown in our results. Third, subjects with type 1 diabetes may have been included in our study population. In a clinical study, 3–4% of the group of nonobese Japanese diabetic patients were positive for the GAD antibody (23). Immune abnormality in pancreatic β-cells is considered to lead to diabetes independently of the E23K polymorphism, suggesting that subjects with type 1 diabetes are distributed equally among the genotypes. Thus, this limitation does not seem to invalidate the association of the E23K polymorphism with the risk of type 2 diabetes.

In conclusion, we confirmed the association between the E23K polymorphism of the K_{ATP} channel subunit Kir6.2 and susceptibility to type 2 diabetes in a follow-up study and a large cross-sectional study in a general Japanese population. Considering that populations throughout the world have a high frequency of the E23K polymorphism, this polymorphism may be a pathogenic gene for diabetes worldwide.

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Prehypertension Increases the Risk for Renal Arteriosclerosis in Autopsies: The Hisayama Study

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ABSTRACT

Information regarding the association between prehypertension BP level and renal arteriosclerosis is limited. In 652 consecutive population-based autopsy samples without hypertension treatment before death, the relationship between the severity of renal arteriosclerosis and BP levels classified according to the criteria of the Seventh Report of the Joint National Committee on Prevention, Detection, Evaluation, and Treatment of High Blood Pressure was examined. The age- and gender-adjusted frequencies of renal arteriosclerosis linearly increased with elevating BP levels; both hypertensive and prehypertensive subjects had significantly higher frequencies of renal arteriosclerosis than subjects with normal BP (normal 11.9%; prehypertension 28.5%; stage 1 hypertension 32.9%; stage 2 hypertension 58.2%; all $P < 0.01$ *versus* normal). In a logistic regression model, prehypertension was significantly associated with renal arteriosclerosis after adjustment for other cardiovascular risk factors (prehypertension multivariate-adjusted odds ratio [mOR] 5.99 [95% confidence interval (CI) 2.20 to 15.97]; stage 1 hypertension mOR 6.99 [95% CI 2.61 to 18.72]; stage 2 hypertension mOR 22.21 [95% CI 8.35 to 59.08]). This significant association was observed for all renal arterial sizes. The similar association was also observed for arteriolar hyalinosis. When the subjects were divided into those with and those without target organ damage, the impact of prehypertension on renal arteriosclerosis was similar for both groups (subjects without target organ damage mOR 5.04 [95% CI 1.36 to 18.62]; subjects with target organ damage mOR 6.42 [95% CI 1.29 to 32.04]). These findings suggest that both hypertension and prehypertension are associated significantly with the severity of renal arteriosclerosis, regardless of the presence or absence of target organ damage.

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Hypertension has been recognized as one of the major risk factors for the development of ESRD.[1,2] Nephrosclerosis is characterized pathologically by focal or global glomerular sclerosis and renal arteriosclerosis and is frequently found in individuals with hypertension.[3–5] Meanwhile, several prospective studies have indicated that the impressive increase in the risk for cardiovascular disease or in the risk for progression to hypertension started at a BP level of ≥120/80 mmHg.[6–8] On the basis of these findings, the Seventh Report of the Joint National Committee on Prevention, Detection, Evaluation, and Treatment of High Blood Pressure (JNC-7) introduced a "prehypertension" category in which BP is 120 to 139/80 to 89 mmHg and for which health-promoting lifestyle modifications are recommended to prevent cardiovascular disease.[9]

A prospective population-based study of cardiovascular disease has been carried out since 1961 in

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the town of Hisayama on Kyushu Island in southern Japan. The most characteristic feature of the Hisayama Study is that the cause of death has been verified by autopsy for approximately 80% of deceased subjects in the study population.[10–13] Our previous autopsy reports of Hisayama residents showed that systolic BP (SBP) was closely related to the progression of glomerular sclerosis, renal arteriolar hyalinosis, and renal arteriosclerosis.[12,13] To our knowledge, the Honolulu Heart Program is the only other population-based study that has examined this issue, although the autopsy rate was not high (20.6%).[14,15] Their findings also suggested that diastolic BP (DBP) was an independent predictor of glomerular sclerosis, renal arteriolar hyalinosis, and renal arteriosclerosis.[14,15] However, the association between categorized BP levels and renal vascular changes was not assessed in these studies or in ours. In this study, we examined the relationship between BP levels and renal arteriosclerosis, focusing on prehypertension, in population-based autopsy samples of the Hisayama Study, taking into account other cardiovascular risk factors as well as renal artery size.

RESULTS

The baseline characteristics of the 652 autopsy subjects are represented according to the BP levels in Table 1. The subjects with stage 1 or stage 2 hypertension were older than those with normal BP. The proportions of women gradually increased with elevating BP levels. The mean GFR values decreased significantly in stage 2 hypertension relative to normal BP level, whereas serum creatinine levels did not change across BP levels. The frequencies of proteinuria and history of cardiovascular disease were significantly higher in subjects with stage 2 hypertension, and that of electrocardiogram (ECG) abnormalities increased linearly with elevating BP levels. The mean values of total cholesterol were significantly higher in subjects with hypertensive or prehypertensive BP levels, whereas the frequency of glucose intolerance and the mean value of body mass index (BMI) did not change across BP levels. The frequency of current smoking decreased gradually with elevating BP levels, but no such tendency was observed for the frequency of alcohol intake.

Figure 1 presents the age- and gender-adjusted frequencies of renal arteriosclerosis, arteriolar hyalinosis, and glomerular sclerosis according to BP classification. The frequencies of renal arteriosclerosis and arteriolar hyalinosis linearly increased with elevating BP levels; not only hypertensive subjects but also prehypertensive subjects had a significantly higher frequency of renal arteriosclerosis and arteriolar hyalinosis compared with subjects with normal BP. Likewise, the age- and gender-adjusted mean values of the wall-lumen ratio of renal arteries decreased linearly (normal 5.10; prehypertension 4.16; stage 1 hypertension 3.96; stage 2 hypertension 3.47; all $P < 0.01$ *versus* normal), and those of the arteriolar hyalinosis index increased gradually with elevating BP levels (normal 1.21; prehypertension 1.29 [$P < 0.05$ *versus* normal]; stage 1 hypertension 1.29 [$P < 0.05$]; stage 2 hypertension 1.38 [$P < 0.01$]). The severity of glomerular sclerosis increased significantly in only stage 2 hypertension. The age- and gender-adjusted odds ratios (OR) of renal arteriosclerosis and arteriolar hyalinosis were significantly higher in prehypertension subjects and in hypertension subjects than in normal ones (Table 2). This association remained substantially unchanged even after adjustment for age at death, gender, total cholesterol, glucose intolerance, BMI, smoking habits, and alcohol intake. Furthermore, we di-

Table 1. Mean values or frequencies of potential risk factors and laboratory variables according to BP classification for 652 autopsy subjects[a]

Variables	BP Classification			
	Normal (n = 106)	Prehypertension (n = 172)	Stage 1 HT (n = 176)	Stage 2 HT (n = 198)
Age at death (yr)	70 ± 12	72 ± 13	75 ± 12[b]	79 ± 11[b]
Women (%)	37.7	40.7	43.2	52.5[c]
SBP (mmHg)	109 ± 8	129 ± 6[b]	148 ± 7[b]	179 ± 18[b]
DBP (mmHg)	66 ± 7	73 ± 10[b]	80 ± 11[b]	91 ± 13[b]
GFR (ml/min per 1.73 m^2)	77.1 ± 15.8	75.5 ± 18.8	76.3 ± 20.6	70.2 ± 19.5[c]
Serum creatinine (μmol/L)	83.5 (57.3 to 121.8)	85.4 (54.7 to 133.4)	84.0 (51.5 to 137.2)	88.2 (49.0 to 158.7)
Proteinuria (%)	9.2	6.8	10.8	23.4[b]
History of cardiovascular disease (%)	4.7	11.6	9.1	12.1[c]
Electrocardiogram abnormalities (%)	6.8	15.4[c]	25.7[b]	40.8[b]
Total cholesterol (mmol/L)	4.25 ± 1.07	4.67 ± 1.07[b]	4.60 ± 1.22[c]	4.63 ± 1.10[c]
Glucose intolerance (%)	13.2	21.5	18.2	22.2
BMI (kg/m^2)	20.2 ± 2.8	20.7 ± 3.2	20.7 ± 3.3	20.7 ± 3.1
Smoking habits (%)	47.6	41.3	39.7	35.4[c]
Alcohol intake (%)	24.0	32.6	31.2	30.6

[a]Data are means ± SD or percentage. GFR determined by Modification of Diet in Renal Disease Study Group formula. Glomerular filtration rate and serum creatinine were measured in 442 subjects who died after 1977. Geometric mean values and 95% confidence intervals (CI) of serum creatinine are shown because of the skewed distribution. BMI, body mass index; DBP, diastolic BP; HT, hypertension; SBP, systolic BP.
[b]$P < 0.01$, [c]$P < 0.05$ *versus* normal.

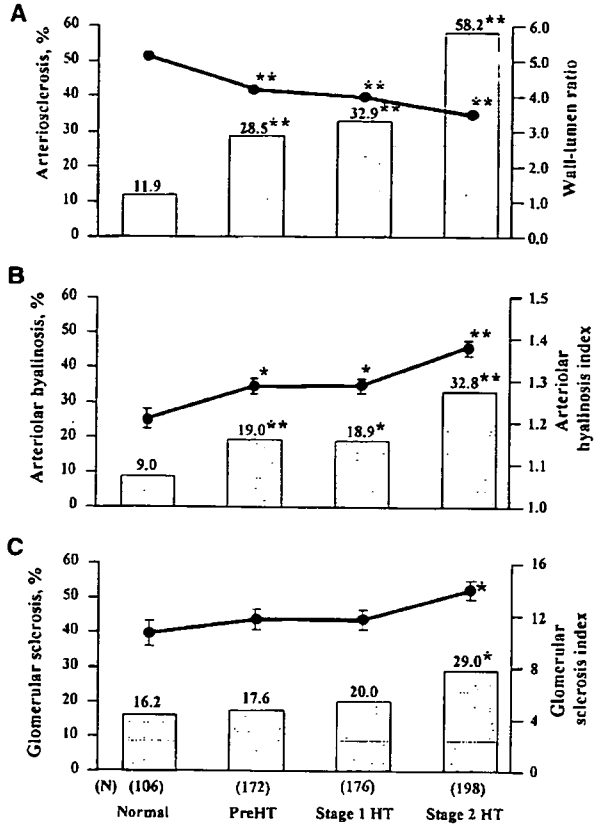

Figure 1. Age- and gender-adjusted frequencies of renal arteriosclerosis (A), arteriolar hyalinosis (B), and glomerular sclerosis (C) according to BP classification among 652 autopsy subjects. Solid lines indicate age- and gender-adjusted mean values of wall-lumen ratio, arteriolar hyalinosis index, and glomerular sclerosis, respectively. PreHT, prehypertension; HT, hypertension. *P < 0.05, **P < 0.01 *versus* normal.

vided prehypertension into two subcategories—BP 120 to 129/80 to 84 mmHg and BP 130 to 139/85 to 89 mmHg—and examined the association between BP level and renal arteriosclerosis. As a result, the risk for having renal arteriosclerosis was significantly increased in both BP subcategories even after adjustment for the previously mentioned cardiovascular risk factors (BP 120 to 129/80 to 84 mmHg OR 5.93 [95% confidence interval (CI) 2.08 to 16.91; $P < 0.01$]; BP 130 to 139/85 to 89 mmHg OR 5.92 [95% CI 2.02 to 17.29; $P < 0.01$]).

We examined whether the association between BP and renal arteriosclerosis differs by the presence or absence of target organ damage. As shown in Figure 2A, the age- and gender-adjusted frequencies of renal arteriosclerosis were higher in the group with target organ damage than in the group without it, regardless of BP level. In both groups, however, the frequencies of renal arteriosclerosis increased significantly with elevating BP levels; the difference was significant between the normal and both the prehypertension and hypertension categories. Likewise, the mean values of the wall-lumen ratio of renal arteries were significantly lower in BP levels of prehypertension and hypertension than in normal BP level for both the target-organ-damaged and target-organ-undamaged groups (Figure 2B). As shown in Table 3, the impact of prehypertension on renal arteriosclerosis was similar for both the damaged and undamaged groups after adjustment for the previously mentioned cardiovascular risk factors (without target organ damage OR 5.04 [95% CI 1.36 to 18.62; $P < 0.05$]; with target organ damage OR 6.42 [95% CI 1.29 to 32.04; $P < 0.05$]).

Finally, we examined the associations between BP levels and renal arteriosclerosis by the size of renal arteries using logistic regression analysis (Table 4). After adjustment for the previously mentioned cardiovascular risk factors, both prehypertension and hypertension significantly increased the risk for having renal arteriosclerosis in all arterial sizes. For smaller arteries (<300 μm), the risk for arteriosclerosis significantly and linearly increased with elevating BP levels, whereas this linear association was diminished for larger arteries (≥300 μm).

DISCUSSION

In this population-based autopsy survey, we histopathologically examined the relationship between categorized BP levels classified according to the JNC-7 criteria and renal arteriosclerosis. The results showed that both hypertension and prehypertension were associated significantly with renal arteriosclerosis, without regard for the presence or absence of target organ damage or for the size of intrarenal arteries. The relationships between BP and renal histopathologic changes also have been reported in the several biopsy-based studies of living subjects. Lhotta *et al.*[16] showed in patients who underwent biopsy that SBP was associated significantly with the frequencies of glomerular sclerosis and arteriolar hyalinosis. According to the study for patients with biopsy-proven IgA nephropathy, patients with hypertension, defined as BP ≥140/90 mmHg, had more severe glomerular sclerosis, interstitial fibrosis/tubular atrophy, interstitial infiltration, and atherosclerosis compared with those without hypertension.[17] In a similar biopsy study for IgA nephropathy, prehypertension (BP 120 to 139/80 to 89 mmHg) was associated significantly with the severity of mesangial proliferation and arteriolar changes, including intimal thickening, intimal duplication or hyalinosis, but not glomerular sclerosis.[18] These findings are in accordance with those of our study.

Several recent reports have shown that the risk for the development of cardiovascular disease or the risk for the progression to hypertension initiates an increase in BP levels of ≥120/80 mmHg. A meta-analysis of individual data for 1 million adults in 61 prospective studies indicated that the mortality from both ischemic heart disease and stroke increased progressively and linearly from BP levels as low as SBP of 115 mmHg and DBP of 75 mmHg in middle and old age.[6] In addi-

Table 2. Age- and gender-adjusted or multivariate-adjusted OR for renal arteriosclerosis, arteriolar hyalinosis, and glomerular sclerosis according to BP classification among 652 autopsy subjects

Parameter	BP Classification			
	Normal	Prehypertension	Stage 1 HT	Stage 2 HT
Arteriosclerosis				
age and gender adjusted				
OR[b]	1.00	4.21[d]	4.97[d]	16.57[d]
CI	Reference	1.85 to 9.60	2.20 to 11.21	7.41 to 37.05
multivariate adjusted				
OR[c]	1.00	5.99[d]	6.99[d]	22.21[d]
CI	Reference	2.20 to 15.97	2.61 to 18.72	8.35 to 59.08
Arteriolar hyalinosis				
age and gender adjusted				
OR[b]	1.00	2.70[e]	2.59[e]	5.84[d]
CI	Reference	1.19 to 6.13	1.14 to 5.89	2.65 to 12.88
multivariate adjusted				
OR[c]	1.00	2.36[e]	2.19	5.42[d]
CI	Reference	1.01 to 5.50	0.93 to 5.16	2.37 to 12.38
Glomerular sclerosis				
age and gender adjusted				
OR[b]	1.00	1.03	1.24	2.06[e]
CI	Reference	0.50 to 2.12	0.62 to 2.49	1.06 to 4.02
multivariate adjusted				
OR[c]	1.00	1.01	1.21	2.21[e]
CI	Reference	0.46 to 2.21	0.56 to 2.61	1.06 to 4.64

[a]OR odds ratio.
[b]Adjusted for age at death and gender.
[c]Adjusted for age at death, gender, total cholesterol, glucose intolerance, BMI, smoking habit, and alcohol intake.
[d]$P < 0.01$, [e]$P < 0.05$ *versus* normal.

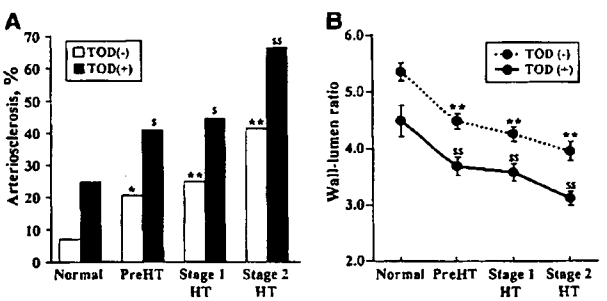

Figure 2. Age- and gender-adjusted frequencies of renal arteriosclerosis (A) and mean values of wall-lumen ratio of the renal arteries (B) according to BP classification by the presence or absence of target organ damage among 652 autopsy subjects. TOD, target organ damage; $^{*}P < 0.05$, $^{**}P < 0.01$ *versus* normal in target organ damage (−); $^{\$}P < 0.05$, $^{\$\$}P < 0.01$ *versus* normal in target organ damage (+).

tion, longitudinal data that were obtained from the Framingham Heart Study indicated that high-normal BP and normal BP, defined by JNC-6, were associated with the occurrence of cardiovascular disease.[7] Moreover, according to the randomized, controlled trial conducted in the Modification of Diet in Renal Disease (MDRD) study, low target BP (mean BP <92 mmHg, equivalent to a BP <125/75 mmHg) reduced the risk for developing kidney failure by approximately 30% compared with the usual target BP (mean BP <107 mmHg, equivalent to a BP <140/90 mmHg).[19] Our findings also showed that prehypertension levels were significantly associated with renal arteriosclerosis and arteriolar hyalinosis. It may be reasonable to suppose that prehypertension promotes systemic arteriosclerosis including renal vascular changes and causes cardiovascular disease and renal dysfunction.

It is possible that prehypertension is not the cause of renal arteriosclerosis but the result of renal vascular changes or organ damages by other cardiovascular risk factors. In this study, however, prehypertension was clearly associated with renal arteriosclerosis, regardless of the presence or absence of target organ damage, and this association was significant even after adjustment for other cardiovascular risk factors. This suggests that a slight increase in BP to prehypertension levels was associated independently with the severity of renal arteriosclerosis. Therefore, it is possible that antihypertensive treatment with BP-lowering <120/80 mmHg prevents the progression of renal arteriosclerosis, regardless of the presence or absence of target organ damage.

In our study, the relationship between BP levels and renal arteriosclerosis differed somewhat according to the size of renal arteries; the risk for renal arteriosclerosis increased significantly and linearly with elevating BP levels in smaller arteries (<300 μm), including arterioles, whereas this phenomenon was diminished in larger arteries (≥300 μm). Instead, the impact of total cholesterol levels was reinforced with elevating renal arterial size in our subjects (data not shown). In autopsy

Table 3. Multivariate-adjusted OR for renal arteriosclerosis according to BP classification by the presence or absence of target organ damage.

Parameter	BP Classification			
	Normal	Prehypertension	Stage 1 HT	Stage 2 HT
Target organ damage (−)[a]				
population at risk	83	109	103	70
OR[b]	1.00	5.04[c]	6.05[d]	18.81[d]
95% CI	Reference	1.36 to 18.62	1.65 to 22.20	4.98 to 71.01
Target organ damage (+)[a]				
population at risk	23	63	73	128
OR[b]	1.00	6.42[c]	7.21[c]	18.02[d]
95% CI	Reference	1.29 to 32.04	1.46 to 35.65	3.75 to 86.47

[a]Target organ damage was defined as the presence of preexisting cardiovascular disease, electrocardiogram abnormalities, proteinuria, or GFR <60 ml/min per 1.73 m^2.
[b]Adjusted for age at death, gender, total cholesterol, glucose intolerance, BMI, smoking habits, and alcohol intake.
[c]$P < 0.05$, [d]$P < 0.01$ *versus* normal.

Table 4. Multivariate-adjusted OR for renal arteriosclerosis according to BP classification by the size of renal arteries among 652 autopsy subjects

Size of Renal Arteries (μm)	BP Classification			
	Normal	Prehypertension	Stage 1 HT	Stage 2 HT
60 to 149				
OR[a]	1.00	4.01[b]	4.13[b]	12.00[b]
95% CI	Reference	1.59 to 10.10	1.64 to 10.39	4.84 to 29.68
150 to 299				
OR[a]	1.00	2.39[c]	4.27[b]	9.94[b]
95% CI	Reference	1.13 to 5.07	2.05 to 8.89	4.77 to 20.72
300 to 499				
OR[a]	1.00	4.21[b]	2.73[c]	6.21[b]
95% CI	Reference	1.68 to 10.60	1.06 to 6.99	2.49 to 15.47
≥500				
OR[a]	1.00	3.80[c]	2.59	3.08
95% CI	Reference	1.09 to 13.30	0.72 to 9.34	0.86 to 11.04

[a]Adjusted for age at death, gender, total cholesterol, glucose intolerance, BMI, smoking habits, and alcohol intake.
[b]$P < 0.01$, [c]$P < 0.05$ *versus* normal.

findings from the Honolulu Heart Program, BP was associated strongly with the intimal thickness of renal arteries with an outer diameter of 80 to 300 μm, but there were no correlations between the intimal thickness of these renal arteries and other cardiovascular risk factors, such as total cholesterol, triglycerides, blood glucose, and smoking.[14] It is feasible to speculate that the degree of the atherogenic effects of risk factors varies according to artery size and that hypertension affects small arteries notably.

Several limitations of our study should be discussed. First, our findings might be biased by the exclusion of 187 subjects who were taking antihypertensive medications. The mean values of SBP, DBP, serum creatinine, and total cholesterol and the frequencies of proteinuria, ECG abnormalities, glucose intolerance, and history of cardiovascular disease were significantly higher and the mean values of wall-lumen ratio were significantly lower in subjects who were taking antihypertensive medications than in the 652 subjects without antihypertensive medications in the present study. This bias has the potential to underestimate the impact of hypertension or other cardiovascular risk factors on renal arteriosclerosis. However, it is unlikely that this bias affects the association between prehypertension and renal arteriosclerosis, because prehypertensive subjects did not use antihypertensive medication. Second, only a single BP measurement was obtained at the baseline examination in the recumbent position. This imperfect measurement of BP might have resulted in a misclassification of our study subjects into different BP categories and a consequent dilution of our estimates of BP's impact on renal arteriosclerosis. Third, this is a cross-sectional study. Therefore, it is difficult to infer causality between prehypertension and risk for progression of renal arteriosclerosis, because it may be presumed that BP increased as a result of renal ischemia by preexisting renal arteriosclerosis, acting mainly through the renin-angiotensin system. In any case, our findings suggest that subjects with prehypertension should be considered as those with more progressive renal arteriosclerosis. Fourth, several variables used in this study were less accurate. We used the MDRD equation to estimate GFR; this formula is notoriously inaccurate in patients with normal kidney function. Proteinuria was established as 1+ or more on the dipstick; this would have missed all subjects with microalbuminuria. In addition,

the definition of glucose tolerance varied depending on when the examination was done. These facts might lead to the misclassification of a normal subgroup without any risk factor and affect the cutoff value of each histologic parameter. However, it seems to be unlikely that this limitation distorted the associations between BP levels and severity of renal arteriosclerosis, because BP levels showed the dosage-dependent association with the continuous values of wall-lumen ratio. Finally, this study is based on autopsy and the proportion of aged people is extremely high. Therefore, its findings cannot be applied to the overall living population. However, we believe that our findings provide useful information toward a better understanding of the pathogenesis of renal arteriosclerosis.

CONCLUSION

Prehypertension level classified by JNC-7 was associated significantly with the severity of renal arteriosclerosis. Therefore, prehypertensive individuals should be considered a high-risk population, regardless of the presence or absence of target organ damage. Our findings emphasize the need to determine whether the lowering of goal BP in hypertension management can prevent the progression of renal and systemic arteriosclerosis.

CONCISE METHODS

Study Population

The population of the town of Hisayama is approximately 7500, and data from the national census show it to be representative of Japan as a whole.[10,11] The study design and characteristics of the subject population have been described in detail elsewhere.[12,13] Briefly, from January 1962 to December 1994, a total of 1742 Hisayama residents of all age groups died, 1394 (80.0%) of whom underwent autopsy. The autopsy rate was not different between men (78.7%) and women (81.6%). Among these consecutive autopsy subjects, 1168 participated in at least one of the six health examinations conducted in 1961, 1967, 1974, 1978, 1983, and 1988. For every examination, the participation rate exceeded >80% of all Hisayama residents 40 yr or older. After exclusion of 98 subjects who lacked preserved renal tissues, 33 with degenerated or small renal tissues, 80 who underwent autopsy at other hospitals, 118 who had no health examination data within 7 yr before death, and 187 who had been treated with antihypertensive medications, 652 subjects (362 men and 290 women) were included in this study. The mean period from the most recent health examination to death was 3.6 ± 1.8 yr.

Morphologic Examination of Renal Tissue

The methods of morphologic examination of renal tissue have been described in detail elsewhere.[13] Briefly, for light microscopic study, paraffin-embedded renal tissues that were obtained by standard autopsy methods were cut at a 2-μm thickness and stained with periodic acid-Schiff reagent. The wall-lumen ratio was evaluated as the severity of arteriosclerosis by the method of Kernohan *et al.*[20] For each specimen, all arteries with an outer diameter >60 μm were examined using an eyepiece micrometer. The outer diameter and the lumen diameter of the least axis of the elliptic profile were directly measured. The wall-lumen ratio was calculated in each artery as lumen diameter/(outer diameter − lumen diameter)/2, and the mean value for all arteries in all subjects was used as the index of arteriosclerosis. We further classified all arteries into four categories according to the outer diameters of the renal arteries—60 to 149, 150 to 299, 300 to 499, and ≥500 μm—and calculated the mean values of the wall-lumen ratio by the previously mentioned categories.

The severity of arteriolar hyalinosis was assessed semiquantitatively by the method of Barder *et al.*[21] For each tissue specimen, 50 arterioles were examined and the severity of the lesion in each arteriole was graded from 1+ to 4+ according to the extent of arteriolar hyalinosis. The arteriolar hyalinosis index was calculated by the following formula: Arteriolar hyalinosis index = $(n_1 \times 1 + n_2 \times 2 + n_3 \times 3 + n_4 \times 4)/50$. Here, n_1, n_2, n_3, and n_4 indicate the number of arterioles showing hyalinosis scores of 1+ to 4+, respectively.

The semiquantitative score was used to evaluate the severity of glomerular sclerosis by the method of Raij *et al.*[22] For each tissue specimen, 100 glomeruli from the superficial to deep cortex were examined uniformly, and the severity of the lesion in each glomerulus was graded from 0 to 4+ according to the percentage of glomerular sclerosis. The glomerular sclerosis index was calculated by the following formula: Glomerular sclerosis index = $(n_0 \times 0 + n_1 \times 1 + n_2 \times 2 + n_3 \times 3 + n_4 \times 4)/4$. Here, n_0, n_1, n_2, n_3, and n_4 indicate the number of glomeruli showing sclerotic lesion scores of 0 to 4+, respectively.

Definition of Renal Arteriosclerosis, Arteriolar Hyalinosis, and Glomerular Sclerosis

To differentiate the effects of cardiovascular risk factors from age-related changes, we selected 103 subjects who had none of the following characteristics: Proteinuria, kidney failure, hypertension, glucose intolerance, or primary renal disease at autopsy. Using this subgroup, the cutoff limits were defined as below the 10th percentile or above the 90th percentile of each histologic parameter distribution; that is, renal arteriosclerosis, arteriolar hyalinosis, and glomerular sclerosis were defined as a wall-lumen ratio <3.37, an arteriolar hyalinosis index >1.44, and a glomerular sclerosis index >17.0, respectively. In the analysis by the size of renal arteries, furthermore, renal arteriosclerosis was defined as below the lower 10th percentile for mean values of the wall-lumen ratio by size (60 to 149 μm: wall-lumen ratio <3.56; 150 to 299 μm: wall-lumen ratio <2.65; 300 to 499 μm: wall-lumen ratio <2.64; ≥500 μm: wall-lumen ratio <2.44).

Risk Factors

BP was measured three times after a single rest period of at least 5 min using a standard mercury sphygmomanometer with the subject in the recumbent position. The mean of the three measurements was used for the analysis. BP levels were categorized according to the criteria recommended by JNC-7[9] (normal: SBP <120 mmHg and DBP <80 mmHg; prehypertension: SBP 120 to 139 mmHg or DBP 80 to 89 mmHg; stage 1 hypertension: SBP 140 to 159 mmHg or DBP 90 to 99

mmHg; stage 2 hypertension: SBP ≥160 mmHg or DBP ≥100 mmHg).

Glucose intolerance was defined by an oral glucose tolerance test in the subjects with glycosuria in 1961 and 1967; by fasting and postprandial glucose concentrations in 1974, 1978, and 1983; and by a 75-g oral glucose tolerance test in 1988, in addition to medical history of diabetes. ECG abnormalities were defined as Minnesota codes 3-1 and/or 4-1, -2, -3. Serum total cholesterol levels were measured by the Zak-Henly method with a modification by Yoshikawa in 1961 and 1967, by the Zurkowski method in 1974, and by the enzymatic method after 1978. Serum creatinine concentration was measured by the Jaffe method after 1974, and GFR was calculated by the MDRD Study Group formula.[23] Freshly voided urine samples were tested by the sulfosalicylic acid method in 1961 and 1967 and by the dipstick method after 1974. Proteinuria was defined as 1+ or more. Body height and weight were measured in light clothing without shoes, and the BMI (kg/m^2) was calculated. Information on antihypertensive medication, alcohol intake, and smoking habits was obtained through a standard questionnaire and classified as current habitual use or a lack thereof. All available information about potential cardiovascular diseases, including stroke, myocardial infarction, and coronary intervention, was gathered and reviewed by a panel of physician members of the Hisayama Study to determine the occurrence of cardiovascular disease under the standard criteria. A history of cardiovascular disease was determined on the basis of this information. Target organ damage was defined as the presence of ECG abnormalities, proteinuria, GFR <60 ml/min per 1.73 m^2, or a history of cardiovascular disease.

Statistical Analyses

SAS software (SAS Institute, Cary, NC) was used to perform all statistical analyses. The crude or age- and gender-adjusted mean values and frequencies of variables were compared among BP levels using Dunnett *t* test or logistic regression analysis as appropriate. The age- and gender-adjusted or multivariate-adjusted OR and 95% CI were calculated by a logistic regression analysis. $P < 0.05$ was considered statistically significant in all analyses.

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DISCLOSURES

None.

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Functional SNP in an Sp1-binding site of *AGTRL1* gene is associated with susceptibility to brain infarction

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Brain infarction is one of the common causes of death and also a major cause of severe disability. To identify a gene(s) susceptible to brain infarction, we performed a large-scale association study of Japanese patients with brain infarction, using 52608 gene-based single nucleotide polymorphism (SNP) markers. Comparison of allele frequencies between 1112 cases with brain infarction and age- and sex-matched control subjects of the same number found an SNP in the 5′-flanking region of angiotensin receptor like-1 (*AGTRL1*) gene (rs9943582, −154G/A) to have a significant association with brain infarction [odds ratio = 1.30, 95% confidence interval (CI) = 1.14–1.47, $P = 0.000066$]. We also found the binding of Sp1 transcription factor to the region including the susceptible G allele, but not the non-susceptible A allele. Luciferase assay and RT–PCR analysis demonstrated that exogenously introduced Sp1 induced transcription of *AGTRL1* and its ligand, *apelin*, as well, indicating direct regulation of apelin/APJ pathway by Sp1. Furthermore, a 14 year follow-up cohort study in a Japanese community in Hisayama town, Japan revealed that the homozygote of the susceptible G allele of this particular SNP had significantly higher risk of brain infarction (hazard ratio = 2.00, 95% CI = 1.22–3.29, $P = 0.006$). Our results indicate that the SNP in the *AGTRL1* gene is associated with the susceptibility to brain infarction.

INTRODUCTION

Stroke is one of the leading causes of death as well as severe physical disability and cognitive dysfunction. In Japan, the mortality rate from stroke has decreased significantly in the last three decades as a result of improvement in medicine and public health, but the incidence of stroke remained still high, especially in the elderly (1). Since we are facing a rapid increase of the elderly population, prevention and better treatment of stroke are becoming more important. For such purpose, identification of genetic and environmental risk factors for stroke is one of the critical steps. Stroke is classified into three major types, namely brain infarction, brain haemorrhage and subarachnoid haemorrhage. Brain infarction is the most common type of stroke and usually occurs because of atherosclerosis of small or large arteries in the brain or because of thromboembolism developed in the heart. Hypertension, diabetes, dyslipidaemia and smoking are well-known risk factors for brain infarction (2,3). In addition, case–control and cohort studies have indicated that a positive family history is a risk factor for brain infarction (4), suggesting involvement of genetic components in the aetiology. However, genes susceptible to brain infarction are still not well understood.

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To clarify genetic factors that increase the risk of brain infarction, a number of candidate genes involved in haemostasis, renin angiotensin system and lipid metabolism have been investigated, but their associations with brain infarction are still controversial (5). Recently, genome-wide approach has been applied to screen genes that were involved in complex traits, and two novel genes, *PDE4D* and *ALOX5AP*, were identified to be associated with brain infarction in the Icelandic population (6,7). Through a large-scale case–control association study in a Japanese population, using 52608 gene-based single nucleotide polymorphism (SNP) markers, we recently reported non-synonymous SNP (Val374Ile) in protein kinase C-eta (*PRKCH*) to be associated with the susceptibility to brain infarction (8). In the present study, using the same approach, we demonstrate that a functional SNP in the 5′-flanking region (SNP30, rs9943582, −154G/A) of angiotensin receptor like-1 gene (*AGTRL1*) is significantly associated with brain infarction.

RESULTS

Case–control association study

To screen a gene(s) involved in susceptibility to brain infarction, we performed a large-scale association study using gene-based SNPs in a step-wise manner. We enrolled 1112 Japanese subjects with brain infarction as well as 1112 age- and sex-matched control subjects in this study. All case subjects were diagnosed by stroke neurologists on the basis of clinical information and brain imaging. First, we genotyped 52608 gene-based tag-SNPs selected from JSNP database (9), using 188 cases and 188 controls, and identified 1098 SNPs that revealed *P*-values of 0.01 or smaller in a comparison of allele frequencies. These 1098 SNPs were further genotyped for the remaining 924 cases and 924 controls. Among these SNPs, SNP32 (rs948847) showed a *P*-value of 0.0061 in the first-step screening, and a *P*-value of 0.0011 in the second screening. Statistical analysis of the combined samples indicated a significant association of SNP32 with brain infarction in the allele frequency model ($P = 0.000043$). This association remained significant after a permutation test for multiple testing ($P = 0.036$), suggesting that this SNP was a good candidate marker associated with brain infarction.

Linkage disequilibrium (LD) analysis of the data from 44 unrelated Japanese individuals in the International HapMap Project (10) revealed that the marker SNP32 was located in an LD block spanning 230 kb on chromosome 11q12 (data not shown). This large LD block included five genes, *P2RX3*, *SSRP1*, *TNKS1BP1*, *AGTRL1* and *LRRC55*. To further define the region of interest, we selected 49 tag-SNPs near and within this LD block from the JSNP and the HapMap databases (Supplementary Material, Table S1) and genotyped these SNPs in 1112 cases and 1112 controls. We constructed a pairwise LD map on the basis of genotype data of 48 SNPs that had minor allele frequencies of 0.15 or higher in 1112 case subjects (Fig. 1A). According to Gabriel's criteria (11), the 230 kb LD block could be divided into six small blocks, and SNP32 was located in block 4. When we compared the allele frequencies of 49 genotyped SNPs, we found that SNP25 in block 3 showed the strongest association with brain infarction ($P = 0.000011$). Among the 10 SNPs in block 3 (SNP16 to SNP25), six SNPs (SNP20–SNP25), including SNP25, were absolutely linked ($D' \geq 0.99$, $r^2 \geq 0.96$) and located in the intergenic region between the two genes, *TNKS1BP1* and *AGTRL1*, where no putative gene was predicted by the GENSCAN program. Other four SNPs in block 3 were located in the *TNKS1BP1* gene (SNP16–SNP18) or in its 3′-flanking region (SNP19), but their associations with brain infarction were less significant ($P = 0.0017$–0.0058). Therefore, we excluded these SNPs for further functional analysis and focused on SNPs in block 4, where only *AGTRL1* gene is present. Since we previously identified 10 SNPs (SNP27–SNP36) in the *AGTRL1* locus by direct sequencing of a region from 2 kb upstream to the last exon, using 48 Japanese individuals (Fig. 2A) (12), we additionally genotyped these SNPs for our case–control subjects (Table 1). Among 11 SNPs in block 4, SNP29 showed the strongest association with brain infarction ($P = 0.000037$) when we compared allele frequencies between cases and controls (Fig. 1B and Table 1). A detailed LD analysis indicated that five SNPs in block 4 (SNP26, SNP28, SNP29, SNP30 and SNP32) were almost absolutely linked to each other ($D' \geq 0.96$, $r^2 \geq 0.87$) and showed similar *P*-values with brain infarction as that of SNP29. Of these five SNPs, four SNPs (SNP28, SNP29, SNP30 and SNP32) were located in the *AGTRL1* gene. APJ, the product of *AGTRL1*, is a G protein-coupled receptor and had been reported to be expressed in the cardiovascular and the central nervous system (13–15). Apelin (*APLN*), the endogenous ligand of APJ, had been reported to have some function in the control of blood pressure (16,17). Therefore, we considered that *AGTRL1* might be a good candidate gene related to brain infarction.

Expression of *AGTRL1* mRNA in human normal organs

We subsequently examined the expression of *AGTRL1* in normal human tissues by northern blot analysis and found a high level of a 3.8 kb *AGTRL1* transcript (V1 mRNA) and a less amount of a 1.8 kb transcript (V2 mRNA) in heart, placenta, spleen and spinal cord (Fig. 2B). These results indicated that *AGTRL1* was highly expressed in the cardiovascular system and the central nervous system consistent with the previous reports (13–15). Although two splicing variants of *AGTRL1* transcript were registered in the GenBank database of the National Center of Biotechnology Information (NCBI) (Fig. 2A), we considered the longer transcript (V1, 3.8 kb) to be the major transcript in normal human tissues.

Sp1 regulates transcription of *AGTRL1* at SNP30

Among the 10 SNPs genotyped in *AGTRL1*, four SNPs, three in the 5′-flanking region (SNP28–SNP30) and one in the coding region (SNP32), showed the strong association with brain infarction. Since SNP32 in the coding region was synonymous (Gly45Gly), we considered that one or a combination of these SNPs might have functional significance to alter the quantity of APJ, the gene product of *AGTRL1*.

We prepared ^{32}P-labelled oligonucleotide probes corresponding to each allele of these four candidate polymorphism