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Functional SNP of *ARHGEF10* confers risk of atherothrombotic stroke

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

INTRODUCTION

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

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

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

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

RESULTS

Case–control association study

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

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

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

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

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

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

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

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

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

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

rs4376531 increases the incidence of ischemic stroke

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

DISCUSSION

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

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

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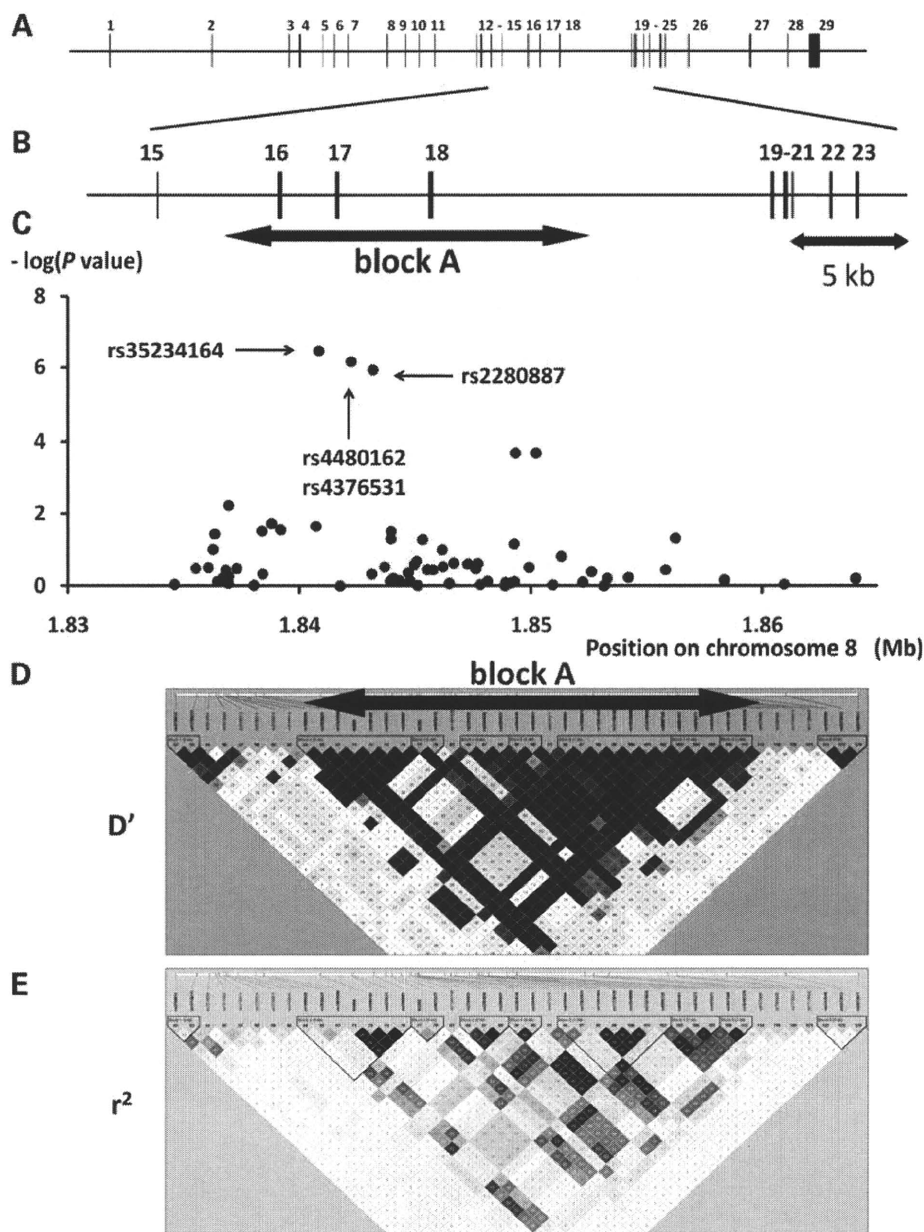

Figure 1. Exon–intron structure, case–control results and LD map in *ARHGEF10*. (**A**) All of the exon–intron structure of *ARHGEF10*. (**B**) Exon–intron structure around the marker SNP, rs2280887. (**C**) Case–control association study results. The $-\log_{10}$-transformed *P*-values for a dominant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

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

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

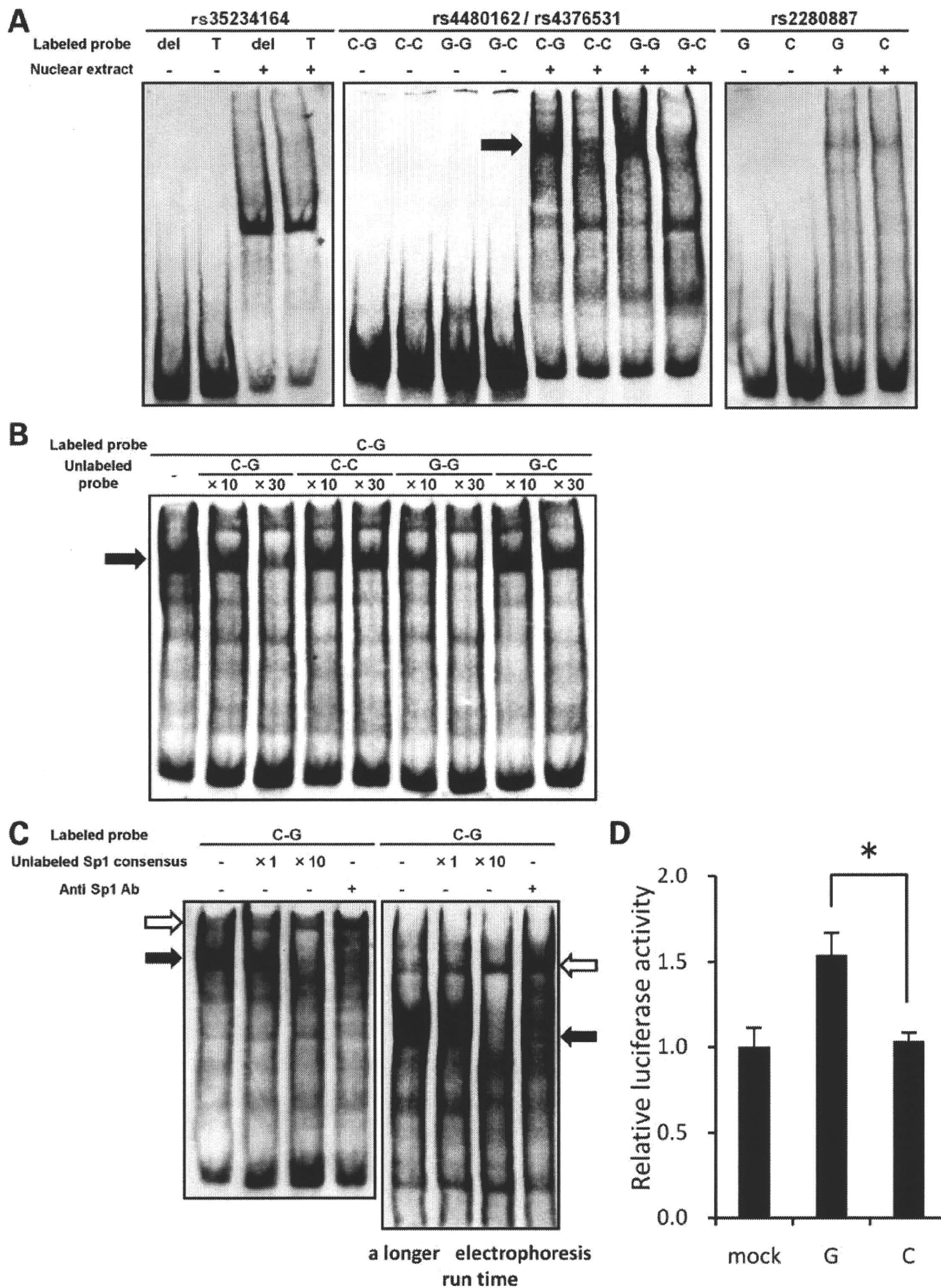

Figure 2. rs4376531 alters the binding affinity of Sp1 and affects *ARHGEF10* transcriptional activity. (**A**) EMSA using 5′ end-labeled 50 bp probes around each allele of SNPs in *ARHGEF10*. A black arrow indicates the shifted band that shows tighter binding of a nuclear protein to the susceptible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

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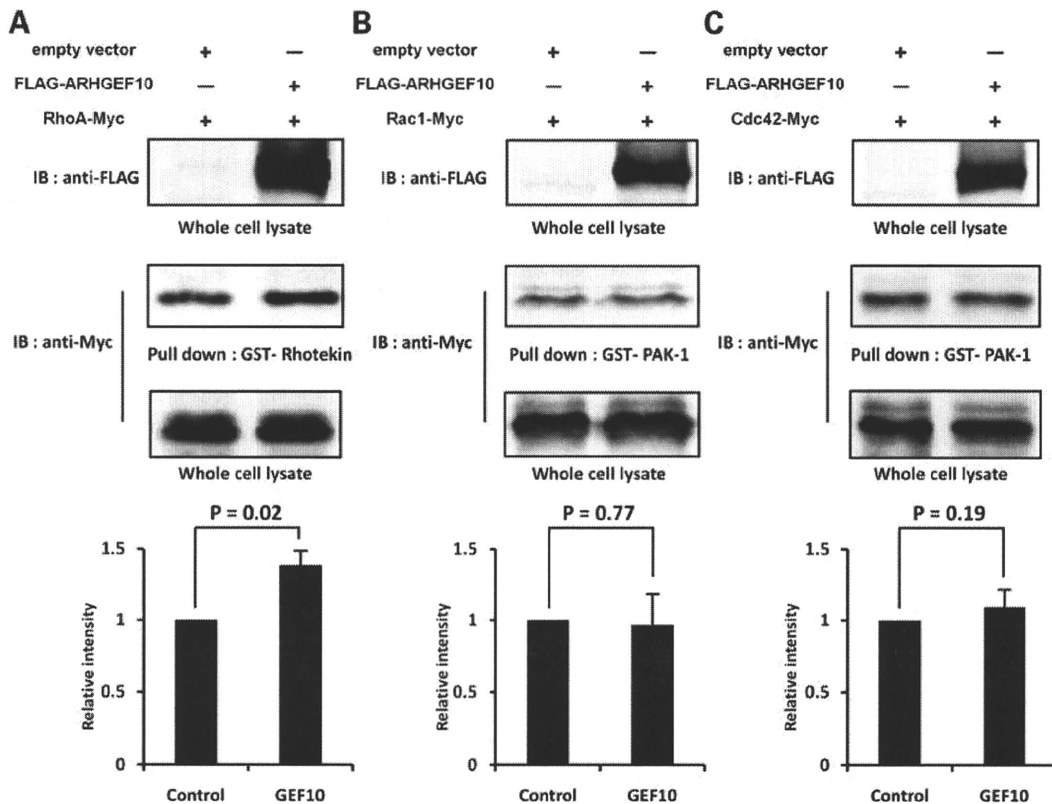

Figure 3. ARHGEF10 activates RhoA specifically. (**A**) RhoA activity assay. 293FT cells were co-transfected with the plasmids as indicated at the top. Cell lysates were pulled down by GST-Rhotekin and subjected to immunoblot with anti-Myc antibody. Whole-cell lysates were analyzed by immunoblot with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

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

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

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

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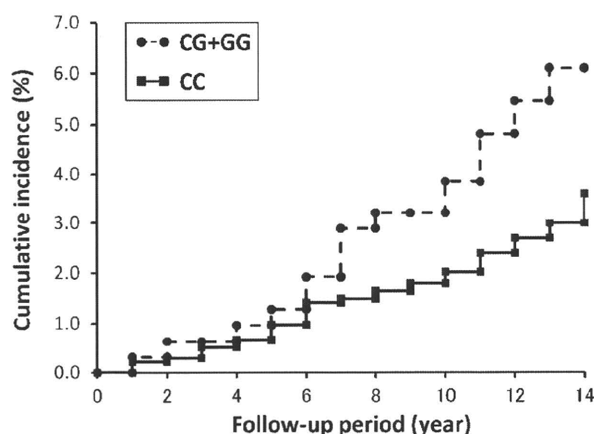

Figure 4. Kaplan–Meier estimates of the incidence of ischemic stroke by rs4376531 during 14-year follow-up period in the Hisayama study.

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

MATERIALS AND METHODS

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

Study populations

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

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

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

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

SNP selection and genotyping

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

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

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

Cell culture

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

Electrophoretic mobility shift assay

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

Luciferase reporter assay

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

Small GTPase activity assay

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

Statistical analysis

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

SUPPLEMENTARY MATERIAL

Supplementary Material is available at *HMG* online.

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Conflict of Interest statement. None declared.

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SHORT COMMUNICATION

Brain-Derived Neurotrophic Factor Treatment Increases the Skeletal Muscle Glucose Transporter 4 Protein Expression in Mice

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Summary

The purpose of the present study was to investigate whether peripheral brain-derived neurotrophic factor (BDNF) treatment induced metabolic adaptations in mouse skeletal muscle. BDNF (20 mg/kg/day) was injected subcutaneously for successive 14 days. BDNF treatment significantly reduced the total food intake and inhibited the weight gain in comparison to the control group. The glucose transporter 4 (GLUT4) protein expression in the gastrocnemius muscle was significantly increased by BDNF treatment in comparison to the control and pair-fed groups. Neither the oxidative nor the glycolytic enzyme activities in the gastrocnemius muscle changed after the BDNF treatment. These results suggest that the peripheral BDNF treatment promotes the skeletal muscle GLUT4 protein expression as well as hypophagia.

Key words

BDNF • GLUT4 • Hypophagia • Skeletal muscle

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Brain-derived neurotrophic factor (BDNF) is a part of the neurotrophin family and is produced in the nervous system and periphery. The BDNF controls the food consumption (Xu *et al.* 2003), lipid and glucose metabolism (Nakagawa *et al.* 2000, Tsuchida *et al.* 2002), and insulin resistance (Kuroda *et al.* 2003). Recent human studies have shown that circulating BDNF is associated with eating disorders (Nakazato *et al.* 2003, Monteleone *et al.* 2004), obesity (Monteleone *et al.* 2004, Suwa *et al.* 2006), glucose and lipid metabolism (Suwa *et al.* 2006, Levinger *et al.* 2008), type II diabetes mellitus (Suwa *et al.* 2006) and metabolic syndrome (Chaldakov *et al.* 2003, 2004). Based on these metabolic contributions, BDNF is considered to be a "metabotrophins" (Chaldakov *et al.* 2007).

Skeletal muscle metabolic characteristics such as glucose transporter 4 (GLUT4) expression and mitochondrial oxidative capacity are associated with skeletal muscle insulin-stimulated glucose uptake, whole body insulin sensitivity and prevalence of type II diabetes mellitus (He *et al.* 2001, Bruce *et al.* 2003, Doehner *et al.* 2010). Chronic BDNF treatment to diabetic mice significantly improves the glucose uptake in skeletal muscle (Yamanaka *et al.* 2007). Based on these results, the BDNF is hypothesized to regulate the skeletal muscle metabolism. This study examined whether chronic BDNF treatment to mice affects skeletal muscle metabolic characteristics such as GLUT4 protein expression and glycolytic and oxidative enzyme activities.

Sixty-nine- to 72-day old female ICR mice were

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used for the current study. All mice were fed a standard rodent chow (CE-2, CLEA Japan, Inc., Tokyo, Japan). All experimental procedures were approved by the Nakamura Gakuen University Animal Experiment Committee.

Because BDNF treatment reduces food intake (Nakagawa *et al.* 2003), the effects of BDNF treatment was studied in comparison with both *ad libitum*-fed control and pair-fed mice. The mice were divided into an *ad libitum*-fed (AL, n=8), a pair-fed (PF, n=8), or a BDNF-treated (BDNF, n=8) group. The mice of the BDNF group were subcutaneously administered daily with 20 mg/kg body mass BDNF (Dainippon Sumitomo Pharma, Osaka, Japan) in saline for 14 successive days. This dose of BDNF has been shown to enhance the skeletal muscle glucose uptake (Yamanaka *et al.* 2007). In the AL and PF groups, a comparable volume of saline was administered subcutaneously.

About 24 h after the last administration, the mice were fasted for 4 h and anesthetized with pentobarbital sodium (60 mg/kg body weight i.p.). The gastrocnemius muscle was rapidly dissected, frozen in liquid nitrogen and stored at –80 °C until the analyses were performed.

The GLUT4 protein expression was determined by Western blotting and the enzyme activities including citrate synthase (CS), malate dehydrogenase (MDH), β–hydroxyacylCoA dehydrogenase (βHAD), hexokinase (HK), and lactate dehydrogenase (LDH) were measured spectrophotometrically as described previously (Suwa *et al.* 2008).

To compare the findings among the three groups, a one-way analysis of variance (ANOVA) was used. Fisher's PLSD was conducted if the ANOVA indicated a significant difference. A value of $P<0.05$ was considered to be significant.

The body mass prior to the treatment was similar in all three groups (AL; 27.5±0.5 g, PF; 27.9±0.6 g, BDNF; 27.6±0.2 g). The changes in the body mass in the PF (0.0±0.7 g) and BDNF (-0.3±0.4 g) groups were significantly lower than AL group (1.7±0.4 g) (Fig. 1A, $P<0.05$). Total food intake in the PF (49.7±2.1 g) and BDNF (49.7±2.1 g) groups were significantly lower than in AL group (58.7±1.9 g) (Fig. 1B, $P<0.01$). These results suggest that BDNF treatment inhibits the body mass increase because of reducing food intake.

The GLUT4 protein expression in the BDNF group was significantly higher by +37 % and +35 % than in the AL and PF groups, respectively (Fig. 2, $P<0.05$). Oxidative (CS, MDH and βHAD) and glycolytic (HK and LDH) enzyme activities were measured, and no differences were observed among the groups in any enzymes (data not shown).

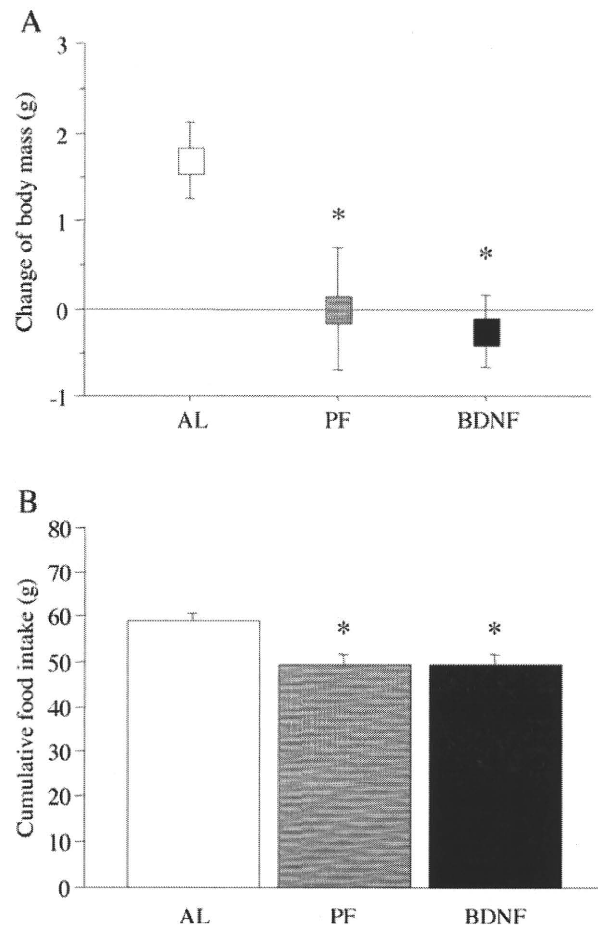

Fig. 1. A: Change of body mass during treatment. **B:** Cumulative food intake during the treatment. N=8 per group. Data are expressed as the mean ± S.E.M. *; P<0.05 vs. AL.

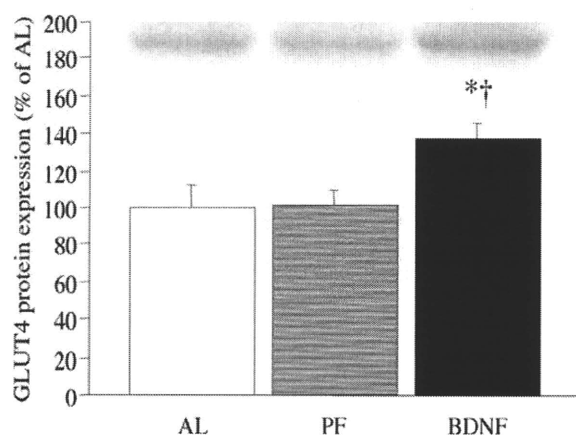

Fig. 2. GLUT4 protein expression of the gastrocnemius muscle in the 3 groups. N=8 muscles per group. Data are expressed as the mean ± S.E.M. *; P<0.05 vs. AL. †; P<0.05 vs. PF.

The current study demonstrated that the subcutaneous BDNF injection to mice significantly decreased the food intake in agreement with the previous study (Nakagawa *et al.* 2003). In humans, the serum BDNF level may demonstrate a possible link with such eating disorders as *bulimia nervosa* and *anorexia nervosa* (Nakazato *et al.* 2003, Monteleone *et al.* 2004). Circulating BDNF may therefore play a role in suppressing food intake. BDNF expressed in ventromedial hypothalamus neurons has been shown to apparently suppress food consumption downstream of the melanocortin-4 receptor (Xu *et al.* 2003). Because BDNF can cross the blood-brain barrier (Pan *et al.* 1998), the subcutaneous injection of BDNF reduces the food intake possibly *via* hypothalamus neurons.

The most important finding in the current study is that the BDNF treatment increases the GLUT4 expression. GLUT4 plays an important role in skeletal muscle glucose uptake (Röckl *et al.* 2008). GLUT4 protein abundance is strongly associated with capacity of skeletal muscle glucose uptake (Doehner *et al.* 2010), suggesting that skeletal muscle GLUT4 abundance is a potential limiting factor of whole body and skeletal muscle glucose metabolism. The increasing GLUT4 protein expression in the current study is thus considered to improve the glucose metabolism.

Although it has been generally accepted that the neurotrophins act by either paracrine or autocrine mechanisms (Davies 1996), BDNF also exists in the blood (Radka *et al.* 1996). More than 90 % of blood BDNF is stored in platelets, and platelets can release the BDNF (Fujimura *et al.* 2002). Platelets are assumed to release BDNF at nerves or other tissues expressing BDNF receptor tyrosine kinase B (Fujimura *et al.* 2002). In addition, circulating BDNF level is associated with eating behavior (Monteleone *et al.* 2004), metabolic disorders (Chaldakov *et al.* 2003, 2004, Suwa *et al.* 2006), physical activity (Nofuji *et al.* 2008), depression (Brunoni *et al.* 2008), Alzheimer's disease (Laske *et al.* 2006), and cognitive function (Gunstad *et al.* 2008). We therefore presume that circulating BDNF might possess several of physiological functions including GLUT4 biogenesis and thereby mimic the endocrine mechanism.

Although this is only a preliminary study, the results presented herein raise the possibility that BDNF treatment may potentially contribute to the therapy of obesity and type II diabetes mellitus, while also helping to treat related cardiometabolic diseases. Further studies are necessary to identify the therapeutic effects of BDNF for such diseases and to clarify the mechanism underlying the effects of BDNF for GLUT4 expression and hypophagia.

Conflict of Interest

There is no conflict of interest.

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Research article

Association of cardiorespiratory fitness with elevated hepatic enzyme and liver fat in Japanese patients with impaired glucose tolerance and type 2 diabetes mellitus

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Abstract
No study has so far determined whether a favorable level of cardiorespiratory fitness (CF) contributes to a reduced risk of elevated hepatic enzymes and a high degree of liver fat in patients having various metabolic risks. This study investigated the association between the maximal oxygen uptake (VO_2 max) and the prevalence of elevated liver enzymes and high liver fat, while considering such factors as abdominal obesity, hyperinsulinemia and the other metabolic risks. The study enrolled newly diagnosed Japanese patients (n = 84; 52 males and 32 females; aged 25-69 years) with impaired glucose tolerance (IGT) and type 2 diabetes mellitus (Type2DM) who did not receive any intervention or pharmacological therapy. The subjects were divided into 3 groups according to the distribution of the VO_2max for each sex. The odds ratios (ORs) for the prevalence of elevated aspartate and alanine aminotransferase (AST and ALT) and high degree of liver fat adjusted for age, sex, disease type, daily ethanol intake, and current smoking were significantly lower in the moderate- and high CF groups in comparison to the low CF group. In addition, a significant OR for AST was maintained in the moderate and high CF group after adjusting for abdominal obesity and/or hyperinsulinemia. The significant ORs for the prevalence of elevated ALT and a high degree of liver fat were attenuated after adjusting for abdominal obesity and/or hyperinsulinemia. No significant OR for the prevalence of elevated gamma-glutamyl transferase (GGT) was recognized in all logistic models. These results indicated that CF was negatively and independently associated with the prevalence of elevated AST even in Japanese diabetic patients having various metabolic risks. It was concluded that the AST level might be useful as a simple marker reflecting physical inactivity in such subjects.

Key words: Cardiorespiratory fitness, hepatic enzyme, non-alcoholic fatty liver, abdominal obesity, insulin resistance.

Introduction

Hepatic enzymes are primary indices for the diagnosis of non-alcoholic fatty liver disease (NAFLD), which is noticed as one of phenotypes of metabolic syndrome (André et al., 2007). Furthermore, elevated hepatic enzymes have been noted as a predictor of metabolic syndrome, type 2 diabetes mellitus (Type2DM) and cardiovascular disease (André et al., 2006; Cho et al., 2007; Doi et al., 2007, Monami et al., 2008; Nakanishi et al., 2004; Rector et al., 2008; Sattar et al., 2004). Hepatic enzymes might therefore be a general marker reflecting the pathology of these diseases.

On the other hand, cardiorespiratory fitness (CF), which is a direct index of physical activity, plays a role of suppressing the onset of type 2 DM, metabolic syndrome, cardiovascular diseases and mortality (LaMonte et al., 2005; Lakka et al., 2002; Sawada et al., 2003; Sui et al., 2007; Lyerly et al., 2009). In addition, recent cross-sectional studies reported an inverse association between CF and NAFLD (Church et al., 2006; Lawlor et al., 2005; Nguyen-Duy et al., 2003; Perseghin et al., 2007). It is therefore naturally expected that a favorable level of CF might be related not only with a low prevalence of NAFLD, but also elevated levels of hepatic enzymes.

A recent study (Messier et al., 2010) has demonstrated that metabolically healthy but obese women who were in the upper quartile of insulin sensitivity values had significantly lower concentrations of ALT, AST, and GGT as well as a lower fatty liver index in comparison to individuals in the lower 3 quartiles. However, this study did not evaluate either the physical activity or CF. A survey performed on adults aged 17 yrs of age or older in US (n = 15676) (Clark et al., 2003) reported unexplained aminotransferase elevation, which was significantly associated with a higher body mass index, waist circumference, triglyceride levels, fasting insulin, and lower HDL. It is well-known that these indices are strongly influenced by physical activity; however, no description regarding lifestyle was made in that report. Furthermore, the most of those studies are conducted in normal populations, and no study has yet investigated the impact of the maximal oxygen uptake on both liver fat and liver enzymes while taking other metabolic risks into consideration in specific subjects having a number of metabolic abnormalities.

The current study therefore investigated whether the prevalence of high degree of liver fat and elevated liver enzymes could be associated with low level of CF in newly diagnosed impaired glucose tolerance (IGT) and Type2DM patients with various metabolic risks but not consuming excessive amounts of alcohol.

Methods

Subjects

One hundred fifty-seven Japanese outpatients (114 males and 43 females, aged 25 to 81 years) who were newly-diagnosed to have IGT and Type2DM based on a 75g oral glucose tolerance test (75g OGTT) participated in the present study. The pathological state was classified based

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on the diagnostic criteria of the Committee of Japan Diabetes Society (Kuzuya et al., 2002). Though 2-24 months passed from the time that the patients were noted to have an elevated blood glucose level at a group medical checkup, none of the subjects had received pharmacological therapy or intervention until the diagnosis.

The patients answered a questionnaire to assess their alcohol consumption and current smoking habits. The type, amount, and frequency of alcohol consumption were assessed, from which the total amount of alcohol consumption was calculated and converted to the daily ethanol intake. Sixty-five subjects whose daily ethanol intake was more than 20g in males and 10g in females (Hashimoto, 2004), were excluded from the analysis. In addition, any cases including missing data needed for an analysis (n = 8) were also excluded. Finally, the data of 84 patients (52 male and 32 female, aged 25 to 69 years) were used for the analysis of the present study. Informed consent was obtained from each patient and the study was approved by The Ethics Committee of Institute of Health Science in Kyushu University.

Anthropometric measurement and protocol for computed tomography

The BMI was calculated as the weight (kilograms) divided by height (meters) squared. The waist circumference was measured at the level of the umbilicus. The visceral (VFA) and subcutaneous fat areas (SFA) were assessed by computed tomography (CT; VIGOR LAU DATOR, Toshiba, Japan). The subjects were examined following overnight fasting and in the supine position. Scanning was performed using the usual clinical assessment settings, i.e., 120kV and 200mA, 400mm field of view, 5mm thickness, and 2sec scanning time. The regions of interest were determined by the clinical specialists by tracing an outline of the adipose tissue on the CT image at the umbilical level. The whole abdominal and visceral fat areas were computed automatically based on the pixels for the X-ray attenuation range of these areas (Tokunaga et al., 1983). The SFA values were derived by subtracting the VFA from the whole abdominal fat area. In addition, liver fat deposition was evaluated using a CT image including both the liver and spleen derived from the twelfth thoracic vertebra level to the second lumbar vertebra level. The analysis of the mean CT attenuation values derived for the liver and spleen were performed by clinical specialists in diagnostic imaging. The ratio of the liver/spleen attenuation value (L/S ratio) was defined as an index of liver fat (Church et al., 2006).

Measurements of clinical data

Following overnight fasting of at least 9 hrs, blood samples were drawn from antecubital vein for the analysis as below; sampling tubes of EDTA 2K-NaF and plain were used. A 75g OGTT was performed on the subjects' blood samples obtained at 30, 60, 120, and 180 minutes. The fasting insulin and fasting blood glucose concentrations were measured using a radioimmunoassay and an enzymatic method, respectively. The levels of aspartate aminotransferase (AST), alanine aminotransferase (ALT), and gamma-glutamyl transferase (GGT) were determined as indices of the hepatic function, using a method recommended by the Japanese Society of Clinical Chemistry for determining the catalytic amounts of enzymes. Tests for hepatitis B or C virus and other liver diseases were performed on the subjects whose AST and/or ALT were over 100 IU/L. The levels of fasting triglyceride, total cholesterol, and high-density lipoprotein cholesterol were assessed using an enzymatic method. The resting systolic (SBP) and diastolic blood pressure (DBP) were determined 3 times following a 30-minute rest period using a mercury sphygmomanometer, with the lowest values used as the resting blood pressure. The subjects newly diagnosed to have IGT or Type2DM were instructed to undergo an anthropometric evaluation and a fitness test within 2 to 3 weeks following the diagnostic tests.

Criteria for abnormalities of parameters

The definition of elevated liver enzymes based on a statement by The Ministry of Health, Labour and Welfare in Japan, 2007. The abnormal criteria for each enzyme were as follows; elevated AST: AST > 30U/L, elevated ALT: ALT > 30U/L, and elevated GGT: GGT > 50U/L. Furthermore, a patient with an L/S ratio less than 0.9, which is a cutoff value usually adopted in domestic medical institutions (Hashimoto, 2006), was regarded as having high liver fat.

Patients whose VFA levels were more than 100 cm^2 were defined as having excess visceral fat (The Examination Committee of Criteria for "Obesity Disease" in Japan, 2002). The fasting insulin equivalent was determined to be 7μU/mL, a 75th percentile value of fasting insulin in Japanese male workers (Tamakoshi et al., 2003) as the basic criteria for hyperinsulinemia in this study.

Evaluation of cardiorespiratory fitness

Graded exercise tests were performed by a skilled examiner using a cycle ergometer (Monark, Stockholm, Sweden) to evaluate the CF. The heart rate, electrocardiogram, and blood pressure were monitored and recorded during the test. The exercise intensity was increased 3 or 4 times every 4 minutes until the heart rate reached 70% of the maximum or higher. Maximal oxygen uptake (VO_2max), which is regarded as an index of CF, was determined according to the nomogram of Åstrand & Rhyming (1954), a modality that is generally used to predict the VO_2max.

The distributions of VO_2max were divided into tertiles in each sex. The details regarding the range in each group were as follows; the lowest tertile (Low-CF group): $VO_2max \leq 31.8$ml/kg/min in males and $VO_2max \leq 26.2$ in females; the intermediate tertile (Moderate-CF group): $31.8 < VO_2max \leq 35.6$ in males and $26.2 < VO_2max \leq 30.2$ in females; and the highest tertile (High-CF group): $VO_2max > 35.6$ in males and $VO_2max > 30.2$ in females.

Statistical analysis

An analysis of variance (ANOVA) was performed to compare continuous variables of the subjects classified by CF level. TG, fasting glucose and insulin, AST, ALT, and GGT had a skewed distribution and were therefore analyzed following log-transformation. A comparison of categorical variables was analyzed using chi-square analysis. The odds-ratio (OR) and 95% confidence inter-

val (95%CI) for the prevalence of any abnormalities in each group were calculated using 4 logistic regression models. First, ORs adjusted for age, sex, disease type, daily ethanol intake, and smoking as basic confounding factors for the prevalence of these abnormalities were calculated (Model-1). After the analysis using Model-1, the ORs were adjusted for abdominal obesity or hyperinsulinemia (Model-2 and 3), finally, adjustments for both abdominal obesity and hyperinsulinemia were added (Model-4). All statistical analyses were performed using the SPSS version 14.0 software program (SPSS Japan Inc.). Statistical significance was set at a value of $p < 0.05$.

Results

Characteristics of the subjects divided by the CF level

Characteristics of all subjects and those classified by CF levels are indicated in Table 1. The distribution of the subjects' VO_2max was observed to have shifted slightly to a lower level and the whole range was narrower than that in the Japanese healthy population.

The mean value of the VFA in all the subjects ($160.4 \pm 63.2 cm^2$) was substantially higher than the Japanese criteria for abdominal obesity ($\geq 100 cm^2$). The mean value of the fasting insulin level ($7.4 \pm 4.7 \mu U/ml$) was as high as the mean value of the top quartile in Japanese male workers (Tamakoshi et al., 2003). Prevalence of elevated AST, ALT and GGT in all subjects was 23, 49 and 31%, respectively. The subjects having elevated AST accounted for 48, 14 and 7% in the high, moderate and low CF group, respectively. The elevated ALT in each group accounted for 74, 41 and 32%, in addition, the elevated GGT was accounted for 37, 35 and 21%, respectively. Further, prevalence of high liver fat in all subjects was 21%, and 41, 14 and 11% in each fitness level, respectively. The Abdominal and liver fatness, fasting insulin, AST and ALT levels showed a gradual decrease according to the increase of CF level.

Analysis of the prevalence of abnormalities in the groups classified by CF level

As indicated in Table 2, The ORs for the prevalence of elevated AST in the moderate- and high CF group were significantly low in all models in comparison to the low CF group; the ORs ranged from 0.06 to 0.14. The ORs for an elevated ALT in the moderate- and high CF group were also significantly low in model 1, which ranged from 0.15 to 0.25. Model 2 showed a significant OR for elevated ALT only in high CF group. However, the significant ORs were attenuated after adjusting for only hyperinsulinemia (model 3), and after adjusting for both abdominal obesity and hyperinsulinemia (model 4). The ORs for an elevated GGT showed no significance in any group. The OR for high liver fat in the high CF group was significantly low in comparison to the low CF group (OR: 0.21) in model 1; however, the ORs in the other models adjusted for abdominal obesity and/or hyperinsulinemia showed no significance in any group.

Discussion

The main finding in the current study was that a favorable level of CF contributed to the attenuation of the elevated

Table 1. Comparison of characteristics of subjects classified by fitness level.

Continuous variables	All subject (M=52, F=32)		Fitness level: Low (M=18, F=9)		Fitness level: Moderate (M=17, F=12)		Fitness level: High (M=17, F=11)		p
	Mean	SD	Mean	SD	Mean	SD	Mean	SD	
Age (yrs)	50.9	10.7	47.4	11.5	53.6	10.4	51.4	9.6	N.S.
BMI ($kg \cdot m^{-2}$)	25.1	4.1	27.7	4.4	24.8	3.2	22.9	3.2	< .001
Waist girth (cm)	88.4	10.1	94.3	10.8	88.0	7.5	83.0	8.8	< .001
Daily ethanol intake (g)	3.0	5.2	1.7	3.6	3.9	5.5	3.4	6.1	N.S.
Type 2 DM (%)	60.0 (71.4)		20.0 (74.1)		21.0 (72.4)		19.0 (67.9)		
Current smoking (%)	26.0 (31.0)		10.0 (37.0)		6.0 (20.7)		10.0 (35.7)		N.S.
Visceral fat area (cm^2)	160.4	63.2	197.6	70.4	155.2	44.9	129.8	55.2	< .001
Subcutaneous fat area (cm^2)	172.1	86.0	202.1	104.1	165.7	78.6	149.8	66.7	N.S.
L / S ratio †	1.03	0.26	0.90	0.28	1.09	0.17	1.08	0.26	< .005
AST (U/L)	26.3	12.5	33.9	14.3	22.6	8.9	22.8	10.6	< .001
ALT (U/L)	38.8	31.0	57.6	39.3	29.3	17.3	30.5	25.3	< .001
GGT (U/L)	43.7	26.8	53.0	31.9	42.2	25.7	36.3	19.7	N.S.
VO_2max ($ml \cdot kg^{-1} \cdot min^{-1}$)	32.1	5.7	27.5	3.8	31.1	3.0	37.5	4.9	< .001
Total cholesterol (mg/dL)	217.6	35.9	211.0	39.1	226.6	31.2	214.7	36.8	N.S.
Triglyceride (mg/dL)	134.4	78.3	140.1	77.8	127.9	67.2	135.5	90.8	N.S.
HDL-C (mg/dL)	51.4	12.5	47.1	11.0	55.3	14.9	51.6	10.1	N.S.
Fasting glucose (mg/dL)	136.1	33.5	136.3	41.8	140.9	32.8	131.0	24.3	N.S.
Fasting insulin (μU/mL)	7.4	4.7	10.3	5.8	6.3	3.3	5.6	3.3	< .001
Systolic blood pressure (mmHg)	127	17	133	16	124	18	124	17	N.S.
Diastolic blood pressure (mmHg)	80	11	84	11	79	11	76	9	< .05

Abbreviations are denoted in text. Data are expressed as means±S.D. or number of patients. The percentage in each group is shown in parenthesis. One-way ANOVA or Chi-square test was performed for statistical analysis. N.S. not significant.

Table 2. **Odds ratios for the elevated hepatic enzymes and NAFL in the groups classified by fitness level (n = 84).**

	Model 1 [a]			Model 2 [b]			Model 3 [c]			Model 4 [d]		
	OR	95%CI	p	OR	95%CI	p	OR	95%CI	p	OR	95%CI	p
Elevated AST												
Moderate CF	**.11**	**.02-.55**	**.007**	**.12**	**.02-.58**	**.009**	**.13**	**.02-.78**	**.025**	**.14**	**.02-.85**	**.033**
High CF	**.06**	**.01-.36**	**.002**	**.06**	**.01-.42**	**.004**	**.07**	**.01-.49**	**.008**	**.07**	**.01-.58**	**.013**
Elevated ALT												
Moderate CF	**.25**	**.06-.94**	**.041**	.28	.07-1.07	.063	.48	.11-2.02	.314	.52	.12-2.29	.390
High CF	**.15**	**.04-.58**	**.006**	**.20**	**.05-.83**	**.027**	.29	.07-1.25	.096	.39	.09-1.79	.226
Elevated GGT												
Moderate CF	.99	.28-3.47	.981	1.08	.31-3.81	.906	1.15	.29-4.65	.842	1.30	.32-5.25	.714
High CF	.52	.14-1.90	.320	.66	.17-2.53	.545	.60	.14-2.52	.488	.78	.18-3.37	.740
High liver fat												
Moderate CF	.35	.08-1.49	.155	.37	.09-1.63	.191	1.04	.18--5.86	.963	1.06	.19-5.92	.950
High CF	**.21**	**.05-.99**	**.048**	.28	.06-1.33	.109	.62	.10-3.63	.592	.77	.12-4.77	.778

These odds ratios are referring for that in the low CF group. Abbreviations are denoted in text. [a]: Adjusted for age, sex, disease type, daily ethanol intake and current smoking. [b]: Added adjusting for abdominal obesity to the Model 1. [c]: Added adjusting for hyperinsulinemia to the Model 1. [d] Added adjusting for abdominal obesity and hyperinsulinemia to the Model 1. CI: confidence interval.

AST, independent of the pathology frequently observed in the diabetic subjects. The prevalence of elevated AST was below one fourth of all subjects, whereas one half of them were included in the low CF group. On the other hand, the association of elevated ALT or high liver fat with CF depended on the presence of abdominal obesity and/or hyperinsulinemia in diabetic subjects. No association found between CF level and elevated GGT.

It is highly important to identify the difference in the strength of association with CF among these enzymes. This remains a matter for speculation, but might be due to a difference in the location of these enzymes. While ALT and GGT exist mainly in hepatic cells, AST exists not only in the hepatic cells, but also in cardiac and muscle cells. In the current study, the prevalence of elevated AST among the subjects with a high degree of liver fat was 50%, which was obviously lower than that in the subjects demonstrating both high liver fat and elevated ALT or GGT (88.9 and 72.2%, respectively). It is speculated that AST might therefore reflect either cell injury or inflammation beside hepatic tissue in such subjects having various metabolic abnormalities. At this point, the robust inverse relationship between CF and elevated AST can be attributed to the findings of recent studies reporting an inverse association of directly measured CF and such inflammation markers as C-reactive protein, fibrinogen and cytokine, etc (Kullo et al., 2007; Jae et al., 2008). In addition, a recent clinincal study showed a significant correlation between the carotid intimal media thickness and hepatic enzymes, including AST (Abdou et al., 2009). However, these explanations remain mere speculation. Further accumulation of evidence is thus needed to clarify the association between the CF and AST levels in the future.

On the other hand, ALT which mainly exists in the hepatic cells might be directly affected by higher levels of liver fat, which is related to both abdominal fat and insulin resistance (Messier et al., 2010). Results from recent animal experiments, which examined the effect of daily aerobic exercise (Rector et al., 2008), the cessation of exercise (Rector et al., 2008) and a genetically low aerobic capacity (Thyfault et al., 2009) to the hepatic oxidative capacity, are all consistent with the hypothesis that regular aerobic exercise or a favorable CF improve the hepatic oxidative capacity. Such evidence could therefore help us to explain both the low prevalence of high liver fat and the elevated ALT levels observed in the high CF group. However, the prevalence of both abnormalities was dependent on abdominal obesity and/or hyperinsulinemia rather than on the CF level in diabetic subjects; the result in the current study agreed with that in the prior-mentioned study (Messier et al., 2010).

No association between CF and elevated GGT found in the logistic model adjusted for basic confounders including disease type. Considerable number of prospective studies reported elevated GGT was a strong predictor of Type 2 DM (André et al., 2005, André et al., 2006, André et al., 2007, Doi et al, 2007, Lee et al., 2003, Nakanishi et al., 2004). The GGT level was closely correlated with the insulin level in the present study ($r = 0.452$, $p < 0.0001$, data not shown). Taking these evidences into consideration, it was speculated that GGT level in diabetic subjects was affected by insulin resistance rather than aerobic capacity strongly reflecting muscle oxidative capacity and cardiac function.

The present study has some limitations. The design of the study was cross-sectional and thus unable to identify causality between CF and elevated hepatic enzymes or high liver fat. In addition, the results of the current study were derived from diabetic patients; it should not be regarded as phenomena in healthy population. The VO_2max data was calculated using heart rate during exercise, thus few errors in the values of VO_2max might occur, though VO_2max measurements were performed by a skilled examiner. The daily ethanol intake was self-reported, and may therefore be biased or inaccurate. Tests for hepatitis B or C virus were only performed for the patients who were suspected of having these viruses. At least a 3-year treatment regimen by the subjects' primary doctor and at least a 1-year follow-up of lifestyle modification was performed for almost all subjects after the assessment of the present study; however, no onset of hepatitis B or C was recognized.

Conclusion

The current study is thus considered to demonstrate, for the first time, a favorable level of cardiorespiratory fitness could contribute to a reduced risk of elevated aminotransferase and high liver fat in Japanese patients newly diagnosed as IGT or type 2 DM. An independent and inverse association between the CF level and the prevalence of an elevated AST level was observed, the possibility that AST may potentially be useful as a simple marker concerning physical inactivity should therefore be assessed. Prospective cohort studies in the general population, exercise-intervention for high-risk populations, and a biochemical approach are required to address the effect of physical activity on both the hepatic enzyme levels and liver fat levels in the future.

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