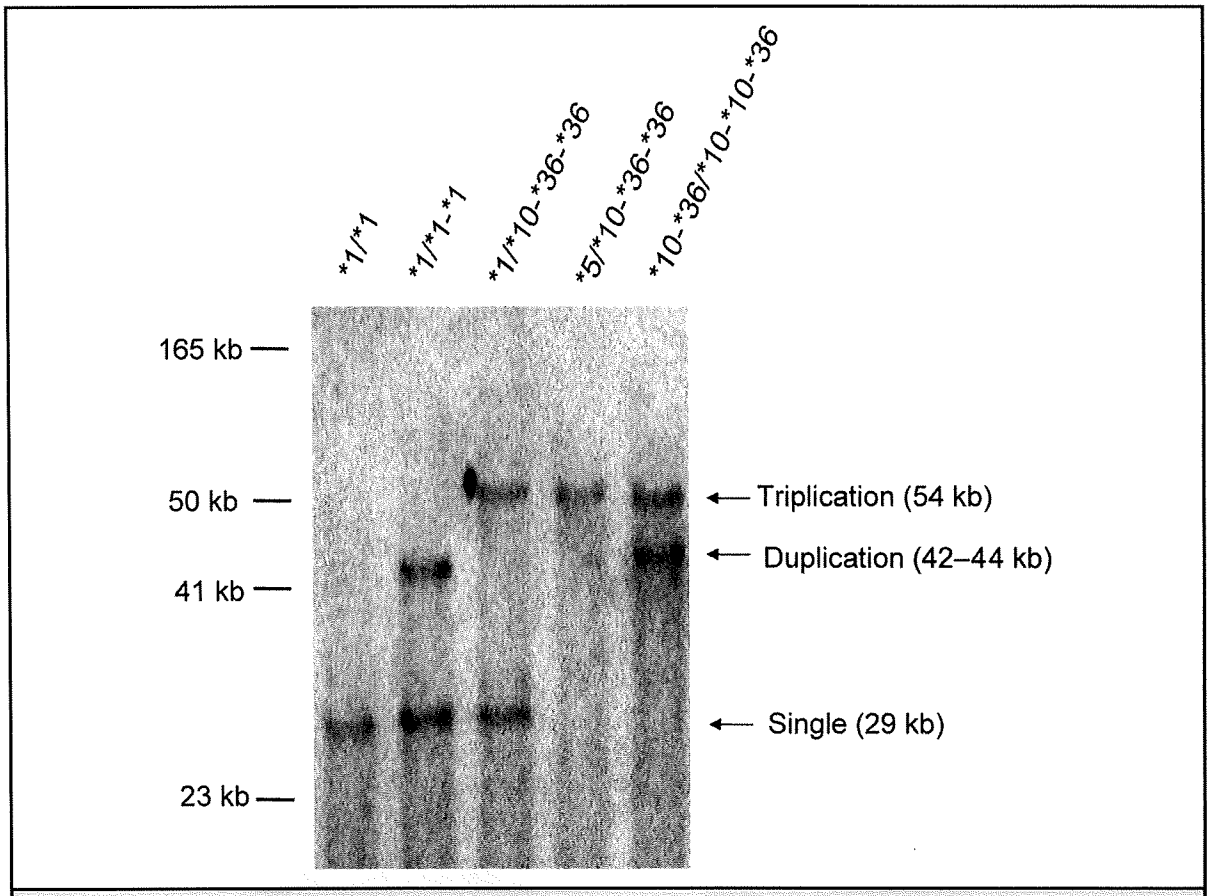

Fig. 4. Confirmation of new *CYP2D6* alleles (triplications) by Southern-RFLP analysis.

Estimated diplotypes of representative samples are indicated above image of blot membrane. Samples estimated as *CYP2D6*1/*1* and *CYP2D6*1/*1-*1* were used as controls for a single gene and *CYP2D6* gene duplication, respectively. Arrows indicate positions of *CYP2D6*-specific fragments from genomic DNA digested with the *XbaI* restriction enzyme. Fragments of 29 kb, 42–44 kb, and 54 kb indicate single-copy, duplication, and triplication fragments, respectively, of the *CYP2D6* gene [Schaeffeler et al. *(14)*, Johansson et al. *(27)*].

primer extension, removal of unincorporated dideoxynucleoside triphosphates, and electrophoresis on a genetic analyzer) *(12)*. All of these methods require approximately 1–2 days or longer to obtain the final results. In contrast, qPCR is a 1-step reaction as a homogeneous assay, and mPCR-RETINA is just a 2-step reaction of PCR combined with the Invader assay. Both can be performed in a 384-well plate format. The qPCR requires 2 h, the triplex PCR requires 3 h, and RETINA requires only 15 min. Because the qPCR and mPCR-RETINA assays can be run on different platforms simultaneously, we can obtain allele copy number data within several hours. Our method has the advantages of labor efficiency and throughput. We believe our method will be a powerful tool for the pharmacogenetic study of CYP2D6.

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

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

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

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

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

Key Words: cerebral infarct ■ genetics ■ PDE4D

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

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

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

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

Materials and Methods

Study Populations

We used 2 independent Japanese case–control samples and a prospective cohort for this study. One is a Kyushu sample consisting of 1112 cases of ischemic stroke and 1112 age- and sex-matched control subjects. Details on this population were described previously.[22] Briefly, patients with ischemic stroke were recruited from 7 medical centers in and around Fukuoka City, Japan, in 2004. These included 491 cases of lacunar infarction, 369 of ATI, 136 of CEI, and 116 of undetermined subtype. Age (within 5 years) and sex-matched control subjects were selected from the 3328 participants of the Hisayama screening survey between 2002 and 2003. All case subjects were diagnosed by stroke neurologists on the basis of detailed clinical features and ancillary laboratory examinations such as brain imaging. The subtypes of ischemic stroke were determined on the basis of the Classification of Cerebrovascular Disease III proposed by the National Institute of Neurological Disorders and Stroke (NINDS-III).[23]

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

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

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

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

SNP Selection and Genotyping

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

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

Statistical Analysis

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

Results

Single-Marker Association Test

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

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

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

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

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

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

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

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

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

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

Haplotypes with frequency >2% are shown.

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

Stratified Analysis by Hypertension Status

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

Haplotype Analysis

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

Tag-SNP Analysis

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

Discussion

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

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

Table 3. Continued

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

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

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

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

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

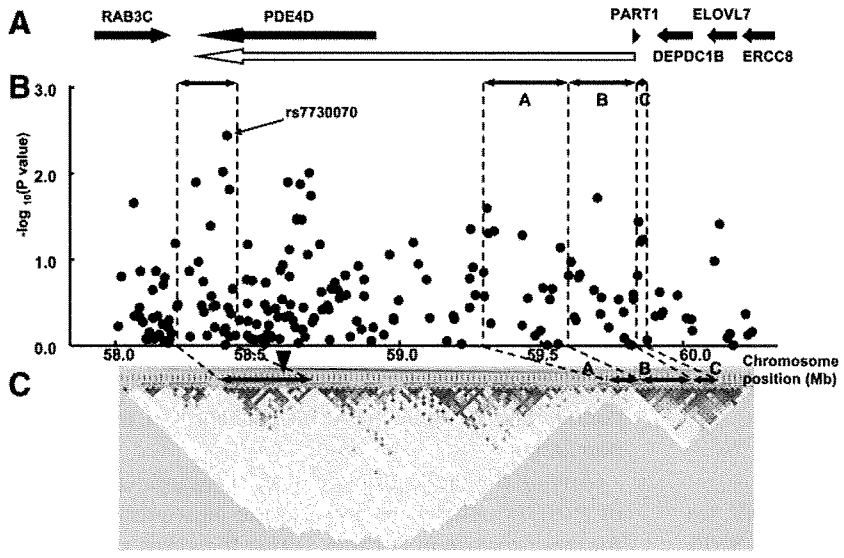

Figure. Genomic structure, case–control results, and linkage disequilibrium map of the 2.2-Mb region, including *PDE4D*. A, Genomic structure around *PDE4D*. The white arrow indicates *PDE4D* reported by the initial study. B, Case–control association results for ischemic stroke among Japanese. The log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

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

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

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Disclosures

None.

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【原　著】

糖尿病患者におけるメタボリックシンドロームとC反応性蛋白質との関連性 —全身持久力と内臓脂肪面積が及ぼす影響—

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【要約】**背景**：メタボリックシンドローム（MS）の出現には，C反応性蛋白質（CRP）の増加が関連する。しかしながら，この関連性には，全身持久力（$\dot{V}O_2max$）や内臓脂肪面積（VFA）が影響するのかどうか明らかでない。

方法：腹部内臓脂肪型肥満を伴う男性が本研究に参加した（平均 50.7±13.1 歳）。彼らは，境界型糖尿病（IGT，18 名）または 2 型糖尿病（T2D，76 名）と新規に診断され，過去に薬物および食・運動による介入治療を受けていない。高感度 CRP（hs-CRP）は，免疫比濁法にて測定した。$\dot{V}O_2max$ は，自転車エルゴメータによる多段階運動負荷試験により推定した。VFA は，CT スキャンにより腹部臍位で測定した。MS の診断基準として，修正した WHO 基準を用いた。

結果：MS と non-MS 群の血中 hs-CRP 濃度には，有意な群間差が認められなかった。すべての被験者を hs-CRP 濃度の低い順に 1st, 2nd および 3rd の 3 群に分けた。3 群間の VFA には有意な群間差が認められなかったけれども，$\dot{V}O_2max$ は有意に異なった。年齢調整後の MS 出現のオッズ比（95%信頼区間）は，1st 群と比較して，2nd および 3rd 群で有意に高値であった。しかし，2 群のオッズ比の有意性は，VFA を調整因子に加えることでその一部が消滅し，更に，$\dot{V}O_2max$ を加えることですべての有意性が消滅した。

結論：MS 出現は，CRP よりも全身持久力や内臓脂肪面積に依存している可能性が示唆された。

Key words: newly diagnosed diabetic patient, estimated $\dot{V}O_2max$, metabolic syndrome, C-reactive protein

1．はじめに

我が国の 2 型糖尿病患者の約 50%は，メタボリックシンドローム（MS）を併発していることが報告されている[1)]。MS 発症の基盤である脂肪細胞の過剰な蓄積は，心血管系疾患（CVD）の発症をも高める要因である[2)]。脂肪細胞は，種々の生理活性を有する蛋白質（アディポサイトカイン）を分泌し，生体内の代謝を調節している。アディポサイトカインには，動脈硬化への進展に関与する前駆炎症性サイトカインが含まれる。前駆炎症性サイトカインは，C 反応性蛋白質（CRP）の分泌を促す[3)]。CRP は，肝臓由来の炎症反応蛋白で，CVD の予測マーカーの 1 つである。このことから，アディポサイトカイン分泌の変化は，肥満や 2 型糖尿病が CVD を併発する機序の 1 つとして考えられている[4)]。CRP に関する研究は，欧米諸国から報告されたものが多く，邦人を対象とした検討は数少ない。

Ishikawa ら[5)]は，30 代から 70 代の一般男女を対象に，MS 出現と CRP との関連を検討し，CRP 濃度高値群の MS 出現は，低値群に比べ約 6 倍高いことを報告している。したがって，CRP の血中濃度は MS であるか否かで異なり，CVD を予測するだけでなく，MS 出現の予測マーカーとしても有用であることが示唆される。しかしながら，彼らの成績は，健常な一般集団が対象であり，比較的発症早期で未治療・未介入な糖尿病患者においては検討されていない。

MS 出現に関連する因子は数多く報告されてい

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る[6]。我々は特に，全身持久力（$\dot{V}O_2max$）や内臓脂肪面積（VFA）の影響に着目している。我々の先行研究では，腹部内臓脂肪型肥満で，かつ発症早期の糖尿病男性を対象に，MS 出現と $\dot{V}O_2max$ および VFA との関連性を横断的に検討した。その結果，$\dot{V}O_2max$ が低く，VFA が大きい群の MS 出現率は，そうでない群に比べ有意に高いことを報告した[7]。更に興味深いことに，VFA が大きくても $\dot{V}O_2max$ が高値であれば，MS 出現率は低いことも報告した[7]。しかしながら，MS 出現と CRP との関連性について，$\dot{V}O_2max$ や VFA が影響するのかどうかを検討した研究は，著者らの知る限りない。

そこで本研究では，発症早期の糖尿病男性患者を対象に，MS 出現と CRP との関連性に及ぼす $\dot{V}O_2max$ と VFA の影響を横断的に検討した。

2．研究方法

2-1．対象者

腹部内臓脂肪型肥満を伴う糖尿病男性が本研究に参加した。対象者は，9 時間以上の絶食後，75g 経口糖負荷試験（OGTT）を施行し，その結果，境界型糖尿病（IGT, 18 名）または 2 型糖尿病（T2D, 76 名）と新規に診断された発症早期の患者である。IGT および T2D の診断基準は，1999 年の日本糖尿病学会で策定された糖尿病診断基準（空腹時血糖値[FPG]≧126mg/dl, および血糖 2 時間値[2h-PG]≧200mg/dl）に基づいている。対象者は，食事や運動による介入治療を受けた経験がなく，更に，糖・脂質代謝に影響を及ぼす薬剤も服用していない。

対象者は，研究の意義，目的，および方法について説明を受けたうえで研究への参加に同意した。本研究は，九州大学健康科学センター倫理委員会で承認を得た。

2-2．測定項目および測定方法

2-2-1．代謝指標

血液サンプルは，早朝空腹の状態で肘前静脈より採取した。空腹時インスリン（FIRI）は，IRI キット（Pharmacia, Uppsala, Sweden）を用いた放射免疫測定法で分析し，空腹時血糖（FPG），総コレステロール（TC），高比重リポ蛋白コレステロール（HDL-c）および中性脂肪（TG）は，酵素法により測定した。ヘモグロビン A1c（HbA1c）は，高速液体クロマトグラフィー（HPLC）により測定した。インスリン抵抗性の指標は，homeostasis model assessment of insulin resistance（HOMA-IR）を用い，FIRI（μU/mL）×FPG（mmol/L）/ 22.5 の式[8]より算出した。CRP の測定は，高感度（hs-CRP）まで測定可能な N-ラテックス'CRP Ⅱ測定キット（デイトベーリング社）を用い，ネフェロメトリー法によって分析した。

2-2-2．形態および肥満指標

身長および体重は，それぞれ 0.1cm，0.01kg 単位まで測定した。body mass index（BMI）は，体重（kg）を身長（m）の二乗で除することで算出した。

腹部内臓脂肪蓄積の評価は，臍位での皮下脂肪面積（SFA）および内臓脂肪面積（VFA）を用いた。測定時，対象者は空腹で仰臥位の状態を維持した。内臓脂肪面積が 100cm^2 以上である場合に腹部内臓脂肪型肥満とした。分析機器は，Computed tomography 画像分析器（東芝社製，Vogor Lau Dator）を用いた。

2-2-3．体力指標

対象者の安静時の収縮期血圧（SBP）および拡張期血圧（DBP）は，少なくとも 30 分の安静後，自転車エルゴメータ（モナーク社製，Stockholm, Sweden）に座位の状態で測定された。運動負荷試験の初動負荷は，対象者の性，年齢および体重を考慮して決定した。運動負荷試験中の目標心拍数は，最大酸素摂取量（$\dot{V}O_2max$）の約 70%相当とし，負荷は多段階式を用いて 4 分おきに 3 回漸増した。第 3 段階実施中，対象者の心拍上昇が不十分であった場合，第 4 段階が追加実施された。心電図は常時記録された。血圧は各段階の終了 1 分前から測定され，心拍数は 15 秒前から記録された。推定 $\dot{V}O_2max$ は，各段階に対する心拍数を，Åstrand と Ryhming のノモグラム[9]に代入し，年齢補正[10]を加え算出した。

2-2-4．MS の診断基準

世界保健機構（WHO）[11]基準をもとに，FPG≧110 mg/dL に加え，次の①から③のうちいずれか 2 つ以上に該当する者とした。①BMI≧25 kg/m^2 あるいは WHR≧0.9，②SBP≧140 mmHg と DBP≧90 mmHg のどちらかまたは双方，③TG≧150

mg/dL と HDL-c＜35mg/dL のどちらかまたは双方である。WHO における BMI の基準値は 30 kg/m^2 以上である[11]。しかし，我々は，人種差を考慮して[12]，日本肥満学会の診断基準[13]を採用した。我が国が策定した MS 診断基準は，腹囲のカットオフ値に関して議論の余地があることに加え[14]，早期耐糖能異常者が本研究の対象であることから，インスリン抵抗性の存在を重視する WHO 基準を一部修正した基準を採用した。

2-2-5. 統計処理

MS の有無（MS 群と non-MS 群）における諸特性は，対応のない t 検定を用いて比較した。群間の人数，喫煙および飲酒者の割合は，χ^2 検定，または Fischer's Exact Test を用いて比較し，等分散性を認めない因子は，Aspin-Welch 検定を用いた。対象者の hs-CRP 濃度は，正規分布でなかったため，対数変換値を解析に用いた。hs-CRP 濃度の違いによる諸特性の差異を検討するため，hs-CRP 濃度の低い順に 1st，2nd および 3rd 群として対象者を 3 群に分けた。諸特性の群間比較には，一元配置の分散分析（One-way ANOVA）用いて解析した。有意性が認められた項目は，Tukey posthoc test により解析した。更に，1st，2nd および 3rd 群の MS 出現率には，ロジスティック回帰分析により算出されたオッズ比（OR）とその 95%信頼区間（95% CI）を用いて比較した。有意水準はすべて 5%未満とし，解析システムは，九州大学情報基盤センターの研究用計算機（SAS バージョン 8.2，SAS Institute，NC，USA）を使用した。

3．結　果

3-1. MS および non-MS 群における諸特性の比較

MS および non-MS 群の年齢には有意差が認められなかった。MS 群の血中 hs-CRP 濃度は，non-MS 群との間に有意な群間差を認めなかった（MS 群：1.1±0.1 mg/L，non-MS 群：1.2±0.2 mg/L，p=0.452）。MS 群の VFA，FIRI，HOMA-IR は，

表 1　General characteristics of subjects with or without MS

	MS	non-MS	p
T2D / IGT number[a]	39 / 4	37 / 14	0.004
hs-CRP (mg/L)[b]	1.1 (0.1)	1.2 (0.2)	0.452
VO_2max (mL/kg/min)	32.3 (0.8)	35.3 (0.9)	0.014
VFA (cm^2)[b]	202.5 (11.3)	148.5 (7.7)	<0.001
Age (yrs)	50.6 (1.7)	53.3 (1.8)	0.293
BMI (kg/m^2)	26.1 (0.6)	24.2 (0.7)	0.043
SBP (mmHg)[b]	137.7 (2.8)	124.8 (1.9)	<0.001
DBP (mmHg)	88.1 (1.7)	77.9 (1.2)	<0.001
HbA1c (%)	7.1 (0.2)	6.4 (0.2)	0.024
TC (mg/dL)	224.3 (5.9)	205.6 (5.0)	0.016
TG (mg/dL)[b]	215.6 (19.9)	122.7 (9.6)	<0.001
HDL-c (mg/dL)	46.8 (1.6)	51.8 (1.8)	0.044
FPG (mg/dL)	153.5 (5.0)	131.3 (4.8)	0.002
2-h PG (mg/dL)	297.4 (11.3)	250.0 (12.6)	0.007
FIRI (μU/mL)[b]	9.6 (1.4)	5.9 (0.6)	0.019
2-h IRI (μU/mL)	50.3 (8.4)	49.3 (8.9)	0.939
SFA (cm^2)[b]	150.9 (11.8)	139.0 (15.2)	0.549
HOMA-IR[b]	3.8 (0.6)	1.8 (0.2)	0.002
Alcohol n(%)[a]	18 (41.9)	19 (37.3)	0.677
Smoking n(%)[a]	12 (27.9)	8 (15.7)	0.206
Number of MS risk factors	4.4 (0.2)	2.1 (0.1)	<0.001

Mean（SE）. p <0.05 by Student's t-test. [a], χ^2 or Fisher's exact test; [b], Aspin-Welch test.
BMI; body mass index, VO_2max; maximal oxygen uptake, SBP; systolic blood pressure, DBP; diastolic blood pressure, HbA1c; hemoglobin A1c, TC; total cholesterol, HDL-c; high density lipoprotein cholesterol, TG; triglyceride, FPG; fasting plasma glucose, FIRI; fasting immunoreactive insulin, VFA; visceral fat area, SFA; subcutaneous fat area, HOMA-IR; homeostasis model assessment of insulin resistance.

non-MS 群と比較して有意に高かったが，$\dot{V}O_2max$ は有意に低かった（表 1）。

3-2. hs-CRP 濃度の違いによる諸特性の比較

対象者を，hs-CRP 濃度の低い順に 1st，2nd および 3rd 群に分けて解析した。1st，2nd および 3rd 群の血中 hs-CRP 濃度は，それぞれ 0.28±0.0 mg/L，0.72±0.0 mg/L，および 2.42±0.2 mg/L であった。3 群間の年齢，糖・脂質代謝指標，VFA，SFA，インスリン抵抗性，喫煙および飲酒頻度には，有意な群間差が認められなかった。3rd 群の BMI は，1st 群と比較して有意に高かった。1st，2nd および 3rd 群の $\dot{V}O_2max$ は，それぞれ 36.0±1.0 mL/kg/min，33.9±0.9 mL/kg/min および 31.9±1.2 mL/kg/min であり，hs-CRP 濃度の高い群ほど有意に低かった（表 2）。

3-3. 1st，2nd および 3rd 群における MS 出現の比較

MS 出現の OR および 95%CI の算出には，年齢を調整したモデル（年齢調整モデル）を用いた。年齢調整モデルによる MS 出現の OR（95%CI）は，1st 群の OR を 1 とした場合，2nd 群で 2.93（1.003～8.577），3rd 群で 5.33（1.622～17.548）と有意に高かった。年齢調整モデルに VFA を追加した際の MS 出現の OR では，2nd 群で 1.38（0.444～4.302）と有意性は消失したが，3rd 群で 3.74（1.122～12.438）と有意性は維持された。一方，年齢調整モデルに $\dot{V}O_2max$ を追加した際の MS 出現の OR は，2nd 群で 2.09（0.633～6.928），3rd 群で 3.06（0.781～11.996）と双方ともに有意性が消失した。年齢調整モデルに VFA と $\dot{V}O_2max$ の 2 因子を同時に投入した際の MS 出現の OR は，2nd 群で 1.15（0.361～3.674），3rd 群で 2.45（0.727～8.280）と双方の有意性が消失した（表 3）。

4．考　察

本研究では，腹部内臓脂肪型肥満を伴う発症早期の糖尿病患者において，MS 出現と C 反応性蛋白質（CRP）との関連性には，全身持久力（$\dot{V}O_2max$）および内臓脂肪面積（VFA）が影響するか否かを横断的に検討した研究である。その結果，以下の 3 点が明らかとなった。

まず 1 点目は，MS の有無で区分された糖尿病

表 2　General characteristics of subjects divided by tertile of CRP

hs-CRP categories	1st (n=32)	2nd (n=31)	3rd (n=31)	p
T2D/IGT number[a]	22 / 10	28 / 3	26 / 5	0.124
hs-CRP (mg/L)	0.28 (0.0)	0.72 (0.0)	2.42 (0.2)*#	<0.001
$\dot{V}O_2max$ (mL/kg/min)	36.0 (1.0)	33.9 (0.9)	31.9 (1.2)*	0.024
VFA (cm^2)	154.7 (13.6)	170.5 (9.9)	195.3 (11.7)	0.063
Age (yrs)	48.3 (1.9)	55.0 (2.0)	53.0 (2.4)	0.073
BMI (kg/m^2)	23.6 (0.5)	24.8 (0.6)	26.8 (1.1)*	0.015
SBP (mmHg)	128.4 (2.8)	132.6 (3.0)	131.4 (3.3)	0.612
DBP (mmHg)	82.1 (1.9)	83.5 (2.1)	82.2 (2.0)	0.867
HbA1c (%)	6.4 (0.3)	6.7 (0.2)	7.0 (0.3)	0.391
TC (mg/dL)	211.4 (7.5)	212.3 (5.1)	218.8 (7.5)	0.701
TG (mg/dL)	161.6 (14.7)	167.4 (20.4)	166.6 (24.3)	0.975
HDL (mg/dL)	49.2 (2.2)	51.3 (2.1)	48.1 (2.2)	0.583
FPG (mg/dL)	133.6 (5.8)	144.1 (5.3)	147.0 (7.6)	0.285
2-h PG (mg/dL)	243.1 (15.6)	285.5 (11.9)	287.5 (17.2)	0.066
FIRI (μU/mL)	6.7 (1.4)	7.4 (1.0)	8.7 (1.4)	0.535
2-h IRI (μU/mL)	39.5 (3.9)	41.2 (5.8)	68.9 (17.0)	0.091
SFA (cm^2)	122.9 (9.9)	133.6 (9.6)	177.0 (24.3)	0.054
HOMA-IR	2.39 (0.6)	2.74 (0.4)	3.05 (0.5)	0.691
Alcohol n (%)[a]	12 (37.5)	13 (42.0)	12 (38.7)	0.965
Smoking n (%)[a]	9 (28.1)	5 (16.1)	6 (19.4)	0.539

Mean（SE）. $p < 0.05$ by One-way ANOVA. [a], χ^2 or Fisher's exact test.
* Comparison of 1st group; #Comparison of 2nd group examined by Tukey posthoc test.
Abbreviations see Table 1.

表 3　Proportions of MS divided by tertile of CRP

	2nd		3rd	
Adjusting factors	OR	95% CI	OR	95% CI
Age	2.93	1.003 - 8.577	5.33	1.622 - 17.548
Age + VFA	1.38	0.444 - 4.302	3.74	1.122 - 12.438
Age + $\dot{V}O_2$max	2.09	0.633 - 6.928	3.06	0.781 - 11.996
Age + VFA+$\dot{V}O_2$max	1.15	0.361 - 3.674	2.45	0.727 - 8.280

OR; odds ratio, 95%CI; 95% confidence interval.

患者の hs-CRP 濃度には，有意な群間差が認められないことであった（表 1)。これは，Ishikawa ら [5] の邦人一般集団における成績と異なった。血中 CRP 濃度を高める要因には，MS，インスリン抵抗性，糖尿病，肥満（特に内臓脂肪蓄積)，およびアディポサイトカイン分泌変化などの関与が報告されている [15,16]。我々の対象者では，non-MS 群に比べ，MS 群の VFA，安静時血圧，糖・脂質代謝指標およびインスリン抵抗性は有意に高いにもかかわらず，血中 CRP 濃度には 2 群の差異が認められない。一般集団における MS および non-MS 男性の血中 CRP 濃度は，平均 0.3 mg/L，0.1 mg/L と我々の対象者よりも低値であった [5]。したがって，発症早期の糖尿病患者のような血中 CRP 濃度レベルの高い集団において，血中 CRP 濃度の調節には，MS，インスリン抵抗性および内臓脂肪蓄積以外の要因の関与が示唆された。

2 点目は，hs-CRP 濃度の低い順に 1st，2nd および 3rd 群の 3 群に分けた集団において，hs-CRP 濃度が高い群ほど $\dot{V}O_2$max のみが有意に低かったことある（表 2)。Kuo ら [17]は，健常男性を CRP 濃度別の 4 群に分けて $\dot{V}O_2$max を検討し，高 CRP 濃度群の $\dot{V}O_2$max は有意に低いことを報告している。また，Aronson ら [18] も同じく，$\dot{V}O_2$max の高低によって血中 CRP 濃度は有意に異なることを報告している。しかし，両者の研究では，交絡因子としての肥満指標に BMI が用いられ，VFA による検討はなされていない。本研究では，肥満指標として BMI に加え VFA を評価した。その結果，血中 CRP 濃度の 1st，2nd および 3rd 群における VFA や糖・脂質代謝指標には有意な群間差が認められなかった。中等度の運動強度での定期的な運動は，CRP を含む抗炎症性サイトカイン分泌を増加させ，局所炎症の沈静化を高める [19]。このことから，筋収縮活動が多い，すなわち $\dot{V}O_2$max レベルが高い状態では，CRP が組織レベルで多く利用されるため，血中レベルでは低い可能性がある。著者らの知る限り，本邦における CRP と $\dot{V}O_2$max との関連性を検討した知見は得られていないことから，我々の成績は，炎症マーカーと全身持久力との関連性を示した有用な知見と考える。

3 点目は，MS 出現は，CRP よりも全身持久力や内臓脂肪面積に依存していることであった（表 3)。このことは，本研究の重要な知見である。血中 hs-CRP 濃度 1st，2nd および 3rd 群における MS 出現 OR の有意性は，VFA を調整因子に加えることで一部消滅し，$\dot{V}O_2$max を調整することですべて消失した。これにより，MS 出現と CRP との関連性における VFA と $\dot{V}O_2$max の相対的貢献度は，$\dot{V}O_2$max のほうがより強いことが示唆された。$\dot{V}O_2$max は，全身持久力の指標であると同時に，骨格筋におけるエネルギー消費能や全身における安静時代謝能の指標でもある [20-22]。更に，トレーニングによる $\dot{V}O_2$max の増加は，インスリン感受性や脂質代謝の改善 [23]，血圧の低下 [24]，体重・体脂肪量の減少 [25] をもたらすことが多くの研究によって実証されている。したがって，発症早期の糖尿病患者において全身持久力が高いことは，たとえ内臓脂肪蓄積が高い状態であっても，MS 出現に対して抑制的に作用することが推察される。

本研究の限界は，横断研究であること，コントロール群が設定されていないことに加え，CRP 分泌に関与する炎症性サイトカインの測定がなされていないことである。今後，前向きあるいは介入研究によって hs-CRP および $\dot{V}O_2$max の変化量と MS 出現率の変化，および性差の検討も必要であろう。

本研究の結論として，発症早期の糖尿病男性患者における MS 出現は，血中 CRP 濃度よりも全身持久力や内臓脂肪面積に依存している可能性が示唆された。

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Relationship between Metabolic Syndrome and C-reactive Protein in Japanese Diabetic Men: Impacts of Cardiorespiratory Fitness and Visceral Fat Area

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Abstract

Background: It is still unknown whether relationship between prevalence of metabolic syndrome (MS) and C-reactive protein (CRP) is affected by caridorespiratory fitness ($\dot{V}O_2$max) and/or visceral fat area (VFA).

Methods: Ninety-four Japanese men with visceral fat accumulation were participated in this study. They were newly diagnosed patients with either impaired glucose tolerance (IGT, n=18) or type 2 diabetes mellitus (T2D, n=76). They have not been received any medical and interventional therapies before participation of this study. High sensitivity CRP (hs-CRP) was measured by immunonephelometry. $\dot{V}O_2$max was estimated by indirectly multistage exercise test using cycle ergometer. VFA was measured using CT scanner. Definition of MS was used a modified WHO criteria.

Results: Concentrations of hs-CRP did not significantly differ in the MS and non-MS groups. All subjects were divided three groups (1st, 2nd, and 3rd groups) based on the hs-CRP concentrations. $\dot{V}O_2$max differed significantly among three groups, while visceral fat area did not. Odds ratio for the prevalence of MS was significantly higher in the 2nd and 3rd groups than that of 1st group as reference. VFA as adjusting factor disappeared a part of these significances, moreover, $\dot{V}O_2$max disappeared all of those.

Conclusions: Our study suggested that prevalence of MS might be depend on cardiorespiratory fitness and/or visceral fat area more than CRP.

Key words: newly diagnosed diabetic patient, estimated $\dot{V}O_2$max, metabolic syndrome, C-reactive protein

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