Table 2 | Mean values or frequencies of risk factors for type 2 diabetes according to age groups in 1988 and 2002 by sex

Variable	Age (years)	Men			Women		
		1988	2002	*P*-value	1988	2002	*P*-value
Body mass index (kg/m^2)	40–49	23.5 (2.9)	23.9 (3.3)	0.05	23.2 (3.1)	22.5 (3.7)	0.008
	50–59	23.4 (2.9)	23.9 (3.0)	0.04	23.3 (3.2)	23.0 (3.5)	0.13
	60–69	22.4 (2.9)	23.6 (2.9)	<0.001	23.0 (3.2)	23.4 (3.3)	0.06
	70–79	21.3 (2.6)	22.8 (2.9)	<0.001	22.0 (3.3)	23.3 (3.4)	<0.001
Overall obesity (%)	40–49	29.1	33.1	0.29	24.6	18.7	0.048
	50–59	29.2	31.4	0.53	25.7	24.7	0.73
	60–69	21.8	30.7	0.01	26.2	30.7	0.16
	70–79	7.6	21.9	<0.001	18.5	30.3	0.003
Waist circumference (cm)	40–49	83.1 (8.1)	83.6 (8.4)	0.45	79.3 (9.5)	76.8 (9.7)	<0.001
	50–59	83.3 (7.7)	84.2 (7.9)	0.11	82.1 (9.9)	79.8 (9.4)	<0.001
	60–69	81.5 (8.4)	84.1 (7.8)	<0.001	83.3 (9.8)	83.2 (9.2)	0.87
	70–79	78.8 (7.7)	83.3 (8.5)	<0.001	80.3 (10.6)	84.8 (9.5)	<0.001
Central obesity (%)	40–49	18.5	23.5	0.13	49.4	32.6	<0.001
	50–59	20.6	23.9	0.28	60.0	47.6	<0.001
	60–69	15.5	21.6	0.052	67.5	68.4	0.79
	70–79	10.6	23.5	0.002	50.8	68.8	<0.001
Regular exercise (%)	40–49	10.0	11.0	0.68	6.5	5.1	0.40
	50–59	6.1	8.2	0.28	7.0	9.8	0.13
	60–69	12.7	10.8	0.45	11.8	10.7	0.63
	70–79	26.4	16.2	0.02	14.2	11.4	0.36

All values are given as the mean (standard deviations) or as a percentage. Overall obesity was defined as a body mass index ≥25.0 kg/m^2. Central obesity was defined as a waist circumference ≥90 cm in men and ≥80 cm in women. Regular exercise was defined as engaging in sports at least three times per week during leisure time.

because the present study was carried out in a suburban population, the generalizability of our results to the entire population of Japan is limited. Third, because of issues of overlap and uniformity with the 1988 survey data, we did not use medical history of diabetes in the estimation of prevalence of diabetes. This might have resulted in the underestimation of the prevalence of diabetes. Fourth, there might have been a selection bias resulting from the exclusion of participants who did not have the OGTT. However, the study participants had a similar age distribution and proportion of men (43.3 vs 45.8% in 1988, and 44.1 vs 46.3% in 2002) compared with the original population. Furthermore, the present study had high participation rates in both surveys (approximately 80%). Therefore, we believe that the findings of the present study reflect the actual secular trends in the prevalence of glucose intolerance in our population. Finally, the use of HbA_{1c} has now been recommended for the diagnosis of diabetes by the international expert committee[31], but in the present study, the values of HbA_{1c} were not used for diagnosing diabetes because of the non-standardized HbA_{1c} assay in our 1988 survey. However, HbA_{1c} measurement has been found to be less sensitive for detecting subjects with diabetes compared with the OGTT[32–34]. Furthermore, glucose tolerance status was evaluated in the same way across the two surveys. Thus, this limitation is not likely to distort the prevalence trends in the present study.

In conclusion, the present analysis showed that the prevalence of type 2 diabetes and prediabetes increased significantly in both sexes from the 1980s to the 2000s in a Japanese population. The increasing prevalence of overall and central obesity, and the decline in physical activity seemed to have an influence on this rising trend. More intense efforts for the prevention of type 2 diabetes by modification of lifestyle are required to reduce the burden of type 2 diabetes in Japan.

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Midlife and late-life handgrip strength and risk of cause-specific death in a general Japanese population: the Hisayama Study

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ABSTRACT

Background Decreased handgrip strength has been reported to be a risk factor for all-cause death among the elderly. However, it is unclear whether handgrip strength measured in midlife is associated with risk of all-cause and cause-specific death in the general population.

Methods We followed, prospectively, a total of 2527 community-dwelling Japanese (1064 men and 1463 women) aged ≥40 years for 19 years. Participants were divided into three groups according to the age-specific and sex-specific tertiles of handgrip strength (T1, lowest; T3, highest).

Results During the follow-up period, 783 participants died, of whom 235 died of cardiovascular disease, 249 of cancer, 154 of respiratory disease and 145 of other causes. In the middle-aged group (40–64 years), multivariable-adjusted HRs (95% CIs) for all-cause death were 0.75 (0.56 to 0.99) in T2 and 0.49 (0.35 to 0.68) in T3 compared with T1 as a reference. Corresponding HRs (95% CI) in the elderly group (≥65 years) were 0.50 (0.40 to 0.62) and 0.41 (0.32 to 0.51), respectively. As regards the cause of death, higher levels of handgrip strength were significantly associated with decreased risks of cardiovascular death, respiratory death and death from other causes, but not of cancer, in the middle-aged and the elderly.

Conclusions Our findings suggest that handgrip strength levels in midlife and late life are inversely associated with the risks of all-cause and non-cancer death in the general Japanese population.

INTRODUCTION

Handgrip strength, one of various indicators which reflect whole-body muscle strength, has been measured in many epidemiologic studies because it is a simple, easy and inexpensive way to evaluate muscle strength. Some population-based prospective studies have shown that handgrip strength levels were inversely associated with increased risks of all-cause death[1–13] and cardiovascular death.[1 2] Similarly, in a meta-analysis of observational studies, higher handgrip strength was associated with a lower risk of all-cause mortality.[14] In general, handgrip strength reaches its peak in the decade between the ages of 30 and 39 years, and then decreases with age after the age of 40 years.[15 16] Therefore, the association of handgrip strength levels with mortality risk may differ between midlife and late life. However, most previous studies have reported the influence of late-life handgrip strength in an elderly population (approx ≥65 years),[2–8 17] and only a small number of studies have examined the association between midlife handgrip strength and mortality risk.[1 10–13 18] Moreover, the influence of midlife handgrip strength on the risk of cause-specific death is still unclear.

The aims of the present study were to investigate the association of levels of handgrip strength with the risks of all-cause and cause-specific death in a general Japanese population, and to compare the influence of handgrip strength in the middle-aged (40–64 years old) and in the elderly (≥65 years old).

METHODS

Study participants

A population-based prospective study of cardiovascular and malignant diseases has been underway since 1961 in the town of Hisayama, a suburb of the Fukuoka metropolitan area of Kyushu Island in southern Japan. In 1988, a baseline examination for the present study was performed in this town. A total of 2742 residents aged ≥40 years (80.9% of the total population in this age group) participated in the examination. Excluded from the study were 168 individuals with a history of stroke, coronary heart disease, or cancer; 45 individuals in whom handgrip strength was not measured; and two individuals who died before follow-up; the remaining 2527 participating individuals (1064 men and 1463 women) were enrolled in the present study.

Baseline examination

At baseline examination, handgrip strength was measured using the Smedley Hand Dynamometer (MIS, Tokyo, Japan) according to instructions provided by a public health nurse. The width of the handle was adjusted such that the second phalanx was against the inner stirrup. The participants were encouraged to exert maximal handgrip strength. Two trials were allowed for each hand alternately, and the maximum value among four measurements was used for the analyses.

Each participant completed a self-administrated questionnaire covering medical history, treatments for hypertension and diabetes, smoking status, alcohol intake and leisure-time physical activity. Smoking status was classified into never smokers, former smokers, current light smokers (<20 cigarettes/day), and current heavy smokers (≥20 cigarettes/day). Alcohol intake was categorised into never drinkers, former drinkers, current light

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drinkers (ethanol intake <34 g/day), and current heavy drinkers (≥34 g/day). Leisure-time physical activity was quantified by the sum of activity time (hours per week) multiplied by the metabolic equivalents (MET) intensity for each activity.[19]

Resting blood pressure was measured three times with the subject in a sitting position, with a mercury sphygmomanometer at the right upper arm after at least five minutes of rest; the mean of the three measurements was used in the analysis. Diabetes was determined by the administration of antidiabetic treatment, plasma glucose levels (fasting glucose level ≥7.0 mmol/L or postprandial glucose level ≥11.1 mmol/L), or a 75 g oral glucose tolerance test using the 1998 WHO criteria,[20] with plasma glucose measured by the glucose-oxidase method. Total cholesterol was determined by an enzymatic autoanalyser. Body height and weight were measured while the subject was wearing lightweight clothing without shoes, and body mass index (BMI) was calculated. Electrocardiogram abnormalities were defined as left ventricular hypertrophy (Minnesota code 3-1), ST segment depression (4-1, 2, or 3), or atrial fibrillation (8-3).

Follow-up survey

The participants were followed prospectively for 19 years from December 1988 to November 2007 by repeated health examinations and by a daily monitoring system established by the study team, local physicians and members of the town's Health and Welfare Office. Vital status was checked annually by mail or telephone for any subjects who did not undergo regular examination or who moved out of town. Information about death was received via this follow-up system. When a resident died, all medical information related to a participant's illness and death, including hospital charts, physician's records and death certificate, were collected. Moreover, autopsy was performed at the Kyushu University Departments of Pathology, provided if consent for autopsy had been obtained. All medical information and autopsy findings were scrutinised, and the underlying causes of death were determined according to the International Classification of Diseases, 10th Revision (ICD-10). Cause of death was classified into the following categories: cardiovascular death (ICD-10 code of I00-I99), cancer death (C00-C97), respiratory death (J00-J99.8), and death from other causes. During the follow-up period, 783 participants (397 men and 386 women) died, of whom 564 (72.0%) underwent autopsy. Of the deceased persons, 235 (106 men and 129 women) died of cardiovascular disease, 249 (149 men and 100 women) of cancer, 154 (81 men and 73 women) of respiratory disease, and 145 (61 men and 84 women) of other causes. All individuals were completely followed-up for 19 years or until death.

Statistical analysis

All statistical analyses were performed with SAS V.9.3 software (SAS Institute, Cary, North Carolina, USA). The participants were divided into three groups on the basis of age-specific and sex-specific tertiles of handgrip strength (T1: 12.0 to 39.5, T2: 40.0 to 46.5, and T3: 47.0 to 64.0 kg for middle-aged (40–64 years) men; T1: 0.5 to 18.5, T2: 19.0 to 23.5, and T3: 24.0 to 27.5 kg for middle-aged women; T1: 3.0 to 29.5, T2: 30.0 to 36.5, and T3: 37.0 to 52.0 kg for elderly (≥65 years) men; T1: 0.5 to 16.0, T2: 16.5 to 20.5, T3: 21.0 to 39.0 kg for elderly women). Age- and sex-adjusted mean values of possible risk factors taken as continuous variables were estimated across handgrip strength levels using analysis of covariance, and the prevalence of risk factors taken as categorical variables were adjusted for age and sex by means of the direct method, where overall study population was used as the standard population. Linear trends in the mean values or prevalence of risk factors across handgrip strength levels were tested using linear or logistic regression analysis. The mortality rate was calculated by the person-year method and adjusted for either age or sex using the direct method. The HR and its 95% CI across the tertiles or per 1 SD increment of handgrip strength were estimated using the Cox proportional hazards model with adjustment for potential confounding factors at baseline, namely, systolic blood pressure, use of antihypertensive agents, diabetes, total cholesterol, BMI, smoking status, alcohol intake and leisure-time physical activity, as well as either age or sex. Here, SD of handgrip strength was 7.5 kg for middle-aged men, 5.5 kg for middle-aged women, 8.4 kg for elderly men and 5.7 kg for elderly women. Heterogeneity in the relationship between men and women or between age groups was tested by adding a multiplicative interaction term to the relevant Cox model. Proportions of missing values were less than 1% for all variables in the multivariable model. A two-sided $p<0.05$ was considered to be statistically significant in all analyses.

Ethical considerations

This study was conducted with the approval of Kyushu University Institutional Review Board for Clinical Research, and written informed consent was obtained from the participants.

RESULTS

Baseline characteristics of the study population are shown in table 1. Higher handgrip strength levels were associated with younger age, higher diastolic blood pressure, higher total cholesterol, higher BMI, lower prevalence of electrocardiogram abnormalities and increased leisure-time physical activity level.

Table 2 shows the association of all-cause and cause-specific death according to handgrip strength level by sex. In both sexes, the age-adjusted all-cause mortality in the second tertile (T2) and the highest tertile (T3) of handgrip strength was significantly lower compared to the lowest tertile (T1) (all $p<0.05$). Handgrip strength was inversely associated with the risk of all-cause death, even after adjustment for potential confounding factors. Compared with the T1 of handgrip strength, the multivariable-adjusted HR (95% CI) for all-cause death was 0.81 (0.64 to 1.03) for T2 and 0.70 (0.53 to 0.92) for T3 in the men, and 0.66 (0.52 to 0.85) for T2 and 0.65 (0.50 to 0.84) for T3 in the women. The HR (95% CI) for all-cause death per 1 SD increment of handgrip strength was 0.72 (0.64 to 0.81) in the men and 0.74 (0.66 to 0.82) in the women. Similar associations were observed for cardiovascular death, respiratory death and death from other causes, but not for cancer death. There was no evidence of heterogeneity in the association between men and women (all P for heterogeneity >0.2).

Next, we examined the association between handgrip strength and the risks of all-cause and cause-specific death in the middle-aged (40–64 years) and the elderly (≥65 years) groups (table 3). Here, the men and women were analysed together due to limited statistical power. The all-cause mortality in T2 and T3 was significantly decreased compared to T1 in both age groups (all $p<0.05$). These associations remained significant even after adjustment for multiple confounding factors. The multivariable-adjusted HR (95% CI) for all-cause death was 0.75 (0.56 to 0.99) for T2 and 0.49 (0.35 to 0.68) for T3 in the middle-aged group and 0.50 (0.40 to 0.62) for T2 and 0.41 (0.32 to 0.51) for T3 in the elderly group. The HR (95% CI) per 1 SD increment for handgrip strength was 0.72 (0.63 to

Table 1 Baseline characteristics according to levels of handgrip strength in 1988, the Hisayama Study

	Handgrip strength levels			
	T1 (n=802)	T2 (n=815)	T3 (n=910)	p for trend
Number of subjects				
Middle-aged men/women (40–64 years), n	244/314	248/302	269/366	
Elderly men/women (≥65 years), n	88/156	111/154	104/171	
Age, years	62 (0.4)	59 (0.4)	56 (0.4)	<0.001
Systolic blood pressure, mmHg	133 (0.7)	134 (0.7)	134 (0.7)	0.71
Diastolic blood pressure, mmHg	77 (0.4)	78 (0.4)	79 (0.4)	0.002
Antihypertensive agents, %	14.6	14.7	16.8	0.34
Diabetes, %	13.1	11.3	11.4	0.32
Total cholesterol, mmol/L	5.25 (0.04)	5.38 (0.04)	5.41 (0.04)	0.003
BMI, kg/m^2	22.2 (0.1)	22.7 (0.1)	23.6 (0.1)	<0.001
Electrocardiogram abnormalities, %	20.2	15.2	15.4	0.02
Smoking status				
Never smoker, %	60.6	59.8	61.7	
Former smoker, %	11.2	15.2	15.5	
Current smoker (<20 cigarettes/day), %	15.2	14.7	13.2	
Current smoker (≥20 cigarettes/day), %	13.1	10.4	9.5	
Alcohol intake				
Never, %	65.0	67.2	65.5	
Former, %	3.7	2.9	2.7	
Current (ethanol <34 g/day), %	19.3	19.6	20.8	
Current (ethanol ≥34 g/day), %	12.1	10.3	10.9	
Leisure-time physical activity, METs hour/week	3.1 (0.4)	4.1 (0.4)	5.8 (0.4)	<0.001

Age- and sex-adjusted mean (SE) or frequencies are shown.
BMI, body mass index; METs, metabolic equivalents.

0.81) in the middle-aged group and 0.63 (0.58 to 0.69) in the elderly group. We observed similar associations for cause-specific mortality, with the exception of cancer death. There was no evidence of heterogeneity in the association between age groups (all p for heterogeneity >0.4).

The sensitivity analysis, which excluded participants who died within 5 years of follow-up, did not generate any substantial discrepancies with the study conclusions (data not shown).

DISCUSSION

Using data from a 19-year follow-up study of a general Japanese population, we demonstrated that greater handgrip strength levels were associated with a reduced risk of all-cause death in men and women, even after adjustments were made for other conventional risk factors. With regard to the causes of death, handgrip strength levels were inversely associated with the risk of cardiovascular death, respiratory death, and death from other causes, but not with the risk of cancer death. These associations were observed in the middle-aged group as well as in the elderly group.

Many prospective studies in Western[2–8 10–13 18] and Asian countries[1 9 17] have examined the association between handgrip strength and the risk of all-cause mortality. Most previous studies have reported that handgrip strength is associated with all-cause death in men[1 2 4 6 9–12] and in women.[1 4–6 8] However, only a few population-based prospective studies have reported the association of handgrip strength levels with risk of cause-specific deaths.[1 2 9] The Adult Health Study in Hiroshima, Japan[1] and a cohort study in the UK[2] found that elevated levels of handgrip strength were associated with a decreased risk of cardiovascular death, while a similar but non-significant association was found in another Japanese study.[9] The Adult Health Study[1] also observed a significantly inverse association between handgrip strength and the risk of death from pneumonia. Our findings are in agreement with those of these previous studies. On the other hand, there is no consensus on the association between handgrip strength and cancer death.[1 2 9] While the study in the UK[2] observed a significantly inverse association between handgrip strength and cancer death, our study and two other Japanese cohort studies[1 9] did not observe any such association.

The inverse associations were recognised concerning lower handgrip strength levels and all-cause death in observational studies of elderly populations.[2–8] By contrast, there have been a small number of studies on this issue in middle-aged populations, and the findings were inconsistent.[1 10–13 18] The Honolulu Heart Program,[10 12] which observed Japanese–American men, and the Adult Health Study in Hiroshima, Japan,[1] showed a significant inverse association of handgrip strength levels with all-cause death in middle-aged populations. The Mini-Finland Health Examination Survey[13] and our present study showed a similar inverse association in the middle-aged and elderly group. On the other hand, the Baltimore Longitudinal Study of Aging[11] and the Canadian Fitness Survey[18] failed to show a significant association in middle-aged populations. The discrepancy between results for middle-aged populations may be due to differences in background characteristics, such as ethnicity and other confounding factors, and differences between statistical methods used.

To the best of our knowledge, there has been no previous study which examined the association of midlife handgrip strength with the risk of cause-specific mortality. In our study, the level of handgrip strength measured in midlife was associated with the risks of cardiovascular, respiratory and other non-cancer death, as was handgrip strength measured in late life. Therefore, even though the absolute risk of death (shown

Table 2 Association of handgrip strength with the risks of all-cause death and cause-specific death by sex, the Hisayama Study, 1988–2007

	Men (n=1064)			Women (n=1463)		
	Number of events/ subjects	Age-adjusted mortality per 1000 person-years	Multivariable-adjusted HR (95% CI)	Number of events/ subjects	Age-adjusted mortality per 1000 person-years	Multivariable-adjusted HR (95% CI)
All-cause death						
T1 (lowest)	164/332	34.8	1.00 (reference)	162/470	22.3	1.00 (reference)
T2	134/359	26.9*	0.81 (0.64 to 1.03)	117/456	15.8*	0.66 (0.52 to 0.85)*
T3 (highest)	99/373	21.7*	0.70 (0.53 to 0.92)*	107/537	14.9*	0.65 (0.50 to 0.84)*
Per 1 SD increment			0.72 (0.64 to 0.81)†			0.74 (0.66 to 0.82)†
Cardiovascular death						
T1 (lowest)	54/332	11.4	1.00 (reference)	50/470	6.9	1.00 (reference)
T2	31/359	6.1*	0.51 (0.32 to 0.82)*	43/456	5.9	0.74 (0.48 to 1.12)
T3 (highest)	21/373	4.4*	0.38 (0.22 to 0.66)*	36/537	5.2	0.67 (0.43 to 1.05)
Per 1 SD increment			0.52 (0.41 to 0.66)†			0.77 (0.64 to 0.93)†
Cancer death						
T1 (lowest)	46/332	9.6	1.00 (reference)	35/470	4.5	1.00 (reference)
T2	51/359	10.1	1.16 (0.77 to 1.75)	29/456	3.9	0.79 (0.47 to 1.31)
T3 (highest)	52/373	10.9	1.43 (0.93 to 2.18)	36/537	4.7	1.07 (0.66 to 1.74)
Per 1 SD increment			1.11 (0.92 to 1.35)			0.98 (0.79 to 1.22)
Respiratory death						
T1 (lowest)	34/332	7.5	1.00 (reference)	36/470	5.1	1.00 (reference)
T2	28/359	5.9	0.91 (0.53 to 1.55)	22/456	3.0*	0.60 (0.35 to 1.05)
T3 (highest)	19/373	4.8*	0.81 (0.43 to 1.51)	15/537	2.2*	0.45 (0.24 to 0.84)*
Per 1 SD increment			0.60 (0.46 to 0.79)†			0.61 (0.49 to 0.78)†
Death from other causes						
T1 (lowest)	30/332	6.2	1.00 (reference)	41/470	5.8	1.00 (reference)
T2	24/359	4.7	0.75 (0.43 to 1.32)	23/456	3.1*	0.55 (0.32 to 0.92)*
T3 (highest)	7/373	1.6*	0.21 (0.09 to 0.50)*	20/537	1.5*	0.48 (0.27 to 0.84)*
Per 1 SD increment			0.54 (0.40 to 0.73)†			0.62 (0.50 to 0.77)†

*p<0.05 vs T1. †p<0.05 for 1 SD increment of handgrip strength.
HRs were adjusted for age, systolic blood pressure, use of antihypertensive agents, diabetes, total cholesterol, body mass index, electrocardiogram abnormalities, smoking status, alcohol intake and leisure-time physical activity.

as mortality rates in table 3) among the middle-aged was much lower than that of the elderly, handgrip strength measured in midlife may be a good predictive marker that could be used to identify people at higher risk of non-cancer diseases, and subsequent risk of mortality.

The mechanisms underlying the association between handgrip strength and risk of all-cause and cause-specific death have not been clearly defined, but a lower level of handgrip strength, which reflects a weaker whole-body muscle strength, is known to be associated with traditional risk factors for death or cardiovascular disease, that is, lower body weight,[21] physical inactivity[22] and chronic diseases, such as diabetes and hypertension.[23] However, our findings showed that the association of handgrip strength levels with all-cause and cardiovascular death remained significant, even after adjustment for these factors. A population-based study reported a positive correlation of handgrip strength with the serum concentration of insulin-like growth factor 1 (IGF-1),[24] which is a key regulator of muscle cell proliferation and differentiation, and an inhibitor of cell apoptosis and necrosis.[25] Other epidemiological studies have shown an association between decreased IGF-1 concentration and elevated risk of insulin resistance,[26] impaired glucose tolerance including type 2 diabetes,[27] ischaemic heart disease,[28] and mortality.[29] Therefore, IGF-1 may potentially mediate the association between muscle strength and risk of cardiovascular death. The mechanisms which account for the link between handgrip strength and respiratory death remain unclear, but a case-control study[30] demonstrated that patients with chronic obstructive pulmonary disease, a common cause of respiratory death, had decreased expiratory muscle endurance and lower handgrip strength, compared to control subjects with normal lung function, indicating that weaker handgrip strength may be a marker of reduced respiratory muscle function. In our cohort, pneumonia was also an important cause of respiratory death, and death from other causes primarily included infectious diseases such as sepsis. Lower handgrip strength may reflect lower body weight,[21] and may be associated with higher risk of pneumonia and sepsis due to undernourished and immunocompromised conditions.

The strengths of the present study include a longitudinal population-based design, long duration of follow-up, perfect follow-up of the participants, and accurate diagnosis for cause of death on the basis of medical information and autopsy. However, some limitations should be noted. First, handgrip strength levels were determined on the basis of measurements at baseline examination only. Possible changes in handgrip strength levels during the follow-up period were not taken into consideration. Therefore, the risk estimates reported in this study might be underestimated. Second, socioeconomic information, such as educational level and occupation, which might affect the association between handgrip strength and the risk of death, was not available in our cohort. Third, we could not

Table 3 Association of handgrip strength with the risks of all-cause death and cause-specific death by age groups, the Hisayama Study, 1988–2007

	Middle-aged (40–64 years) (n=1743)			Elderly (≥65 years) (n=784)		
	Number of events/ subjects	Sex-adjusted mortality per 1000 person-years	Multivariable-adjusted HR (95% CI)	Number of events/ subjects	Sex-adjusted mortality per 1000 person-years	Multivariable-adjusted HR (95% CI)
All-cause death						
T1 (lowest)	118/558	12.7	1.00 (reference)	208/224	88.2	1.00 (reference)
T2	84/550	8.6*	0.75 (0.56 to 0.99)*	167/265	46.6*	0.50 (0.40 to 0.62)*
T3 (highest)	58/635	5.0*	0.49 (0.35 to 0.68)*	148/275	36.3*	0.41 (0.32 to 0.51)*
Per 1 SD increment			0.72 (0.63 to 0.81)†			0.63 (0.58 to 0.69)†
Cardiovascular death						
T1 (lowest)	32/558	3.5	1.00 (reference)	72/224	30.5	1.00 (reference)
T2	20/550	2.0	0.62 (0.35 to 1.11)	54/265	14.8*	0.44 (0.31 to 0.64)*
T3 (highest)	14/635	1.2*	0.41 (0.21 to 0.80)*	43/275	10.3*	0.32 (0.22 to 0.48)*
Per 1 SD increment			0.65 (0.50 to 0.84)†			0.60 (0.51 to 0.70)†
Cancer death						
T1 (lowest)	40/558	4.3	1.00 (reference)	41/224	17.7	1.00 (reference)
T2	37/550	3.8	0.97 (0.61 to 1.53)	43/265	12.1*	0.69 (0.44 to 1.08)
T3 (highest)	36/635	3.1	0.94 (0.59 to 1.51)	52/275	13.0*	0.76 (0.49 to 1.18)
Per 1 SD increment			0.99 (0.81 to 1.22)			0.92 (0.76 to 1.11)
Respiratory death						
T1 (lowest)	13/558	1.4	1.00 (reference)	57/224	24.8	1.00 (reference)
T2	10/550	1.0*	0.85 (0.37 to 1.98)	40/265	11.3*	0.43 (0.28 to 0.65)*
T3 (highest)	1/635	0.1*	0.08 (0.01 to 0.59)*	33/275	8.2*	0.35 (0.22 to 0.56)*
Per 1 SD increment			0.46 (0.31 to 0.70)†			0.55 (0.45 to 0.66)†
Death from other causes						
T1 (lowest)	33/558	3.5	1.00 (reference)	38/224	15.3	1.00 (reference)
T2	17/550	1.7*	0.58 (0.32 to 1.05)	30/265	8.3*	0.57 (0.35 to 0.94)*
T3 (highest)	7/635	0.6*	0.21 (0.09 to 0.49)*	20/275	4.8*	0.33 (0.19 to 0.59)*
Per 1 SD increment			0.52 (0.40 to 0.68)†			0.56 (0.45 to 0.70)†

*p<0.05 vs T1. †p<0.05 for 1 SD increment of handgrip strength.
HRs were adjusted for sex, systolic blood pressure, use of antihypertensive agents, diabetes, total cholesterol, body mass index, electrocardiogram abnormalities, smoking status, alcohol intake and leisure-time physical activity.

determine the cut-off level of handgrip strength to predict the mortality risk (which might be clinically useful information), because we did not have an adequate number of mortality events to analyse.

In conclusion, handgrip strength levels were associated with the risk of all-cause and cause-specific mortality, except for cancer mortality, in middle-aged and elderly subjects in a Japanese population. Our results suggest that handgrip strength measured in midlife may be a good predictive marker that could be used to identify individuals at high risk for death from non-cancer diseases.

What is already known on this subject

- Most previous studies have reported an inverse association between handgrip strength and all-cause mortality in elderly populations (approx 65 years or older).
- A small number of studies have evaluated the association of handgrip strength with all-cause mortality in a middle-aged population, and their findings have been inconsistent.
- The association of handgrip strength with cause-specific mortality in the middle-aged population has been unknown.

What this study adds

- Handgrip strength measured in midlife, as well as in late life, was significantly and inversely associated with the risk of cardiovascular, respiratory and other non-cancer death, and these associations were independent of other potential risk factors.
- Handgrip strength levels in midlife may be a good predictive marker for the future risk of non-cancer death.

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Contributors We would like to include nine authors as listed in the title page of the manuscript. This study was a collaboration study among three institutes, and each author made an important and remarkable contribution to this study. With regard to author's contributions, Hiro Kishimoto contributed to the study concept, design, data collection, interpretation of data, statistical analysis, and writing the manuscript. Jun Hata contributed to study concept, design, data collection, interpretation of data, and revising the manuscript. Toshiharu Ninomiya, Hajnalka Nemeth, Yoichiro Hirakawa, and Daigo Yoshida contributed to data collection, interpretation of data, and revising the manuscript. Shuzo Kumagai, Takanari Kitazono, and Yutaka Kiyohara were study coordinators and contributed to the study performance, obtaining funding, study concept, design, interpretation of data, and revising of manuscript.

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Competing interests None.

Patient consent Obtained.

Ethics approval This study was conducted with the approval of Kyushu University Institutional Review Board for Clinical Research, and written informed consent was obtained from the participants.

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Original Article

Down-regulation of MET in hippocampal neurons of Alzheimer's disease brains

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We found that mRNA of MET, the receptor of hepatocyte growth factor (HGF), is significantly decreased in the hippocampus of Alzheimer's disease (AD) patients. Therefore, we tried to determine the cellular component-dependent changes of MET expressions. In this study, we examined cellular distribution of MET in the cerebral neocortices and hippocampi of 12 AD and 11 normal controls without brain diseases. In normal brains, MET immunoreactivity was observed in the neuronal perikarya and a subpopulation of astrocytes mainly in the subpial layer and white matter. In AD brains, we found marked decline of MET in hippocampal pyramidal neurons and granule cells of dentate gyrus. The decline was more obvious in the pyramidal neurons of the hippocampi than that in the neocortical neurons. In addition, we found strong MET immunostaining in reactive astrocytes, including those near senile plaques. Given the neurotrophic effects of the HGF/MET pathway, this decline may adversely affect neuronal survival in AD cases. Because it has been reported that HGF is also up-regulated around senile plaques, β-amyloid deposition might be associated with astrocytosis through the HGF signaling pathway.

Key words: Alzheimer's disease, HGF, MET, neurotrophic factor, senile plaque.

INTRODUCTION

Alzheimer's disease (AD) is a neurodegenerative disorder that accounts for half or more of dementia cases. Since 1961, we have been conducting a long-term prospective cohort study of cerebro-cardiovascular diseases in the town of Hisayama, a suburb of Fukuoka in Japan. Careful surveillance of cognitive impairment was started in 1985. As a part of the Hisayama study, we have shown trends in the prevalence of AD and vascular dementia, and that both insulin resistance and dyslipidemia may be independent risk factors for plaque-type pathology.[1,2] Furthermore, we have extended our observations toward molecular pathological alterations in AD brains by microarray analyses of post mortem human brains donated for the Hisayama study.[3] The full-length study revealed that MET mRNA was most drastically declined in AD hippocampus.

MET is a receptor of hepatocyte growth factor (HGF), a multifunctional protein in hepatocytes. HGF/MET signaling induces glucose metabolism, cell proliferation, neuroprotection and neuroregeneration.[4–7] In the CNS in particular, HGF signaling works as a neuroprotective pathway in ischemic damage,[8–10] neuronal injury,[11] and sporadic and familial ALS with SOD1 gene mutation,[12] and as a recovery pathway from neuronal damage.[13,14] Recent studies revealed that HGF/MET signaling is involved in synaptic plasticity,[15] memory function[16] and neurogenesis in the adult mammalian brain.[17] In recent years, the interaction between diabetes and AD has received much attention.[18,19] AD is related to insulin resistance. In the liver, MET directly engages insulin receptors to form a MET-insulin receptor hybrid complex, culminating in a robust signal output.[20] However, the alteration of MET expression in AD brains is not well understood. In the present study, we performed immunohistochemical staining for MET in AD brains to determine characteristic changes in MET expression in AD brains, especially in the hippocampus.

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MATERIALS AND METHODS

Post mortem brain tissues

We examined 26 autopsy samples, including 12 AD (mean age: 90.3 ± 7.0 years; sex: male = 4, female = 8, mean post mortem interval: 19.1 ± 12.2 h) and 14 controls (74.4 ± 11.7 years; mean post mortem interval: 21.7 ± 16.0 h, sex: male = 7, female = 7) from Hisayama residents. In AD cases, 10 pure AD, one mixed AD and vascular dementia, one mixed AD and dementia with Lewy bodies were included. In all control cases, the concomitant AD-related changes were less than Consortium to Establish a Registry for Alzheimer Disease (CERAD) sparse or Braak stage III. The specimens in each case included middle frontal gyrus, superior and middle temporal gyri, inferior parietal lobule, hippocampus with entorhinal cortex and transentorhinal cortex (at the level of the lateral geniculate body) and calcarine cortex. The study was approved by the Ethics Committee of the Faculty of Medicine, Kyushu University. Written informed consent was obtained from the families of all subjects. Neuropathologic changes in brain specimens were examined as previously described.[1] Sections were routinely stained using HE, KB stain, modified Bielschowsky silver impregnation and immunohistochemistry for phosphorylated tau protein. AD pathology was assessed according to the CERAD guidelines[21] and Braak staging guidelines.[22]

Immunohistochemistry

We used anti-MET rabbit monoclonal antibody raised against the N-terminal region of MET (EP1454Y; Abcam, Cambridge, UK) at a dilution of 1:250 as a primary antibody, and Envision1 System Labeled Polymer-HRP (horseradish peroxidase) anti-rabbit and anti-mouse (Dako Cytomation, Carpinteria, CA, USA) antibody as the secondary antibody. The specimens were deparaffinized in xylene and rehydrated in ethanol, then autoclaved in 0.01 mol/L citrate buffer, pH 8 to unmask the epitope. Endogenous peroxidase activity was blocked with methanol containing 0.3% H_2O_2. Specimens were incubated with the primary antibody at 4°C overnight. After rinsing, the specimens were incubated with the secondary antibody for 1 h at room temperature. Immunoreactivity was detected using 3, 3′-diaminobenzidine (DAB, Dojindo, Kumamoto, Japan) and specimens were lightly counterstained with hematoxylin.

Immunofluorescence

We performed double immunofluorescence labeling using the anti-MET rabbit monoclonal antibody and an anti-GFAP mouse monoclonal antibody (GA5; Sigma-Aldrich, St Louis, MO, USA). Fluorescein isothiocyanate-labeled anti-rabbit IgG (N1034; Amersham, BKM, Amersham, UK), Alexa488-labeled anti-mouse IgG (Invitrogen, Carlsbad, CA, USA), Alexa546-labeled anti-mouse IgG (Invitrogen), and Alexa546-labeled anti-rabbit IgG (Invitrogen) were used as secondary antibodies. Both primary and secondary antibodies were used at a dilution of 1:50. The specimens were deparaffinized and unmasked as previously described. Specimens were incubated with primary antibodies at 4°C overnight. After rinsing, the specimens were incubated with the appropriate secondary antibodies for 1 h at room temperature. The specimens were counterstained with 4′,6-diamidino-2-phenylindole (Invitrogen). We observed the specimens using a Nikon A1R-A1 Confocal Microscope System (Nikon, Tokyo, Japan).

RESULTS

Immunohistochemical findings for MET in control cases

In the normal brains, we found distinct perikaryal staining for MET in all pyramidal neurons of the hippocampi (Fig. 1). There was no difference in immunoreactivity between CA1 region and CA2 region, but CA4 was weakly stained. Subpopulations of astrocytes, especially those in the subpial layer and white matter, were also immunopositive for MET. In the dentate gyrus, we found MET immunoreactivity in the granule cells and long slender processes extending to the molecular layer, some of which were co-localized with GFAP (Fig. 2). Astrocytes in the granular layer, subgranular layer and CA4 were also MET immunopositive, but those in the molecular layer were negative. In neocortices, we also found similar perikaryal MET immunoreaction, mainly in the pyramidal neurons (Fig. 1).

Immunohistochemical findings for MET in AD cases

In AD brains, we observed marked decline of cytoplasmic MET immunoreactivity in hippocampal neurons compared with normal controls. This decline of neuronal immunoreactivity in pyramidal cells shows a similar tendency of the reduction of MET mRNA (Fig. 3).[3] The extent of the decline of MET immunoreactivity was similar in both CA1 and CA2 regions. The reduction of MET immunoreactivity was also observed in neocortical neurons; however, the reduction appeared milder than that of hippocampal neurons (Fig. 4). In contrast, we observed strong MET immunoreactivity in reactive astrocytes including those near senile plaques in AD

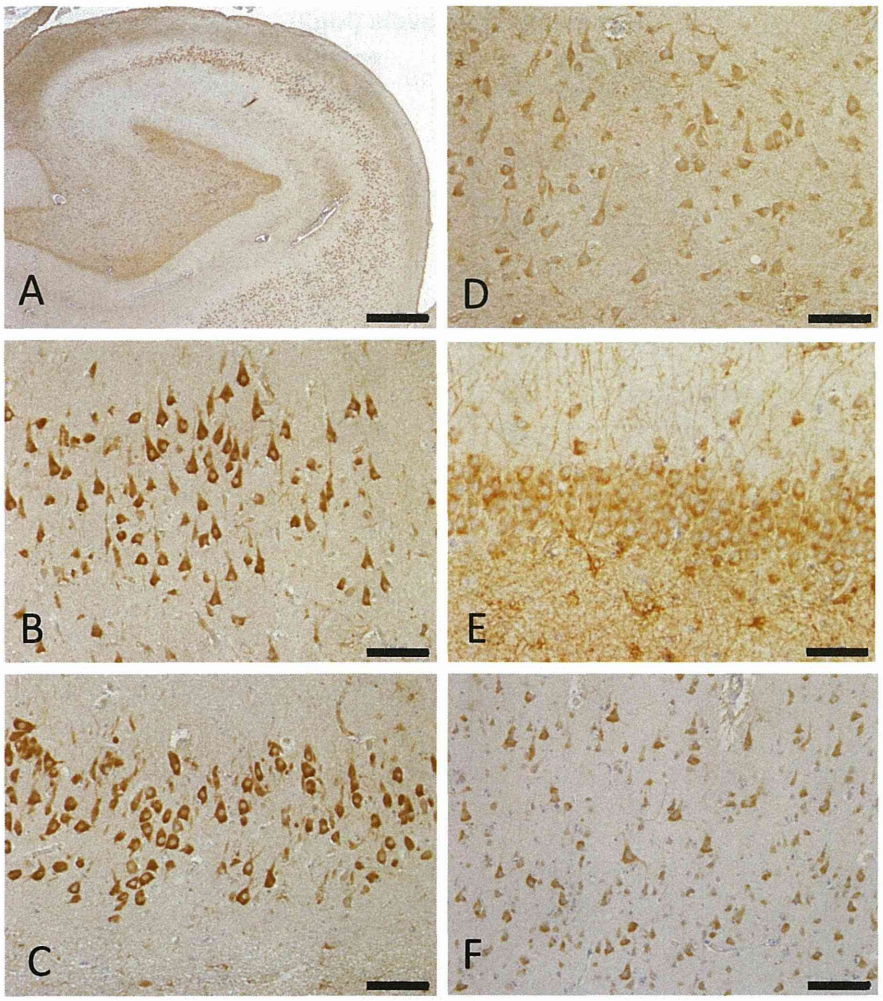

Fig. 1 Cellular distribution of MET immunoreactivity in the hippocampus of a normal control case. (A) Low-power view of the hippocampus shows MET immunoreactivity in the pyramidal layer, the dentate gyrus, and the alveus. Pyramidal neurons show strong MET immunoreactivity in the perikarya (B: cornu ammonis 1 (CA1) region, C: CA2 region, D: CA4 region and E: dentate gyrus, F: frontal cortex) Scale bar = 1 mm (A), 100 μm (B,C,D,F), 50 μm (E).

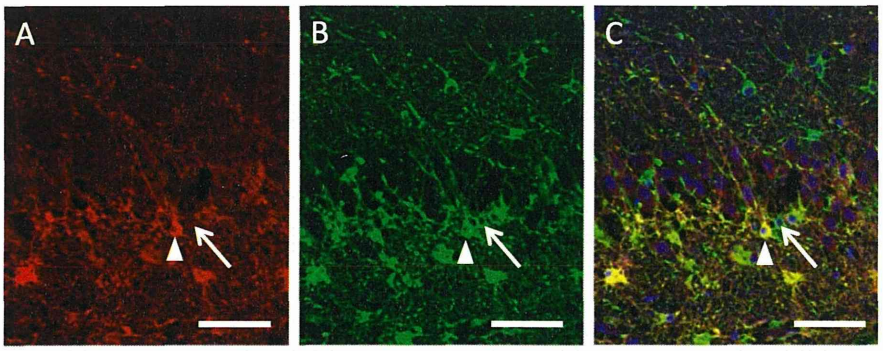

Fig. 2 Immunofluorescence for MET(A) and GFAP(B) in the dentate gyrus of a normal control case. (A) Both dentate granule cells and astrocytes show MET immunoreactivity. (B) Note the long slender cytoplasmic processes from the subgranular zone are also labeled by GFAP immunostaining. (C) Some GFAP positive cells co-express MET (arrowhead) while others do not (arrow). Scale bar = 50 μm.

brains (Figs 5,6). In the dentate gyrus, we found MET immunoreactivity both in granule cells and astrocytes was similar to control cases. The amyloid core, neurofibrillary tangles and Lewy bodies were immunonegative for MET.

DISCUSSION

Previous studies have reported that MET is expressed throughout the brain, preferentially in neurons in the CA1 area of the hippocampus.[23] Most of these neurons are MET positive, and HGF stimulates tyrosine phosphorylation of MET in the neurons.[24] Our study also shows that MET is mainly expressed in neurons and astrocytes of normal brains, while in AD brains neuronal MET is markedly declined, especially in the hippocampal pyramidal cells. The reduction in MET mRNA observed in our previous study may be ascribed to the down-regulation in hippocampal neurons.[3] HGF/MET signaling is an important

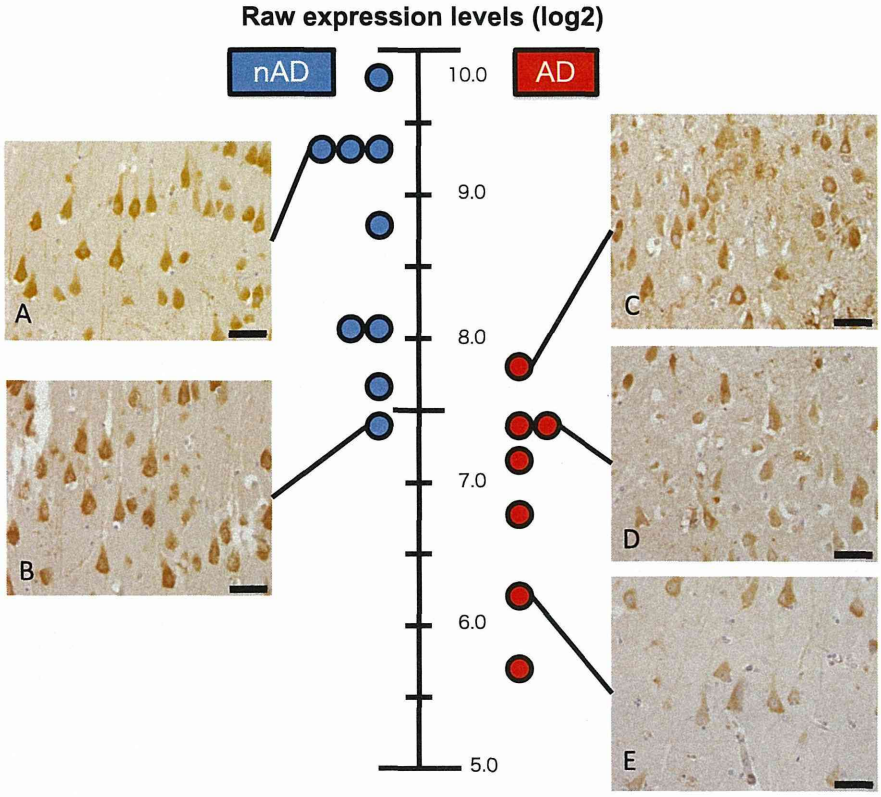

Fig. 3 Comparison of the raw expression levels of MET mRNA and the corresponding MET immunoreactivity in the CA1 region of the hippocampus of non-AD cases (A,B) and AD cases (C,D,E). The vertical bar in the center represents a logarithm of the amount of MET mRNA determined by microarray analysis.[3] Immunohistochemistry reveals decreased labeling in the affected pyramidal neurons of the AD cases (D,E). Scale bar = 50 μm.

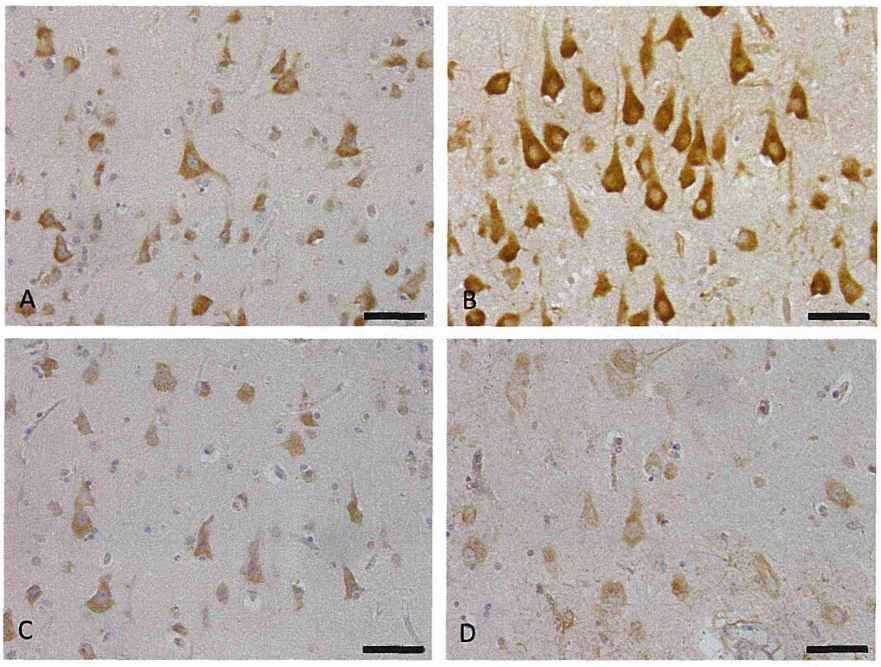

Fig. 4 Immunohistochemistry for MET in the frontal cortex (A,C) and the hippocampus (B,D). In the normal brain (A,B), MET is strongly expressed both in the neocortical neurons and in the pyramidal cells of the hippocampus. In the AD brain (C,D), reduced MET immunoreactivities of the neurons are apparent in both the frontal lobe and hippocampus. Scale bar = 50 μm.

neurotrophic factor in the brain that can help to protect from neuronal death. The decline of MET in pyramidal neurons leads to the dysfunctions of HGF/MET signaling. Our results suggest that dysfunction of HGF/MET signaling in AD cases may adversely affect neuronal survival.

HGF/MET signaling is associated with learning and memory function. According to experiments using a transgenic mouse model, overexpression of HGF in neurons resulted in enhanced memory function.[25] The hippocampus is well known as an important region for learning and

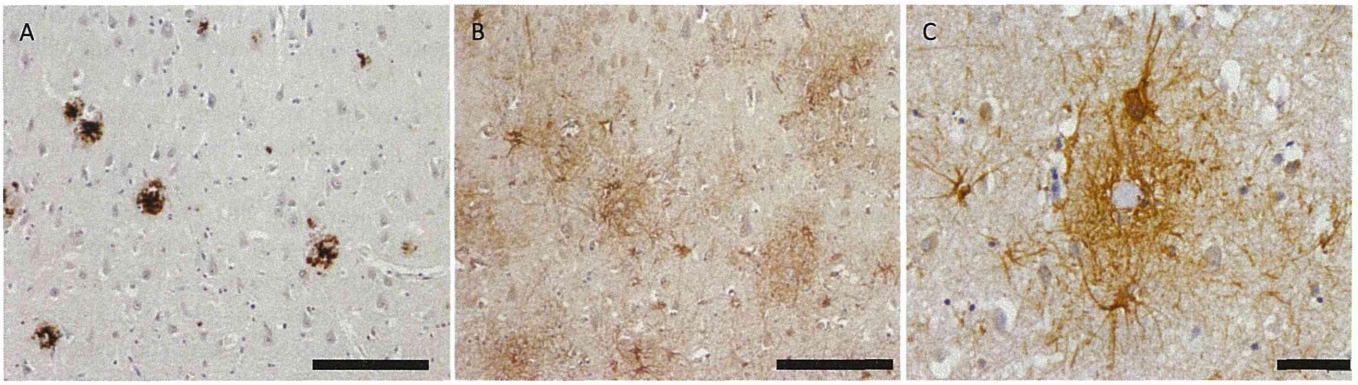

Fig. 5 Elevated MET immunoreactivities of reactive astrocytes near the senile plaques. Low-power views of consecutive specimens show similar patchy distributions of Aβ immunoreactivity (A) and MET immunoreactivity (B). (C) Higher magnification view of the MET immunoreactivity delineates fine processes surrounding the amyloid core as well as astrocyte cell bodies. Scale bar = 200 μm (A,B), 50 μm (C).

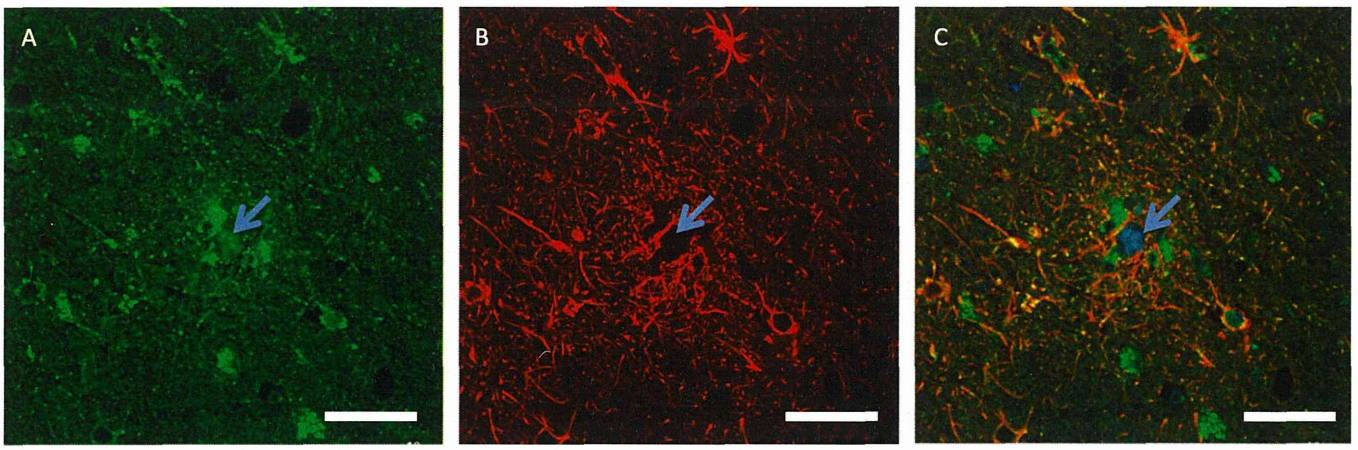

Fig. 6 Double immunofluorescence of a senile plaque for MET (A), GFAP (B) and merged image (C). (A) MET is up-regulated in reactive astrocytes around an amyloid core (arrow). (B) GFAP reactivity is also detected in the astrocytes. (C) MET and GFAP are colocalized in the astrocytes around an amyloid core. Scale bar = 20 μm.

memory, so the depletion of neuronal MET in the hippocampus may be implicated in cognitive impairment and dementia in AD. In the liver, MET is relevant to glycometabolism. MET makes a hybrid complex with insulin receptors to regulate metabolism by promoting hepatic glucose uptake and suppressing hepatic glucose output.[20] Although the function of brain MET is not completely understood, it is plausible that the loss of neuronal MET immunoreactivity may contribute to insulin resistance in AD brains.

We observed increased expression of MET in reactive astrocytes, notably near amyloid deposits. In the mammalian brains, HGF is present not only in hippocampal neurons, but also at high levels in ependymal cells, choroid plexus,[23] astrocytes,[26,27] microglia[28] and oligodendrocyte precursor cells.[29] Previous studies showed that HGF/MET signaling was stimulated in reactive astrocytes in the process of post-damaged regeneration in injury models, ischemic stroke and neuroinflammation.[30] Considering that HGF was also up-regulated around senile plaques,[31] astrocytes near the senile plaques may play a role in reparative defense mechanisms against Aβ deposition, possibly through both paracrine and autocrine signaling of the HGF pathway.

In the human hippocampus, we also observed diffuse MET expression in granule cells of the dentate gyrus. Wang *et al.* reported that HGF/MET signaling works on multiple steps in postnatal forebrain neurogenesis.[17] The hippocampus is known to be one of the neurogenic regions of the adult brain. In the hippocampus, neural stem cells localize in the subgranular zone (SGZ). Ming and Song reported that proliferating radial glia-like precursors and nonradial precursors give rise to intermediate progenitors, which in turn generate neuroblasts. The radial glia-like cell can be identified by GFAP immunostaining in the SGZ.[32] We observed MET- and GFAP-immunopositive SGZ cells

of a similar morphology. We thus suggest that MET is expressed in GFAP-positive radial glia-like cells, and plays a role in neurogenesis. Contrary to our expectation, there was little difference in MET immunopositivity in the dentate gyrus between normal brains and AD brains. Because many factors are involved in adult neurogenesis, further examination of factors related to neurogenesis will be needed.

ACKNOWLEDGMENTS

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Original Article

Elevated expression of fatty acid synthase and nuclear localization of carnitine palmitoyltransferase 1C are common among human gliomas

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Fatty acid synthase (FASN) and carnitine palmitoyltransferase 1C (CPT1C), a brain-specific isoform of the CPT1 family, are upregulated in certain types of cancers, including gliomas. Acetyl-CoA carboxylase (ACC) catalyzes the carboxylation of acetyl-CoA to malonyl-CoA, the rate-limiting step in fatty acid synthesis, and its phosphorylated form inhibits lipid synthesis. We examined the expression and subcellular localization of these fatty acid metabolism-related molecules in human gliomas. We performed immunostaining of two glioma cell lines (U373MG and U87MG) and 41 surgical specimens of diffuse gliomas with various histological grades (21 with the isocitrate dehydrogenase 1(IDH1) R132H mutation and 20 without the mutation). In the cultured glioma cells, CPT1C and phosphorylated ACC (p-ACC) were mainly localized to the nuclei, whereas FASN localized to the cytoplasm. In the surgical specimens, most glioma tissues showed nuclear staining for CPT1C and p-ACC, and cytoplasmic staining for FASN, regardless of the genetic status of IDH1 and the histological grade. Therefore, elevated cytoplasmic expression of FASN and nuclear localization of CPT1C are common among human diffuse gliomas, which may be regulated by the differential phosphorylation status of ACC in the cellular compartment.

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Key words: acetyl-CoA carboxylase, carnitine palmitoyltransferase, fatty acid synthase, glioma, isocitrate dehydrogenase 1.

INTRODUCTION

Otto Warburg observed that cancer cells preferentially consume glucose to produce lactic acid even under aerobic conditions (the Warburg effect).[1] Furthermore, isocitrate dehydrogenase 1 (IDH1) and 2 (IDH2), enzymes that function in the tricarboxylic acid cycle, lipid synthesis and carbohydrate use, are frequently mutated in low-grade glioma and secondary glioblastoma.[2,3] Therefore, cancer metabolism differs from normal cell metabolism. Fatty acids (FAs) are important substrates for energy metabolism and are the building blocks for lipids. To synthesize FAs, acetyl-CoA carboxylase (ACC) catalyzes the conversion of acetyl-CoA into malonyl-CoA. Fatty acid synthase (FASN) then uses malonyl-CoA to synthesize long-chain fatty acids (LCFAs). Many types of tumor cells, including glioblastoma cells, show increased expression of FASN.[4]

Also, various types of tumor cells show an increased level of mRNA expression of carnitine palmitoyltransferase 1C (CPT1C).[5] Carnitine palmitoyltransferase 1 (CPT1) has three isoforms, including CPT1A, CPT1B and CPT1C. CPT1 plays an essential role in the regulation of FA oxidation in normal cells. CPT1A and CPT1B are associated with the outer mitochondrial membrane and mediate the transport of acylated LCFAs into mitochondria by binding them to carnitine, followed by β-oxidation of LCFAs and production of acetyl-CoA. CPT1 activity is inhibited by ACC and malonyl-CoA, and promoted when ACC is inactivated by phosphorylation.[6,7] CPT1C is a brain-specific isoform of

CPT1 that is found only in the microsomal fraction of neurons, and shows a much lower carnitine acyltransferase activity than other CPT1s.[8] CPT1C is associated with regulation of food intake and energy metabolism in the hypothalamus[6] and ω-oxidation of very long-chain fatty acids (VLFAs).[9] However, the function of CPT1C in physiological and pathological conditions largely remains unknown.

In this study, we examined the expression and subcellular localization of FASN and CPT1C in human glioma cell lines and surgical specimens of gliomas by immunostaining, which should lead to clues in understanding the metabolic state of glioma cells.

MATERIALS AND METHODS

Cell culture

Human glioma cell lines (U373MG and U87MG) were cultured in DMEM supplemented with 10% fetal bovine serum at 37°C and 5% CO_2.

Tissue samples

Forty-one glioma tissues, five neuronal and mixed neuronal-glial tumor tissues, and five non-neoplastic adult cerebral tissues from epileptic patients, were obtained at surgery. The glioma samples included seven grade II gliomas (three diffuse astrocytomas (DAs), two oligodendrogliomas (OLs) and two oligoastrocytomas (OAs)), 12 grade III gliomas (three anaplastic astrocytomas (AAs), six anaplastic oligodendrogliomas (AOLs) and three anaplastic oligoastrocytomas (AOAs)), and 22 grade IV gliomas (22 glioblastomas (GBMs)). The neuronal and mixed neuronal-glial tumor tissues included three central neurocytomas and two gangliogliomas. All specimens were fixed in 10% formalin and processed into paraffin sections. Sections were routinely stained with HE and histopathological diagnoses were made at the Department of Neuropathology, Kyushu University, according to the WHO classification of tumors of the nervous system.[10] This study was approved by the Ethics Committee of the Faculty of Medicine, Kyushu University and conformed to the provisions of the Declaration of Helsinki.

Immunohistochemistry

The antibodies used for immunohistochemistry in this study included mouse monoclonal anti-mutant IDH1R132H (H09; Dianova, Hamburg, Germany 1:100), anti-p-ACC1 (F-2; Santa Cruz Biotechnology, Dallas, TX, USA; 1:500) and anti-FASN (A-5; Santa Cruz Biotechnology; 1:500), rabbit polyclonal anti-CPT1C (E-19; Santa Cruz Biotechnology; 1:500) and goat polyclonal anti-ACC1 (T-18; Santa Cruz Biotechnology; 1:100). All sections were deparaffinized in xylene and rehydrated in an ethanol gradient. Sections were subjected to antigen retrieval by autoclaving in 0.01 mol/L citrate buffer, pH 6.0. Endogenous peroxidase activity was blocked with 0.3% H_2O_2/methanol. Then the sections were incubated with primary antibodies at 4°C overnight before being subjected to the enhanced indirect immunoperoxidase method using Envision (DakoCytomation, Glostrup, Denmark) for IDH1R132H mutation, p-ACC1, FASN and CPT1C, or Histofine Simple Stain (Nichirei, Tokyo, Japan) for ACC1. Immunoreactivity was detected using 3,3′diaminobenzidine (DAB), and sections were counterstained with hematoxylin.

For semiquantitative analysis of the FASN immunostaining, we scored the cytoplasmic staining as: ±, a few positive cells; +, <50% positive cells; and ++, ≥50% positive cells (Tables S1, S2). For semiquantitative analyses of the CPT1C and p-ACC1 immunostaining, we scored the nuclear staining as: ±, a few positive cells; +, <50% positive cells; and ++, ≥50% positive cells (Tables S1, S3, S4).

Double immunofluorescence

The human glioma cells (U373MG and U87MG) were fixed in 4% paraformaldehyde and extracted with 0.2% Triton X-100. Indirect double immunofluorescence was performed using combinations of the aforementioned primary antibodies against CPT1C, FASN, p-ACC1 and ACC1. Alexa Fluor 488- or Alexa Fluor 546-conjugated secondary antibodies (Invitrogen, Carlsbad, CA, USA) were used for signal detection. The samples were examined with a confocal laser microscope (A1 Confocal Laser Microscope, Nikon Corporation, Tokyo, Japan).

Western blotting

U373MG cells were harvested in 50% NuPAGE LDS sample buffer (Life Technologies, Waltham, MA, USA) containing 5% 2-mercaptoethanol. The sample was crushed using a Micro Smash MS-100 homogenizer (Tomy, Tokyo, Japan) and centrifuged. Then, the supernatant was heated to 100°C. The lysate was separated in a 10% Mini-PROTEAN® TGX Stain-Free ™ gel (Bio-Rad, Hercules, CA, USA) and transferred onto polyvinylidene difluoride membrane (Bio-Rad). FA-related proteins were detected using mouse monoclonal anti-FASN (A-5; Santa Cruz Biotechnology; 1:1000), anti-p-ACC1 (F-2; Santa Cruz Biotechnology; 1:1000), rabbit polyclonal anti-CPT1C (E19 and N18; Santa Cruz Biotechnology; 1:1000) and peroxidase-conjugated anti-mouse IgG (AP192P; Chemicon, Temecula, CA, USA; 1:3000) or anti-rabbit IgG (AP187P; Chemicon; 1:3000) secondary antibodies. Immunoreactivity was visualized using enhanced chemiluminescence plus Western blot-

ting Detection System (GE Healthcare; Chalfont St. Giles, Buckinghamshire, UK) according to the manufacturer's instructions.

RESULTS

Expression of FA metabolism–related proteins in non-neoplastic cerebral tissues

We first stained non-neoplastic human cerebral tissues with anti-FASN, p-ACC1 and CPT1C antibodies (Fig. 1). FASN was detected in the perikarya of many neurons and some glial cells, including astrocytes and oligodendrocytes. CPT1C was detected mainly in the perikarya of neurons. A few neurons and some glial cells also showed nuclear staining for CPT1C. p-ACC1 was detected in the nuclei of many of the neurons and some astrocytes and oligodendrocytes. In summary, in the cortical neurons, FASN and CPT1C were mainly expressed in the perikarya and p-ACC1 was expressed in the nucleus. Some of the glia also expressed these proteins.

Expression of the FA metabolism-related proteins in human glioma cells

We next examined the expression and subcellular localization of FASN, CPT1C, ACC1 and p-ACC1 in human glioma cell lines (U373MG and U87MG). We performed double immunofluorescence with combinations of anti-CPT1C/anti-FASN and anti-ACC1/anti-p-ACC1 antibodies.

U373MG and U87MG showed a similar expression pattern of these proteins (Figs 2,3). FASN was expressed in the perinuclear area of the cytoplasm. CPT1C was similarly expressed in the perinuclear area; however, it was also localized to the nucleus. While ACC1 expression was mainly seen in the perinuclear area and partially seen in the nucleus, p-ACC1 was expressed exclusively in the nucleus.

Western blotting of FASN and CPT1C in U373MG cells

Western blot analysis of U373MG lysates using the monoclonal antibody against FASN (A-5) showed a band at the reported molecular weight (250–270 kDa)[4] (Fig. 4).

E-19 and N-18 rabbit polyclonal anti-CPT1C antibodies, the epitopes for which are the internal region and the N-terminal region of CPT1C, respectively, both detected a band with a molecular weight of around 60 kDa (Fig. 5). We attempted to perform Western blotting for p-ACC1; however, we failed to detect a specific band for p-ACC1.

Expression of FA metabolism-related proteins in glioma tissues

The 41 gliomas in this study were composed of 21 cases with the IDH1R132H mutation and 20 cases without the mutation (Table 1). Immunophenotypes of the FA metabolism-related proteins are summarized in Table 2. FASN was expressed in 21 gliomas with IDH1R132H mutation (100%) and in 18 gliomas without the mutation (90%). FASN was mainly localized to the cytoplasm of glioma cells (Figs 6B, 7B, 8B, 9B). CPT1C was expressed in

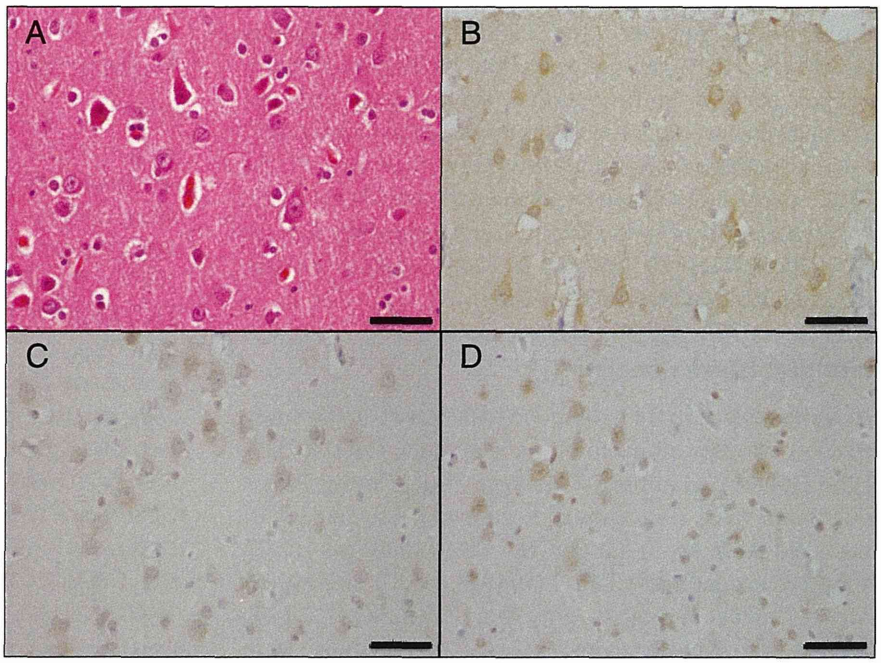

Fig. 1 Expression of fatty acid synthase (FASN), carnitine palmitoyltransferase 1C (CPT1C) and phosphorylated acetyl-CoA carboxylase 1 (p-ACC1) in non-neoplastic temporal cortex from an epileptic patient. (A) HE staining. FASN (B) and CPT1C (C) are mainly expressed in the perikarya and nuclei of neurons, respectively. p-ACC1 (D) is expressed in the nuclei of neurons. Bar: 50 μm.

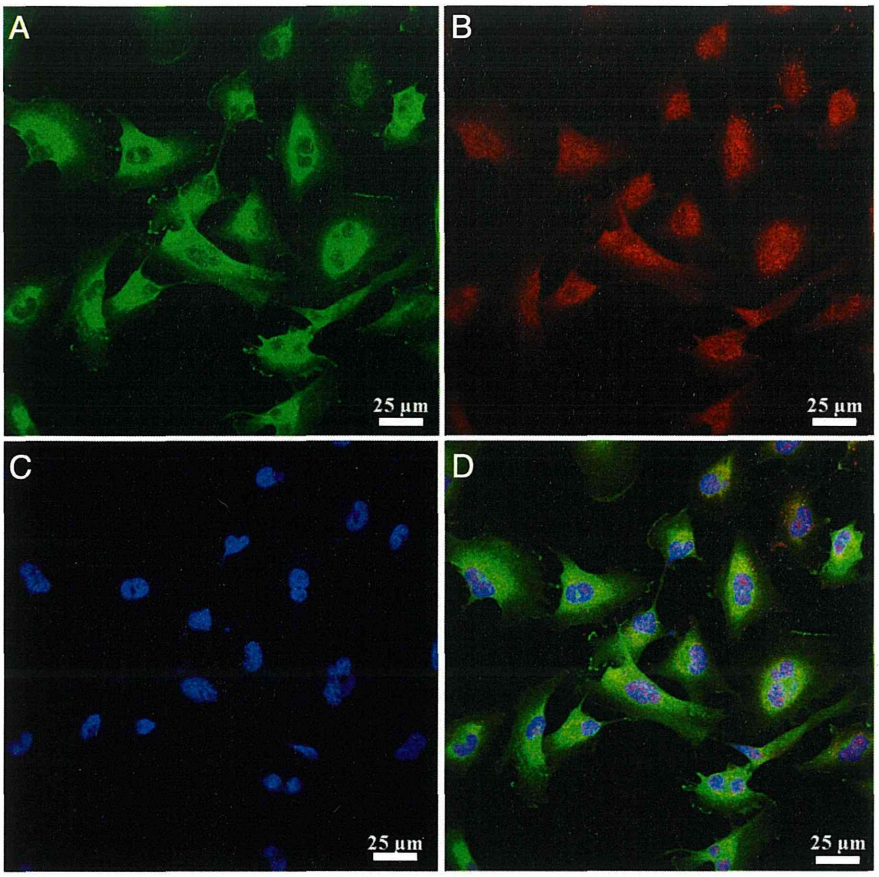

Fig. 2 Double immunofluorescence for fatty acid synthase (FASN) and carnitine palmitoyltransferase 1C (CPT1C) in U373MG cells. FASN is localized to the cytoplasm (A. *green*), while CPT1C is found in both the nucleus and the cytoplasm (B. red). D: merged image, C: nuclear staining with 4′,6-diamidino-2-phenylindole (DAPI). *Bar*: 25 μm.

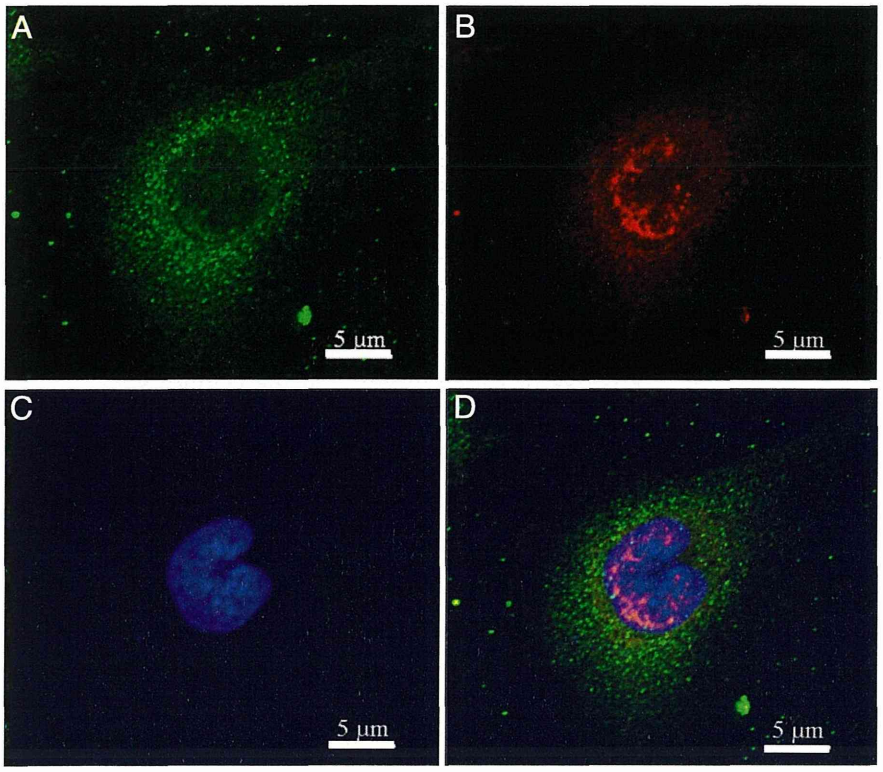

Fig. 3 Double immunofluorescence for acetyl-CoA carboxylase 1 (ACC1) and phosphorylated ACC1 (p-ACC1) in U373MG cells. ACC1 is localized to the cytoplasm (A. green), while p-ACC1 is found in the nucleus (B. red). D: merged image, C: nuclear staining with 4′,6-diamidino-2-phenylindole (DAPI). Bar: 5 μm.