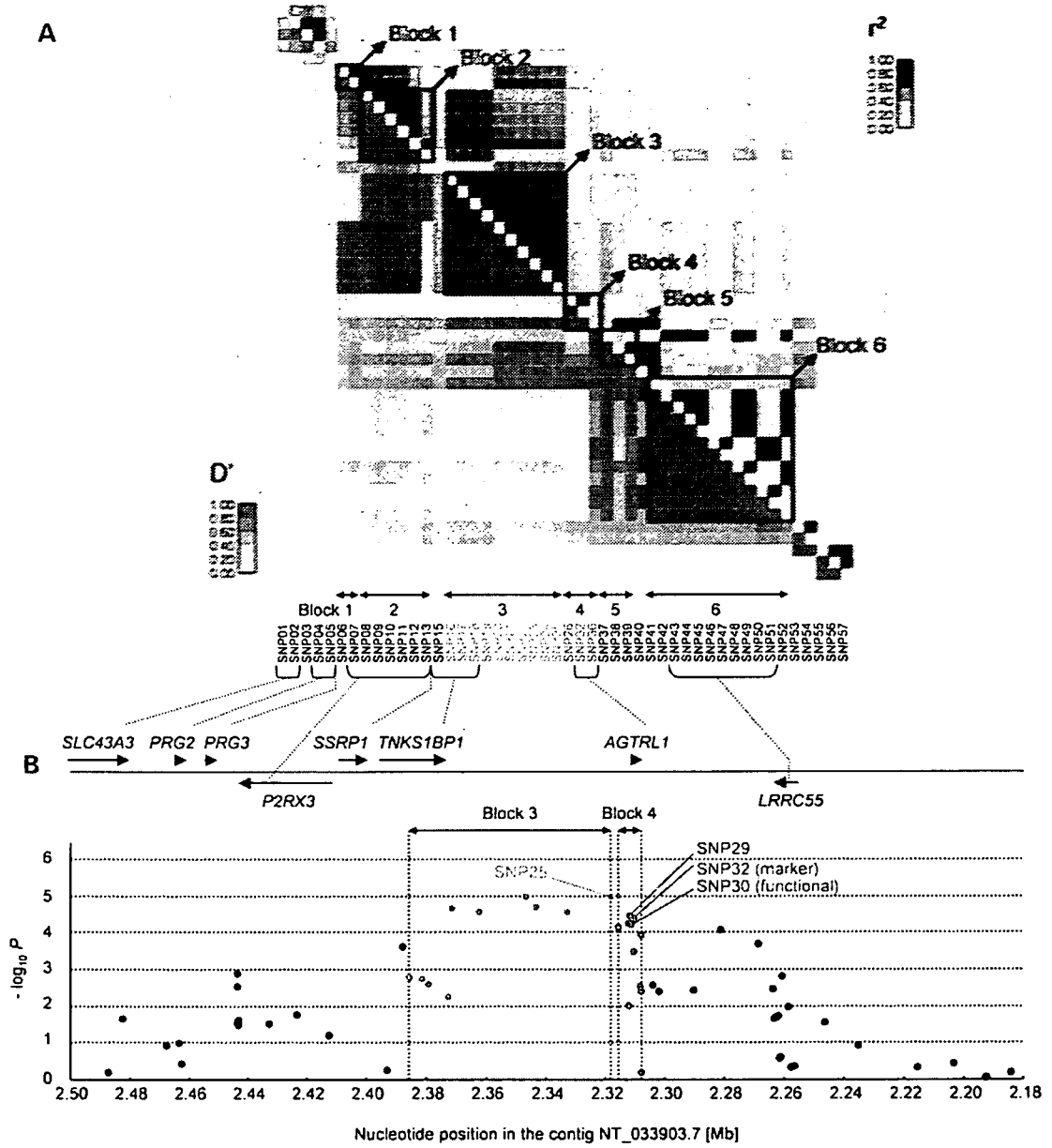

Figure 1. Case–control association study and LD map. (A) Pairwise LD map around *AGTRL1* gene, as measured by D' (lower left triangle) and r^2 (upper right triangle) in the case subjects with brain infarction. Among 49 genotyped SNPs, SNP14 was excluded because of minor allele frequency <15%. An LD block spanning 230 kb defined by the Japanese data from the HapMap Project (SNP06–SNP54) was divided into six blocks according to Gabriel's criteria (11). The marker SNP32 was located in block 4. (B) Case–control association plots for 57 genotyped SNPs. In each SNP, allele frequency between 1112 cases and 1112 control subjects was compared using chi-square test, and $-\log_{10} P$-values are plotted. Blue dots indicate six SNPs in block 3 that revealed the highest association and are located in the intergenic region between *TNKS1BP1* and *AGTRL1* (SNP20–SNP25). Green dots indicate other four SNPs in block 3 that showed less significant association and are located in *TNKS1BP1* gene (SNP16–SNP18) and in its 3′-flanking region (SNP19). Red dots indicate 11 SNPs in block 4 that are located in the intergenic region (SNP26) and in *AGTRL1* gene (SNP27–SNP36). All genotype data were evaluated Hardy–Weinberg equilibrium and no significant deviation ($P > 0.01$) was found.

loci and performed the gel-shift assay using nuclear extract of SBC-3 cells, in which *AGTRL1* was expressed abundantly. The gel-shift assay demonstrated a shifted band of a DNA–protein complex, with a very strong intensity in a lane corresponding to the G allele of SNP30 (rs9943582, −154G/A), whereas that to the A allele was very weak

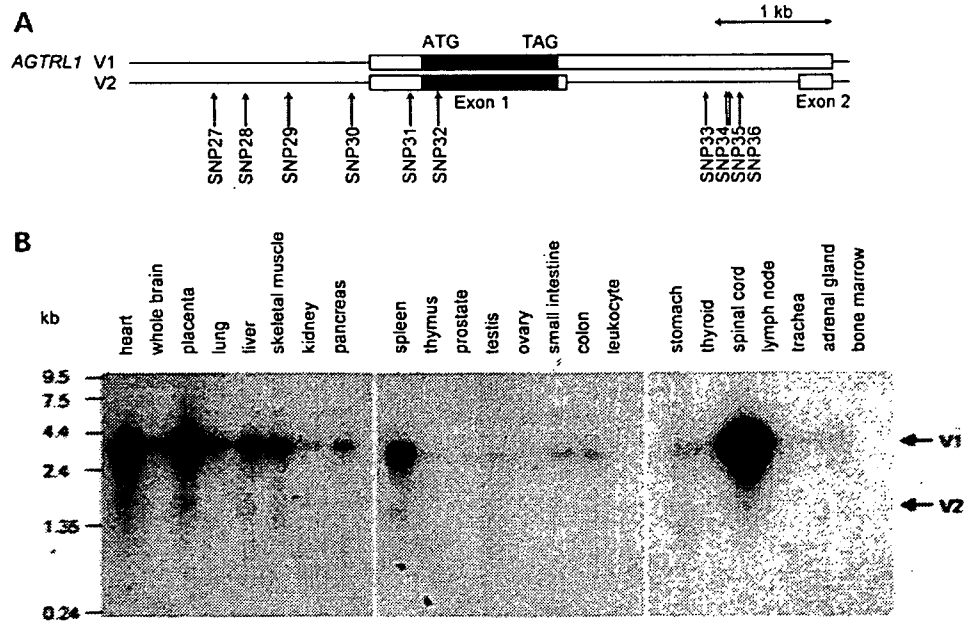

Figure 2. Genomic structure and expression of *AGTRL1* gene. (A) Alternative splicing variants and SNPs of *AGTRL1* gene (3.8 kb of V1, NM_005161.3 and 1.8 kb of V2, X89271.1 in GenBank database). ATG indicates the initiation codon. TAG indicates the stop codon. (B) Multiple tissue northern blotting for human normal tissues. Arrows show two splicing variants of *AGTRL1*.

Table 1. Case–control association study of 10 SNPs in *AGTRL1* gene

		Number of genotype								Comparison of allele frequency (Chi-square test)	
		Case				Control					
SNP ID	Allele 1/2	11	12	22	Total	11	12	22	Total	OR (95% CI)	*P*-value
SNP27	−1308T/A	9	133	964	1106	2	107	1001	1110	1.39 (1.08–1.79)	0.0099
SNP28	−1051C/T	576	447	85	1108	482	508	115	1105	1.30 (1.14–1.48)	0.000062
SNP29	−674G/A	577	445	84	1106	482	510	115	1107	1.31 (1.15–1.49)	0.000037
SNP30	−154G/A	574	448	85	1107	486	507	118	1111	1.30 (1.14–1.47)	0.000066
SNP31	+337G/A	641	400	67	1108	557	463	88	1108	1.28 (1.12–1.46)	0.00035
SNP32	+570A/C	551	455	99	1105	452	523	128	1103	1.30 (1.15–1.48)	0.000043
SNP33	+2803T/C	212	503	393	1108	145	543	423	1111	1.20 (1.06–1.35)	0.0031
SNP34	+2986C/T	499	483	125	1107	481	511	119	1111	1.03 (0.91–1.16)	0.67
SNP35	+3003G/A	243	517	348	1108	161	558	393	1112	1.26 (1.12–1.42)	0.00012
SNP36	+3088A/G	213	505	391	1109	147	541	417	1105	1.19 (1.06–1.34)	0.0044

Transcription-initiation site of *AGTRL1* gene (the first nucleotide of NM_005161.3) is denoted nucleotide + 1. All genotype data were evaluated Hardy–Weinberg equilibrium and no significant deviation was found ($P > 0.01$). 1, at-risk allele; 2, non-risk allele; OR, odds ratio.

(Fig. 3A); the G allele was considered to be the risk allele and the A allele to be the protective allele in the association study [Table 1, odds ratio = 1.30, 95% confidence interval (CI) = 1.14–1.47, $P = 0.000066$]. The competition assay with the unlabelled oligonucleotides demonstrated that the self (G allele) oligonucleotide inhibited DNA–protein complex formation in a dose-dependent manner but the non-self oligonucleotide (A allele) did not (Fig. 3B), suggesting that some nuclear protein(s) specifically bound to the DNA fragment corresponding to the G allele. The computer simulation using MATCH program based on TRANSFAC database indicated that Sp1 transcription factor was likely to bind to the G allele of SNP30 (Fig. 3C). Unlabelled Sp1-binding consensus oligonucleotide also inhibited the formation of the DNA–protein complex (Fig. 3B). Moreover, when we added anti-Sp1 antibody to the mixture, the band was further shifted to a higher molecular position, indicating the specific binding of the Sp1 protein to the at-risk G allele of SNP30 (Fig. 3B). We also carried out the chromatin immunoprecipitation (ChIP) assay, using HEK293T cells, which were found to be the heterozygote at SNP30. We transfected HEK293T cells with HA-tagged Sp1 expression vector (pCAGGS-Sp1-HA) and then DNA–protein complex were precipitated using anti-HA antibody. Subsequent PCR

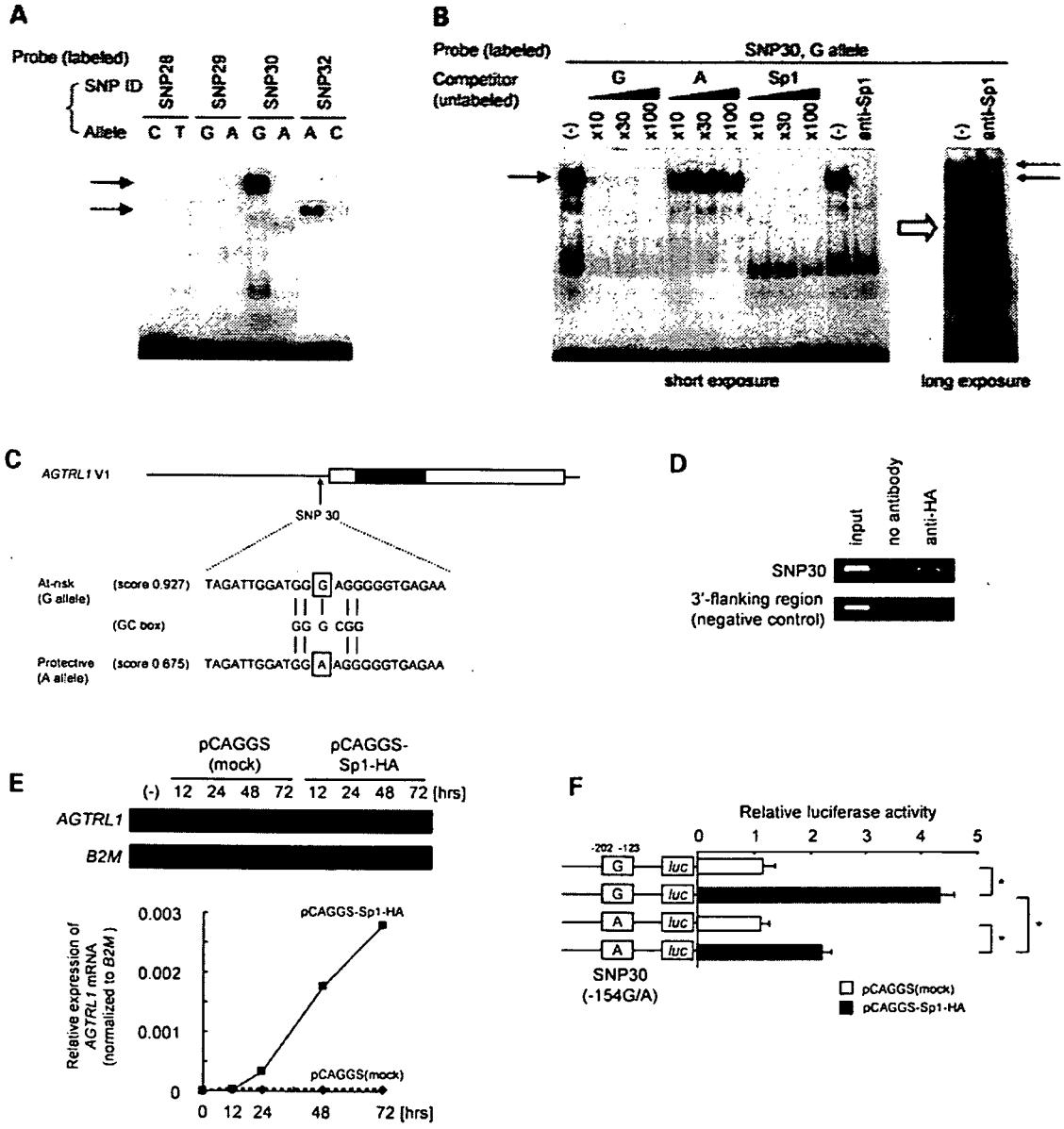

Figure 3. Sp1 regulates transcription of *AGTRL1* gene at the G allele of SNP30. (A) Gel-shift assay using end-labelled 25 bp probes around each allele of four SNPs of *AGTRL1* gene and nuclear extract of SBC-3 cells. A solid arrow indicates the shifted band that shows tighter binding of a nuclear factor to the G allele of SNP30 than to the A allele. A broken arrow indicates the shifted band that shows tighter binding of a nuclear factor to the A allele of SNP32 than to the C allele. (B) Gel-shift assay using end-labelled probes around the G allele of SNP30 and nuclear extract of SBC-3 cells. DNA–protein complex (solid arrow) was competed by unlabelled oligonucleotide with the G allele but not by oligonucleotide with the A allele. This complex was also strongly competed by unlabelled Sp1-consensus oligonucleotide. When we added anti-Sp1 antibody to the mixture, additional shifted band was observed in a long exposure image (broken arrow). (C) DNA sequences of oligonucleotide used in gel-shift assay. The sequence around the G allele of SNP30 is more similar to GC box (GGGCGG), the Sp1-consensus sequence, than the A allele. The core match scores were calculated using MATCH program. (D) ChIP, using formaldehyde-treated HEK293T cells that were ectopically expressed HA-tagged Sp1 protein. DNA was immunoprecipitated with (anti-HA) or without (no antibody) anti-HA antibody. DNA sample before precipitation was used as a control (input). For each sample, PCR for genomic fragment around SNP30 was performed. PCR for the 3′-flanking region of *AGTRL1* was also performed as a negative control that had no putative Sp1-binding site. (E) Exogenously introduced Sp1 induced *AGTRL1* mRNA. HEK293T cells were transfected with mock pCAGGS or HA-tagged Sp1 expression vector (pCAGGS-Sp1-HA). RNAs were extracted in various time spans, and semi-quantitative (upper) and real-time quantitative (lower) RT–PCR for *AGTRL1* and *B2M* mRNA were performed. (F) Luciferase assay. Eighty base pair fragments around each allele of SNP30 in the 5′-flanking region of *AGTRL1* were inserted into pGL3-basic vector. Luciferase assay was performed using U-2OS cells with co-transfection of mock pCAGGS or pCAGGS-Sp1-HA vector. Luciferase activity is indicated relative to the activity of pGL3-basic vector. Each sample was studied in triplicate and data shown are the mean ± SD (*$P < 0.01$).

experiments indicated that Sp1 bound to a genomic fragment corresponding to SNP30 *in vivo* (Fig. 3D). To evaluate allele-specific binding of Sp1 in the ChIP assay, we subcloned the PCR product from anti-HA ChIP sample into pCR2.1-TOPO vector and transformed *Escherichia coli* competent cells. Then, we genotyped SNP30 in 20 colonies of these transformed cells and found all colonies had the G allele, indicating the specific binding of Sp1 to the genomic region including the G allele, but not the A allele. Furthermore, we found that introduction of Sp1 expression vector to HEK293T cells remarkably induced transcription of *AGTRL1* mRNA (Fig. 3E).

To further evaluate the promoter or enhancer activity of the genomic region around this SNP, we performed a luciferase assay using U-2OS cells, which expressed Sp1 at a very low level (18). An 80 bp DNA fragment (−202/−123) including the SNP30 locus was inserted into the pGL3-basic vector. Exogenously introduced Sp1 enhanced the luciferase activity in the cells transfected with the reporter vector containing the G allele, but the enhancement was relatively low in the cells transfected with the A allele vector (Fig. 3F). These findings implied that Sp1 enhanced the transcription of *AGTRL1* through the binding to the G allele of SNP30. As Sp1 is abundantly expressed in multiple tissues, the subjects with the disease-susceptible G allele are expected to have higher expression of APJ and might result in the higher activity in the apelin/APJ-signalling pathway.

We also found a DNA–protein complex formation specific to the A-allele oligonucleotide of SNP32 (rs948847, +570A/C) (Fig. 3A), but no transcription factor was predicted to bind to a region corresponding to this SNP. We also constructed luciferase vector containing an 18 bp DNA fragment (+566/+583) corresponding to each allele of SNP32, but no difference in the reporter activities was found (data not shown).

The association of SNP30 with brain infarction susceptibility in the prospective cohort study

We then examined the effect of SNP30 on the incidence of brain infarction, using the data obtained from the population-based prospective cohort study in Hisayama town (1). During a 14 year follow-up period, 67 events of first-ever brain infarction were observed among 1659 subjects without a history of stroke at baseline examination. The 14 year cumulative incidence of brain infarction was 5.58% in the subjects with GG genotype and 2.79% in the other genotypes (GA and AA) (Fig. 4). Age- and sex-adjusted risk of brain infarction was significantly higher in the GG genotype than in the other genotypes (hazard ratio = 2.00, 95% CI = 1.22–3.29, $P = 0.006$).

Transcription of apelin, the ligand of APJ, was also activated by Sp1

Since MATCH program also predicted Sp1-binding motif (GC box) in the 5′-flanking region of *APLN* gene (Fig. 5A, −147/−142), we examined the effect of Sp1 on *APLN* transcription. Introduction of Sp1 expression vector to HEK293T cells significantly increased *APLN* mRNA

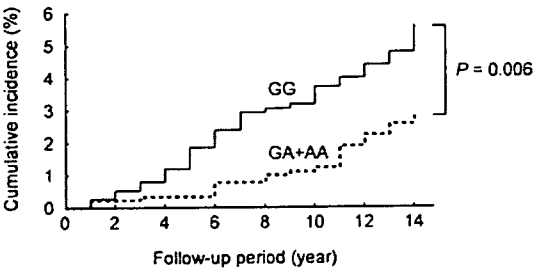

Figure 4. Kaplan–Meier estimates of incidence of brain infarction during 14 years of follow-up in the Hisayama study, stratified by the GG genotype and the other genotypes (GA and AA).

(Fig. 5B). Furthermore, we constructed a 468 bp DNA fragment (−336/+132) including this putative Sp1-binding site into the upstream of the luciferase gene. The reporter-gene assay by co-transfection of this clone with Sp1 expression vector in U-2OS cells indicated remarkable enhancement of the luciferase activity (Fig. 5C), indicating *APLN* was also a transcriptional target of Sp1. These results demonstrated that Sp1 is a key regulator of the apelin/APJ-signalling pathway.

DISCUSSION

In spite of the recent advances in medicine, stroke is still one of the leading causes of death as well as severe physical disability. A genome-wide approach is believed to be useful to find novel genes underlying complex traits. In fact, genetic variations in several genes enhancing susceptibility to common diseases such as myocardial infarction (19), IgA nephropathy (20,21), Crohn's disease (22) and osteoarthritis (23) were successfully identified. We recently reported that a non-synonymous SNP in *PRKCH* gene was associated with susceptibility to brain infarction through the large-scale gene-based SNP analysis (8). Using the same approach, the present study identified an SNP in the 5′-flanking region of the *AGTRL1* gene (SNP30, −154G/A) to be significantly associated with brain infarction. We demonstrated that the genomic fragment including the G allele of this particular SNP had much higher binding affinity to Sp1 transcription factor and higher enhancer activity than that of the A allele. It is also found that the subjects with homozygote of the G allele of SNP30 had higher incidence of brain infarction in the 14 year follow-up study of the population-based cohort. Furthermore, we showed that both *AGTRL1* and *APLN* genes were likely to be regulated by Sp1. From these results, it is reasonable to consider that the apelin/APJ-signalling pathway might be highly activated in the subjects with the G allele of SNP30 through the binding of Sp1 and might contribute to the pathogenesis of brain infarction.

APJ was first identified as a novel orphan G protein-coupled receptor in 1993 (24), and the endogenous ligand for APJ, apelin, was found in 1998 (25). Recent accumulated lines of evidence revealed that the apelin/APJ signalling plays an important role to maintain homeostasis of cardiovascular system. APJ is abundantly expressed in hypothalamus and medulla oblongata, which play key roles in cardiovascular

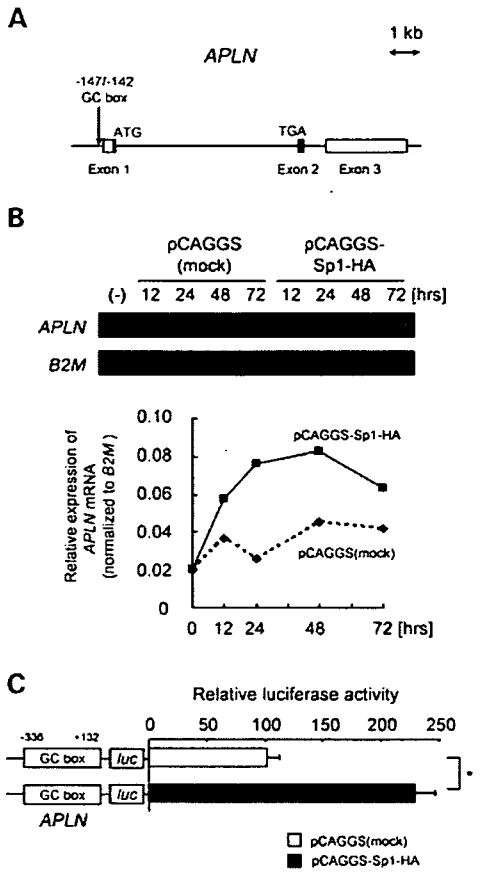

Figure 5. Transcriptional regulation of *APLN* gene by Sp1. (A) Genomic structure of *APLN*. A putative Sp1-binding site (GC box) was located in the upstream (−147/−142) of the transcription-initiation site based on NM_017413.3. ATG indicates the initiation codon. TGA indicates the stop codon. (B) Exogenously introduced Sp1 induced *APLN* mRNA. HEK293T cells were transfected with mock pCAGGS or HA-tagged Sp1 expression vector (pCAGGS-Sp1-HA). RNAs were extracted in various time spans, and semi-quantitative (upper) and real-time quantitative (lower) RT–PCR for *APLN* mRNA and *B2M* were performed. (C) Luciferase assay. A 468 bp fragment around the GC box of *APLN* was inserted into pGL3-basic vector. Luciferase assay was performed using U-2OS cells with co-transfection of mock pCAGGS or pCAGGS-Sp1-HA vector. Luciferase activity is indicated relative to the activity of pGL3-basic vector. Each sample was studied in triplicate and data shown are the mean ± SD (*$P < 0.01$).

regulation (14–17). It was recently reported that mean arterial pressure was increased after administration of apelin into cerebral ventricle or medulla oblongata in rats (16,17). Furthermore, apelin/APJ signalling was shown to activate the phosphatidylinositol 3-kinase (PI3K)/Akt pathway (26), which was indicated to promote the development of atherosclerotic lesion (27). Thus, we considered that disease-susceptible SNP in the *AGTRL1* gene might be associated with the disorder of blood pressure and the progression of atherosclerosis, which are the major causes of brain infarction, although the relationship between this SNP and cardiovascular diseases should be examined in the future studies.

In conclusion, we identified *AGTRL1* as a susceptible gene to brain infarction by means of a large-scale SNP screening. A functional SNP in the 5′-flanking region of *AGTRL1* gene appears likely to regulate its transcription level through the allele-specific binding of Sp1 transcription factor. We also found that this functional SNP increased the risk of brain infarction in the population-based prospective cohort study. Despite the recent advances in medicine, brain infarction is still one of the major causes of death. Prediction of brain infarction risk by the analysis of *AGTRL1* genotypes may be useful for the prevention and primary care of stroke. Although the mechanisms of apelin/APJ signalling in the pathogenesis of brain infarction are still to be elucidated, our finding might contribute to a better understanding of brain infarction in the future.

MATERIALS AND METHODS

Study populations

For the large-scale case–control association study, we registered patients with brain infarction from seven medical centres in and around Fukuoka City, Japan (Kyushu University Hospital, National Hospital Organization Kyushu Medical Center, National Hospital Organization Fukuoka Higashi Medical Center, Fukuoka Red Cross Hospital, Hakujuji Hospital, Imazu Red Cross Hospital and Seiai Rehabilitation Hospital) in 2004. Details of the registration were described previously (8). In brief, all case subjects were diagnosed by stroke neurologists on the basis of detailed clinical features and brain imaging including computed tomography and magnetic resonance imaging. Control subjects were enrolled from the participants of the Hisayama study, a population-based cohort study for cardiovascular diseases in Hisayama Town, started in 1961. Details of this study have been described previously (1,3,8). Between 2002 and 2003, we performed a screening examination for Hisayama residents, and 3328 individuals of 40 years or higher (78% of the total population of this age group) participated in the examination. After excluding the subjects with a history of stroke or coronary heart disease, we randomly selected age- and sex-matched control subjects by 1:1, using random numbers. Mean age ± SD was 70 ± 10 years and 60.7% of subjects were male in both case and control groups.

For the prospective cohort study, we used a cohort population of the Hisayama study established in 1988 (1,8). In this cohort, 2637 Hisayama residents aged 40 years or over without a history of stroke or coronary heart disease were enrolled in 1988 and continuously followed up for 14 years until the occurrence of cardiovascular diseases or death. Among them, the 1683 subjects participated in the examination between 2002 and 2003 were used in the present study. Mean age ± SD at baseline was 56 ± 10 years and 40.3% of subjects were male in this cohort.

All subjects were Japanese and provided written informed consent to participate in the study. This study was approved by human ethics committees of Graduate School of Medical Sciences, Kyushu University and Institute of Medical Science, the University of Tokyo.

SNP genotyping

We extracted genomic DNA from peripheral blood leukocytes by a standard method. We genotyped SNPs, using the multiplex PCR-based invader assay (Third Wave Technologies), as described previously (28), or TaqMan SNP genotyping assays (Applied Biosystems).

Northern blotting

cDNA probe was constructed from full-length coding region of *AGTRL1* gene. The probe was labelled with [α-^{32}P]-CTP (GE Healthcare), using Megaprime DNA labelling systems (GE Healthcare), and hybridized with Human Multiple Tissue Northern Blot I, II and III membranes (Takara Bio), using a standard protocol.

Cell culture

Human lung cancer SBC-3 cells were grown in RPMI medium 1640 (Invitrogen) with 10% fetal bovine serum (FBS). Human embryonic kidney fibroblasts HEK293T were grown in Dulbecco's modified Eagle's medium (Invitrogen) with 10% FBS. Human osteosarcoma U-2OS cells were grown in McCoy's 5a medium (Invitrogen) with 15% FBS. All cells were incubated in a humidified atmosphere with 5% CO_2 at 37°C.

Gel-shift assay

The sequence of each probe is listed in Supplementary Material, Table S2. Oligonucleotides were annealed and end-labelled with [γ-^{32}P]-ATP (GE Healthcare), using T4-polynucleotide kinase (Toyobo). We prepared nuclear extract from SBC-3 cells, as described previously (29). Ten micrograms of nuclear extract was incubated for 30 min at room temperature with 500 000 c.p.m. of labelled probe in a reaction mixture of 15 mM Tris–HCl (pH 7.5), 6.5% glycerol, 50 mM KCl, 0.7 mM EDTA-2Na (pH 8.0), 0.2 mM dithiothreitol, 0.1% bovine serum albumin, 1 μg of poly(dI–dC) and 0.1 μg of salmon sperm DNA. For competition assays, 10-, 30- or 100-fold molar excess of unlabelled oligonucleotide was added and incubated for another 30 min at room temperature. For supershift assay, 2 μg of goat polyclonal anti-human Sp1 antibody (SantaCruz, sc-59X) was added and incubated for another 30 min at room temperature. The mixture was subjected to electrophoresis on a 4% polyacrylamide gel in 0.5× Tris–Borate–EDTA buffer. The gel was dried up before exposure to X-ray film.

ChIP assay

We subcloned the full-length human Sp1 cDNA with HA tag in the C-terminus into pCAGGS expression vector (pCAGGS-Sp1-HA). HEK293T cells were transfected with the vector using FuGENE 6 Transfection Reagent (Roche). Forty-eight hours later, cells were treated with 1% formaldehyde and immunoprecipitated by rabbit polyclonal anti-HA antibody (SantaCruz, sc-805), using Chromatin Immunoprecipitation Assay Kit (Upstate) according to the manufacturer's protocol. We also performed the same protocol without antibody. Precipitated DNAs were analysed via PCR using primer pairs listed in Supplementary Material, Table S3. To evaluate allele-specific binding of Sp1, we subcloned the PCR product from anti-HA ChIP sample into pCR2.1-TOPO vector (Invitrogen) and transformed *E. coli* competent cells (DH10B strain) with this vector by electroporation. Then we incubated these *E. coli* on a Luria Bertani agar plate overnight and picked up 20 colonies from the plate. For each colony, we performed PCR, using the same primer sets, and genotyped SNP30 by direct sequencing, using ABI 3700 (Applied Biosystems).

Semi-quantitative RT–PCR and real-time quantitative RT–PCR

We transfected pCAGGS-Sp1-HA or mock pCAGGS vector to HEK293T cells. Total RNAs were collected in various time spans, using RNeasy Mini Kit and RNase-free DNase Set (Qiagen). cDNAs were synthesized by SuperScript II Reverse Transcriptase (Invitrogen). Expression levels of human *AGTRL1*, *APLN* and housekeeping gene *B2M* were determined by semi-quantitative RT–PCR, using primer pairs listed in Supplementary Material, Table S3. For real-time quantitative RT–PCR, cDNAs were amplified using SYBR Premix ExTaq (Takara Bio) and analysed by ABI PRISM 7700 (Applied Biosystems), using primer pairs listed in Supplementary Material, Table S3. The normalized amount of *AGTRL1* or *APLN* expression was obtained by dividing the *AGTRL1* or *APLN* value, respectively, by the *B2M* value.

Luciferase assay

DNA fragment corresponding to −202 to −123 of *AGTRL1*, including either allele of SNP30 and DNA fragment corresponding to −336 to +132 of *APLN* were subcloned into pGL3-basic luciferase vector (Promega). We transfected U-2OS cells with 90 ng of each reporter construct, 10 ng of pRL-TK vector (Promega) and 100 ng of either pCAGGS-Sp1-HA or mock pCAGGS vector, using FuGENE 6 Transfection Reagent (Roche). After 48 h, we collected the cells and measured luciferase activities, using Dual Luciferase Assay System (Toyo B-Net).

Statistical analysis

We assessed case–control association and Hardy–Weinberg equilibrium by chi-square test and Fisher's exact test. For adjustment of multiple testing, we performed a random permutation test with 10 000 replications, using MULTTEST procedure of SAS software version 9.1.2 (SAS Institute). LD coefficients (D' and r^2) were calculated using the expectation-maximization algorithm, and haplotype blocks were defined by Gabriel's criteria (11), using Haploview version 3.32 (Broad Institute). In the prospective cohort study, the cumulative incidence of brain infarction was estimated by Kaplan–Meier product limit method, and age- and sex-adjusted hazard ratio and its 95% CI were estimated by Cox

proportional hazards model using SAS software. The relative luciferase activities were compared using *t*-test.

URLs

The JSNP database can be found at http://snp.ims.u-tokyo.ac.jp/. The International HapMap Project can be found at http://www.hapmap.org/. The dbSNP and GenBank databases provided by the NCBI of the USA can be found at http://www.ncbi.nlm.nih.gov/. The Haploview software (Broad Institute) can be found at http://www.broad.mit.edu/mpg/haploview/. The GENSCAN program (Stanford University) can be found at http://genes.mit.edu/GENSCAN.html. The MATCH program (Biobase) can be found at http://www.gene-regulation.com/.

SUPPLEMENTARY MATERIAL

Supplementary Material is available at HMG Online.

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Conflict of Interest statement. None declared.

REFERENCES

1. Kubo, M., Kiyohara, Y., Kato, I., Tanizaki, Y., Arima, H., Tanaka, K., Nakamura, H., Okubo, K. and Iida, M. (2003) Trends in the incidence, mortality, and survival rate of cardiovascular disease in a Japanese community, the Hisayama Study. *Stroke*, **34**, 2349–2354.
2. Sacco, R.L., Benjamin, E.J., Broderick, J.P., Dyken, M., Easton, J.D., Feinberg, W.M., Goldstein, L.B., Gorelick, P.B., Howard, G., Kittner, S.J. *et al.* (1997) Risk factors. *Stroke*, **28**, 1507–1517.
3. Tanizaki, Y., Kiyohara, Y., Kato, I., Iwamoto, H., Nakayama, K., Shinohara, N., Arima, H., Tanaka, K., Ibayashi, S. and Fujishima, M. (2000) Incidence and risk factors for subtypes of cerebral infarction in a general population, the Hisayama Study. *Stroke*, **31**, 2616–2622.
4. Floßmann, E., Schulz, U.G.R. and Rothwell, P.M. (2004) Systematic review of methods and results of studies of the genetic epidemiology of ischemic stroke. *Stroke*, **35**, 212–227.
5. Hassan, A. and Markus, H.S. (2000) Genetics and ischaemic stroke. *Brain*, **123**, 1784–1812.
6. Gretarsdottir, S., Thorleifsson, G., Reynisdottir, S.T., Manolescu, A., Jonsdottir, S., Jonsdottir, T., Gudmundsdottir, T., Bjarnadottir, S.M., Einarsson, O.B., Gudjonsdottir, H.M. *et al.* (2003) The gene encoding phosphodiesterase 4D confers risk of ischemic stroke. *Nat. Genet.*, **35**, 131–138.
7. Helgadottir, A., Manolescu, A., Thorleifsson, G., Gretarsdottir, S., Jonsdottir, H., Thorsteinsdottir, U., Samani, N.J., Gudmundsson, G., Grant, S.F.A., Thorgeirsson, G. *et al.* (2004) The gene encoding 5-lipoxygenase activating protein confers risk of myocardial infarction and stroke. *Nat. Genet.*, **36**, 233–239.
8. Kubo, M., Hata, J., Ninomiya, T., Matsuda, K., Yonemoto, K., Nakano, T., Matsushita, T., Yamanaka-Yamazaki, K., Ohnishi, Y., Saito, S. *et al.* (2007) Nonsynonymous SNP in *PRKCH* encoding protein kinase c-η increases the risk of cerebral infarction. *Nat. Genet.*, **39**, 212–217.
9. Tsunoda, T., Lathrop, G.M., Sekine, A., Yamada, R., Takahashi, A., Ohnishi, Y., Tanaka, T. and Nakamura, Y. (2004) Variation of gene-based SNPs and linkage disequilibrium patterns in the human genome. *Hum. Mol. Genet.*, **13**, 1623–1632.
10. The International HapMap Consortium (2005) A haplotype map of the human genome. *Nature*, **437**, 1299–1320.
11. Gabriel, S.B., Schaffner, S.F., Nguyen, H., Moore, J.M., Roy, J., Blumenstiel, B., Higgins, J., DeFelice, M., Lochner, A., Faggart, M. *et al.* (2002) The structure of haplotype blocks in the human genome. *Science*, **296**, 2225–2229.
12. Saito, S., Iida, A., Sekine, A., Kawauchi, S., Higuchi, S., Ogawa, C. and Nakamura, Y. (2003) Catalog of 178 variations in the Japanese population among eight human genes encoding G protein-coupled receptors (GPCRs). *J. Hum. Genet.*, **48**, 461–468.
13. Kleinz, M.J., Skepper, J.N. and Davenport, A.P. (2005) Immunocytochemical localisation of the apelin receptor, APJ, to human cardiomyocytes, vascular smooth muscle and endothelial cells. *Regul. Pept.*, **126**, 233–240.
14. O'Carroll, A.-M., Selby, T.L., Palkovits, M. and Lolait, S.J. (2000) Distribution of mRNA encoding B78/apj, the rat homologue of the human APJ receptor, and its endogenous ligand apelin in brain and peripheral tissues. *Biochim. Biophys. Acta.*, **1492**, 72–80.
15. Matsumoto, M., Hidaka, K., Akiho, H., Tada, S., Okada, M. and Yamaguchi, T. (1996) Low stringency hybridization study of the dopamine D4 receptor revealed D4-like mRNA distribution of the orphan seven-transmembrane receptor, APJ, in human brain. *Neurosci. Lett.*, **219**, 119–122.
16. Kagiyama, S., Fukuhara, M., Matsumura, K., Lin, Y., Fujii, K. and Iida, M. (2005) Central and peripheral cardiovascular actions of apelin in conscious rats. *Regul. Pept.*, **125**, 55–59.
17. Seyedabadi, M., Goodchild, A.K. and Pilowsky, P.M. (2002) Site-specific effects of apelin-13 in the rat medulla oblongata on arterial pressure and respiration. *Auton. Neurosci.*, **101**, 32–38.
18. Liu, F., Pore, N., Kim, M., Voong, K.R., Dowling, M., Maity, A. and Kao, G.D. (2006) Regulation of histone deacetylase 4 expression by the SP family of transcription factors. *Mol. Biol. Cell*, **17**, 585–597.
19. Ozaki, K., Ohnishi, Y., Iida, A., Sekine, A., Yamada, R., Tsunoda, T., Sato, H., Sato, H., Hori, M., Nakamura, Y. *et al.* (2002) Functional SNPs in the lymphotoxin-α gene that are associated with susceptibility to myocardial infarction. *Nat. Genet.*, **32**, 650–654.
20. Obara, W., Iida, A., Suzuki, Y., Tanaka, T., Akiyama, F., Maeda, S., Ohnishi, Y., Yamada, R., Tsunoda, T., Takei, T. *et al.* (2003) Association of single-nucleotide polymorphisms in the polymeric immunoglobulin receptor gene with immunoglobulin A nephropathy (IgAN) in Japanese patients. *J. Hum. Genet.*, **48**, 293–299.
21. Ohtsubo, S., Iida, A., Nitta, K., Tanaka, T., Yamada, R., Ohnishi, Y., Maeda, S., Tsunoda, T., Takei, T., Obara, W. *et al.* (2005) Association of a single-nucleotide polymorphism in the immunoglobulin μ-binding protein 2 gene with immunoglobulin A nephropathy. *J. Hum. Genet.*, **50**, 30–35.
22. Yamazaki, K., McGovern, D., Ragoussis, J., Paolucci, M., Butler, H., Jewell, D., Cardon, L., Takazoe, M., Tanaka, T., Ichimori, T. *et al.* (2005) Single nucleotide polymorphisms in *TNFSF15* confer susceptibility to Crohn's disease. *Hum. Mol. Genet.*, **14**, 3499–3506.
23. Mototani, H., Mabuchi, A., Saito, S., Fujioka, M., Iida, A., Takatori, Y., Kotani, A., Kubo, T., Nakamura, K., Sekine, A. *et al.* (2005) A functional single nucleotide polymorphism in the core promoter region of *CALM1* is associated with hip osteoarthritis in Japanese. *Hum. Mol. Genet.*, **14**, 1009–1017.
24. O'Dowd, B.F., Heiber, M., Chan, A., Heng, H.H.Q., Tsui, L.-C., Kennedy, J.L., Shi, X., Petronis, A., George, S.R. and Nguyen, T. (1993) A human gene that shows identity with the gene encoding the angiotensin receptor is located on chromosome 11. *Gene*, **136**, 355–360.
25. Tatemoto, K., Hosoya, M., Habata, Y., Fujii, R., Kakegawa, T., Zou, M.-X., Kawamata, Y., Fukusumi, S., Hinuma, S., Kitada, C. *et al.*

(1998) Isolation and characterization of a novel endogenous peptide ligand for the human APJ receptor. *Biochem. Biophys. Res. Commun.*, **251**, 471–476.
26. Masri, B., Morin, N., Cornu, M., Knibiehler, B. and Audigier, Y. (2004) Apelin (65–77) activates p70 S6 kinase and is mitogenic for umbilical endothelial cells. *FASEB. J.*, **18**, 1909–1911.
27. Chen, C.-N., Li, Y.-S.J., Yeh, Y.-T., Lee, P.-L., Usami, S., Chien, S. and Chiu, J. (2006) Synergistic roles of platelet-derived growth factor-BB and interleukin-1β in phenotypic modulation of human aortic smooth muscle cells. *Proc. Natl Acad. Sci. USA.* **103**, 2665–2670.
28. Ohnishi, Y., Tanaka, T., Ozaki, K., Yamada, R., Suzuki, H. and Nakamura, Y. (2001) A high-throughput SNP typing system for genome-wide association studies. *J. Hum. Genet.*, **46**, 471–477.
29. Andrews, N.C. and Faller, D.V. (1991) A rapid micropreparation technique for extraction of DNA-binding proteins from limiting numbers of mammalian cells. *Nucleic Acids Res.*, **19**, 2499.

Impact of Metabolic Syndrome on the Development of Cardiovascular Disease in a General Japanese Population

The Hisayama Study

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Background and Purpose—The metabolic syndrome (MetS) is associated with an increased risk of cardiovascular disease (CVD) events in general populations. However, well-designed prospective studies in Asian populations are very limited.

Methods—We prospectively evaluated a total of 2452 community-dwelling Japanese individuals aged 40 years or older from 1988 to 2002 and examined the effects of MetS defined by the modified National Cholesterol Education Program Adult Treatment Panel III criteria on incident CVD.

Results—The prevalence of the MetS was 21% in men and 30% in women at baseline. During the follow up, 307 CVD events occurred. Compared with those without MetS, the age-adjusted incidence of CVD (per 1000 person-years) was significantly higher in subjects with the MetS in both men (21.8 versus 11.6, $P<0.01$) and women (12.9 versus 6.5, $P<0.01$). The risk of CVD events was significantly higher even after adjusting for the following confounding factors: age, proteinuria, electrocardiographic abnormalities, serum total cholesterol, smoking habits, alcohol intake, and regular exercise (hazard ratio, 1.86; 95% CI, 1.32 to 2.62 in men and hazard ratio, 1.70; 95% CI, 1.22 to 2.36 in women). The risk of incident CVD was found to increase with the number of components of MetS and became significantly predictive when the number of components reached 3. Similar associations were also observed when CVD was divided into coronary heart disease and stroke.

Conclusions—Our findings suggest that MetS is a significant risk factor for the development of CVD in the Japanese middle-aged population. (***Stroke*. 2007;38:2063-2069.)**

Key Words: cardiovascular disease ■ epidemiology ■ metabolic syndrome ■ myocardial infarction ■ stroke

Metabolic syndrome (MetS), also known as syndrome X,[1] the insulin resistance syndrome,[2] and deadly quartet,[3] is a constellation of dyslipidemia, central obesity, elevated blood pressure, and impaired glucose tolerance. It is associated with high risk for the development of type 2 diabetes mellitus and cardiovascular disease (CVD).[4–7] In the past several years, a great deal of attention has been directed to it attributable to increases in its prevalence worldwide[6] and its association with CVD morbidity and mortality. Although each of the components of MetS has been shown to increase CVD risk,[8–12] the presence of MetS has been reported to identify additional risk.[7] Different prospective studies[7,13–25] based on the definitions from the National Cholesterol Education Program's (NCEP) Third Adult Treatment Panel Report III[5] and World Health Organization[26] showed that subjects with MetS are at increased risk of incident CVD, CVD mortality, and all-cause mortality in the general population with or without diabetes mellitus. However, most of these studies were based on Western populations, and well-designed prospective studies in Asian populations are very limited.[27–29] Thus, there is a dearth of literature regarding the relationship of MetS with incident CVD based on general population cohorts with a reasonable length of follow-up time in ethnic groups other than whites. In this study, we examined the impact of MetS on CVD events in a general Japanese population cohort based on 14-year prospective follow-up data.

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Statistical Analysis

The SAS software package (SAS Institute, Inc, Cary, NC) was used to perform all statistical analyses. Serum triglycerides were transformed into logarithms to improve the skewed distribution. The statistical significance of differences in mean values of continuous variables and frequencies of categorical variables was examined using the Student t test and χ^2 test as appropriate. The incidences were calculated by the person-year method. Differences in incidences between MetS status were tested by the Cox proportional hazards regression analysis after adjustment for age. The age- or multivariate-adjusted hazard ratios (HRs) and 95% CIs were also estimated with the use of the Cox proportional hazards model. $P<0.05$ was considered statistically significant in all analyses.

Results

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During the 14-year follow up, 307 first-ever CVD events (158 men and 149 women) occurred. Of these, there were 125 CHD (78 men and 47 women) and 209 stroke events (94 men and 115 women). The age-adjusted incidences of CVD were significantly higher in subjects with MetS compared with those without MetS for both sexes (men: 21.8 versus 11.6 per 1000 person-years, $P<0.01$; women: 12.9 versus 6.5, $P<0.01$) (Table 2). The same was true for CHD incidence in both sexes (men: 9.2 versus 5.7, $P<0.01$; women: 5.1 versus 1.5, $P<0.01$) and for stroke in men (14.1 versus 6.4, $P<0.01$). When we divided strokes into ischemic and hemorrhagic type, the age-adjusted incidences of ischemic stroke were

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TABLE 1. Clinical Characteristics of Study Population in 1988

Variables	Men		Women	
	MetS (−) (N=834)	MetS (+) (N=216)	MetS (−) (N=983)	MetS (+) (N=419)
Age, years	58±11	58±11	57±11	62±10†
Systolic blood pressure, mm Hg	132±20	145±18†	126±19	145±19†
Diastolic blood pressure, mm Hg	79±11	87±10†	74±10	81±11†
Antihypertensive medication, %	11.8	21.3†	9.0	29.6†
Hypertension, %	37.4	70.4†	24.0	67.5†
Proteinuria, %	7.0	11.6*	3.0	6.9†
Electrocardiogram abnormalities, %	18.7	19.7	12.5	14.6
Waist circumference, cm	80.3±7.6	88.7±6.9†	78.1±9.2	88.2±8.4*
Body mass index, kg/m^2	22.3±2.8	25.0±2.6†	22.1±2.9	24.9±3.1†
Fasting blood glucose, mmol/L	5.7±1.1	6.7±1.7†	5.5±1.0	6.3±1.7†
Diabetes, %	6.7	29.2†	3.0	17.7†
Serum total cholesterol, mmol/L	5.09±1.06	5.19±1.14	5.47±1.05	5.78±1.09†
Serum triglycerides, mmol/L	1.13 (0.43–2.95)	2.46 (0.83–7.32)†	0.90 (0.44–1.86)	1.58 (0.61–4.10)†
Serum high-density lipoprotein cholesterol, mmol/L	1.31±0.29	1.06±0.27†	1.42±0.28	1.14±0.22†
Smoking habits, %	51.6	45.8	6.2	7.9
Alcohol intake, %	59.5	69.4*	8.6	9.8
Regular exercise, %	12.2	8.8	9.2	9.3

Values are mean±SD or percentage.
Electrocardiogram abnormalities are defined as left ventricular hypertrophy (Minnesota code, 3–1) and/or ST depression (Minnesota code, 4–1, 2, 3).
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*$P<0.05$, †$P<0.01$ vs MetS (−).
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significantly higher in subjects with MetS than in those without MetS for both sexes (men: 9.0 versus 4.8, $P=0.03$; women: 6.2 versus 3.4, $P=0.01$). The similar tendency was observed for hemorrhagic stroke only in men (5.1 versus 1.6, $P=0.01$).

Age- and multivariate-adjusted hazard ratios of MetS for the development of CVD were estimated for both sexes (Table 3). The age-adjusted analysis showed that MetS was a significant risk factor for CVD in men and women. These

TABLE 2. Age-Adjusted Incidence Rates of CVD, CHD, and Stroke According to MetS Status in 2452 Subjects During a 14-Year Follow Up by Sex

	Men				Women			
	Person-Years at Risk	No. of Events	Age-Adjusted Incidence Rate	*P* Value	Person-Years at Risk	No. of Events	Age-Adjusted Incidence Rate	*P* Value
Cardiovascular disease								
MetS (−)	9958	108	11.6		12 759	78	6.5	
MetS (+)	2416	50	21.8	<0.01	5078	71	12.9	<0.01
Coronary heart disease								
MetS (−)	10 213	53	5.7		13 010	17	1.5	
MetS (+)	2533	25	9.2	<0.01	5279	30	5.1	<0.01
Stroke								
MetS (−)	10 099	63	6.4		12 817	65	5.3	
MetS (+)	2477	31	14.1	<0.01	5122	50	8.8	0.06
Ischemic stroke								
MetS (−)	10 099	46	4.8		12 817	40	3.4	
MetS (+)	2477	20	9.0	0.03	5122	39	6.2	0.01
Hemorrhagic stroke								
MetS (−)	10 099	17	1.6		12 817	25	2.0	
MetS (+)	2477	11	5.1	0.01	5122	11	2.6	0.72

TABLE 3. Age- or Multivariate-Adjusted HRs for Development of CVD, CHD, or Stroke According to MetS Status in 2452 Subjects During a 14-Year Follow Up by Sex

	Men						Women					
	Age-Adjusted			Multivariate-Adjusted*			Age-Adjusted			Multivariate-Adjusted*		
	HR	(95% CI)	*P* Value	HR	(95% CI)	*P* Value	HR	(95% CI)	*P* Value	HR	(95% CI)	*P* Value
Cardiovascular disease												
MetS (−)	1.00	(reference)		1.00	(reference)		1.00	(reference)		1.00	(reference)	
MetS (+)	1.93	(1.38–2.70)	<0.01	1.86	(1.32–2.62)	<0.01	1.68	(1.22–2.33)	<0.01	1.70	(1.22–2.36)	<0.01
Coronary heart disease												
MetS (−)	1.00	(reference)		1.00	(reference)		1.00	(reference)		1.00	(reference)	
MetS (+)	1.95	(1.21–3.13)	<0.01	1.94	(1.19–3.17)	<0.01	3.11	(1.71–5.65)	<0.01	2.86	(1.56–5.24)	<0.01
Stroke												
MetS (−)	1.00	(reference)		1.00	(reference)		1.00	(reference)		1.00	(reference)	
MetS (+)	2.04	(1.33–3.14)	<0.01	1.92	(1.23–2.98)	<0.01	1.43	(0.99–2.08)	0.06	1.50	(1.03–2.19)	0.03
Ischemic stroke												
MetS (−)	1.00	(reference)		1.00	(reference)		1.00	(reference)		1.00	(reference)	
MetS (+)	1.80	(1.07–3.05)	0.03	1.68	(0.98–2.89)	0.06	1.77	(1.14–2.76)	0.01	1.78	(1.13–2.79)	0.01
Hemorrhagic stroke												
MetS (−)	1.00	(reference)		1.00	(reference)		1.00	(reference)		1.00	(reference)	
MetS (+)	2.67	(1.25–5.69)	0.01	2.54	(1.18–5.49)	0.02	0.88	(0.43–1.80)	0.72	0.99	(0.48–2.05)	0.91

*Adjusted for age, proteinuria, electrocardiogram abnormalities, serum total cholesterol, smoking habits, alcohol intake, and regular exercise.

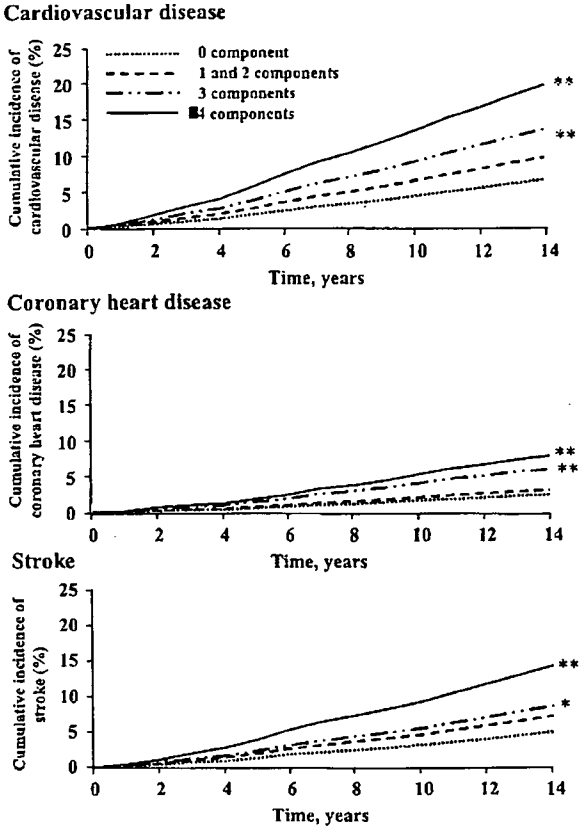
relationships remained substantially unchanged even after adjustment for the following confounding factors: age, proteinuria, electrocardiographic abnormalities, serum total cholesterol, smoking habits, alcohol intake, and regular exercise. Furthermore, MetS was found to be an independent risk factor for the development of CHD and stroke after adjustment for the confounding factors in men and women. When strokes were divided into ischemic and hemorrhagic type, multivariate-adjusted HR of MetS for ischemic stroke was marginally higher in men and significantly higher in women, whereas MetS is an independent risk factor for hemorrhagic stroke only in men.

The age- and sex-adjusted cumulative incidences of CVD, CHD, and stroke according to the number of MetS components are shown in the Figure. Because the cumulative incidence curves for one and 2 components overlapped, we combined these components. The incidences of CVD, CHD, and stroke were significantly higher among the subjects with 3 or more MetS components compared with those without any MetS component. A significant graded relationship between the number of components of MetS and the HR for developing CVD was identified from 3 MetS components and onward (Table 4). Compared with individuals with no MetS component, individuals with one, 2, 3, and 4 or more components had gradually increased HRs, respectively, for developing CVD after adjusting the confounding factors. A similar relationship was found when CVD was divided into CHD and stroke.

Because hypertension and diabetes are strong risk factors for CVD, we examined the combined as well as separate effects of MetS and hypertension or diabetes on the development of CVD. As shown in Table 5, the age- and sex-adjusted HR of CVD was significantly higher in normotensive subjects with MetS, hypertensive subjects without MetS, and hypertensive subjects with MetS compared with those without hypertension and MetS. Furthermore, there was a significant excess risk of CVD in hypertensives with MetS than in those without MetS. Similarly, the age- and sex-adjusted HR of CVD was significantly higher in nondiabetic subjects with MetS and diabetic subjects with MetS compared with those without diabetes and MetS. However, no significant difference was found in the risk of CVD in diabetic subjects without MetS. Among diabetic subjects, the risk of CVD was significantly higher in subjects with MetS than in those without MetS. These relationships remained substantially unchanged even after adjusting for the confounding factors. Furthermore, we examined the association of MetS with CVD by the multivariate analysis using hypertension and diabetes in addition to the previously mentioned risk factors as confounding factors. As a result, MetS remained a significantly independent risk factor for the development of CVD (HR, 1.38; 95% CI, 1.07 to 1.78, $P=0.01$). The risks of other risk factors were as follows: age (HR, 2.00 [per increment of 10 years]; 95% CI, 1.79 to 2.26, $P<0.01$), male sex (1.45; 1.07 to 1.97, $P=0.02$), hypertension (1.64; 1.26 to 2.12, $P<0.01$), diabetes (1.55; 1.14 to 2.13, $P<0.01$), smoking habits (1.69; 1.28 to 2.23, $P<0.01$), regular exercise (0.58; 0.39 to 0.87, $P<0.01$), proteinuria (1.64; 1.13 to 2.38, $P<0.01$), electrocardiographic abnormalities (1.29; 0.98 to 1.69, $P=0.07$), serum total cholesterol (0.99 [per increment of 1 mmol/L]; 0.89 to 1.11, $P=0.92$), and alcohol intake (0.97; 0.73 to 1.30, $P=0.84$).

Discussion

To our knowledge, our study is the first prospective cohort study of a general Japanese population with a long duration of follow up reporting the association of MetS with incident CVD using the modified NCEP definition. The sole study from Japan, which examined a similar association, was based on a diabetic population.[27] We found a clearly increased incidence of CVD during 14 years of follow up in both men



Age- and sex-adjusted cumulative incidences of CVD, CHD, and stroke according to the number of the metabolic syndrome components in 2452 subjects during a 14-year follow up. *$P<0.05$, **$P<0.01$ versus 0 component.

and women with MetS compared with those without MetS. Besides, the risk of MetS for the development of CVD remained significant even after adjustment for hypertension, diabetes, and other potentially confounding factors.

In our study, subjects with MetS had little over 70% increases in CVD risk compared with those without MetS. Similar or higher HRs (1.4- to 5.0-fold) of MetS for CVD/CHD were reported from different European and American studies.[7,13–25] Differences in the study populations, prevalence of individual components of MetS, follow-up length, and MetS definition used seem to be the main causes behind the variation in the HRs. In our study, CHD risk related to MetS is higher in women than in men, which is consistent with the studies from the Western world.[17]

Our study showed that the risk of incident combined CVD, and CHD and stroke separately, was found to increase with the number of components of MetS and increased by 3-fold or more in those with 4 or more MetS components compared with those without any component. It also revealed that the risk of CVD increased in incremental fashion with the number of components of MetS and became predictive of CVD (also CHD and stroke separately) when the number of components reached 3. This phenomenon gives credence to the requirement of ≥3 components in the NCEP definition for establishing the diagnosis of MetS. Thereby, it can be assumed that the modified NCEP definition of MetS is well predictive for CVD in the general Japanese population.

One prospective study based on a Japanese diabetic population mentioned that MetS based on the NCEP definition was predictive for CVD in men and was not in women.[27] The same authors again reported that the new International Diabetes Federation definition[34] was not predictive for CVD in either male or female patients with diabetes.[35] On the other hand, in our study, MetS based on the NCEP definition was consistently predictive of CVD not only in both men and women, but also in subjects with diabetes. We speculate that this discrepancy resulted from the difference in the cutoff point of the waist circumference between the 2 studies. The former used the waist circumference definition for abdominal obesity proposed by the Japan Society for the Study of Obesity (85 cm for men and 90 cm for women),[36] whereas in our study, we used the waist circumference definition for Asian populations (90 cm for men and 80 cm for women), which was recommend by the International Diabetes Federation to use for the Japanese population.[37] Further research is needed to refine the MetS definition, which would be applicable to various populations, including Japanese.

There was a possibility that the increased risk of MetS for CVD resulted from the influences of hypertension or diabetes, which are components of MetS and major risk factors for developing CVD. However, our stratified analysis showed that the MetS was a significant risk factor for CVD in normotensive subjects as well as in nondiabetic individuals and has a similar risk for CVD as hypertension; the risk is even higher than that of diabetes. Moreover, in the multivariate analysis, MetS was found to be a significant risk factor for CVD independent of hypertension, diabetes, and other confounding risk factors. These results imply the significant roles of MetS in the development of CVD and the need for prevention and early management of the MetS components. In addition, diabetes is not predictive of CVD in subjects without MetS in our study. This finding might suggest that good diabetic control is useful. However, because the number of our diabetic subjects without MetS is small, further studies are necessary to elucidate this issue in detail.

The strengths of our study include its longitudinal population-based study design, long duration of follow up, sufficient number of CVD events and almost perfect follow up of subjects, examining the data in men and women separately, and exclusion of patients with CVD at baseline. Moreover, it is the first study to examine prospectively CVD in relation to MetS based on a general Japanese population. One limitation of our study is that the diagnosis of MetS was based on a single measurement of its components at baseline as was the case in other epidemiological studies.[13–25,27–29] During the follow up, risk factor levels could be changed attributable to modification of lifestyle or medication, and misclassification of the MetS is possible. Thus, it would weaken the association found in this study, biasing the results toward the null hypothesis. Therefore, the true association may be stronger than that shown in our findings.

In conclusion, we have shown that the prevalence of MetS is sizeable in Japanese middle-aged men and women and it is predictive of future CVD in both sexes based on a prospective study with 14 years of follow up. Our findings suggest that early identification of MetS and appropriate behavioral and therapeutic intervention may reduce the burden of CVD in the long run.

TABLE 4. Age- or Multivariate-Adjusted HRs for Development of CVD, CHD, and Stroke According to the Number of the MetS Components in 2452 Subjects During a 14-Year Follow Up

	Population at Risk	No. of Events	Age- and Sex-Adjusted			Multivariate-Adjusted*		
			HR	(95% CI)	*P* Value	HR	(95% CI)	*P* Value
Cardiovascular disease								
No. of MetS components								
0	436	30	1.00	(reference)		1.00	(reference)	
1	756	84	1.49	(0.98–2.26)	0.06	1.45	(0.95–2.20)	0.08
2	625	72	1.47	(0.96–2.26)	0.08	1.39	(0.91–2.15)	0.15
3	394	65	2.12	(1.37–3.28)	<0.01	1.95	(1.25–3.04)	<0.01
≥4	241	56	3.19	(2.03–5.02)	<0.01	2.99	(1.89–4.73)	<0.01
Coronary heart disease								
No. of MetS components								
0	436	13	1.00	(reference)		1.00	(reference)	
1	756	35	1.41	(0.75–2.67)	0.29	1.38	(0.72–2.62)	0.33
2	625	22	1.05	(0.53–2.09)	0.89	0.95	(0.47–1.90)	0.88
3	394	32	2.55	(1.33–4.89)	<0.01	2.29	(1.18–4.47)	0.01
≥4	241	23	3.36	(1.68–6.72)	<0.01	2.96	(1.45–6.01)	<0.01
Stroke								
No. of MetS components								
0	436	20	1.00	(reference)		1.00	(reference)	
1	756	58	1.52	(0.91–2.53)	0.11	1.48	(0.89–2.47)	0.14
2	625	50	1.50	(0.89–2.53)	0.13	1.45	(0.86–2.46)	0.16
3	394	41	1.89	(1.10–3.25)	0.02	1.78	(1.03–3.09)	0.04
≥4	241	40	3.16	(1.83–5.46)	<0.01	3.05	(1.75–5.31)	<0.01

*Adjusted for age, sex, proteinuria, electrocardiogram abnormalities, serum total cholesterol, smoking habits, alcohol intake, and regular exercise.

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TABLE 5. Age- and Sex-Adjusted or Multivariate-Adjusted HRs of the MetS for Development of CVD According to the Presence or Absence of Hypertension or Diabetes in 2452 Subjects During a 14-Year Follow Up

	Population at Risk	No. of Events	Age- and Sex-Adjusted		Multivariate-Adjusted*	
			HR	(95% CI)	HR	(95% CI)
Hypertension						
HT (−)+MetS (−)	1269	89	1.00	(reference)	1.00	(reference)
HT (−)+MetS (+)	200	25	1.79	(1.14–2.79)*	1.75	(1.12–2.75)*
HT (+)+MetS (−)	548	97	1.81	(1.35–2.43)†	1.75	(1.29–2.37)†
HT (+)+MetS (+)	435	96	2.59	(1.93–3.48)†‡	2.45	(1.81–3.32)†‡
Diabetes						
DM (−)+MetS (−)	1732	171	1.00	(reference)	1.00	(reference)
DM (−)+MetS (+)	498	84	1.60	(1.23–2.09)†	1.54	(1.17–2.02)†
DM (+)+MetS (−)	85	15	1.35	(0.80–2.30)	1.38	(0.81–2.34)
DM (+)+MetS (+)	137	37	2.75	(1.93–3.93)†‡	2.60	(1.81–3.74)†‡

*Adjusted for age, sex, proteinuria, electrocardiogram abnormalities, serum total cholesterol, smoking habits, alcohol intake, and regular exercise.

*$P<0.05$, †$P<0.01$ vs reference.

‡$P<0.05$ vs HT(+)+MetS (−) or DM (+)+MetS (−).

HT indicates hypertension; DM, diabetes mellitus.

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Disclosures

None.

References

1. Reaven GM. Banting lecture 1988. Role of insulin resistance in human disease. *Diabetes*. 1988;37:1595–1607.
2. DeFronzo RA, Ferrannini E. Insulin resistance: a multifaceted syndrome responsible for NIDDM, obesity, hypertension, dyslipidemia, and atherosclerotic cardiovascular disease. *Diabetes Care*. 1991;14:173–194.
3. Kaplan NM. The deadly quartet. Upper-body obesity, glucose intolerance, hypertriglyceridemia, and hypertension. *Arch Intern Med*. 1989; 149:1514–1520.
4. Grundy SM, Brewer HB Jr, Cleeman JI, Smith SC Jr, Lenfant C. Definition of metabolic syndrome: report of the National Heart, Lung, and Blood Institute/American Heart Association conference on scientific issues related to definition. *Circulation*. 2004;109:433–438.
5. Expert Panel on Detection, Evaluation, and Treatment of High Blood Cholesterol in Adults. Executive Summary of the Third Report of the National Cholesterol Education Program (NCEP) Expert Panel on Detection, Evaluation, and Treatment of High Blood Cholesterol in Adults (Adult Treatment Panel III). *JAMA*. 2001;285:2486–2497.
6. Lorenzo C, Okoloise M, Williams K, Stern MP, Haffner SM. The metabolic syndrome as predictor of type 2 diabetes: the San Antonio Heart Study. *Diabetes Care*. 2003;26:3153–3159.
7. Lakka HM, Laaksonen DE, Lakka TA, Niskanen LK, Kumpusalo E, Tuomilehto J, Salonen JT. The metabolic syndrome and total and cardiovascular disease mortality in middle-aged men. *JAMA*. 2002;288:2709–2716.
8. Zimmet P, Alberti KG, Shaw J. Global and societal implications of the diabetes epidemic. *Nature*. 2001;414:782–787.
9. McGill HC, McMahan A, Herderick EE, Zieske AW, Malcom GT, Tracy RE, Strong JP. Pathobiological Determinants of Atherosclerosis in Youth (PDAY) Research Group: obesity accelerates the progression of coronary atherosclerosis in young men. *Circulation*. 2002;105:2712–2718.
10. The DECODE Study Group. Is the current definition for diabetes relevant to mortality risk from all causes and cardiovascular and noncardiovascular diseases? *Diabetes Care*. 2003;26:688–696.
11. Eberly LE, Stamler J, Neaton JD. Multiple Risk Factor Intervention Trial Research Group: relation of triglyceride levels, fasting and nonfasting, to fatal and nonfatal coronary heart disease. *Arch Intern Med*. 2003;163:1077–1083.
12. Grundy SM, Balady GJ, Criqui MH, Fletcher G, Greenland P, Hiratzka LF, Houston-Miller N, Kris-Etherton P, Krumholz HM, LaRosa J, Ockene IS, Pearson TA, Reed J, Washington R, Smith SC Jr. Primary prevention of coronary heart disease: guidance from Framingham. A statement for healthcare professionals from the AHA Task Force on Risk Reduction. *Circulation*. 1998;97:1876–1887.
13. Girman CJ, Rhodes T, Mercuri M, Pyorala K, Kjekshus J, Pedersen TR, Beere PA, Gotto AM, Clearfield M, 4S Group, AFCAPS/TexCAPS Research Group. The metabolic syndrome and risk of major coronary events in the Scandinavian Simvastatin Survival Study (4S) and the Air Force/Texas Coronary Atherosclerosis Prevention Study (AFCAPS/TexCAPS). *Am J Cardiol*. 2004;93:136–141.
14. Hu G, Qiao Q, Tuomilehto J, Balkau B, Borch-Johnsen K, Pyorala K, the DECODE Study Group. Prevalence of the metabolic syndrome and its relation to all-cause and cardiovascular mortality in non-diabetic European men and women. *Arch Intern Med*. 2004;164:1066–1076.
15. Malik S, Wong ND, Franklin SS, Kamath TV, L'Italien GJ, Pio JR, Williams GR. Impact of the metabolic syndrome on mortality from coronary heart disease, cardiovascular disease, and all causes in United States adults. *Circulation*. 2004;110:1245–1250.
16. Ford ES. The metabolic syndrome and mortality from cardiovascular disease and all-causes: findings from the National Health and Nutrition Examination Survey II Mortality Study. *Atherosclerosis*. 2004;173:309–314.
17. Hunt KJ, Resendez RG, Williams K, Haffner SM, Stern MP. National Cholesterol Education Program versus World Health Organization metabolic syndrome in relation to all-cause and cardiovascular mortality in the San Antonio Heart Study. *Circulation*. 2004;110:1251–1257.
18. Isomaa B, Almgren P, Tuomi T, Forsen B, Lahti K, Nissen M, Taskinen MR, Groop L. Cardiovascular morbidity and mortality associated with the metabolic syndrome. *Diabetes Care*. 2001;24:683–689.
19. Bonora E, Kiechl S, Willeit J, Oberhollenzer F, Egger G, Bonadonna RC, Muggeo M. Carotid atherosclerosis and coronary heart disease in the metabolic syndrome: prospective data from the Bruneck study. *Diabetes Care*. 2003;26:1251–1257.
20. Bonora E, Targher G, Formentini G, Calcaterra F, Lombardi S, Marini F, Zenari L, Saggiani F, Poli M, Perbellini S, Raffaelli A, Gemma L, Santi L, Bonadonna RC, Muggeo M. The metabolic syndrome is an independent predictor of the cardiovascular disease in type 2 diabetic subjects. Prospective data from the Verona Diabetes Complications Study. *Diabet Med*. 2004;21:52–58.
21. Rutter MK, Meigs JB, Sullivan LM, D'Agostino RB Sr, Wilson PW. C-reactive protein, the metabolic syndrome, and prediction of cardiovascular events in the Framingham Offspring Study. *Circulation*. 2004;110: 380–385.
22. Sundstrom J, Riserus U, Byberg L, Zethelius B, Lithell H, Lind L. Clinical value of the metabolic syndrome for long term prediction of total and cardiovascular mortality: prospective, population based cohort study. *BMJ*. 2006;332:878–882.
23. Dekker JM, Girman C, Rhodes T, Nijpels G, Stehouwer CD, Bouter LM, Heine RJ. Metabolic syndrome and 10-year cardiovascular disease risk in the Hoorn Study. *Circulation*. 2005;112:666–673.
24. McNeill AM, Rosamond WD, Girman CJ, Golden SH, Schmidt MI, East HE, Ballantyne CM, Heiss G. The metabolic syndrome and 11-year risk of incident cardiovascular disease in the Atherosclerosis Risk in Communities Study. *Diabetes Care*. 2005;28:385–390.
25. Scuteri A, Najjar SS, Morrell CH, Lakatta EG; Cardiovascular Health Study. The metabolic syndrome in older individuals: prevalence and prediction of cardiovascular events: the Cardiovascular Health Study. *Diabetes Care*. 2005;28:882–887.
26. *Definition, Diagnosis and Classification of Diabetes Mellitus and Its Complications. Report of a WHO Consultation. Part 1: Diagnosis and Classification of Diabetes Mellitus*. Geneva: World Health Organization; 1999 (WHO/NCD/NCS99.2).
27. Sone H, Mizuno S, Fujii H, Yoshimura Y, Yamasaki Y, Ishibashi S, Katayama S, Saito Y, Ito H, Ohashi Y, Akanuma Y, Yamada N; Japan Diabetes Complications Study. Is the diagnosis of metabolic syndrome useful for predicting cardiovascular disease in Asian diabetic patients? Analysis from the Japan Diabetes Complications Study. *Diabetes Care*. 2005;28:1463–1471.
28. Chen HJ, Bai CH, Yeh WT, Chiu HC, Pan WH. Influence of metabolic syndrome and general obesity on the risk of ischemic stroke. *Stroke*. 2006;37:1060–1064.
29. Chien KL, Hsu HC, Sung FC, Su TC, Chen MF, Lee YT. Metabolic syndrome as a risk factor for coronary heart disease and stroke: an 11-year prospective cohort in Taiwan community. *Atherosclerosis*. 2006 (in press).
30. Katsuki S. Epidemiological and clinicopathological study on cerebrovascular disease in Japan. *Prog Brain Res*. 1966;21B:64–89.
31. Ohmura T, Ueda K, Kiyohara Y, Kato I, Iwamoto H, Nakayama K, Nomiyama K, Ohmori S, Yoshitake T, Shinkawa A, Hasuo Y, Fujishima M. Prevalence of type 2 (non-insulin-dependent) diabetes mellitus and impaired glucose tolerance in the Japanese general population: the Hisayama Study. *Diabetologia*. 1993;36:1198–1203.
32. Kubo M, Kiyohara Y, Kato I, Tanizaki Y, Arima H, Tanaka K, Nakamura H, Okubo K, Iida M. Trends in the incidence, mortality, and survival rate of cardiovascular disease in a Japanese community: the Hisayama study. *Stroke*. 2003;34:2349–2354.
33. World Health Organization/International Association for the Study of Obesity/International Obesity Task Force. *The Asia-Pacific Perspective: Redefining Obesity and its Treatment*. Available at: http://www.diabetes.com.au/pdf/obesity_report.pdf.
34. The International Diabetes Federation: The IDF consensus worldwide definition of metabolic syndrome, 2005. Available at: www.idf.org/webdata/docs/IDF_metasyndrome_definition.pdf. Accessed March 21, 2006.
35. Sone H, Tanaka S, Ishibashi S, Yamasaki Y, Oikawa S, Ito H, Saito Y, Ohashi Y, Akanuma Y, Yamada N; Japan Diabetes Complications Study (JDCS) Group. The new worldwide definition of metabolic syndrome is not a better diagnostic predictor of cardiovascular disease in Japanese diabetic patients than the existing definitions: additional analysis from the Japan Diabetes Complications Study. *Diabetes Care*. 2006;29:145–147.
36. Examination Committee of Criteria for 'Obesity Disease' in Japan; Japan Society for the Study of Obesity. New criteria for 'obesity disease' in Japan. *Circ J*. 2002;66:987–992.
37. Alberti KG, Zimmet P, Shaw J. Metabolic syndrome—a new world-wide definition. A consensus statement from the International Diabetes Federation. *Diabet Med*. 2006;23:469–480.

Liver Enzymes as a Predictor for Incident Diabetes in a Japanese Population: The Hisayama Study

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Abstract

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Objective: We studied the relationship between liver enzymes and the development of diabetes in a general Japanese population.

Research Methods and Procedures: A total of 1804 nondiabetic subjects 40 to 79 years of age were followed-up prospectively for a mean of 9.0 years.

Results: During the follow-up, 135 subjects developed diabetes. In both sexes, the age-adjusted cumulative incidence of diabetes increased significantly with elevating quartiles of serum γ-glutamyltransferase (GGT) and alanine aminotransferase (ALT) levels. This pattern was also observed in aspartate aminotransferase (AST) quartiles for men but not for women. In multivariate analyses after adjusting for comprehensive risk factors and other liver enzymes, the risk of developing diabetes was significantly higher in the highest GGT quartile than in the lowest quartile [odds ratio (OR), 2.54; 95% confidence interval (CI), 1.03 to 6.26 for men; OR, 5.73; 95% CI, 1.62 to 20.19 for women]. Similar results were observed in ALT quartiles (OR, 2.32; 95% CI, 0.91 to 5.92 for men; OR, 4.40; 95% CI, 1.38 to 14.06 for women) but not in AST quartiles in either sex. Significant positive associations of GGT and ALT with diabetes were seen within each stratified category of risk factors, namely fasting insulin, BMI, waist-to-hip ratio, high-sensitivity C-reactive protein, and alcohol consumption. In receiver operating characteristic analyses, the areas under the receiver operating characteristic curve of GGT and ALT were significantly larger than that of AST, fasting insulin, waist-to-hip ratio, or C-reactive protein.

Discussion: Our findings suggest that serum GGT and ALT concentrations are strong predictors of diabetes in the general population, independent of known risk factors.

Key words: liver, longitudinal, C-reactive protein, diabetes, visceral obesity

Introduction

The liver, a major site of insulin clearance, plays an important role in maintaining normal glucose concentrations during fasting and postprandially (1). Recently, several cohort studies have shown that serum γ-glutamyltransferase (GGT)[1] (2–6), alanine aminotransferase (ALT) (7–9), and aspartate aminotransferase (AST) (10) levels are predictors of diabetes. In one of these reports, a study on Pima Indians (8) found that high serum ALT levels were a significant risk factor for diabetes, although no clear association between serum GGT and diabetes was seen. On the other hand, serum GGT levels, but not AST levels, have been identified as an independent predictor of incident diabetes in British men selected from lists of general practitioners (2). Moreover, the Mexico City Diabetes Study

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[1] Nonstandard abbreviations: GGT, γ-glutamyltransferase; ALT, alanine aminotransferase; AST, aspartate aminotransferase; HS-CRP, high-sensitivity C-reactive protein; HDL-C, high-density lipoprotein cholesterol; OR, odds ratio; CI, confidence interval; ROC, receiver operating characteristic.

found that serum AST is an independent risk factor for future diabetes in multivariable adjustment, whereas no association was observed between serum GGT or ALT and the development of diabetes (10). These reports suggest that the liver is associated with the development of diabetes; however, to the best of our knowledge, there have been no studies to date to determine which of these three enzymes is the best marker for incident diabetes. Furthermore, it also remains unknown whether liver enzyme markers are stronger predictors of future diabetes than well-known risk factors for diabetes, such as adiposity, insulin resistance, and inflammation. The purpose of this study is to examine the effects of serum liver enzymes, i.e., GGT, ALT, and AST, on the development of diabetes in a prospective study of a defined Japanese population, taking into account comprehensive risk factors, including BMI, waist-to-hip ratio, fasting insulin, and high-sensitivity C-reactive protein (HS-CRP) levels.

Research Methods and Procedures

Study Population and Follow-up Survey

A population-based prospective study of cardiovascular disease has been underway since 1961 in the town of Hisayama, a suburb in the Fukuoka metropolitan area on Kyushu Island in Japan. The age and occupational distributions of the town population were almost identical to those of Japan as a whole from 1961 to the present based on data from the national census. A screening survey for this study was performed in 1988. A detailed description of this survey has been published previously (11,12). Briefly, of all 3227 residents 40 to 79 years of age listed in the town registry, 2587 (80.2%) consented to take part in a comprehensive assessment, including an interview covering medical history (including diabetes, hypertension, and other chronic diseases) and current medical treatment with insulin and oral anti-diabetic agents. The baseline classification of subjects as either having or not having diabetes was based on the fasting criteria of the American Diabetes Association (13): subjects with a fasting plasma glucose level of ≥7.0 mM or those who were taking anti-diabetic medications were defined as having diabetes. A total of 2274 subjects (963 men and 1311 women) were enrolled in the baseline examination after the exclusion of 1 subject for whom no blood sample was obtained, 75 subjects who had already taken breakfast before the examination, 233 subjects with diabetes, and 4 subjects who had died before starting our follow-up.

After the initial screening in 1988, fasting glucose levels were again measured between 1993 and 1998. Of the 2274 subjects, 1804 (719 men and 1085 women) underwent a follow-up examination (follow-up rate, 79.3%). We considered a subject to have developed diabetes when his/her fasting glucose level met the above-mentioned American Diabetes Association criteria or if the subject started taking anti-diabetic medication during the follow-up period. During this period, 135 subjects (71 men and 64 women) developed diabetes.

Clinical Evaluation and Laboratory Measurements

Blood samples were collected after at least 12 hours of fasting for the determination of serum liver enzymes, plasma glucose, and other parameters. Serum GGT concentrations were measured using a modified version of the method of Orlowski and Meister (14). Both serum ALT and AST concentrations were determined by a kinetic ultraviolet ray method based on the rate of reduced nicotinamide adenine dinucleotide oxidation. Plasma glucose levels were determined by a glucose-oxidase method, and serum insulin levels were measured by double-antibody, solid-phase radioimmunoassay. Hemoglobin A_{1c} levels were measured by high-pressure liquid chromatography. Total cholesterol, high-density lipoprotein cholesterol (HDL-C) and triglycerides were determined enzymatically. HS-CRP concentrations were analyzed using a modified latex-enhanced HS-CRP assay (Behring Diagnostics, Westwood, MA). Serum hepatitis B surface antigen was detected by an immunoprecipitation method (Shino-test, Tokyo, Japan), and presence of hepatitis C virus antibody was assessed by both particle agglutination assay (Serodia-HCV; Fujirebio, Tokyo, Japan) and recombinant immunoblot assay (RIBA 2.0; Ortho Diagnostic Systems, Raritan, NJ).

Blood pressure was obtained three times using a mercury sphygmomanometer with the subject in a sitting position; the averages of the three values were used in this analysis. Hypertension was defined as a systolic blood pressure of ≥140 mm Hg and/or a diastolic blood pressure of ≥90 mm Hg and/or current treatment with anti-hypertensive agents. The height and weight of each subject were recorded with the subject wearing light clothes but no shoes, and BMI (kg/m^2) was calculated. Abdominal girth at the umbilical level and hip circumference at 5 cm below the spina iliaca anterior superior were measured and used to calculate the waist-to-hip ratio.

On baseline examination, each participant completed a self-administered questionnaire covering medical history, anti-hypertensive treatment, alcohol intake, and smoking habits, and the questionnaire was checked by trained interviewers at the screening. Diabetes in first- or second-degree relatives was taken to indicate a family history of diabetes. Subjects engaging in sports at least three times per week during their leisure time were defined as the regular exercise group. Alcohol intake and smoking habits were used to classify subjects as having current habits or not.

Statistical Analysis

Because the distributions of GGT, ALT, AST, fasting insulin, HS-CRP, and triglycerides were skewed, these variables were natural log-transformed for statistical analyses.

Table 1. Characteristics of subjects by sex

	Men (n = 719)	Women (n = 1085)
Age (yrs)	58 ± 10	58 ± 10
GGT (units/L)	22 (11 to 95)	13 (8 to 35)
ALT (units/L)	14 (7 to 38)	11 (6 to 24)
AST (units/L)	22 (14 to 45)	19 (12 to 33)
Fasting plasma glucose (mM)	5.6 ± 0.5	5.5 ± 0.5
Hemoglobin A_{1c} (%)	5.5 ± 0.5	5.4 ± 0.5
Family history of diabetes (%)	9.2	7.2
Fasting insulin (pM)	30.0 (18.0 to 72.0)	36.0 (18.0 to 72.0)
BMI (kg/m^2)	22.9 ± 2.9	23.0 ± 3.1
Waist-to-hip ratio	0.92 ± 0.05	0.91 ± 0.07
Total cholesterol (mM)	5.07 ± 1.03	5.54 ± 1.04
HDL-cholesterol (mM)	1.25 ± 0.30	1.34 ± 0.29
Triglycerides (mM)	1.24 (0.57 to 3.49)	1.02 (0.49 to 2.32)
HS-CRP (mg/L)	0.49 (0.07 to 7.14)	0.36 (0.06 to 3.22)
Systolic blood pressure (mm Hg)	131 ± 17	130 ± 20
Diastolic blood pressure (mm Hg)	82 ± 11	76 ± 11
Hypertension (%)	42.8	33.2
Current drinking (%)	60.8	8.6
Current smoking (%)	47.3	5.5
Regular exercise (%)	15.9	4.9

HDL, high-density lipoprotein; HS-CRP, high-sensitivity C-reactive protein; GGT, γ-glutamyltransferase; AST, aspartate aminotransferase; ALT, alanine aminotransferase. Variables of GGT, AST, ALT, fasting insulin, triglycerides, and CRP are median values (95% confidence intervals). All other values are given as mean ± standard deviation or as a percentage.

To analyze liver enzyme levels as categorical variables, these levels were divided into four groups on the basis of quartiles by sex: GGT, men, 6 to 16, 17 to 22, 23 to 37, and 38 to 529 U/L; GGT, women, 6 to 10, 11 to 13, 14 to 17, and 18 to 261 U/L; ALT, men, 5 to 10, 11 to 13, 14 to 18, and 19 to 354 U/L; ALT, women, 5 to 8, 9 to 11, 12 to 14, and 15 to 153 U/L; AST, men, 8 to 17, 18 to 21, 22 to 27, and 28 to 424 U/L; AST, women, 7 to 16, 17 to 18, 19 to 22, and 23 to 273 U/L. The age-adjusted cumulative incidences of diabetes were calculated by the direct method using all subjects, and the results were compared by the Mantel-Haenszel χ^2 test using 10-year age-groupings. Age- and multivariate-adjusted odds ratios (ORs) and 95% confidence intervals (CIs) were calculated by logistic regression analysis. The sensitivity of cut-off points was defined as their ability to correctly identify individuals who later developed diabetes, and their specificity was defined as their ability to correctly identify individuals who did not develop diabetes. To compare the prognostic abilities of risk factors including liver enzymes and to detect the presence or absence of future diabetes across a range of the values for each risk factor, we plotted receiver operating characteristic (ROC) curves and compared the areas under them (15,16). The diagnostic properties of specific cut-off levels of each risk factor were defined by maximizing the sensitivity and specificity to identify future diabetes. A value of $p < 0.05$ was considered statistically significant in all analyses.

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 participants.

Results

The clinical characteristics of all subjects by sex are shown in Table 1. The mean age was 58 years for both sexes. The mean values of GGT, ALT, AST, fasting plasma glucose, hemoglobin A_{1c}, waist-to-hip ratio, triglycerides, HS-CRP, systolic and diastolic blood pressures, frequency of hypertension, alcohol intake, smoking habits, and regular exercise were higher in men than in women, whereas women had higher concentrations of fasting insulin, total cholesterol, and HDL-C. The frequency of family history of diabetes and mean BMI levels did not differ between the sexes.