Table 1b. Baseline Characteristics at the First Visit According to %dBMI

variables	%dBMI-Q1 (range: −21.8–−1.9)	%dBMI-Q2 (range: −1.9–−0.2)	%dBMI-Q3 (range: −0.2–1.4)	%dBMI-Q4 (range: 1.4–15.7)	*p* value
Women					
n	284	268	305	342	
Age, years	53 (52–54)	54 (52–54)	52 (51–53)	49 (49–51)	0.002
Height, cm	156 (156–157)	157 (156–157)	158 (157–158)	157 (157–158)	0.005
Weight, kg	52 (52–54)	51 (52–53)	51 (51–53)	51 (51–53)	0.325
WC, cm	77 (76–78)	76 (76–78)	75 (75–77)	75 (75–77)	0.115
BMI, kg/m^2	21.3 (21.3–22.0)	20.9 (21.1–21.8)	20.5 (20.6–21.2)	20.7 (20.7–21.3)	0.002
Systolic blood pressure, mmHg	117 (118–123)	115 (115–119)	114 (115–119)	113 (115–118)	0.060
Diastolic blood pressure, mmHg	74 (73–76)	73 (72–75)	71 (72–74)	71 (71–74)	0.057
Pulse rate, bpm	63 (63–65)	64 (63–65)	61 (62–64)	63 (63–65)	0.106
LDL-cholesterol, mg/dL	133 (127–135)	132 (129–136)	125 (123–129)	117 (119–125)	<0.001
HDL-cholesterol, mg/dL	67 (66–70)	68 (67–71)	69 (68–71)	67 (67–70)	0.647
Trigryceride, mg/dL	79 (87–102)	76 (80–89)	74 (79–89)	68 (73–81)	0.002
Uric acid, mg/dL	4.5 (4.4–4.7)	4.4 (4.4–4.6)	4.6 (4.5–4.7)	4.4 (4.4–4.6)	0.408
Fasting glucose, mg/dL	88 (88–91)	88 (88–93)	88 (88–91)	88 (88–90)	0.933
Hemoglobin A1c, %	5.1 (5.1–5.2)	5.2 (5.1–5.3)	5.1 (5.1–5.2)	5.1 (5.0–5.1)	0.028
Blood urea nitrogen, mg/dL	13.0 (13.2–14.0)	13.0 (13.1–13.9)	13.0 (13.3–14.2)	13.0 (12.8–13.4)	0.174
Serum creatinine, mg/dL	0.60 (0.61–0.63)	0.60 (0.61–0.63)	0.60 (0.62–0.73)	0.60 (0.61–0.63)	0.002
Anti-dyslipidemic medication, *n* (%)	12 (4.2)	10 (3.7)	12 (3.9)	12 (3.5)	0.972
Anti-hypertensive medication, *n* (%)	23 (8.1)	15 (5.6)	16 (5.2)	17 (5.0)	0.352
Current smoker, *n* (%)	21 (7.4)	22 (8.2)	23 (7.5)	41 (12.0)	0.130
Men					
n	504	531	495	484	
Age, years	54 (53–55)	55 (54–55)	54 (53–54)	51 (51–52)	<0.001
Height, cm	169 (169–170)	169 (168–169)	170 (169–170)	170 (169–171)	0.012
Weight, kg	69 (68–70)	67 (67–68)	68 (68–69)	68 (67–69)	0.097
WC, cm	87 (86–87)	85 (85–86)	86 (85–87)	85 (85–86)	0.011
BMI, kg/m^2	24.0 (23.8–24.3)	23.4 (23.4–23.9)	23.7 (23.6–24.1)	23.5 (23.3–23.8)	0.012
Systolic blood pressure, mmHg	126 (127–130)	124 (125–128)	126 (125–129)	123 (123–126)	0.011
Diastolic blood pressure, mmHg	81 (81–83)	79 (79–81)	80 (80–82)	78 (78–80)	0.019
Pulse rate, bpm	62 (62–64)	62 (62–63)	62 (63–64)	62 (61–63)	0.106
LDL-cholesterol, mg/dL	133 (130–135)	129 (128–133)	130 (126–132)	125 (125–130)	0.014
HDL-cholesterol, mg/dL	54 (54–56)	54 (55–57)	54 (55–58)	54 (54–57)	0.437
Trigryceride, mg/dL	111 (126–141)	108 (123–136)	111 (120–135)	107 (118–132)	0.285
Uric acid, mg/dL	6.1 (6.1–6.3)	6.1 (6.0–6.2)	6.0 (6.0–6.2)	6.1 (6.0–6.3)	0.344
Fasting glucose, mg/dL	95 (97–100)	95 (97–100)	95 (95–97)	93 (94–96)	0.002
Hemoglobin A1c, %	5.3 (5.3–5.5)	5.3 (5.3–5.4)	5.2 (5.2–5.3)	5.2 (5.2–5.3)	<0.001
Blood urea nitrogen, mg/dL	14.0 (14.3–15.0)	14.0 (14.3–14.8)	14.0 (13.9–14.4)	14.0 (14.3–15.0)	0.130
Serum creatinine, mg/dL	0.80 (0.84–0.92)	0.80 (0.85–0.87)	0.80 (0.83–0.86)	0.90 (0.85–0.87)	0.303
Anti-dyslipidemic medication, *n* (%)	20 (4.0)	18 (3.4)	28 (5.7)	21 (4.3)	0.334
Anti-hypertensive medication, *n* (%)	72 (14.3)	81 (15.3)	47 (9.5)	61 (12.6)	0.035
Current smoker, *n* (%)	155 (30.8)	167 (31.5)	148 (29.9)	157 (32.4)	0.851

underwent a general health screening during this period (first visit) and again the following year (second visit). Among these 3325 individuals, 3213 (2014 men, 1199 women) who reported not taking antidiabetic drugs at either visit were enrolled in the current study. The mean ± standard deviation (SD) of the interval between the two visits of the individuals enrolled was 356 ± 51 days. The percent difference in

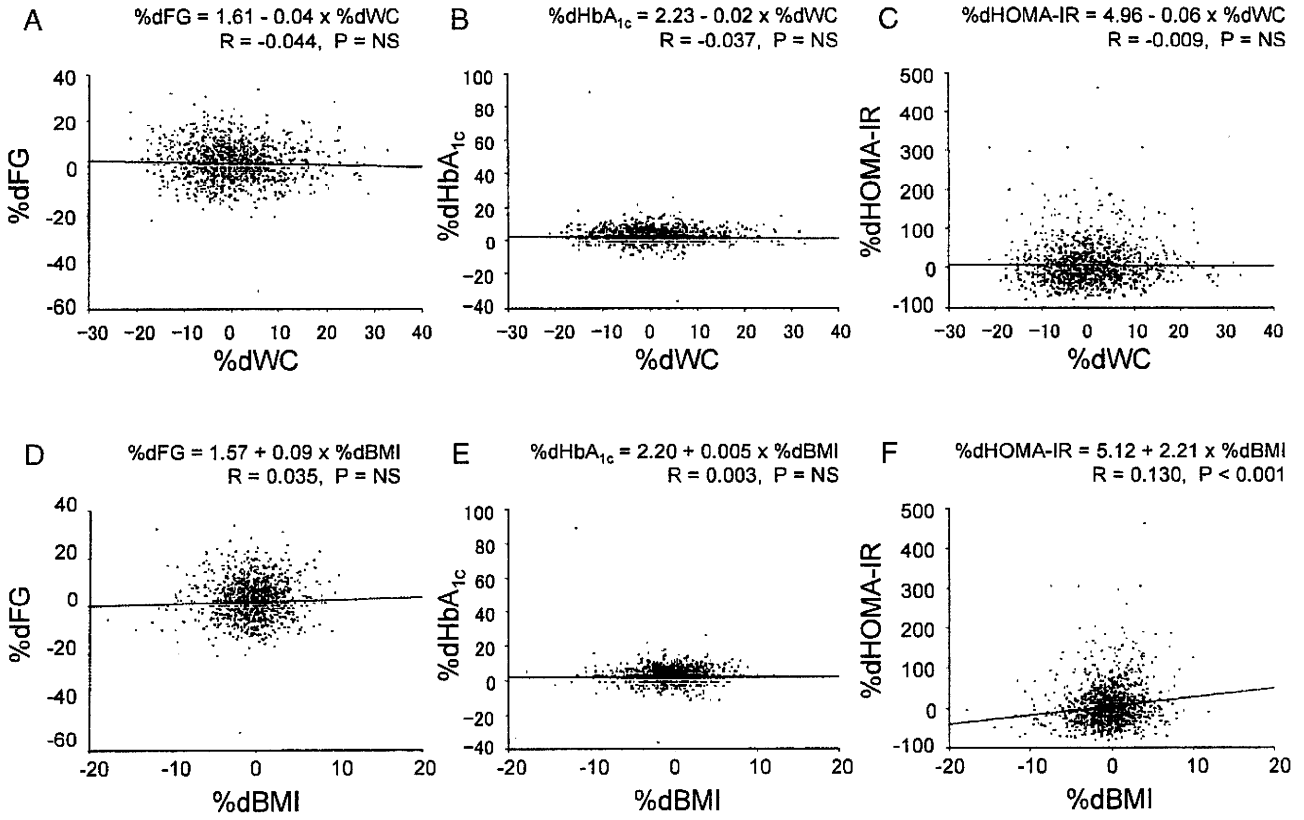

Fig. 1. Scatter plot and linear regression between %dWC and %dFG (A), %dHbA1c (B), and %dHOMA-IR (C) and between %dBMI and %dFG (D), %dHbA1c (E), and %dHOMA-IR (F) in women.

the value of WC, BMI, serum levels of fasting glucose (FG), HbA1c, and HOMA-IR between the first and second visits was designated %dWC, %dBMI, %dFG, %dHbA1c, and %dHOMA-IR, respectively. Blood samples were taken from all subjects after an overnight fast. BMI was expressed as weight (in kilograms) divided by the square of height (in meters). WC was measured at the umbilical level to the nearest 1 cm by trained physicians and technicians[11].

Laboratory Analysis

Serum levels of TC, HDL-C, and TG were determined enzymatically. Serum uric acid was measured by the uricase-peroxidase method; hemoglobin A1c was determined by a latex agglutination immunoassay. Creatinine was measured by TBA-200FR (Toshiba Medical Systems, Tochigi, Japan) using a commercial kit. The homeostasis model assessment of insulin resistance (HOMA-IR) was calculated by the equation: HOMA-IR = (immunoreactive insulin (IRI)) × FBS/405. Blood pressure was measured after about 10 min of rest by an automated sphygmomanometer.

Statistical Analysis

Data are expressed as the median (95% confidence interval (95%CI)) unless stated otherwise. The Kruskal-Wallis test, χ^2 test, logistic regression analysis, and multivariate linear regression analysis were applied as appropriate to assess the statistical significance of differences between groups using computer software, Dr. SPSS II (SPSS Inc., Chicago, IL). A value of $p < 0.05$ was taken to be statistically significant.

Results

Baseline Characteristics

We enrolled 1199 women and 2014 men in this study. The mean age of the individuals enrolled was 51.9 years in women and 53.4 years in men at the first visit. The sex-nonspecific range of the first to fourth %dWC quartiles was −21.3/−3.4, −3.4/−0.1, 0.0/3.3, and 3.3/33.4, respectively, and that of the first to fourth %dBMI quartiles was −21.8/−1.9, −1.9/−0.2, −0.2/1.4, and 1.4/15.7, respectively. Subject characteristics at the first visit are shown according to the

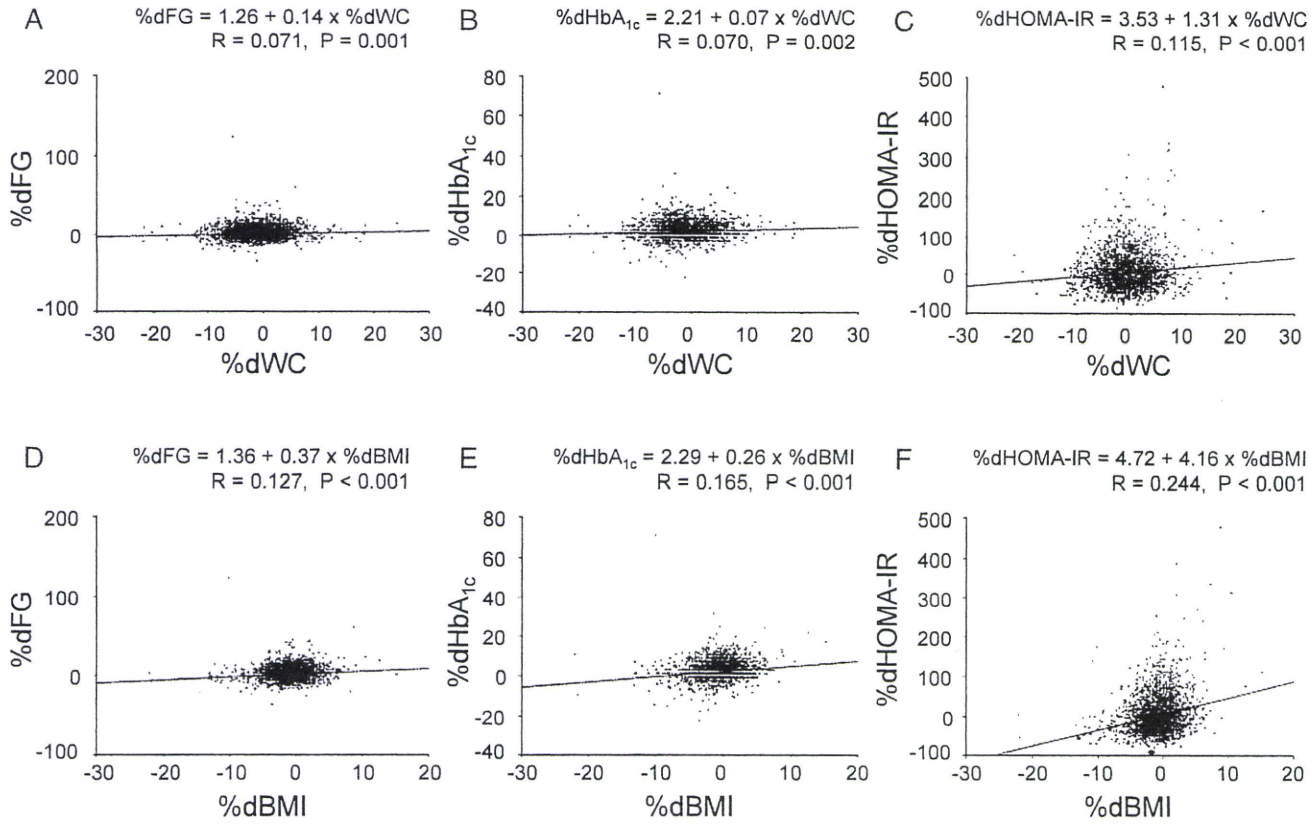

Fig. 2. Scatter plot and linear regression between %dWC and %dFG (A), %dHbA1c (B), and %dHOMA-IR (C) and between %dBMI and %dFG (D), %dHbA1c (E), and %dHOMA-IR (F) in men.

%dWC and %dBMI quartiles in **Table 1**. No statistically significant trends in the rate of anti-dyslipidemic medication or of current smoking were found across the four %dWC or %dBMI quartiles in either gender. The correlation coefficient between %dWC and %dBMI was 0.24 in women and 0.46 in men.

Association between Percent Changes in Obesity Parameters and Percent Changes in Diabetic Parameters

Scatter plots of %dWC and %dBMI versus %dFG, %dHbA1c and %dHOMA-IR, coupled with results of linear regression analyses, are shown in **Fig. 1** and **2**. In women, only the relationship between %dBMI and %dHOMA-IR was significant. In men, by contrast, the relationship was significant between both %dWC and %dBMI and the percent change in each of the diabetic parameters.

Fig. 3 and **4** show the percent changes in diabetic parameters according to the %dWC and %dBMI quartiles. In women, %dHOMA-IR increased with increasing %dBMI. In men, not only %dHOMA-IR but also %dFG and %dHbA1c increased with increasing %dWC and %dBMI.

Logistic Regression Analysis

A multivariate logistic regression analysis, adjusted for age at the first visit, of the second, third, and fourth %dBMI quartiles, showed that the first, second, third, and fourth %dBMI quartiles in men were associated with the highest %dHOMA-IR quartile (%dHOMA-IR >24.3%) with an odds ratio of 1.00 (reference), 1.47 (95%CI 1.08-2.01), 1.51 (95%CI 1.11-2.07), and 2.87 (95%CI 2.13-3.87), respectively. In women, on the other hand, the first, second, third, and fourth %dBMI quartiles were not significantly related to the highest %dHOMA-IR quartile (%dHOMA-IR >24.3%) with an odds ratio of 1.00 (reference), 1.23 (95%CI 0.82-1.85), 1.45 (95%CI 0.98-2.14), and 1.89 (95%CI 1.30-2.74), respectively.

Multivariate Linear Regression Analysis

In a multivariate linear regression analysis with age at the first visit and %dWC as independent vari-

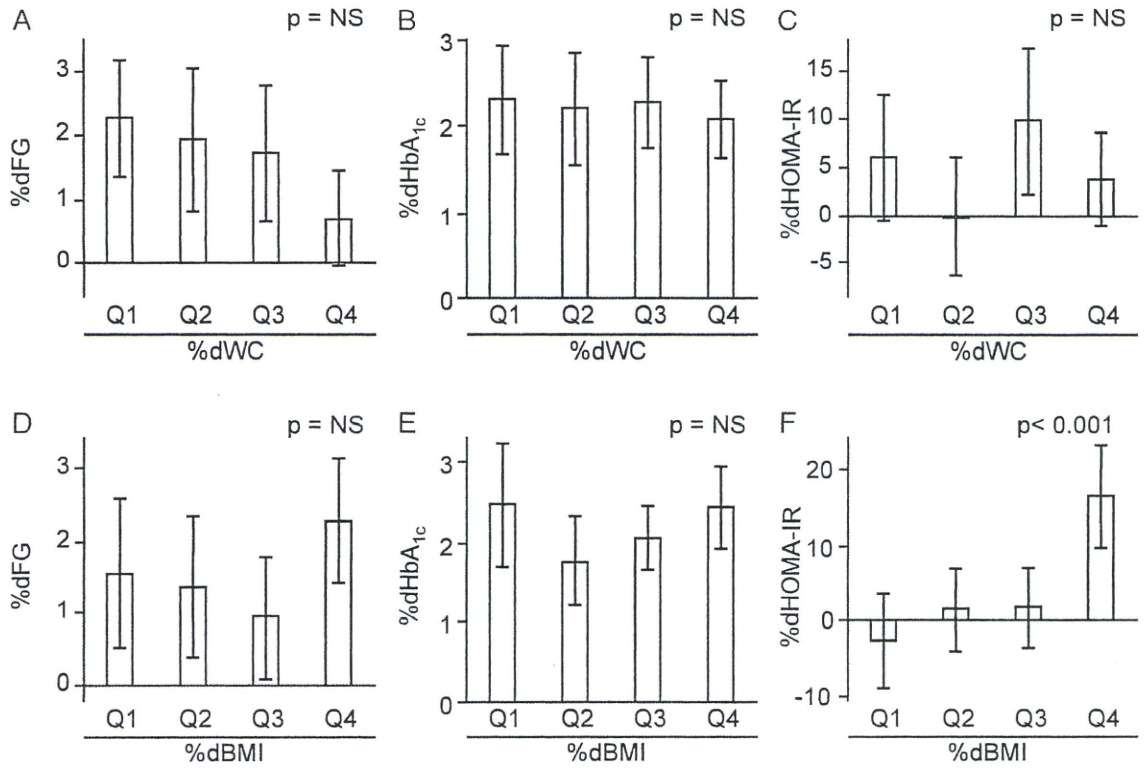

Fig. 3. %dFG (A), %dHbA$_{1c}$ (B), and %dHOMA-IR (C) according to %dWC quartiles, and %dFG (D), %dHbA$_{1c}$ (E), and %dHOMA-IR (F) according to %dBMI quartiles in women. The mean ± 95% confidence interval is shown in each group.

ables (**Table 2**, model 1), %dWC was an independent predictor for %dHOMA-IR in men, but not in women. However, when %dBMI was used as an additional covariate in the statistical model, %dWC did not remain significant (**Table 2**, model 2). In model 2, %dBMI was found to be an independent predictor for %dHOMA-IR, %dFG and %dHbA$_{1c}$ in men, but for only %dHOMA-IR in women.

Discussion

In the current study, we demonstrated that percent changes in obesity parameters (%dWC, %dBMI) were positively correlated with percent changes in glucose metabolism-related parameters (%dFG, %dHbA$_{1c}$, %dHOMA-IR) in men. In women, by contrast, there was no significant relationship between %dWC and percent changes in diabetic parameters, and %dBMI was not significantly associated with %dFG or %dHbA$_{1c}$. In the multivariate linear regression analysis, %dWC was a predictor for %dHOMA-IR in men, although it did not remain significant when %dBMI was used as an additional covariate in the statistical model, suggesting that changes in WC are not a predictor for changes in glucose-metabolism-related parameters independent of changes in BMI.

Obesity is associated with a cluster of specific metabolic abnormalities that may be related to cardiovascular risk factors[8, 12]. Wahrenberg *et al.* have reported that WC, which was found to be the strongest regressor among WC, BMI, log-plasma triglycerides, systolic blood pressure, and high-density lipoprotein cholesterol, is a risk factor for insulin resistance[13]. On the other hand, Onat *et al.* prospectively analyzed 1638 men and found that the age-adjusted waist-to-hip ratio (WHR) was significant in predicting diabetes mellitus[14]. Furthermore, Colditz *et al.* analyzed data from 114281 women who did not have diagnosis of diabetes mellitus, coronary heart disease, stroke, or cancer, and showed that BMI was the dominant predictor of risk for diabetes mellitus, although weight gain was also a risk factor for diabetes[15]. It has been shown that even small gains in weight during adulthood lead to a significantly increased risk of many chronic diseases[16]. Several studies showed that weight loss reduced regional depots of adipose tissue and

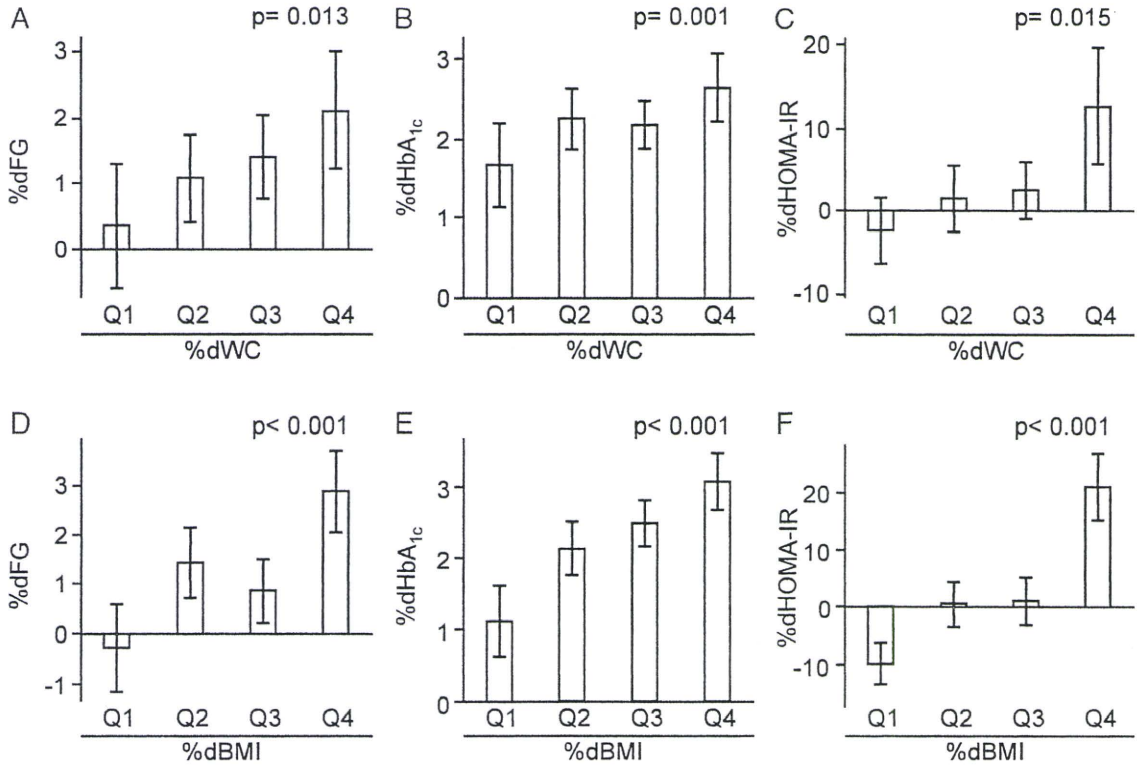

Fig. 4. %dFG (A), %dHbA1c (B), and %dHOMA-IR (C) according to %dWC quartiles, and %dFG (D), %dHbA1c (E), and %dHOMA-IR (F) according to %dBMI quartiles in men. The mean ± 95% confidence interval is shown in each group.

improved insulin sensitivity and cardiovascular risk factors[17, 18]. Pascale *et al.* analyzed 60 women and 33 men participating in a year-long weight loss program and concluded that improvements in FG, fasting insulin, and HbA1c were significantly related to weight loss[19].

Besides body weight, visceral fat has also been reported to be associated with β-cell function in individuals with impaired fasting glycemia and impaired glucose tolerance[9]. In general, BMI is strongly associated with subcutaneous fat area. As parameters of obesity, BMI and WC may have different meanings but similar associations. BMI may have a weaker association with visceral fat; by contrast, WC has a stronger correlation with visceral fat area in both genders[10]. It has been suggested that WC better reflects the accumulation of visceral fat than WHR[20, 21]. Therefore, it is possible that changes in WC have a stronger impact on changes in glucose metabolism as compared with changes in BMI.

In the current study, however, %dBMI was an independent factor predicting %dFG, %dHbA1c, and %dHOMA-IR in men, and %dHOMA-IR in women. %dWC was an independent factor predicting %dHOMA-IR in men, only without adjustment for %dBMI. Why %dBMI had a stronger association with %dFG, %dHbA1c and %dHOMA-IR is not clear.

Because Asian women are relatively lean, subcutaneous fat may have a relatively greater influence on WC[22]. For example, Sakurai *et al.* analyzed 2935 men and 1622 women between 35 and 59 years of age: in a multiple logistic regression analysis, WC was associated with FG in both genders. However, the risk ratio of having two or more metabolic disorders was higher for BMI than for WC in women, suggesting WC to be a relatively poor discriminator of visceral fat, and BMI to be a more appropriate index of total and abdominal fat, especially in women[22, 23].

It has recently been demonstrated that the association between WC and cardiovascular risk markers, such as insulin resistance, weakens with age[24]. Janssen *et al.* reported that, although individuals with a moderate and high WC were likely to have elevated cardiometabolic risk markers irrespective of age, there seemed to be a significant correlation between age and WC, indicating that the relation between WC and insulin

Table 2. Multivariate linear regression analysis between percent changes in diabetic parameters and age, %dWC, and %dBMI

		β	95% CI		Standardized β	p value
Women	Model 1					
	Dependent variable, %dFG					
	age	−0.02	−0.06	0.03	−0.02	0.494
	%dWC	−0.05	−0.10	0.01	−0.05	0.118
	Dependent variable, %dHbA$_{1c}$					
	age	−0.01	−0.04	0.01	−0.03	0.353
	%dWC	−0.02	−0.06	0.01	−0.04	0.181
	Dependent variable, %dHOMA-IR					
	age	0.00	−0.30	0.31	0.00	0.993
	%dWC	−0.06	−0.44	0.32	−0.01	0.753
	Model 2					
	Dependent variable, %dFG					
	age	−0.01	−0.06	0.03	−0.02	0.605
	%dWC	−0.06	−0.12	0.00	−0.06	0.059
	%dBMI	0.12	−0.03	0.27	0.05	0.119
	Dependent variable, %dHbA$_{1c}$					
	age	−0.01	−0.04	0.02	−0.03	0.374
	%dWC	−0.03	−0.06	0.01	−0.04	0.168
	%dBMI	0.02	−0.08	0.11	0.01	0.741
	Dependent variable, %dHOMA-IR					
	age	0.08	−0.22	0.38	0.01	0.610
	%dWC	−0.28	−0.67	0.10	−0.04	0.152
	%dBMI	2.41	1.42	3.40	0.14	<0.001
Men	Model 1					
	Dependent variable, %dFG					
	age	−0.02	−0.06	0.01	−0.03	0.223
	%dWC	0.14	0.05	0.22	0.07	0.002
	Dependent variable, %dHbA$_{1c}$					
	age	−0.01	−0.03	0.01	−0.03	0.250
	%dWC	0.07	0.03	0.12	0.07	0.002
	Dependent variable, %dHOMA-IR					
	age	−0.08	−0.29	0.14	−0.02	0.479
	%dWC	1.30	0.80	1.80	0.11	<0.001
	Model 2					
	Dependent variable, %dFG					
	age	−0.01	−0.05	0.02	−0.02	0.434
	%dWC	0.03	−0.07	0.13	0.02	0.544
	%dBMI	0.35	0.20	0.49	0.12	<0.001
	Dependent variable, %dHbA$_{1c}$					
	age	−0.01	−0.03	0.01	−0.01	0.592
	%dWC	−0.01	−0.06	0.04	−0.01	0.740
	%dBMI	0.26	0.18	0.34	0.17	<0.001
	Dependent variable, %dHOMA-IR					
	age	0.02	−0.19	0.23	0.00	0.840
	%dWC	0.02	−0.52	0.57	0.00	0.932
	%dBMI	4.15	3.33	4.97	0.24	<0.001

For model 1, independent variables include age at the first visit and %dWC. For model 2, independent variables include age at the first visit, %dWC, and %dBMI.

resistance was attenuated in the elderly[24]. With regard to our study, the mean age of the individuals enrolled was 51.9 years in women and 53.4 years in men at the first visit. We may have to analyze the relationship between %dWC or %dBMI and changes in glucose metabolism in a younger population in future studies. In addition, WC measurements may be less reliable or reproducible than weight and height measurements, which might relate to the finding that although %dWC is a predictor for the change in diabetic parameters, the correlation between %dWC and %dBMI was weaker in women, the latter of which is a predictor for the changes in diabetic parameters also in women.

In the current study, interestingly, there was a gender difference in the relationship between %dWC and changes in diabetic parameters. Wing *et al.* reported that the relationship between changes in WHR and changes in lipid parameters differed between women and men: they showed that changes in WHR were associated with changes in total cholesterol and triglycerides levels in men, but not in women[18].

Although we did not look into the mechanisms that may explain the differences in the association of changes in obesity indexes and those in glucose metabolism-related markers between men and women, several explanations may exist. Adipose tissue has been recognized as a significant endocrine organ that releases biologically important cytokines, such as adiponectin, leptin, and vaspin[25, 26]. In several clinical studies, certain gender differences have existed in the serum levels of such adipokines (adiponectin[27, 28], leptin[29], and vaspin[30]), which may account, in part, for the difference in the association between changes in obesity indexes and those in glucose metabolism-related parameters in the current study. Such sexual dimorphism in adipocytokines may be related to the difference in the levels of sex hormones, such as dehydro-epiandrosterone-sulphate (DHEAS), oestradiol, and testosterone[27, 31, 32].

We previously analyzed the relationship between percent changes in obesity parameters and percent changes in serum lipid parameters, uric acid, and systolic blood pressure[33-35]. We found that, as in the current study, the impact of %dBMI was greater than that of %dWC from the viewpoint of changes in serum uric acid and blood pressure.

Our study has several potential limitations. First, we enrolled only individuals who underwent a general health screening at our institute for 2 consecutive years. Second, we analyzed data from participants without considering alcohol consumption or the number of cigarettes smoked. Third, we excluded individuals who were taking antidiabetic drugs at either visit. It has been suggested that these individuals are generally more motivated to improve their own health than those who are not taking such drugs. In addition, a longer follow-up would be required to draw more convincing conclusions in future studies.

In summary, over a one-year period, %dBMI was found to be an independent predictor for %dHOMA-IR in both genders and for %dFG and %dHbA$_{1c}$ only in men. Although %dWC was also associated with percent changes in these diabetic parameters, this relationship did not remain significant after controlling for %dBMI. Conversely, the relationship between %dBMI and percent changes in glucose-related metabolism parameters, especially in men, was independent of %dWC. These findings collectively suggest that controlling body weight, rather than WC, may be the primary target for improving glucose metabolism at least over a one-year period.

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Expression of α-taxilin in hepatocellular carcinoma correlates with growth activity and malignant potential of the tumor

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Abstract. The membrane traffic system has been recognized to be involved in carcinogenesis and tumor progression in several types of tumors. α-taxilin is a newly identified membrane traffic-related molecule, and its up-regulation has been reported in embryonic and malignant tissues of neural origin. In the present study, we analyzed the expression of α-taxilin in relation to clinicopathological features of hepatocellular carcinomas (HCC) and proliferative activity of the tumor determined by proliferating cell nuclear antigen labeling index (PCNA-LI). Twenty-nine surgically resected nodules of HCC (8 well-, 11 moderately-, and 10 poorly-differentiated) were studied. Fifteen cases showed 'strong staining', while 14 cases showed 'weak staining' for α-taxilin. A significantly higher expression of α-taxilin was observed in less-differentiated (p=0.005), and more invasive (p=0.016) HCCs. The 'strong staining' group showed significantly higher PCNA-LI than the 'weak staining' group (the medians of PCNA-LI were 59.4% vs. 14.4%, p<0.0001). We also evaluated the expression of α-taxilin in hepatoma cell lines (PLC/PRF/5, Hep G2 and HuH-6) in association with cell proliferation. The expression levels of α-taxilin protein were correlated with their growth rates. In conclusion, the expression of the α-taxilin protein was related with an increased proliferative activity and a less-differentiated histological grade of HCC. α-taxilin may be involved in cell proliferation of HCC, and its expression can be a marker of malignant potential of HCC.

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Key words: hepatocellular carcinoma, taxilin, vesicular transport proteins

Introduction

The molecular mechanisms of carcinogenesis and tumor progression have been extensively investigated to find novel targets for anti-tumor therapy as well as useful predictors of tumor growth and biological aggressiveness. Involvement of intracellular signaling pathways, cell cycle regulators, growth factors and angiogenic factors has been shown in a variety of carcinomas (1,2). Furthermore, recent studies have revealed that membrane traffic-related molecules play a role in the processes of carcinogenesis and tumor progression in several types of tumors (3-5).

Membrane traffic is a fundamental intracellular transport system in eukaryotic cells (6). Small transport vesicles bud from membranes of a donor compartment, and subsequently fuse with membranes of a target compartment. Cargo molecules in the vesicles as well as biomembranes are dynamically exchanged between organelles with temporal and spatial selectivity. These processes are essential for multiple cellular functions such as endo- and exocytosis, maintenance of organelle homeostasis and cell growth, division, and motility. Soluble *N*-ethylmaleimide-sensitive factor attachment protein receptors (SNAREs), located on vesicles and the target membranes, are the central coordinators of membrane traffic (7), and syntaxin family proteins are their main components.

Recently, taxilin was identified as a novel binding partner of syntaxins (8). α-taxilin, one of the isoforms, was proposed to be involved in Ca^{++}-dependent exocytosis in neuroendocrine cells, although its actual function *in vivo* is not yet known. Over-expression of *α-taxilin* mRNA has been reported in human glioblastoma compared to normal tissues of the central nervous system (CNS) (9). Prominent up-regulation of α-taxilin protein has also been reported in proliferating neural stem cells during embryonic development in rats followed by a rapid decrease of the expression level as development proceeds (10). These findings imply that α-taxilin is related to cell proliferation of mesenchymal cells, especially in the CNS. However, significance of the expression of α-taxilin protein has not yet been studied in tissues or malignancies of epithelial origin.

In the present study, we analyzed the expression of α-taxilin in hepatocellular carcinomas (HCC), which is one of the most common malignancies of epithelial origin worldwide and is known to have wide varieties of differentiation and growth activity. We found a correlation of α-taxilin expression with proliferative activity as well as with malignant potential of HCC.

Patients and methods

Patients. Twenty-nine patients of HCC were studied who underwent curative or non-curative hepatectomy at the Hepato-Biliary-Pancreatic and Transplantation Division between May 2003 and August 2008. No patients received preoperative treatments for HCC. Diagnosis of HCC was confirmed by histology based on sections stained with hematoxylin and eosin. The degree of tumor differentiation and other pathological features were assessed according to the Liver Cancer Study Group of Japan (11), and reviewed by a pathologist who was not informed of the results of α-taxilin expression. When there were multiple nodules of HCC in the resected tissues, the largest one was considered to be representative. The clinical and pathological features of the study population are shown in Table I. Informed consent was obtained from each participant. This study protocol was approved by the institutional review board of the University of Tokyo.

Immunohistochemistry of α-taxilin. The polyclonal anti-human α-taxilin antibody raised in a rabbit was used for immunohistochemistry, as previously described (12).

The formalin-fixed, paraffin-embedded liver specimens containing HCC nodules were sliced into 3 μm-thick sections, immersed in xylene and graded alcohols, and washed in 10 mM PBS. The sections were heated using a microwave processor MI-77 (Azumaya, Tokyo, Japan) in 10 mM citrate buffer (pH 6.0) at 95˚C for 30 min. The sections were treated with 0.3% v/v H_2O_2 in methanol at room temperature for 30 min, blocking solution (10% v/v normal goat serum and 1% w/v BSA in 10 mM PBS) at room temperature for 1 h, and then incubated with a primary antibody diluted in the blocking solution overnight at 4˚C.

A standard avidin-biotin-peroxidase complex (ABC) technique with Vectastain® ABC elite kit (Vector Laboratories, Burlingame, CA, USA) was applied. Briefly, a biotinylated antibody against rabbit IgG diluted in the blocking solution and ABC solution were applied at room temperature for 25 and 30 min, respectively. Staining was visualized with 3,3'-diaminobenzidine working solution (Vector Laboratories) with nuclear counterstaining in Mayer's hematoxylin (Wako Pure Chemical Industries Ltd., Osaka, Japan).

The sections were studied with a light microscope Eclipse 80i (Nikon, Tokyo, Japan) at magnifications x40, x100 and x200 with a digital camera DXM1200F (Nikon). Intensity of α-taxilin staining was evaluated independently by three of the authors. The intensity of staining in HCC tissues was classified into two categories: 'strong staining' where almost all the cancerous cells were stained (Fig. 1A), and 'weak staining' where no stained cells or few weakly stained cells were observed in cancerous tissues (Fig. 1B).

Immunohistochemistry of proliferating cell nuclear antigen (PCNA) in HCC. Immunohistochemistry of PCNA was performed on the serial section of the specimens as mentioned above except for blocking solution (10% v/v normal horse serum and 1% w/v BSA in 10 mM PBS), the primary antibody (mouse monoclonal anti-PCNA antibody (clone PC10; dilution 1:200; Dako, Glostrup, Denmark)), the secondary antibody (a biotinylated anti-mouse IgG) and the duration of microwave heating (10 min).

PCNA labeling index (PCNA-LI) was determined by random evaluation of at least 1,000 HCC cell nuclei at magnification x100. All of the stained nuclei were regarded as positive. PCNA-LI was expressed as the percentage of positive nuclei (13,14).

Preparation of frozen tissue samples of HCC for Western blotting. Western blotting was performed on fresh frozen tissue samples from one representative case showing 'strong staining' of α-taxilin. The samples obtained at hepatectomy from the HCC nodule and adjacent non-cancerous liver tissue were snap-frozen in liquid nitrogen, and stored at -80˚C. They were homogenized in ice-cold buffer containing 20 mM Tris/HCl (pH 7.5), 150 mM NaCl, 1 mM EDTA, Protease inhibitor cocktail Complete® (Roche Diagnostics, Basel, Switzerland), 1 mM DTT, and 2% w/v Triton X-100, incubated for 1 h with intermittent vortexing.

Preparation of lysates of hepatoma cell lines for Western blotting. The human hepatoma cell lines, Hep G2 (cell no. JCRB1054), PLC/PRF/5 (JCRB0406) and HuH-6 Clone 5 (JCRB0401) were purchased from Health Science Research Resources Bank (Osaka, Japan) in November 2008. The cell lines were authorized by Multiplex PCR method using short tandem repeat by the cell bank.

The cells were plated on plastic culture dishes (BD, Franklin Lakes, NJ, USA) at a density of 3,000 cells/cm^2 in DMEM with 10% v/v FCS and grown to 50-70% confluency. The cells were lysed in ice-cold buffer containing 50 mM Tris/HCl (pH 8.0), 120 mM NaCl, 20 mM NaF, 1 mM EDTA, 6 mM EGTA, 0.5 mM DTT, protease inhibitor cocktail, and 1% v/v NP-40, incubated for 10 min with intermittent vortexing.

Western blotting for α-taxilin. Protein concentrations of the tissue homogenates and the cell lysates were determined by the Lowry's method with DC™ protein assay kit (Bio-Rad Laboratories, Hercules, CA, USA). The samples were adjusted to the same protein concentration, boiled at 95˚C for 5 min with a half volume of SDS buffer (186 mM Tris/HCl (pH 6.7), 9% SDS, 15% glycerol, 6% 2-mercaptoethanol, bromophenol blue), separated by SDS-PAGE on a 10% polyacrylamide gel, and transferred to PVDF membranes (GE Healthcare, Buckinghamshire, UK). The membranes were treated with blocking buffer (10 mM PBS containing 5% w/v skim milk and 0.1% w/v Tween-20) for 1 h at room temperature, and incubated with 0.1 μg/ml of anti-human α-taxilin antibody in the blocking buffer overnight at 4˚C, and subsequently with HRP-conjugated anti-rabbit IgG (GE Healthcare) in the blocking buffer (dilution 1:2,000) for 45 min at room temperature. The chemiluminescent signals were visualized

Table I. α-taxilin expression in HCC and clinical and pathological features.

	All	Intensity of α-taxilin expression		P-value
		Weak (%)	Strong (%)	
Age[a]	68.0 (45-76)	68.0 (51-76)	68.0 (45-76)	
<68	14	7 (50)	7 (50)	0.860
≥68	15	7 (47)	8 (53)	
Gender				
Male	24	10 (42)	14 (58)	0.125
Female	5	4 (80)	1 (20)	
Background liver[b]				
Liver cirrhosis	17	10 (59)	7 (41)	0.395
Chronic hepatitis	10	4 (40)	6 (60)	
Normal liver	1	0 (0)	1(100)	
Number of tumors				
Solitary	20	10 (50)	10 (50)	0.786
Multifocal	9	4 (44)	5 (56)	
Tumor diameter cm)[a]	3.0 (0.9-7.5)	2.6 (1-5.5)	3.7 (0.9-7.5)	
<3.0	15	9 (60)	6 (40)	0.199
≥3.0	14	5 (36)	9 (64)	
Degree of tumor differentiation				
Well	8	7 (88)	1 (12)	0.005
Moderately	11	6 (55)	5 (45)	
Poorly	10	1 (10)	9 (90)	
Tumor invasiveness (vascular invasion and/or intrahepatic metastasis)				
Negative	16	11 (69)	5 (31)	0.016
Positive	13	3 (23)	10 (77)	
Fibrous capsular formation/infiltration				
Negative	11	3 (27)	8 (73)	0.216
Positive/negative	3	2 (67)	1 (33)	
Positive/positive	15	9 (60)	6 (40)	

Data shown are number of cases, and the numbers in parenthesis are percentage of cases unless otherwise indicated. [a]Data are expressed as median (range). [b]Data are not available in 1 case.

with ECL plus™ (GE Healthcare) and detected by LAS-1000 (Fujifilm, Tokyo, Japan).

Cell proliferation assay. The human hepatoma cell lines mentioned above were seeded into 96-well tissue culture plates (BD) at 1,000 cells/well in DMEM supplemented with 10% v/v FCS. After 24, 48 and 72 h, the numbers of viable cells were determined by a soluble tetrazolium/formazan assay using Cell Counting Kit-8 (Dojindo Laboratories, Kumamoto, Japan) (15).

Statistical analysis. Statistical analysis was performed using StatView 5.0J software (SAS Institute Inc., Cary, NC, USA). The Mann-Whitney U test was performed to compare variables of two categories. The Kruskal-Wallis test followed by the Scheffé's *post hoc* test was performed to compare variables of three or more categories. All statistical analyses were considered to be significant at $p<0.05$.

Results

Immunohistochemical staining of α-taxilin in HCCs and non-cancerous liver tissues. Diffuse granular staining of α-taxilin was observed in the cytoplasm of HCC cells. Fifteen cases were classified as 'strong staining', where all the tumor cells

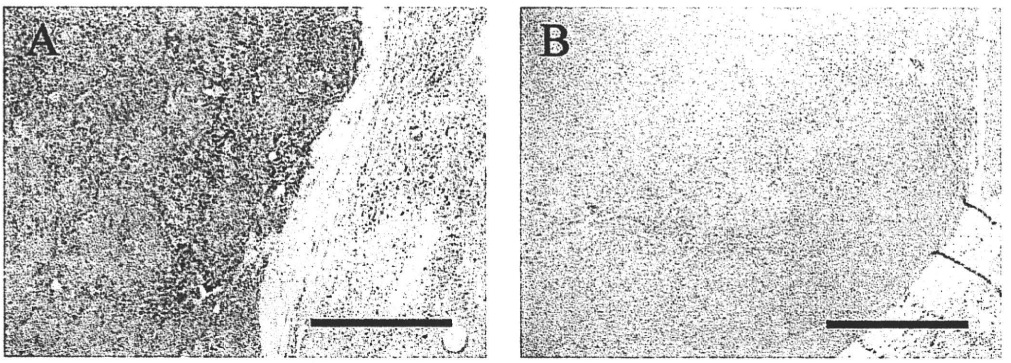

Figure 1. Expression of α-taxilin in HCCs. Intensities of staining were classified into 2 categories: (A) strong staining, almost all the cancerous cells were stained; (B) weak staining, no stained cells or few weakly stained cells were observed in cancerous tissues. Bar, 200 μm.

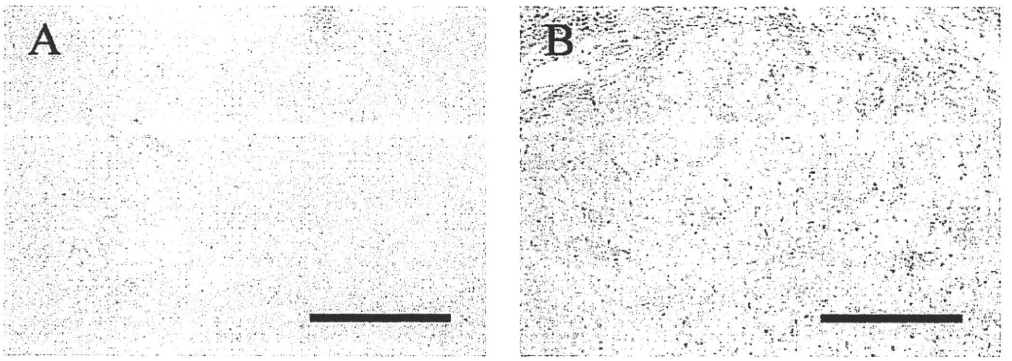

Figure 2. Expression of α-taxilin in non-cancerous tissues. Intensities of staining were evaluated as undetectable (A), or weakly detectable (B). Bar, 200 μm.

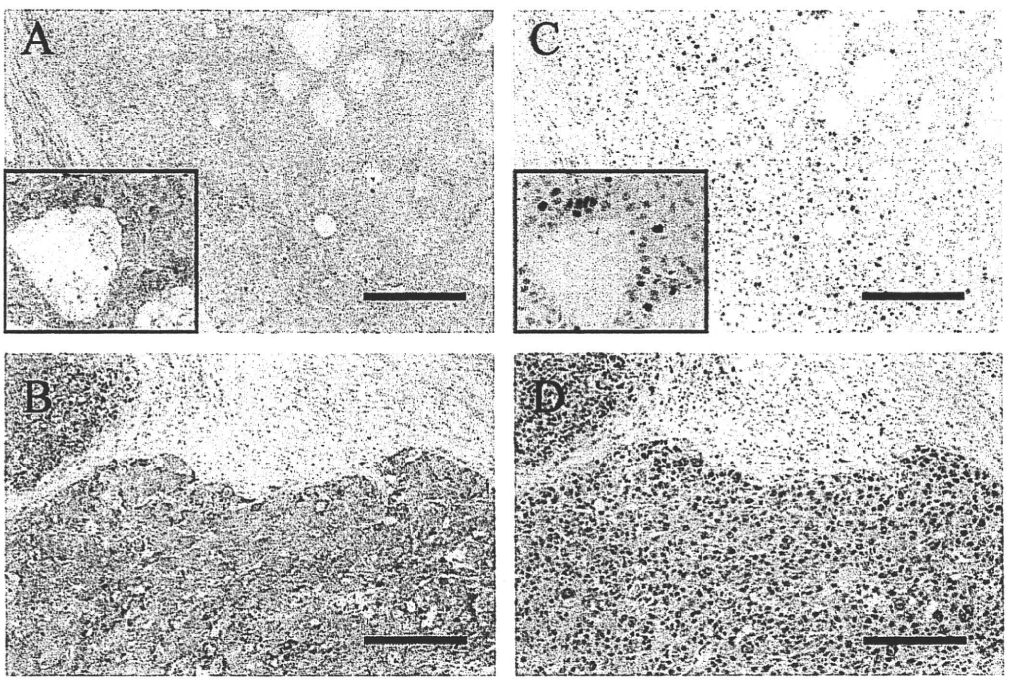

Figure 3. Expression of α-taxilin (A and B) and PCNA (C and D) in HCCs. Intensities of α-taxilin staining and PCNA-LI were determined in serial sections. Two representative cases are shown. Case 1, weak staining of α-taxilin (A) and 7.5% of PCNA-LI (C). Case 2, strong staining of α-taxilin (B) and 94.2% of PCNA-LI (D). Magnified pictures are shown in boxes (A and C). Bar, 500 μm.

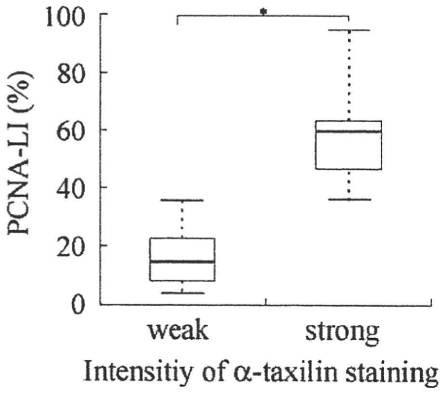

Figure 4. Relationship between α-taxilin expression and PCNA-LI. Tukey's box-and whisker plot are shown. Asterisk indicates a significant difference ($p<0.0001$ by the Mann-Whitney U test).

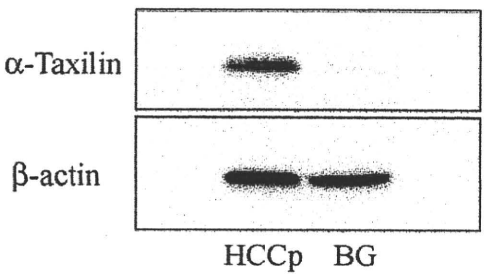

Figure 5. Expression of α-taxilin in HCC and surrounding non-cancerous liver tissue, determined by Western blotting. HCCp, cancerous tissue obtained from poorly differentiated HCC; BG, non-cancerous background liver tissue.

were clearly stained with or without strongly stained foci (Fig. 1A). Fourteen cases were classified as 'weak staining', where only a part of tumor cells showed weak staining mainly in periphery of the tumor nodules (Fig. 1B). Relationships between the α-taxilin expression and various clinicopathological features were shown in Table I. Significantly higher expression of α-taxilin was observed in less differentiated HCCs ($p=0.005$), and more invasive HCCs ($p=0.016$).

In non-cancerous liver tissues surrounding HCCs, the staining was much weaker compared to HCCs. Eleven cases showed no detectable staining (Fig. 2A), 17 cases showed weak and scattered staining in the cytoplasm of hepatocytes in periportal and periseptal areas (Fig. 2B), and 1 case was not appropriate for evaluation because of the small specimen size of non-cancerous tissue.

Relationship between expressions of α-taxilin and PCNA in HCCs. PCNA-LI in HCCs ranged from 3.4 to 94.2%, and the median was 36.0%. α-taxilin-positive cells and PCNA-positive cells showed very similar distribution in the tumor nodules when comparing serial sections (Fig. 3A and C). The sections of 'strong staining' group of α-taxilin showed significantly higher PCNA-LI than the 'weak staining' group (the medians of PCNA-LI were 59.4% vs. 14.4%, $p<0.0001$) (Fig. 4).

Detection of α-taxilin expression in HCC and non-cancerous tissues by Western blot analysis. Western blot analysis showed a single band consistent with α-taxilin (75 kDa) (8) in the tissue of poorly differentiated HCC (Fig. 5), which was strongly stained for α-taxilin by immunohistochemistry as shown in Fig. 3B. Much weaker signal was detected by Western blotting in the surrounding non-cancerous tissue.

Relationship between α-taxilin expression and cellular proliferation in hepatoma cell lines. All the cell lines in logarithmic-growth phase expressed a single band consistent with α-taxilin at various intensities by Western blotting (Fig. 6A and B). The rate of cell growth correlated with the intensity of α-taxilin expression (Fig. 6C).

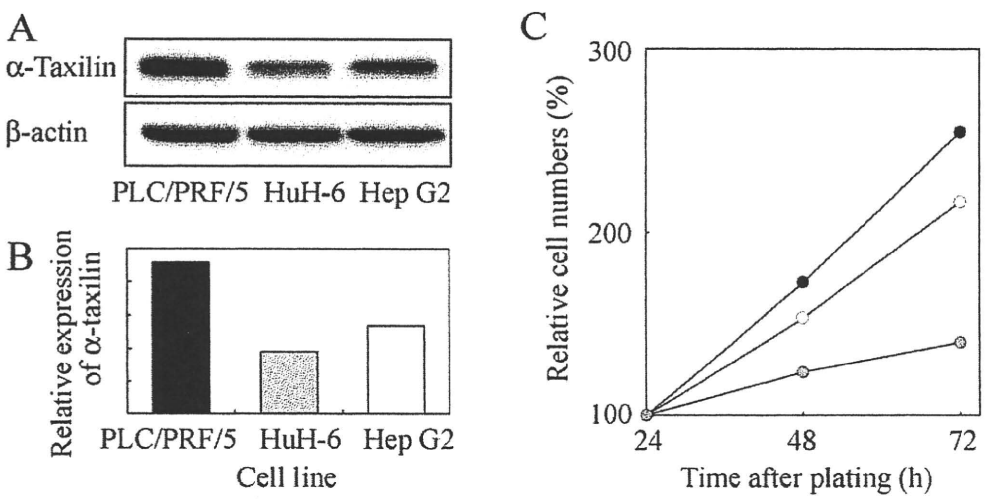

Figure 6. Expression of α-taxilin in hepatoma cell lines (A and B) and cell proliferation rate (C). Closed circles, open circles and shaded circles indicate PLC/PRF/5, Hep G2 and HuH-6, respectively. The expression levels of α-taxilin in the cell lines were correlated with their growth rates.

Discussion

We demonstrated the expression of α-taxilin in HCCs, and its expression levels correlated with dedifferentiation, invasiveness and growth activity. We used a rabbit polyclonal anti-human α-taxilin antibody. Previous reports have shown a single band in lysates of HeLa and COS-7 cells expressing human recombinant α-taxilin by Western blotting using this antibody (12). In the present study, a similar single band consistent with α-taxilin molecular weight was detected in HCC specimen and hepatoma cell lines by Western blotting. This antibody detects α-taxilin specifically in HCC cells.

We determined proliferative activity of HCC using PCNA-LI. PCNA accumulates in the nuclei during S-phase of the cell cycle (16), and is considered as a marker of proliferative activity of various tumors including HCC (13,14,17-20). Previous studies have reported that PCNA-LI in HCCs ranged from 0.2 to 73.3% (13), from 1.0 to 89.4% (17), and from 1.2 to 91.6% (18). In the present study, PCNA-LI was ranged from 3.9 to 94.2%, which is in line with previous reports. The grade of α-taxilin expression in HCCs showed a significant positive correlation with PCNA-LI. In addition, the spatial distribution of α-taxilin positive cells was similar with that of PCNA. These findings suggest that expression levels of α-taxilin relate to proliferative activity of HCC. This notion is supported by the data of *in vitro* experiments showing the relationship between the extent of α-taxilin and proliferative activity of hepatoma cell lines.

Enhanced α-taxilin expression in HCCs was also significantly correlated with less-differentiated histological grade, and more invasive characteristics indicated by positivity for vascular invasion and/or intrahepatic metastasis (21,22). Dedifferentiation of HCCs is usually considered to be associated with higher proliferative activity of the tumor and higher risk of vascular invasion and metastases (2). In addition, in the 'weak staining' cases, α-taxilin positive cells tended to distribute in the periphery of tumor nodules. This feature is also reported for several proteins associated with tumor progression and angiogenesis in HCC (23,24). These findings may suggest that α-taxilin expression in HCC is associated with tumor aggressiveness represented by rapid proliferation and dedifferentiation.

In non-cancerous liver tissues, α-taxilin was weakly expressed in small numbers of hepatocytes. These hepatocytes were mainly distributed in periportal and periseptal area of liver lobule, where mitogenic activity of hepatocytes is considered to be high (25). The expression of α-taxilin in background liver might relate to the potential of hepatocyte proliferation.

At present, limited data are obtained about taxilin, and the precise function still remains unclear. Taxilin is well preserved between species, and three isoforms (α-, ß-, γ-) were reported in mammals (26). The structure of α-taxilin is characterized by a long coiled-coil domain and a leucine zipper motif. Both of them are known to be necessary for a protein-protein interaction or protein dimerization (27), and commonly found in proteins involved in important biological functions such as regulation of gene expression. The long coiled-coil domain is also a characteristic structure of proteins supporting SNARE-mediated membrane fusion (28). The SNARE proteins are localized on the restricted membrane components, and supposed to have selective function in specific intracellular trafficking steps (7). *In vitro* binding assay has shown that α-taxilin binds with some isoforms of syntaxins, specific members of SNAREs (8), which are predominantly localized on the plasma membrane and involved in post-Golgi vesicle transport. These findings lead one hypothesis that α-taxilin is involved in post-Golgi membrane traffic through its association with syntaxins localized on the plasma membrane. In addition, recent studies suggest the involvement of SNAREs and these related molecules in cell proliferation through various steps requiring membrane fusion events, such as nuclear envelope reassembly (29), cytokinesis (29-31), and organelle inheritance. α-taxilin might affect tumor growth through membrane fusion event. Furthermore, α-taxilin has been reported to have another possible binding partner unrelated to SNAREs. *In vitro* binding assay has shown that α-taxilin binds to nascent polypeptide-associated complex (NAC) (12), a ubiquitous factor of eukaryotic cells. NAC reversibly binds to newly synthesized polypeptide chains, and prevent them from improper folding or unwanted interactions with other proteins (32). This finding suggests the association of α-taxilin with translating ribosomes. Diverse functions of α-taxilin should be considered to reveal a possible mitogenic effect of α-taxilin in tumors.

New aspects have been highlighted about the function of factors associated with membrane traffic, especially SNAREs and these related proteins, in cell growth and organogenesis other than membrane fusion event. While ZW10 binds to syntaxin-18 and play a role in vesicle transport between Golgi and the endoplasmic reticulum (33), it is also known as a mitotic checkpoint protein, controlling attachment of microtubules to kinetochores of chromosomes in *Drosophila* (34). Syntaxin-2, which is localized on plasma membrane and involved in post-Golgi transport, has also been identified as an extracellular molecule playing a role in morphogenesis of epithelial organs (35) including liver (36). Overexpression of syntaxin-2 in the mouse mammary gland promotes alveolar hyperplasia and mammary adenocarcinoma (5). Syntaxin-7, which locates on endosome and mediates endosomal/lysosomal fusion events, has been reported to be associated with more aggressive phenotype of malignant melanoma (3). Syntaxin-1 has been associated with more aggressive forms of colorectal carcinomas (37). The relationship between cell proliferation and membrane traffic related proteins would be further investigated in the future.

In conclusion, the expression of the α-taxilin protein is enhanced in HCC, and related with increased proliferative activity and dedifferentiation of HCC. In addition, α-taxilin can be utilized as a marker of malignant potential of HCC.

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Original Article

Association between Gamma-Glutamyltransferase Levels and Insulin Resistance According to Alcohol Consumption and Number of Cigarettes Smoked

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Aim: Alcohol intake may increase serum gamma-glutamyltransferase (GGT) but reduce insulin resistance. We analyzed the association between GGT and a marker of insulin resistance, homeostasis model assessment for insulin resistance (HOMA-IR), according to the drinking and smoking status.
Methods: After excluding former smokers and/or former drinkers, the data of 10,482 men who underwent general health screening were analyzed.
Results: Alcohol consumption showed a graded association with GGT. In men with current alcohol consumption of ≥40 g per day, ≥20 cigarettes per day further increased GGT levels. Alcohol consumption showed a U-shaped association with HOMA-IR. In contrast, smoking 20–39 and ≥40 cigarettes per day increased HOMA-IR as compared with never smokers. An interaction between alcohol consumption and smoking was present for GGT ($p<0.001$) and HOMA-IR ($p=0.059$). GGT was not a significant negative predictive value for HOMA-IR regardless of the drinking or smoking status.
Conclusions: Although alcohol intake showed a graded association with GGT and a U-shaped association with HOMA-IR, serum GGT can be utilized as a predictor of insulin resistance in current drinkers.

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Key words; Drinking, Cigarette smoking, Epidemiology, Insulin resistance, Liver function

Introduction

Recent epidemiological studies have shown that, besides being a biomarker of alcohol intake[1-4], elevated gamma-glutamyltransferase (GGT) may be a predictor of cardiovascular events[5], stroke[6], liver cancer[7], metabolic syndrome and type 2 diabetes[8], associations that may also be present in nondrinkers[9]. Several factors other than alcohol are known to affect serum GGT levels, including coffee consumption[10, 11] and obesity[12]. In addition, a recent study has demonstrated that cigarette smoking may also increase serum GGT levels, especially in men with moderate to heavy alcohol consumption[13]. Furthermore, alcohol consumption may improve insulin sensitivity and lower the incidence of metabolic syndrome[14-19]; therefore, drinking may increase GGT and decrease insulin resistance. On the other hand, it has been reported that serum GGT has a positive association with insulin resistance[20, 21]. To this end, we investigated the effect of drinking and smoking on GGT and HOMA-IR values, and whether the mode of association between GGT and insulin resistance was affected by drinking and smoking in Japanese men who underwent general health screening.

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Methods

Study Population

The study was approved by the Ethics Commit-

tee of Mitsui Memorial Hospital and the Faculty of Medicine, University of Tokyo. Between January 2004 and April 2007, 33914 individuals underwent general health screening, among which information on alcohol consumption was available in 26952. Of these 26952 individuals, information on smoking behavior was further available in 24811, of which 15183 were male individuals and were enrolled in the current study. We were unable to identify any specific reasons to explain why some subjects failed to complete the questionnaire about their smoking and drinking status. Among 15183 individuals enrolled in the current study, data on hepatitis C core antigen (HCcAg) and hepatitis B surface antigen (HBsAg) were available in 14829 individuals (98%), of which 71 were positive for HCcAg and 175 were positive for HBsAg. Individuals who were positive for either type of chronic hepatitis virus infection were significantly older (56 ± 10 years) than hepatitis-negative subjects (53 ± 10 years), although GGT levels were not different between hepatitis-positive (52 ± 52 IU/L) and -negative (58 ± 84 IU/L) individuals. We did not exclude individuals who were taking antihypertensive, antidiabetic, or antidyslipidemic drugs, which might have affected insulin resistance and serum GGT levels, from the current study population.

In Japan, regular health check-ups for employees are a legal requirement; all or most of the costs of the screening are paid for either by the employee's company (about two thirds of individuals attending our institute) or by the subject themselves (about one third of individuals attending our institute). Blood pressure was measured after about 10 min of rest by an automated sphygmomanometer. Individuals were judged to be former smokers and/or former drinkers, if they had stopped cigarette smoking and/or alcohol drinking, respectively, more than one month before their attendance.

Laboratory Analysis

Blood samples were taken from the subjects after an overnight fast. Serum levels of total cholesterol (TC), HDL-cholesterol (HDL-C), and triglycerides (TG) were determined enzymatically. Serum GGT levels were measured enzymatically. Hemoglobin A1c was determined by latex agglutination immunoassay. Plasma glucose was measured by the hexokinase method and serum insulin by enzyme immunoassay. Homeostasis model assessment for insulin resistance (HOMA-IR) was calculated according to the following formula: HOMA-IR = [fasting immunoreactive insulin (μU/mL) × fasting plasma glucose (FPG; mg/dL)]/405.

Statistical Analysis

Data are expressed as the mean ± SD unless stated otherwise. Analyses of variance with trend analysis, Dunnett's post-hoc analysis and multiple linear regression analysis were appropriate to assess the statistical significance of differences between groups using computer software, StatView ver. 5.0 (SAS Institute, NC) and Dr. SPSS II (SPSS Inc., Chicago, IL). A value of $p < 0.05$ was significant.

Results

Baseline Characteristics

The baseline characteristics of the study subjects are described in **Table 1**. Among 15183 men, 4534 were former smokers and 416 were former drinkers. Individuals who were former smokers and/or drinkers ($n = 4701$) were significantly older than the remaining 10482 individuals.

GGT and HOMA-IR According to Smoking and Drinking Status

Current smokers who smoked 1-9, 10-19, and 20-39 cigarettes per day were significantly younger than never smokers (**Fig. 1A**). The daily amount of alcohol consumption showed a negative graded association with age. The number of cigarettes smoked showed a positive graded association with GGT (**Fig. 1B**) and, as compared with never smokers, individuals who currently smoked 1-9, 10-19, 20-39, and ≥40 cigarettes per day had significantly higher GGT levels (by Dunnett's post-hoc analysis). Similarly, the daily amount of alcohol consumption showed a graded association with GGT, and individuals who drank 1-19, 20-39, 40-59, and ≥60 g per day had significantly higher GGT levels than never drinkers (by Dunnett's post-hoc analysis). Individuals who smoked 20-39 and ≥40 cigarettes per day had significantly higher HOMA-IR than never-smokers (**Fig. 1C**). On the other hand, as compared with never drinkers, individuals who drank 1-19, 20-39, and 40-59 g alcohol per day had significantly lower HOMA-IR levels (by Dunnett's post-hoc analysis), demonstrating a U-shaped association.

GGT and HOMA-IR According to Cross Strata of Number of Cigarettes Smoked and Alcohol Consumption

In the following analysis, we analyzed the data from 10482 individuals after excluding former smokers and/or former drinkers. The mean GGT levels and HOMA-IR values according to the smoking and drinking category are shown in **Table 2**. Current

Table 1. Baseline characteristics

Variables	Whole	Former smokers and/or drinkers [A]	Except former smokers and drinkers [B]	*p* value ([A] vs. [B])
N	15,183	4,701	10,482	
Age, years	52.9 ± 10.4	55.6 ± 9.9	51.7 ± 10.4	< 0.001
Height, cm	169.6 ± 6.0	169.1 ± 5.9	169.7 ± 6.0	< 0.001
Weight, kg	68.3 ± 9.5	68.5 ± 8.9	68.2 ± 9.7	0.117
Body mass index, kg/m^2	23.7 ± 2.8	23.9 ± 2.7	23.6 ± 2.9	< 0.001
Systolic blood pressure, mmHg	124.7 ± 18.6	127.6 ± 18.5	123.3 ± 18.4	< 0.001
Diastolic blood pressure, mmHg	79.0 ± 11.3	81.0 ± 11.0	78.2 ± 11.3	< 0.001
Heart rate, bpm	63.3 ± 9.5	63.4 ± 9.6	63.2 ± 9.5	0.373
LDL-cholesterol, mg/dL	126.7 ± 30.5	127.3 ± 30.0	126.5 ± 30.8	0.112
HDL-cholesterol, mg/dL	55.3 ± 13.4	56.9 ± 13.4	54.6 ± 13.3	< 0.001
Triglycerides, mg/dL	133.7 ± 94.2	129.8 ± 83.9	135.5 ± 98.4	0.001
AST, IU/L	23.8 ± 12.1	24.0 ± 10.5	23.7 ± 12.7	0.208
ALT, IU/L	27.3 ± 19.4	26.5 ± 18.8	27.6 ± 19.6	0.001
GGT, IU/L	58.2 ± 82.9	58.3 ± 67.0	58.1 ± 89.1	0.926
Fasting glucose, mg/dL	100.3 ± 20.5	101.7 ± 20.8	99.7 ± 20.4	< 0.001
Hemoglobin A1c, %	5.38 ± 0.74	5.41 ± 0.72	5.36 ± 0.75	< 0.001
HOMA-IR	1.69 ± 1.52	1.74 ± 1.31	1.67 ± 1.60	0.007
Antihypertensive medication, N (%)	1,909 (12.6)	831 (17.7)	1,078 (10.3)	< 0.001
Antidiabetic medication, N (%)	474 (3.1)	169 (3.6)	305 (2.9)	0.026
Antidyslipidemic medication, N (%)	674 (4.4)	276 (5.9)	398 (3.8)	< 0.001
Smoking and drinking status				
Never smoker				
Never drinker, N (%)	791 (14.1)	0 (0)	791 (14.3)	
Former drinker, N (%)	90 (1.6)	90 (100)	0 (0)	
Current drinker, N (%)	4,744 (84.3)	0 (0)	4,744 (85.7)	
Former smoker				
Never drinker, N (%)	263 (1.7)	263 (1.7)	0 (0)	
Former drinker, N (%)	249 (1.6)	249 (1.6)	0 (0)	
Current drinker, N (%)	4,022 (26.5)	4,022 (26.5)	0 (0)	
Current smoker				
Never drinker, N (%)	416 (8.3)	0 (0)	416 (8.4)	
Former drinker, N (%)	77 (1.5)	77 (100)	0 (0)	
Current drinker, N (%)	4,531 (90.2)	0 (0)	4,531 (91.6)	

BMI, body mass index; LDL, low density lipoprotein; HDL, high density lipoprotein; AST, aspartate aminotransferase; ALT, alanine aminotransferase; GGT, gamma-glutamyl transpeptidase; HOMA-IR, homeostasis model assessment for insulin resistance

drinking showed a graded association with GGT regardless of the smoking status. Cigarette smoking was also positively associated with GGT in some drinking categories: smoking 10–19 ($p<0.01$), 20–39 ($p<0.001$) and ≥40 ($p<0.001$) cigarettes per day was associated with greater GGT values than never smoking in individuals who drank 40–59 g/day, and smoking 20–39 ($p<0.001$) and ≥40 ($p<0.001$) cigarettes per day was associated with greater GGT values than never smoking in individuals who drank ≥60 g/day.

Individuals with alcohol consumption of 1–19, 20–39, or 40–59 g/day had lower HOMA-IR value than never drinkers, showing a U-shaped association between current drinking and HOMA-IR. This U-shaped relationship was absent or not significant in current smoking of 20–39 or ≥40 cigarettes per day (**Table 2**). Individuals who smoked 20–39 ($p<0.001$) and ≥40 ($p<0.001$) cigarettes per day had higher HOMA-IR than never smokers (**Table 2**).

Multiple Linear Regression Analysis

Next, multiple linear regression analysis using GGT and HOMA-IR as a dependent variable and age, BMI, amount of smoking, and alcohol consump-

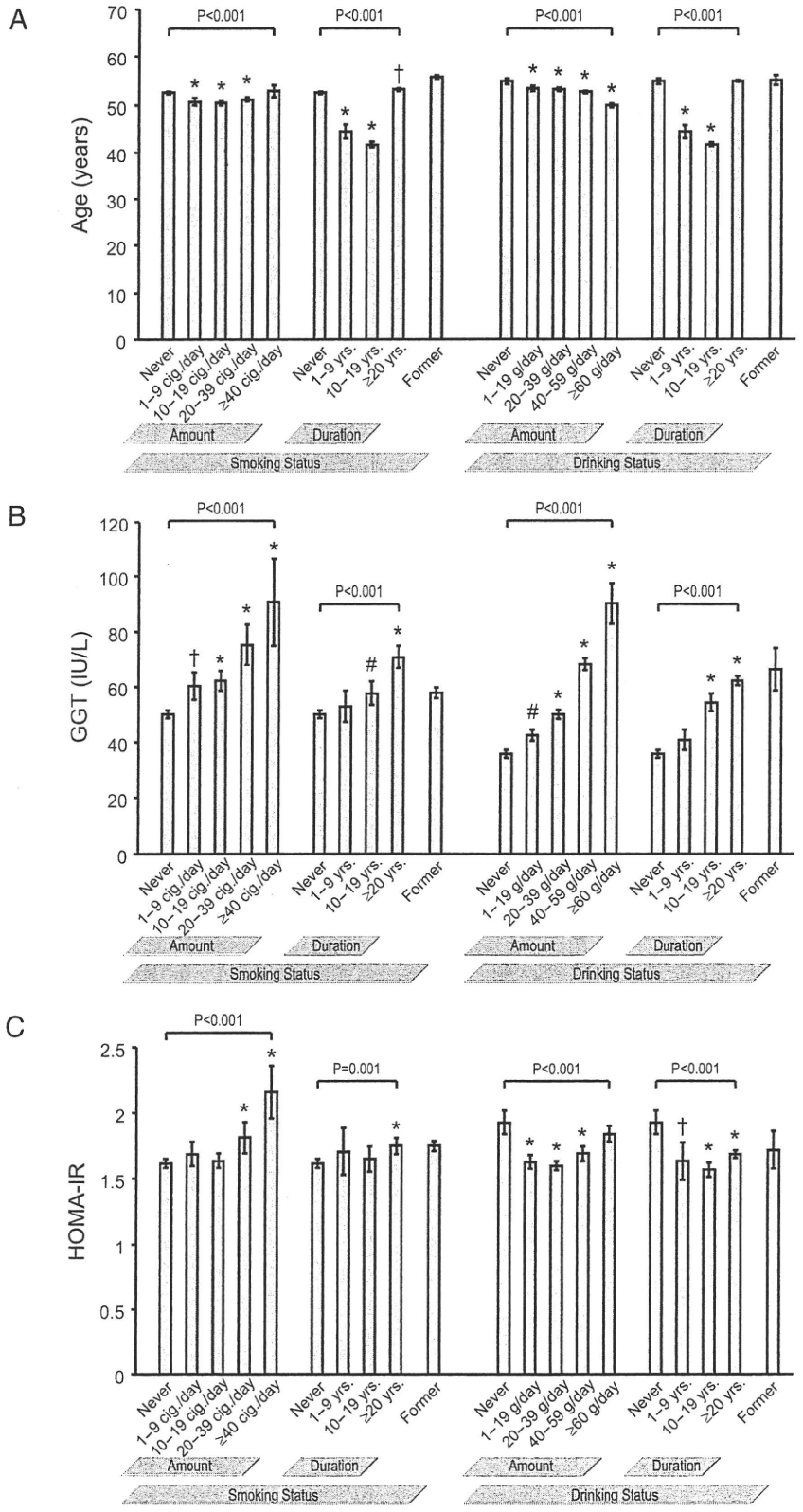

Fig. 1. Age, GGT, and HOMA-IR according to smoking and drinking status.

Bar graphs indicate the mean and 95% CI of age (A), GGT (B), and HOMA-IR. *P* values are for ANOVA trend tests. #, †, and * indicate $p<0.05$, $p<0.01$, and $p<0.001$, respectively, versus never smokers or never drinkers by Dunnett's post-hoc analysis.