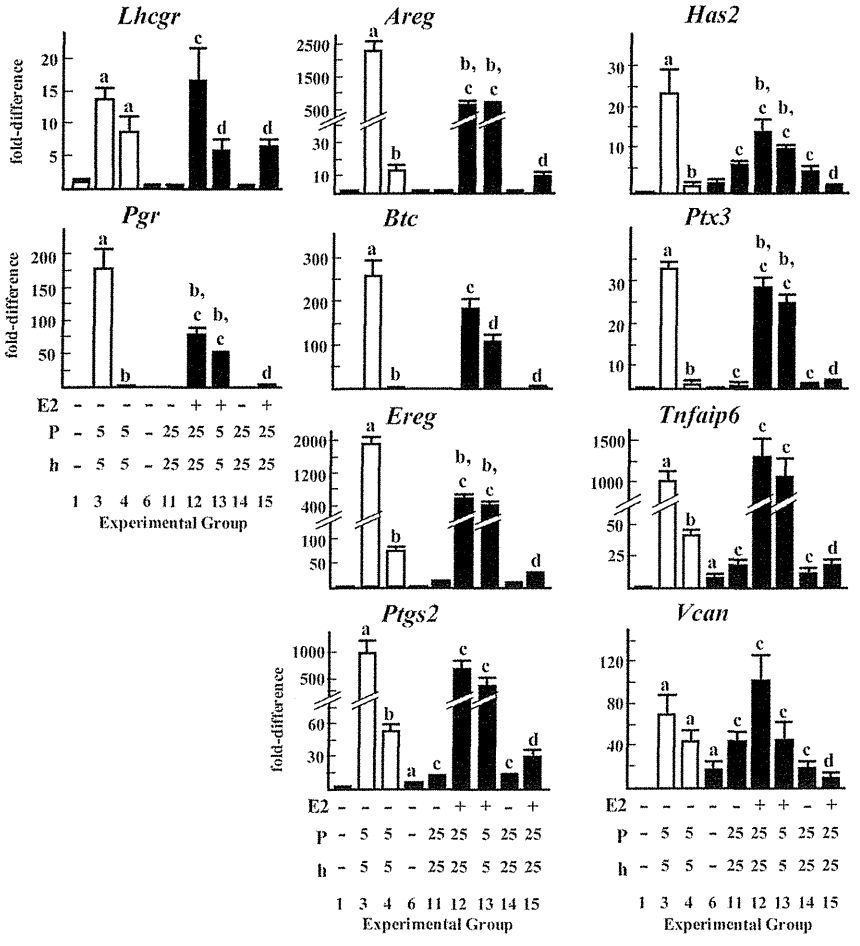

FIG. 3. RT-QPCR analysis of the ovarian mRNA expressions at preovulatory differentiation stage and after ovulation. WT (*open bar*) and ArKO (*closed bar*) mice were treated according to the schedule shown in Table 1 (9–12 mice per group, n = 3). *Bar* illustrates fold difference compared with the expression level in WT ovaries without treatment (mean ± SEM). Numbers for experimental groups presented in Table 1 were marked under the graphs. E2, 10 mg/kg body weight. P, PMSG, h, hCG; *numeral*, dosage (IU)/mouse. a, $P < 0.05$ against untreated WT levels (group 1); b, $P < 0.05$ against WT levels at 4 h after hCG injection (group 3); c, $P < 0.05$ against untreated ArKO levels (group 6); d, $P < 0.05$ against ArKO levels at 4 h after hCG injection (group 12). The absence of *letters* on a *bar* indicates that difference does not reach significance statistically.

those in the untreated WT ovaries ($P < 0.001$) (Fig. 4, groups 1 and 6). In agreement with the ovulatory response, the phosphorylation levels increased significantly at 4 h after stimulation with gonadotropin at ovulatory doses (Fig. 4, group 12) ($P < 0.001$ *vs.* group 6). The phosphorylation levels in the ovary treated with nonovulatory doses of gonadotropin (group 13) were significantly lower compared with the levels in the ovary treated with the ovulatory doses ($P < 0.05$ *vs.* group 12).

Discussion

Recent studies on ArKO mice demonstrated that ovarian follicles enabled progression up to the antral stage (8, 9) and that ArKO oocytes successfully matured and developed to blastocysts after fertilization *in vitro* (12). Thus, it is tentatively concluded that estrogen is not an indispensable component for growth and maturation of the oocytes (10, 12). In contrast, estrogens have been anticipated to be essential for ovulation, because perturbation of estrogen actions resulted in anovulatory. However, because ovulation is a complex process, including multiple factors and signal transduction cascades acting in a cell type-specific manner (21), elaborate schedules of hormonal supplementation appear to be necessary for restoring the anovulatory phenotype *in vivo*. Here, for the first time, we describe ovulatory induction in ArKO mice by treatment with sequential administrations of adequate amounts of E2 and gonadotropins. Gonadotropin treatment alone did not reverse the anovulatory phenotype of ArKO mice, whereas E2 and gonadotropins normalized the phenotype. Thus, this provides a promising experimental system to gain new insights into physiological contributions of E2 in the gonadotropin-stimulated ovaries.

E2 target tissues of ArKO mice responded to exogenously administrated E2 in a tissue-specific fashion (22), indicating that circulating E2 levels might not necessarily reflect its tissue contents. Recently no significant correlations have reported between ovarian steroid concentrations and circulating levels of the steroids (15). Thus, we considered that it is important to determine ovarian steroid contents to study how the steroids associate with the ovarian physiology. The steroid analysis employing LC-MS/MS revealed an unexpected result that a trace amount of E2 is present in the ArKO ovary, although we cannot exclude the possibility that the picolinyl derivative is derived from steroids other than E2. Even if the derivative represents E2, it is unclear whether E2 in the ArKO ovary is a product of endogenous synthesis catalyzed by aromatase activity; nevertheless, the activity was at a negligible level in the ArKO ovaries as assessed using a tritiated water-release assay (8) or enzymes other than aromatase. Because E2 content in the ArKO ovary was not altered by treatments with gonadotropins, the activity for synthesis or accumu-

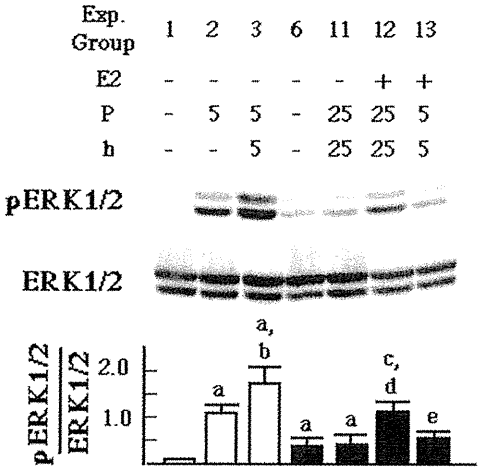

FIG. 4. Western blot analysis of phosphorylation of ERK1/2. WT (*open bar*) and ArKO (*closed bar*) mice were treated according to the schedule shown in Table 1 (five mice per group, n = 3). *Bar* illustrates ratios between phosphorylated and unphosphorylated forms of ERK1/2 (mean ± SEM). Numbers for experimental groups presented in Table 1 were marked above the graph. a, $P < 0.001$ against untreated WT levels (group 1); b, $P < 0.01$ against WT levels at 48 h after PMSG injection (group 2); c, $P < 0.001$ against untreated ArKO levels (group 6); d, $P < 0.001$ against ArKO levels at 4 h after hCG injection (group 11); e, $P < 0.05$ against ArKO levels at 4 h after hCG injection (group 12). Exp, Experimental; p, phosphorylated.

lation of E2 appears to be independent of gonadotropin. Because the uterine size was markedly reduced and the serum levels of FSH and LH were significantly elevated in ArKO females compared with those in WT mice (7, 8, 11), E2 in the ArKO ovary does not appear to serve as an endocrine hormone. However, it might function physiologically within the ovary in a paracrine or autocrine manner, because ArKO mice displayed less severe ovarian phenotypes compared with those of estrogen receptor α KO mice (23). Furthermore, trans-differentiation of ovarian granulosa cells to Sertoli-like cells was reported in one line of ArKO mice (24) but not in our line. This phenotypic variation might be related to differences in the amounts of or sensitivity to the ovarian E2. The variation might also be attributable to differences in the breeding conditions.

One could argue that the presence of E2 in the ArKO ovaries after exogenous administration might be due to blood contamination. Ovarian vascularization was shown to be positively regulated by hCG stimulation through induction of vascular endothelial growth factor gene expression (25). Our ovarian steroid analysis revealed marked decline in E2 contents after hCG administration in ArKO mice, inferring that the E2 contents in ArKO mice exogenously supplemented with E2 might not represent contaminated blood E2 levels, despite of using a whole organ as an experimental source that includes microvascular networks.

The present steroid analysis at the follicular growth stage further demonstrated that E2 amounts associated with the ovaries of ArKO mice treated with the ovulatory doses of E2 plus PMSG were nearly equal to the untreated WT levels, corresponding to less than 10% of the PMSG-primed WT levels. These findings suggest that the poor ovulation rates observed in ArKO mice might be attributable to the low amounts of E2 in addition to the increased T levels at the follicular growth stage.

In addition to E2, actions of P4 and T through their respective nuclear receptors are relevant for normal follicular development and fertility (17, 26–30). The present steroid analysis demonstrated that the presence of suitable amounts of P4, T, and E2 in the ovary is associated with ovulatory induction in ArKO mouse. Furthermore, the analysis suggested that the ratios of T to E2 contents seem to be also important for ovulatory induction (Table 2). The ratios in the WT and ArKO ovaries treated with ovulatory stimulation were calculated to be between 2.4 and 3.5 and between 10.8 and 13.8, respectively. However, the ratios in the ArKO mice treated with nonovulatory stimulations were more than 39. Thus, it is tempting to speculate that treatment of ArKO mice with adequate doses of E2 and gonadotropins might produce ovarian conditions with suitable amounts of P4, T, and E2 and also attain proper intraovarian ratios of androgen to estrogen contents at follicular growth and preovulatory differentiation stages, by which some ovarian follicles proceed to ovulation.

The expression of *Areg*, *Btc*, and *Ereg* has been shown to be regulated by LH and essential for ovulation (18). Furthermore, *Ptgs2*, *Has2*, *Ptx3*, *Tnfaip6*, and *Vcan* are involved in the formation and stabilization of the cumulus matrix. The levels of mRNA of these factors are markedly enhanced at the preovulatory differentiation stage (31, 32). We detected significant mRNA induction of these genes in the ArKO ovary treated with the superovulation regimen. Notably, marked induction of these genes was also observed in the ArKO ovaries treated with nonovulatory doses of gonadotropins. These findings indicate that unknown factors or events critical for ovulatory induction are lacking in the ArKO ovaries treated with E2 plus nonovulatory dose of gonadotropins. It was reported that hCG is a very potent inhibitor of FSH/diethystilbestrol stimulation of ovarian growth at extremely low concentrations, but at far higher concentrations, the hormone behaves as a stimulator (33). Thus, intrinsic sensitivity of ArKO ovary to hCG might explain the requirement of a high dose of gonadotropins for ovulatory induction. Characterization of the missing components in detail, which might be functional in a hormonal milieu with an appropriate ratio of T to E2 contents, allows us to elucidate more precise regulatory mechanisms leading to ovulation, which might have some clinical implications for

effective ovulatory induction in patients who are anovulatory due to estrogen insufficiency.

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ORIGINAL ARTICLE—LIVER, PANCREAS, AND BILIARY TRACT

Platelet count for predicting fibrosis in nonalcoholic fatty liver disease

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Abstract
Background The severity of liver fibrosis is known to be a good indicator for surveillance, and for determining the prognosis and optimal treatment of nonalcoholic fatty liver disease (NAFLD). However, it is virtually impossible to carry out liver biopsies in all NAFLD patients. The purpose of this study was to investigate the clinical usefulness of measuring the platelet count for predicting the severity of liver fibrosis in a large retrospective cohort of Japanese patients with NAFLD.
Methods A total of 1,048 patients with liver-biopsy-confirmed NAFLD seen between 2002 and 2008 were enrolled from nine hepatology centers in Japan. Laboratory evaluations were performed for all patients.
Results A linear decrease of the platelet count with increasing histological severity of hepatic fibrosis was revealed. The area under the receiver operating characteristic curve estimating the diagnostic performance of the platelet count for hepatic fibrosis Stage 3 was 0.774 (optimal cutoff value, 19.2 × 10^4/μl; sensitivity, 62.7%; specificity, 76.3%), and that for Stage 4 was 0.918 (optimal cutoff value, 15.3 × 10^4/μl; sensitivity, 80.5%; specificity, 88.8%).
Conclusions The platelet count may be an ideal biomarker of the severity of fibrosis in NAFLD patients, because it is simple, easy to measure and handle, cost-effective, and accurate for predicting the severity of fibrosis. Furthermore, by using the platelet count cutoff value validated in our multiple large trials, efficient recruitment of NAFLD patients may be facilitated.

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Keywords Nonalcoholic fatty liver disease · Platelet · Fibrosis

Abbreviations

NAFLD	Nonalcoholic fatty liver disease
AST	Aspartate aminotransferase
ALT	Alanine aminotransferase
AUROC	Area under the receiver operating characteristic
BMI	Body mass index
APRI	Aspartate aminotransferase to platelet ratio index

Introduction

Nonalcoholic fatty liver disease (NAFLD) is an important cause of chronic liver injury in many countries around the world [1]. The histological changes range over a wide spectrum, extending from simple steatosis, which is generally nonprogressive, to nonalcoholic steatohepatitis (NASH), liver cirrhosis, liver failure, and sometimes even hepatocellular carcinoma [2]. The severity of liver fibrosis must be estimated for surveillance and to determine the prognosis and optimal treatment of NAFLD, similar to the situation for other liver diseases such as chronic hepatitis C [3]. Liver biopsy is recommended as the gold standard for the diagnosis and staging of fibrosis in patients with NASH [1, 2, 4]. This procedure, however, is invasive and is associated with a high risk of complications [5]. The biopsy procedure results in pain in 24.6% of all patients [6], and the estimated risk of severe complications is reported to be 3.1 per 1,000 procedures [7]. Furthermore, it is impossible to carry out liver biopsies in all NAFLD patients because of the large numbers of these patients; the number of NAFLD patients has reached 80–100 million in the United States and it is estimated that there are about 10 million NAFLD patients in Japan [8].

Because liver cirrhosis with advanced tissue fibrosis gives rise to portal hypertension and enlargement of the spleen, advanced chronic liver disease has long been known to be accompanied by thrombocytopenia [9, 10]. Especially, platelet counts have been used in scoring systems, such as the age-platelet index (AP index) [11], aspartate aminotransferase (AST)-to-platelet ratio index (APRI) [12], NAFLD fibrosis score [13], and the FIB4 (based on age, AST, alanine aminotransferase [ALT] levels, and platelet counts) index [14], used for various liver diseases.

The purpose of this study was to investigate the clinical usefulness of measuring the platelet count for predicting the occurrence of significant liver fibrosis in a large retrospective cohort of Japanese patients with NAFLD.

Patients and methods

Patients

We carried out a large multicenter retrospective cohort study. We measured the diagnostic utility of platelet counts for predicting fibrosis in NAFLD. Written informed consent was obtained from all patients at the time of liver biopsy, and the study was conducted in accordance with the Helsinki Declaration.

A total of 1,048 patients with liver-biopsy-confirmed NAFLD seen between 2002 and 2008 were enrolled from institutes affiliated to the Japan Study Group of NAFLD (JSG-NAFLD), represented by the following nine hepatology centers in Japan: Nara City Hospital, Yokohama City University, Hiroshima University, Kochi Medical School, Saga Medical School, Osaka City University, Kyoto Prefectural University of Medicine, Asahikawa Medical College, and Saiseikai Suita Hospital. All patients had also been involved in the previous JSG-NAFLD study [15]. The histological criterion used for the diagnosis of NAFLD was the presence of macrovesicular fatty changes in the hepatocytes with displacement of the nucleus to the edge of the cells [16]. The criteria for exclusion from this study included a history of hepatic disease, such as chronic hepatitis C (all patients had been checked for anti-hepatitis C virus [HCV] antibody) or concurrent active hepatitis B (seropositive for hepatitis B surface antigen), autoimmune hepatitis, primary biliary cirrhosis, sclerosing cholangitis, hemochromatosis, α1-antitrypsin deficiency, Wilson's disease, or hepatic injury caused by substance abuse, as well as a current or past history of consumption of more than 20 g of alcohol daily. None of the patients suffered from hepatocellular carcinoma or had undergone splenectomy.

Anthropometric and laboratory evaluation

Venous blood samples were obtained in the morning after the patients had fasted for 12 h overnight. Laboratory evaluations in all patients included determination of the blood cell counts, and measurement of the serum levels of AST, ALT, γ-glutamyl transpeptidase (GGT), alkaline phosphatase, cholinesterase (ChE), albumin, immunoreactive insulin (IRI), ferritin, type IV collagen 7s domain, and hyaluronic acid levels and fasting plasma glucose. All of the parameters were measured using standard techniques.

Histologic evaluation

All patients enrolled in this study had undergone a percutaneous liver biopsy under ultrasonic guidance. The liver specimens were embedded in paraffin and stained with hematoxylin and eosin, Masson's trichrome, and reticulin silver stains. Fatty liver was defined as the presence of >5% steatosis, while steatohepatitis was defined as the presence of steatosis, inflammation, and hepatocyte ballooning [17–19]. The individual parameters of NASH histology, including fibrosis, were scored independently according to the scoring system developed by the NASH Clinical Research Network (CRN) [20]. Severe fibrosis was classified as Stage 3 or 4 (bridging fibrosis or cirrhosis).

Statistical analysis

Statistical analysis was conducted using SPSS 12.0 software (SPSS, Chicago, IL, USA). Continuous variables were expressed as means ± standard deviation (SD). Qualitative data are expressed as numbers, with percentages shown in parentheses. Statistical differences in quantitative data were determined using the *t*-test or Mann–Whitney's *U*-test. Because the variables were often not normally distributed, group comparisons of more than two independent groups were performed using the Kruskal–Wallis test. The diagnostic performance of the platelet count was assessed by analyzing the receiver-operating characteristic (ROC) curves. The probability of a true positive (sensitivity) and true negative (specificity) assessment was determined for selected cutoff values, and the area under the ROC curve (AUROC) was calculated. The Youden index was used to identify the optimal cutoff points. Multivariate analysis was performed by using a binary logistic regression analysis. Differences were considered to be statistically significant at $p < 0.05$.

Results

Patients' characteristics

A total of 1,048 patients with biopsy-proven NAFLD were included in the analysis. The characteristics of the patients are summarized in Table 1. The age, serum AST, alkaline phosphatase, GGT, ferritin, fasting glucose, fasting insulin, hyaluronic acid levels, and type IV collagen 7s domain were significantly higher in the NAFLD patients with severe fibrosis (Stage 3, 4) as compared with those with no or mild fibrosis (Stage 0–2). Serum cholinesterase, albumin, and hemoglobin, and the platelet count, were significantly lower in the NAFLD patients with severe fibrosis as compared with these values in NAFLD patients with no or mild fibrosis (Table 2).

Table 1 Characteristics of the patient population

Variable	
Age (years)	51.6 ± 15.0
Gender (male:female)	575:473
Body mass index (kg/m^2)	27.8 ± 4.9
AST (IU/l)	58.7 ± 40.0
ALT (IU/l)	91.3 ± 64.6
Alkaline phosphatase (IU/l)	260.8 ± 98.4
GGT (IU/l)	88.5 ± 93.2
Cholinesterase (IU/l)	379.8 ± 97.9
Albumin (mg/dl)	4.39 ± 0.43
Ferritin (ng/ml)	254.4 ± 248.4
Fasting glucose (mg/dl)	113.7 ± 39.8
Fasting insulin (μU/ml)	14.9 ± 13.7
Hemoglobin (g/dl)	14.4 ± 1.63
Platelet count (×10^4/μl)	22.2 ± 6.8
Hyaluronic acid (ng/ml)	58.4 ± 88.5
Type IV collagen 7s domain	4.79 ± 3.60
Fibrosis stage (0/1/2/3/4)	216/334/270/187/41

Values are means ± SD

AST aspartate aminotransferase, *ALT* alanine aminotransferase, *GGT* γ-glutamyl transpeptidase

Relationship between the platelet count and the severity of fibrosis in NAFLD patients

The platelet counts in the NAFLD patients ranged from 3.3 to 45.7 × 10^4/μl. The counts stratified by the fibrosis stage were as follows: Stage 0: 24.8 ± 6.8 (95% confidence interval [CI] 23.8–25.7) × 10^4/μl; Stage 1: 23.7 ± 6.1 (95% CI 23.0–24.3) × 10^4/μl; Stage 2: 22.0 ± 5.8 (95% CI 21.3–22.7) × 10^4/μl; Stage 3: 18.9 ± 6.4 (95% CI 18.0–19.9) × 10^4/μl; and Stage 4: 12.4 ± 4.4 (95% CI 10.5–13.5) × 10^4/μl. To classify the platelet counts according to the histological fibrosis staging, a bar chart was used, as shown in Fig. 1. When the platelet counts were analyzed in relation to the histological stage of fibrosis, a linear decrease in the platelet counts with increasing severity of hepatic fibrosis was observed ($p < 0.0001$ by Kruskal–Wallis test) (Fig. 1).

Multiple logistic regression analysis of factors associated with no or mild fibrosis (Stage 0–2) compared to severe fibrosis (Stage 3–4)

We performed a multiple logistic regression analysis by using the factors – age, serum AST, alkaline phosphatase, GGT, ferritin, fasting glucose, fasting insulin, hyaluronic

Table 2 Characteristics of the patient population (fibrosis Stage 0–2 vs. Stage 3, 4)

Variable	No or mild fibrosis (Stage 0–2)	Severe fibrosis (Stage 3, 4)	*p* value
Age (years)	49.8 ± 15.3	57.6 ± 12.6	<0.001
Gender (male:female)	361:363	114:110	0.2857
Body mass index (kg/m^2)	27.7 ± 5.0	28.2 ± 4.5	0.1900
AST (IU/l)	54.6 ± 36.7	73.4 ± 47.6	<0.001
ALT (IU/l)	90.5 ± 63.8	94.2 ± 67.2	0.4396
Alkaline phosphatase (IU/l)	253.2 ± 91.3	285.8 ± 115.4	<0.001
GGT (IU/l)	85.2 ± 93.0	100.2 ± 92.9	0.0322
Cholinesterase (IU/l)	388.0 ± 93.5	351.6 ± 107.5	<0.001
Albumin (mg/dl)	4.45 ± 0.38	4.21 ± 0.55	<0.001
Ferritin (ng/ml)	242.2 ± 238.2	300.4 ± 279.5	0.0038
Fasting glucose (mg/dl)	110.3 ± 36.2	125.0 ± 48.5	<0.001
Fasting insulin (μU/ml)	13.6 ± 9.8	20.1 ± 22.9	<0.001
Hemoglobin (g/dl)	14.5 ± 1.6	14.1 ± 1.7	0.0019
Platelet count (×10^4/μl)	23.4 ± 6.3	17.8 ± 6.6	<0.001
Hyaluronic acid (ng/ml)	4.39 ± 3.73	6.31 ± 2.56	<0.001
Type IV collagen 7s domain	1.00 ± 0.18	1.04 ± 0.13	0.0119

Values are mean ± SD

AST aspartate aminotransferase, *ALT* alanine aminotransferase, *GGT* γ-glutamyl transpeptidase

p values from Student's *t*-test, Mann–Whitney test, or χ^2 test, as appropriate

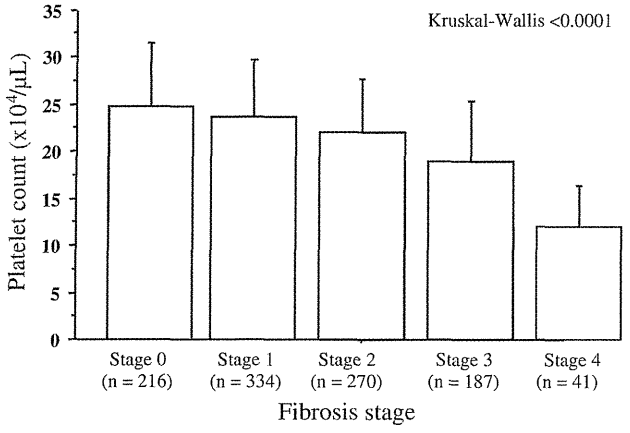

Fig. 1 Bar chart of the platelet count classified by the stage of fibrosis. A steady decrease of the platelet count was observed with the increasing severity of hepatic fibrosis ($p < 0.0001$ by Kruskal–Wallis test)

acid level, type IV collagen 7s domain, serum cholinesterase, albumin, hemoglobin, and the platelet count—which were found to be significantly elevated or decreased in NAFLD patients with severe fibrosis (Stage 3–4) compared to levels in NAFLD patients with no or mild fibrosis (Stage 0–2) in the univariate analysis. In the multiple logistic regression analysis, fasting insulin, platelet counts, and type IV collagen 7s domain were still significantly different in the NAFLD patients with severe fibrosis as compared with the levels in the NAFLD patients with no or mild fibrosis (Table 3).

Table 3 Multiple logistic regression analysis of factors associated with no or mild fibrosis (Stage 0–2) compared to severe fibrosis (Stage 3, 4)

Factor	Odds ratio	95% CI	*p* value
Age (years)	1.008	0.988–1.028	0.4393
AST (IU/l)	1.002	0.994–1.010	0.5949
Alkaline phosphatase (IU/l)	1.002	0.999–1.010	0.2047
GGT (IU/l)	1.000	0.997–1.003	0.9593
Cholinesterase (IU/l)	0.999	0.995–1.002	0.5002
Albumin (mg/dl)	1.591	0.848–2.986	0.1481
Ferritin (ng/ml)	1.000	0.999–1.001	0.4109
Fasting glucose (mg/dl)	1.001	0.995–1.008	0.7195
Fasting insulin (mU/ml)	1.034	1.011–1.057	0.003
Hemoglobin (g/dl)	0.964	0.811–1.147	0.6803
Platelet count (×10^4/ml)	0.913	0.869–0.959	0.0003
Hyaluronic acid (ng/ml)	1.000	0.997–1.003	0.9128
Type IV collagen 7s domain	1.626	1.313–2.015	<0.0001

R^2 for entire model = 0.240

CI confidence interval

Receiver-operating characteristic curves

The AUROC estimating the diagnostic performance of the platelet count for hepatic fibrosis stages equal to or greater than Stage 3 was 0.774 (optimal cutoff value, 19.2 × 10^4/μl; sensitivity, 62.7%; specificity, 76.3%) (Fig. 2). The AUROC estimating the diagnostic performance of the platelet count for the diagnosis of Stage 4 fibrosis was

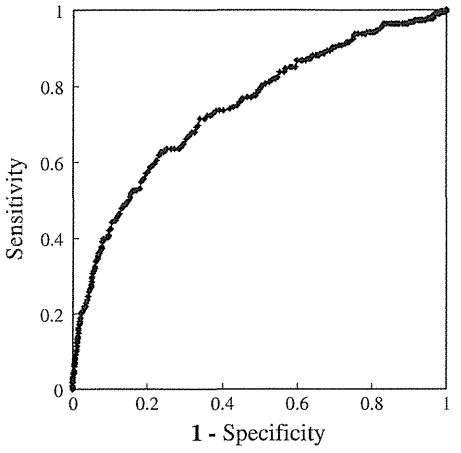

Fig. 2 Receiver-operating characteristic (ROC) curve for detecting nonalcoholic fatty liver disease (NAFLD) with severe fibrosis (Stage 3 and 4) based on the platelet count

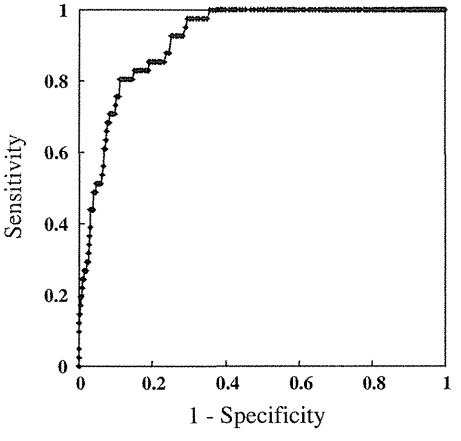

Fig. 3 Receiver-operating characteristic (ROC) curve for detecting NAFLD with liver cirrhosis (Stage 4) based on the platelet count

0.918 (optimal cutoff value, 15.3 × 10^4/μl; sensitivity, 80.5%; specificity, 88.8%) (Fig. 3).

Discussion

We demonstrated a significant negative correlation between the platelet count and the severity of liver fibrosis in patients with NAFLD in this large multicenter retrospective cohort of Japanese patients with NAFLD.

The incidence of NAFLD is rising rapidly in both adults and children because of ongoing epidemics of obesity and type 2 diabetes [21]. In Western countries, the estimated prevalence of NAFLD in the general population ranges from 15 to 39% [22]. In Japan, approximately 10–20% of Japanese adults have NAFLD. Thus, a rapid and noninvasive method for the detection of fibrosis in NAFLD patients is of major clinical interest.

Especially in patients with hepatitis C, a correlation has been reported to exist between the stage of liver fibrosis and the platelet count [23]. There are also some reports of investigations of the correlation between the severity of NAFLD and the platelet count in patients with NAFLD [24, 25]. However, in these studies, the sample sizes were small in comparison with those in the studies of patients with chronic hepatitis C. Therefore, a multicenter study involving a large number of NAFLD patients is required for establishing the clinical usefulness of the measurement of the platelet counts in these patients.

The peripheral blood platelet count is regulated by the balance between the production and destruction of platelets. Splenomegaly in chronic liver disease has been considered to cause thrombocytopenia by increasing the sequestration and enhancing the destruction of platelets [26, 27]. In NASH (as occurs with alcoholic liver disease), fibrosis begins around the central veins; therefore, the symptoms of portal hypertension are thought to appear first as a result of central vein occlusion [28, 29]. The spleen volume is speculated to increase as the portal blood pressure increases in chronic liver disease [30], and we previously reported that splenic enlargement may be a distinct feature of NASH [31].

The platelet count has been included in many scoring systems for the noninvasive prediction of the severity of hepatic fibrosis in patients with chronic hepatitis C, and we have reported that noninvasive laboratory tests proposed for predicting cirrhosis in patients with chronic hepatitis C were also useful in patients with NASH [32]. In recent years Shah et al. [33] reported the utility of a scoring system for NAFLD patients in a multicenter trial. In their study, they evaluated 541 NAFLD patients and reported that the AUROCs of the FIB4 index, NAFLD fibrosis score, APRI, and AST-to-platelet ratio (in which the platelet count is included) for estimating the diagnostic performance for hepatic fibrosis stages equal to or greater than Stage 3 were 0.802, 0.768, 0.730, and 0.720, respectively. From the results of our present study, the AUROC of the platelet count for detecting NAFLD with hepatic fibrosis equal to or greater than Stage 3 was 0.774. Therefore, the platelet count had a diagnostic performance almost equal to those of the other biomarkers of hepatic fibrosis noted above. Furthermore, the platelet count can be easily determined, not only by hepatologists but also by general physicians.

It is not uncommon for patients to present with the complications of previously unrecognized cirrhosis despite being under long-standing medical care, because these patients often do not manifest the classic physical changes associated with cirrhosis. We found that the optimal platelet count for the diagnosis of NAFLD with severe fibrosis (Stage 3–4) was 19.2 × 10^4/μl, and that for the

diagnosis of cirrhosis (Stage 4) was 15.3 × 10^4/μl. Thus, in the future, NAFLD patients with platelet counts of less than 19.2 × 10^4/μl should be closely followed up, because it is likely that they could progress to NAFLD with severe fibrosis. In the present study, if liver biopsies had been performed only in patients with platelet counts of less than 19.2 × 10^4/μl, 710 (67.7%) of the 1,048 biopsies could have been avoided.

Furthermore, NAFLD patients with platelet counts of less than 15.3 × 10^4/μl are likely to already have liver cirrhosis. If these NAFLD patients have liver cirrhosis, they need to be kept under surveillance for the early detection of hepatocellular carcinoma and gastroesophageal varices.

One limitation of our study is the relatively small number of patients compared with the number of patients in Japan assumed to have NAFLD. Selection bias is another limitation, because in this study we did not investigate patients who had any clinical evidence of hepatic decompensation.

In conclusion, from the results of this large multicenter retrospective cohort study, it seems that the platelet count in NAFLD patients would be an ideal biomarker of the severity of fibrosis, because it is simple, easy to measure and handle, cost-effective, and accurate for diagnosis of the severity of fibrosis. Measurement of the platelet count appears to be a clinically useful method for assessing the severity of liver fibrosis in patients with NAFLD. Further, by using the platelet count cutoff values validated in this study, efficient recruitment of NAFLD patients may be facilitated.

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Conflict of interest The authors declare that they have no conflict of interest.

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Original Article

Angiotensinogen gene haplotype is associated with the prevalence of Japanese non-alcoholic steatohepatitis

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Aim: Non-alcoholic steatohepatitis (NASH) patients frequently have hypertension, which is considered to be an important predictive factor for the subsequent development of hepatic fibrosis. The renin-angiotensin system is also known to contribute to the progression of NASH. Various types of functional single-nucleotide polymorphisms (SNPs) involved in the development of NASH have been proposed. Angiotensinogen (AGT) gene SNPs related to cardiovascular diseases have been reported. We aimed to evaluate the involvement of the AGT gene haplotype in Japanese NASH patients.

Methods: Previously described genotypes of SNPs of the AGT gene, rs4762 C/T polymorphism (T207M), rs699 C/T polymorphism (T268M), and rs7079 C/A polymorphism (C11537A), were determined in 124 Japanese biopsy-proven NASH patients and 150 healthy volunteers (controls).

Results: The allele and genotype frequencies in rs4762 and rs699 SNPs in NASH patients were similar to those in controls, while the frequency of the A allele and A/- genotype in rs7079 SNPs were much higher in NASH patients than in controls. In addition, the 3-SNP haplotype CTA was significantly overrepresented in NASH patients compared with controls. Regarding clinical features of NASH patients, diastolic blood pressures in patients with the CTA/- genotype were much higher than in patients with other genotypes.

Conclusions: We found a 3-SNP haplotype of the AGT gene that is involved in the development of NASH and influences hypertension in NASH patients. These results provide new insight into the therapy of NASH patients with the CTA haplotype using ACE inhibitors or angiotensin II type 1 receptor blockers.

Key words: angiotensinogen gene haplotype, non-alcoholic steatohepatitis, single-nucleotide polymorphisms

INTRODUCTION

NON-ALCOHOLIC STEATOHEPATITIS (NASH) is an important chronic liver disease worldwide. The pathogenesis of NASH has been considered to involve metabolic syndrome, obesity, hypertension, dyslipidemia, and genetic influences. Various genetic factors and functional gene polymorphisms involved in the development of hepatic steatosis and NASH have been proposed.[1,2] We also reported the functional gene single-nucleotide polymorphisms (SNPs) in Japanese NASH.[3–5] These reports indicate that the SNPs of genes are important predictive factors in the pathogenesis of NASH.

It is well known that more than half of NASH patients have hypertension.[6] The renin-angiotensin system (RAS) has been considered to be involved in both hypertension and the progression of hepatic fibrosis in chronic hepatitis, including NASH.[7] In addition, the therapeutic efficacy of an angiotensin II type 1 receptor blocker (ARB) in NASH patients was reported.[8] Furthermore, we clarified the mechanisms by which ARB attenuated the progression of hepatic fibrosis in a rat model of NASH.[9] These reports indicate that activation of RAS is an important predictive factor for the development of NASH.

Angiotensinogen (AGT) and its cleaved forms, angiotensin I and angiotensin II, are important regulators of blood pressure. The SNPs of the AGT gene have

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Contributions: All listed authors contributed intellectually to the work presented here either through study concept and design, data acquisition, data analysis and interpretation, critical revision of the manuscript for important intellectual content; statistical analysis; funding or study supervision.

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reportedly been associated with serum AGT levels[10–15] and hypertension.[16,17] However, whether SNPs of the AGT gene are involved in the pathogenesis of NASH has not yet been clarified.

We hypothesized that the SNPs of the AGT gene play an important role in determining an individual's susceptibility to NASH. Therefore, we genotyped three of the most well-known SNPs of the AGT gene, T207M (rs4762), T268M (rs699), and C11537A (rs7079) polymorphisms,[18] and used a regression-based 3-SNP haplotype analysis to evaluate the associations of AGT gene haplotypes with NASH. The aims of this study were to examine the frequency of the SNPs of the AGT gene and regression-based 3-SNP haplotype in Japanese NASH patients, and to compare these frequencies with those of healthy controls.

METHODS

Patients and healthy controls

A TOTAL OF 124 Japanese biopsy-proven NASH patients and 150 healthy volunteers (controls) were enrolled in this study. Liver biopsies had been obtained in patients with non-alcoholic fatty liver disease (NAFLD) after thorough clinical evaluation and signed informed consent by each patient. Liver histology was analyzed, and the diagnosis of NASH was based on the NASH Clinical Research Network (CRN) scoring system[19] and Brunt's criteria.[20]

Patients with known use of methotrexate, tamoxifen, corticoids, insulin, or alcohol in excess of 20 g per day and patients with other known causes of liver disease including viral hepatitis, hemochromatosis, Wilson disease, and autoimmune liver diseases were excluded from this study. After obtaining written informed consent from all subjects, SNPs of the AGT gene and laboratory blood tests were analyzed in both NASH patients and healthy volunteers. The study protocol conformed to the ethical guidelines of the 1975 Declaration of Helsinki and was approved by the Research Committee of Kochi Medical School.

Clinical and laboratory evaluation

Venous blood samples were obtained in the morning after a 12-h overnight fast. Laboratory tests in all NASH patients and healthy volunteers included measurements of serum aspartate transaminase (AST), alanine transaminase (ALT), total cholesterol, triglyceride, high-density lipoprotein cholesterol (HDL-C), low-density lipoprotein cholesterol (LDL-C), fasting plasma glucose (FPG), fasting immunoreactive insulin (fIRI), creatinine (Cr), and blood urea nitrogen (BUN). These parameters were measured using standard clinical chemistry techniques. Body mass index was also calculated in all NASH patients and healthy volunteers. Systolic blood pressure (SBP) and diastolic blood pressure (DBP) were calculated as the average of three measurements at 1-min intervals with an automated device.

Determination of AGT gene genotypes

Genomic DNA was extracted from white blood cells, and genotyping of AGT gene polymorphisms was performed as previously described.[21–23] To confirm the genotype assignment, agarose gel patterns were analyzed twice. Each SNP was in Hardy–Weinberg equilibrium (data not shown). Haplotype frequencies were calculated using PHASE 2.0 software.[24]

Data and statistical analysis

Statistical analysis was performed by using the two-way ANOVA test in StatView (SAS Institute, Inc., Cary, NC, USA). Distribution normality of the groups was preliminarily evaluated by the Kolmogorov-Smirnov test. Relative risk and association analysis were performed using odds ratios and χ^2 tests. Results were considered significant when the *P*-value was less than 0.05.

RESULTS

Three-SNP haplotypes of the AGT gene

WE GENOTYPED THREE SNPs of the AGT gene as previously reported: rs4762 (C/T allele), rs699 (C/T allele), and rs7079 (C/A allele),[18] and examined the three-SNP haplotype of the gene in 150 healthy volunteers. Four haplotypes were defined, as shown in Figure 1. Of these haplotypes, CCC was the prototype of the gene with the highest frequency (Haplotype 1, $n = 212$). Another two haplotypes were TCC, in which C was converted to T in rs4762 (Haplotype 2, $n = 32$), and CTC, in which C was converted to T in rs699 (Haplotype 3, $n = 17$). Interestingly, C was converted to A in rs7079 only when C was converted to T in rs699. As a result, the fourth haplotype, CTA, was formed (Haplotype 4, $n = 39$).

Frequency of rs4762 SNP (T207M) and rs699 SNP (T268M)

The frequency of the rs4762 SNP (T207M) resulting in a C/T substitution was determined in 150 healthy volunteers and 124 patients with biopsy-proven NASH

	SNP rs4762	SNP rs699	SNP rs7079	Frequency (*n*)
Haplotype 4	C	T	A	39
			↑C11537A	
Haplotype 3	C	T	C	17
		↑T268M		
Haplotype 1	C	C	C	212
	↓T207M			
Haplotype 2	T	C	C	32

Figure 1 Three-single nucleotide polymorphisms (SNPs) haplotype of angiotensinogen (AGT) gene in Japanese people. Three SNPs of the AGT gene, rs4762 (C/T allele), rs699 (C/T allele), and rs7079 (C/A allele), were genotyped in 150 healthy Japanese volunteers. Four haplotypes were defined: Haplotype 1 was CCC ($n = 212$), Haplotype 2 was TCC ($n = 32$), Haplotype 3 was CTC ($n = 17$), and Haplotype 4 was CTA ($n = 39$).

(Table 1). The genotype distribution in healthy volunteers was in Hardy–Weinberg equilibrium, and the allele frequency was similar to that previously reported.[25] There was no significant difference in the T allele frequency between healthy volunteers (10.7%) and NASH patients (9.3%) ($P = 0.691$; odds ratio, 0.86; 95% confidence interval (CI), 0.49–1.15). In addition, the T/- genotype frequency in NASH patients (22.6%) was not significant compared with that in healthy volunteers (16.7%) ($P = 0.280$; odds ratio, 1.46; 95% CI, 0.8–2.66).

The frequency of the rs699 SNP (T268M) resulting in a C/T substitution was also determined (Table 1). The genotype distribution in healthy volunteers was in Hardy–Weinberg equilibrium, and the allele frequency was similar to that previously reported.[25] There was no significant difference in the T allele frequency between healthy volunteers (18.7%) and NASH patients (25.8%) ($P = 0.056$; odds ratio, 1.52; 95% CI, 1.01–2.28). In addition, the T/- genotype frequency in NASH patients (45.2%) was not significant compared with that in healthy volunteers (34.7%) ($P = 0.099$; odds ratio, 1.55; 95% CI, 0.95–2.53).

Frequency of rs7079 SNP (C11537A)

Furthermore, we determined the frequency of the rs7079 SNP (C11537A) resulting in an A/C substitution

Table 1 Frequency of single nucleotide polymorphisms (SNPs) in rs4762 (T207M), rs699 (T268M), and rs7079 (C11537A) of the angiotensinogen (AGT) gene

			Control ($n = 150$)	NASH ($n = 124$)	$P =$	Odds ratio
rs4762 (T207M)	Allele Frequency	T	0.107	0.093	NS	0.86 (0.49–1.51)
		C	0.893	0.907		
	Genotype Frequency	T/–	0.167	0.226	NS	1.46 (0.8–2.66)
		C/C	0.833	0.774		
Rs699 (T268M)	Allele Frequency	T	0.187	0.258	NS	1.52 (1.01–2.28)
		C	0.813	0.742		
	Genotype Frequency	T/–	0.347	0.452	NS	1.55 (0.95–2.53)
		C/C	0.653	0.548		
Rs7079 (C11537A)	Allele Frequency	A	0.13	0.21	<0.05	1.78 (1.13–2.8)
		C	0.87	0.79		
	Genotype Frequency	A/–	0.247	0.371	<0.05	1.8 (1.07–3.03)
		C/C	0.753	0.629		

The frequencies of SNPs (rs4762 C/T substitution, rs699 C/T substitution, and rs7079 A/C substitution) of the AGT gene between 150 healthy volunteers and 124 patients with biopsy-proved non-alcoholic steatohepatitis (NASH) were compared.
In the rs4762 SNP, no significant difference was found in the T allele frequency between NASH patients and healthy volunteers (9.3% vs. 10.7%, respectively) ($P = 0.691$; odds ratio, 0.86; 95% CI, 0.49–1.15). The T/- genotype frequency in NASH patients was not significant compared with that in healthy volunteers (22.6% vs. 16.7%, respectively) ($P = 0.280$; odds ratio, 1.46; 95% CI, 0.8–2.66).
In the rs699 SNP, no significant difference was found in the T allele frequency between NASH patients and healthy volunteers (25.8% vs. 18.7%, respectively) ($P = 0.056$; odds ratio, 1.52; 95% CI, 1.01–2.28). The T/- genotype frequency in NASH patients was not significant compared with that in healthy volunteers (45.2% vs. 34.7%, respectively) ($P = 0.099$; odds ratio, 1.55; 95% CI, 0.95–2.53).
In the rs7079 SNP, the A allele frequency in NASH patients was significantly higher than that in healthy volunteers (21.0% vs. 13.0%, respectively) ($P < 0.05$; odds ratio, 1.78; 95% CI, 1.13–2.8). The A/- genotype frequency in NASH patients was significantly higher than that in healthy volunteers (37.1% vs. 24.7%, respectively) ($P < 0.05$; odds ratio, 1.8; 95% CI, 1.07–3.03).

Table 2 CTA haplotype frequency in non-alcoholic steatohepatitis (NASH) patients

		Control ($n = 150$)	NASH ($n = 124$)	$P =$	Odds ratio
Allele frequency	CTA	0.130	0.210	<0.05	1.78
	- -C	0.870	0.790		(1.13–2.8)
Genotype frequency	CTA/-	0.247	0.371	<0.05	1.8
	others	0.753	0.629		(1.07–3.03)

The frequency of the CTA haplotype of the angiotensinogen (AGT) gene in NASH patients and healthy volunteers was determined. The CTA allele frequency in NASH patients was significantly higher than that in healthy volunteers (21.0% vs. 13.0%, respectively) ($P < 0.05$; odds ratio, 1.78; 95% CI, 1.13–2.8). The CTA/- genotype frequency in NASH patients was also significantly higher than that in healthy volunteers (37.1% vs. 24.7%, respectively) ($P < 0.05$; odds ratio, 1.8; 95% CI, 1.07–3.03).

(Table 1). The genotype distribution in healthy volunteers was in Hardy–Weinberg equilibrium, and the allele frequency was similar to that previously reported.[18] The A allele frequency in NASH patients (21.0%) was significantly higher than that in healthy volunteers (13.0%) ($P < 0.05$; odds ratio, 1.78; 95% CI, 1.13–2.8), and the A/- genotype frequency in NASH patients (37.1%) was significantly higher than that in healthy volunteers (24.7%) ($P < 0.05$; odds ratio, 1.8; 95% CI, 1.07–3.03).

Relationship between CTA haplotype and NASH frequency

Next, we determined the frequency of the CTA haplotype (Haplotype 4, Fig. 1) in NASH patients and healthy volunteers (Table 2). The CTA allele frequency in NASH patients (21.0%) was significantly higher than that in healthy volunteers (13.0%) ($P < 0.05$; odds ratio, 1.78; 95% CI, 1.13–2.8); this frequency was identical to the A allele frequency of the rs7079 SNP (Table 1). In addition, the CTA/- genotype frequency in NASH patients (37.1%) was also significantly higher than that in healthy volunteers (24.7%) ($P < 0.05$; odds ratio, 1.8; 95% CI, 1.07–3.03); this frequency was identical to the A/- genotype frequency of the rs7079 SNP (Table 1).

Clinical features of NASH patients

The clinical features between NASH patients with the CTA/- genotype and NASH patients with other genotypes were studied (Table 3). The DBP in NASH patients with the CTA/- genotype (82.2 ± 9.2 mmHg, $n = 46$) was significantly higher than that in NASH patients with other genotypes (78.6 ± 8.5 mmHg, $n = 78$) ($P < 0.05$). Other clinical features were not significantly different.

DISCUSSION

THE PATHOGENESIS OF NASH involves metabolic syndrome, obesity, dyslipidemia, and hypertension. The influences of gene SNPs are also considered to be important predictive factors in the pathogenesis of NASH. Various functional gene SNPs involved in the development of NASH have been proposed.[1,2] In Japanese NASH, the SNPs of the W64R variant of the β-3 adrenergic receptor gene,[3] the V175M variant of the phosphatidylethanolamine *N*-methyltransferase (PEMT) gene,[4] the G-493T variant of the microsomal triglyceride transfer protein (MTP) gene,[5] and the T-1031C and C-856A variants of the tumor necrosis factor-α (TNF-α) gene[26] have been reported. According to these reports, it is obvious that functional gene SNPs are important predictive factors in the pathogenesis of NASH.

Here, we genotyped three SNPs of the AGT gene and evaluated the frequency of these SNPs in NASH patients compared with healthy volunteers. In all SNPs in this article, the genotype distribution in healthy volunteers was in Hardy–Weinberg equilibrium. In NASH patients, the frequency of the rs4762 and rs699 SNPs of the AGT gene were similar to those in healthy volunteers (Table 1), even though these SNPs were reported to be important polymorphisms for hypertension,[16,27,28] left ventricular hypertrophy,[29,30] coronary heart disease,[31–33] atrial fibrillation,[34] and brain lesions.[17] This may be a result of the affinity of the SNPs of AGT gene products to the receptors. Taken together with our results, these findings suggest that neither the rs4762 SNP nor the rs699 SNP of the AGT gene is an important polymorphism for the development of NASH.

On the other hand, the rs7079 SNP of the AGT gene is likely important for the development of NASH. The A allele frequency in NASH patients (21.0%) was significantly higher than that in healthy volunteers (13.0%)

Table 3 Clinical features of non-alcoholic steatohepatitis (NASH) patients with the CTA/– genotype and those with other genotypes

	CTA/—— ($n = 46$)	Others ($n = 78$)	Statistics
Age	44.7 ± 14.8	48.6 ± 13.9	n.s.
Sex (M/F)	23/23	14/37	n.s.
BMI	29.7 ± 3.8	29.3 ± 4.3	n.s.
ALT (IU/L)	115.8 ± 54.4	99.8 ± 46.4	n.s.
HDL-C (mg/dL)	49.1 ± 11.7	46.8 ± 10.8	n.s.
LDL-C (mg/dL)	114.8 ± 24.3	122.9 ± 27.1	n.s.
FPG (mg/dL)	106.2 ± 14.6	107.9 ± 16.9	n.s.
fIRI (mIU/L)	12.2 ± 5.1	13.2 ± 6.9	n.s.
HOMA-IR	3.22 ± 1.58	3.93 ± 2.52	n.s.
BP (mmHg)			
Systolic	132.2 ± 17.9	134.3 ± 14.5	n.s.
Diastolic	82.2 ± 9.2	78.6 ± 8.5	$P < 0.05$
Cr (mg/dL)	0.67 ± 0.1	0.68 ± 0.1	n.s.
BUN (mg/dL)	14.3 ± 2.4	14.7 ± 2.4	n.s.

The clinical features of NASH patients with the CTA/– genotype ($n = 46$) and those with other genotypes ($n = 78$) were compared. The diastolic blood pressure (DBP) in NASH patients with the CTA/– genotype was significantly higher than that in NASH patients with other genotypes (82.2 ± 9.2 mmHg vs. 78.6 ± 8.5 mmHg, respectively) ($P < 0.05$). ALT, alanine aminotransferase; BMI, body mass index; BP, blood pressure; BUN, blood urea nitrogen; Cr, creatinine; fIRI, fasting immunoreactive insulin; FPG, fasting plasma glucose; HDL-C, high-density lipoprotein cholesterol; HOMA-IR, Homeostasis model assessment-Insulin Resistance; LDL-C, low-density lipoprotein cholesterol; n.s., not significant.

($P < 0.05$; odds ratio, 1.78; 95% CI, 1.13–2.8) (Table 1), and the A/- genotype frequency in NASH patients (37.1%) was significantly higher than that in healthy volunteers (24.7%) ($P < 0.05$; odds ratio, 1.8; 95% CI, 1.07–3.03). These results indicate that the rs7079 SNP of the AGT gene is an important predictive factor for the development of NASH.

In addition, we identified four types of three-SNP haplotypes of the AGT gene in the Japanese population, as previously reported.[18] The frequencies of these haplotypes were 70.7%, 10.7%, 5.7%, and 13.0% for the CCC, TCC, CTC, and CTA haplotypes, respectively. Of these haplotypes, it was clarified that C was converted to A in rs7079 only when C was converted to T in rs699 (Fig. 1). Interestingly, even though these two genes, rs699 and rs7079, are genetically far from each other, the SNPs of these genes are in complete linkage disequilibrium.[18] Furthermore, the frequencies of the A/- genotype and A allele in rs7079 in NASH were identical to the haplotype frequencies of the CTA/- genotype and CTA allele in NASH (Tables 1,2). The CTA allele frequency in NASH patients (21.0%) was significantly higher than that in healthy volunteers (13.0%) ($P < 0.05$; odds ratio, 1.78; 95% CI, 1.13–2.8). In addition, the CTA/- genotype frequency in NASH patients (37.1%) was also significantly higher than that in healthy volunteers (24.7%) ($P < 0.05$; odds ratio, 1.8; 95% CI, 1.07–3.03). These results indicate that the CTA haplotype of the AGT gene is an important predictive factor for the development of NASH.

Next, we compared the clinical features in NASH patients with the CTA/- genotype and patients with other genotypes (Table 3). Age, gender balance, obesity, alanine aminotransferase (ALT), lipid metabolism, insulin resistance, and renal functions did not differ between these two groups. In addition, the progression of hepatic fibrosis did not differ between the two groups (data not shown). Although SBP did not differ between these groups (132.2 ± 17.9 mmHg vs. 134.3 ± 14.5 mmHg, respectively) (P = NS), DBP in NASH patients with the CTA/- genotype (82.2 ± 9.2 mmHg) was significantly higher than that in patients with other genotypes (78.6 ± 8.5 mmHg) ($P < 0.05$). Male NASH patients had diastolic hypertension compared with female patients in our study (data not shown). Although the gender balance between NASH patients with the CTA/- genotype and patients with other genotypes was not statistically different, the number of males was slightly higher than that of females in patients of other genotypes (Table 3). However, NASH patients with the CTA/- genotype had much higher DBP. Therefore, the CTA/- genotype strongly influences the DBP in NASH patients. Previously, it was reported that only the CTA haplotype among four haplotypes showed a trend

toward a greater DBP reduction in response to ACE inhibitor therapy.[18] Taken together with our results, these findings indicate that NASH therapy using ACE inhibitors or ARB would be reasonable, especially for NASH patients with the CTA haplotype.

Activation of RAS was recently shown to be an important predictive factor for the development of NASH based on the therapeutic efficacy of ARB in NASH patients[8] and in a rat model of NASH.[9] In addition, angiotensin II type 1 receptor polymorphisms were shown to influence the risk of development of NAFLD and liver fibrosis in NAFLD.[35] The SNPs of the AGT gene have been clarified as important predictive factors not only for cardiovascular disease, but also for other diseases. The prevalence of diabetes mellitus type 2,[36] chronic kidney disease,[37] idiopathic pulmonary fibrosis disease progression,[38] and breast cancer[39] were shown to be closely related to the SNPs of the AGT gene. In addition to these diseases, NASH is not directly related to cardiovascular diseases, which may be related to SNPs of the AGT gene, as mentioned above. Furthermore, which SNPs of the AGT gene are related to the development of these diseases, including NASH, has not yet been clarified. However, in this article, we demonstrated for the first time the relationship between the prevalence of NASH and SNPs of the AGT gene or the AGT haplotype. Our results provide new insight into the therapy of NASH patients with the CTA haplotype using ACE inhibitors or ARBs.

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ORIGINAL ARTICLE

Overexpression of GalNAc-transferase GalNAc-T3 promotes pancreatic cancer cell growth

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O-linked glycans of secreted and membrane-bound proteins have an important role in the pathogenesis of pancreatic cancer by modulating immune responses, inflammation and tumorigenesis. A critical aspect of O-glycosylation, the position at which proteins are glycosylated with N-acetyl-galactosamine on serine and threonine residues, is regulated by the substrate specificity of UDP-GalNAc:polypeptide N-acetylgalactosaminyl-transferases (GalNAc-Ts). Thus, GalNAc-Ts regulate the first committed step in O-glycosylated protein biosynthesis, determine sites of O-glycosylation on proteins and are important for understanding normal and carcinoma-associated O-glycosylation. We have found that one of these enzymes, GalNAc-T3, is overexpressed in human pancreatic cancer tissues and suppression of GalNAc-T3 significantly attenuates the growth of pancreatic cancer cells *in vitro* and *in vivo*. In addition, suppression of GalNAc-T3 induces apoptosis of pancreatic cancer cells. Our results indicate that GalNAc-T3 is likely involved in pancreatic carcinogenesis. Modification of cellular glycosylation occurs in nearly all types of cancer as a result of alterations in the expression levels of glycosyltransferases. We report guanine the nucleotide-binding protein, α-transducing activity polypeptide-1 (GNAT1) as a possible substrate protein of GalNAc-T3. GalNAc-T3 is associated with O-glycosylation of GNAT1 and affects the subcellular distribution of GNAT1. Knocking down endogenous GNAT1 significantly suppresses the growth/survival of PDAC cells. Our results imply that GalNAc-T3 contributes to the function of O-glycosylated proteins and thereby affects the growth and survival of pancreatic cancer cells. Thus, substrate proteins of GalNAc-T3 should serve as important therapeutic targets for pancreatic cancers.

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Introduction

Pancreatic ductal adenocarcinoma (PDAC) is the fourth leading cause of death by cancer in the western world. It shows the worst mortality among common malignancies, with a 5-year survival rate of less than 5% (Wray *et al.*, 2005; Jemal *et al.*, 2009). Thus, development of novel approaches to prevent and treat PDAC is important.

Pancreatic *O*-glycosylated proteins have important biological functions, including protection, lubrication and moisturization of the surfaces of epithelial tissues that line the ductal structures within the pancreas. Both glycosylation and expression of *O*-glycosylated proteins become deregulated during the development and progression of PDAC (Moniaux *et al.*, 2004). The *O*-glycosylation of glycoproteins is critical for their function to protect and control the local environment of the cell surface, whereby aberrant glycosylation of *O*-glycosylated proteins contributes to the transformation and metastatic capacity of cancer cells (Hollingsworth and Swanson, 2004). *N*-acetylgalactosaminyl-transferases (GalNAc-Ts) are localized in the Golgi apparatus where mucin-type linkages (GalNacα1-*O*-Ser/Thr) are initiated. Once synthesized, GalNacα1-*O*-Ser/Thr can be additionally processed by different glycosyltransferases to form different core structures, such as Core-1 (Galβ1-3GalNAcα-*O*-Thr/Ser) and Core-2 (GlcNAcβ1-6(Galβ1-3)GalNAcα-*O*-Thr/Ser). The carbohydrates of glycoproteins, which are displayed on cell membranes, are structurally changed during carcinogenesis (Hakomori, 1989). This carbohydrate heterogeneity has been applied to tumor markers for clinical diagnosis. For example, carbohydrate Antigen 19-9 (CA19-9) and carcinoembrionic antigen are commonly used as markers of PDAC. Structural changes to the carbohydrates of glycoproteins may occur as a result of alterations in the expression

levels of glycosyltransferases, which are implicated in the biosynthesis of glycoforms (Kondo *et al.*, 2006). Analysis of tissue-specific expression of these enzymes showed that GalNAc-T3 is overexpressed by some PDAC cell lines (Sutherlin *et al.*, 1997). Thus, overexpression of GalNAc-T3 may contribute to the function of *O*-glycosylated proteins produced by PDAC cells and thereby affect the ability of these cells to transform and survive in different organ environments.

In this study, we show that GalNAc-T3 is overexpressed in human PDAC tissues and that overexpression of GalNAc-T3 correlates with oncogenic activity. Suppression of GalNAc-T3 expression in PDAC cells by RNA interference (RNAi) results in the growth suppression of cancer cells *in vitro* and *in vivo*. We also report that α-transducing activity polypeptide-1 (GNAT1) may be a substrate protein of GalNAc-T3 and have a similarly important role in the viability of PDAC cells. It is likely that GalNAc-T3 acts on substrate proteins that are important for the growth and/or survival of PDAC cells and contributes to cell growth/survival in an epigenetic manner based on their substrate specificities.

Results

Overexpression of GalNAc-T3 in PDAC

We compared the expression levels of GalNAc-Ts in human PDAC cell lines with that in the normal pancreatic ductal cell line, HPNE, by semi-quantitative reverse transcription–PCR (RT–PCR). We found high levels of GalNAc-T3 expression in six of eight PDAC cell lines when compared with HPNE cells (Figure 1a). Overexpression of GalNAc-T3 was confirmed by immunoblotting (Figure 1a).

Immunohistochemical analysis of GalNAc-T3 expression in human PDAC tissues using an anti-GalNAc-T3 antibody showed strong signals in the cytoplasm of PDAC cells in four PDAC tissue sections from five patients. The expression of GalNAc-T3 in two cases of PDAC is shown in Figure 1b. In normal pancreatic tissues, GalNAc-T3 is weakly expressed in the acinar and ductal epithelium (Figure 1c). Furthermore, tissue-microarray analysis of 32 PDAC tissues shows that 21 of 32 PDACs express high levels of GalNAc-T3 (66%; Supplementary Table 1). Taking together all of the GalNAc-T3-positive PDAC specimens and the tissue microarray, immunohistochemical patterns show granular staining mostly restricted to the perinuclear areas of the cytoplasm in the center and in the surrounding areas of the tumors (Figures 1d and e).

Knockdown effects of GalNAc-T3 on the viability of PDAC cells

To assess whether GalNAc-T3 is essential for the growth of PDAC cells, we produced three independent RNAi constructs using the pSUPER system that targets GalNAc-T3. These constructs were transiently expressed in the moderately differentiated human PDAC cell lines S2-013 and BxPC3 in which GalNAc-T3 is highly expressed, and in the poorly differentiated human PDAC cell line PANC-1, which is GalNAc-T3-null. A knockdown effect produced by siT3-1 and siT3-2 constructs was confirmed by RT–PCR and immunoblotting in S2-013 cells (Figure 2a). The effects of GalNAc-T3 on cell growth were determined by *in vitro* tissue culture assays. We found by colony formation (Figure 2b) and MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assays (Figure 2c) that S2-013 cells transfected with siT3-1 and siT3-2 were drastically reduced in number when compared with those transfected with siT3-3, of which only a weak knockdown effect was apparent. Similar effects were obtained with the BxPC3 cell line; however, no significant differences were observed in PANC-1 cells in which GalNAc-T3 was not expressed (Figure 2d). These results suggest that GalNAc-T3 has a role in PDAC cell viability.

Stable knockdown of GalNAc-T3 in PDAC cell lines by RNAi

To further examine the effects of GalNAc-T3 on cell growth/survival, motility and invasion, we generated clones that stably suppressed GalNAc-T3 expression by vector-based transfection of the siT3-2 plasmid in S2-013 cells, which express high levels of GalNAc-T3. Western blot analysis validated that stable GalNAc-T3 RNAi clones (siT3-clone1 and siT3-clone2) significantly suppressed GalNAc-T3 in S2-013 cells (Figure 3a). We also prepared control S2-013 cells transfected using a mock and a scrambled control vector (Neo-clone1 and Scr-clone1) to compare cell growth, motility and invasion by *in vitro* culture assays and by an *in vivo* xenograft model. MTT assays showed that siT3-clone1 and siT3-clone2 grew much more slowly than the control Neo-clone1 or Scr-clone1 (Figure 3b), in accordance with the results of MTT assays using transiently suppressed GalNAc-T3 (Figure 2c). These results indicate that lower levels of GalNAc-T3 expression suppress cell growth. Suppression of GalNAc-T3 fails to enhance or inhibit motility, as assessed by wound healing and transwell motility assays, as well as by Matrigel invasion assays (data not shown).

We next examined the effect of GalNAc-T3 suppression on tumor xenograft growth in nude mice. GalNAc-T3-silenced S2-013 clones showed significantly decreased tumor growth kinetics as compared with control xenografts (Figure 3c; 10 xenografts of 2 clones per group). This suggests that loss of function of GalNAc-T3 suppresses the growth of xenografted PDAC tumors and that GalNAc-T3 might be involved in accelerating tumorigenesis *in vivo*.

Knockdown effects of GalNAc-T3 on apoptosis

We characterized the function of GalNAc-T3 in cell survival and used flow cytometric analysis to determine the percentage of cells undergoing apoptosis between stable control and GalNAc-T3 S2-013 clones. The ModFit cell-cycle analysis software was used to analyze the percentage of cells undergoing apoptotic cell death.

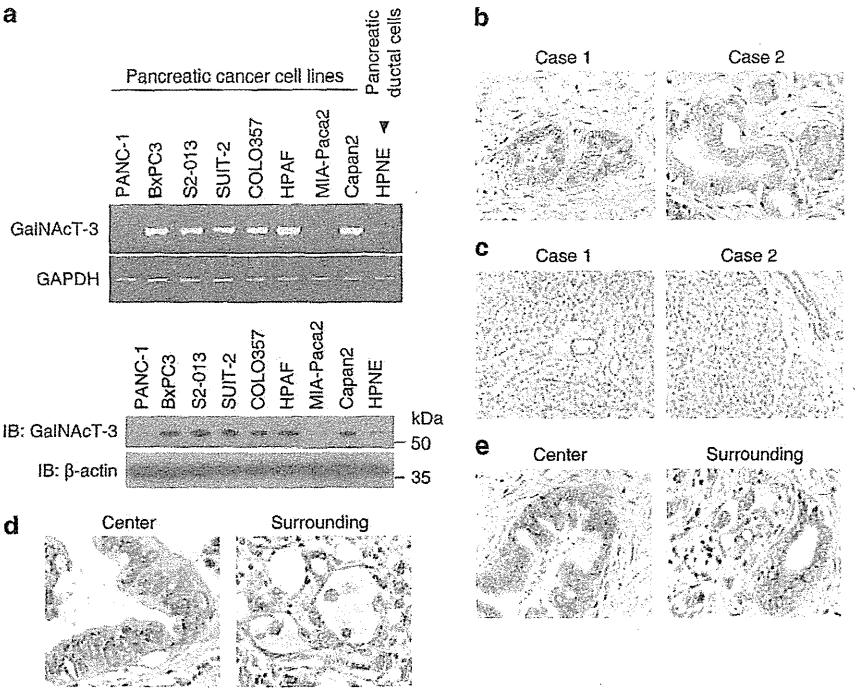

Figure 1 Overexpression of GalNAc-T3 in PDAC. (**a**) Expression of endogenous GalNAc-T3 in PDAC cells as compared with the HPNE cell line, determined by RT–PCR (upper panels) and western blotting using anti-GalNAc-T3 antibody (lower panels). (**b**) Immunohistochemical staining of PDAC tissues using anti-GalNAc-T3 antibody. Original magnification: ×200. (**c**) Immunohistochemical staining of normal pancreas tissues using anti-GalNAc-T3 antibody. Original magnification: ×200. (**d**, **e**) A representative expression pattern of GalNAc-T3 in two PDAC cases. In these cases, granular staining was seen at the center and surrounding the tumors. Original magnification: ×200. GalNAc-T, UDP-GalNAc:polypeptide *N*-acetylgalactosaminyl-transferase; PDAC, pancreatic ductal adenocarcinoma; RT–PCR, reverse transcription–PCR.

We found that the percentage of apoptotic cells increased when GalNAc-T3 was knocked down (Figures 4a and b). We calculated that 11.3 ± 0.3% of siT3-clone1 and 7.1 ± 2.9% of siT3-clone2 of S2-013 cells were undergoing apoptosis, whereas in control mock and scrambled cells, a basal level of 0.8 ± 0.4 and 0.8 ± 0.8% apoptotic cells, respectively, was detected (Figure 4c). Moreover, in fluorescence-activated cell sorting analysis, suppression of GalNAc-T3 was found to have increased the number of cells at sub-G_1, which coincides with the increased apoptotic cell population (Figure 4d). Thus, suppression of GalNAc-T3 drastically attenuates the growth of PDAC cells and induces apoptosis, suggesting an essential role for GalNAc-T3 in maintaining the viability of PDAC cells.

GalNAc-T3 has a role in inducing cell growth and survival

As shown in Figure 5a and in accordance with our flow cytometric data, we found a significantly higher number of apoptotic cells among GalNAc-T3 RNAi S2-013 cells as determined by *in situ* terminal deoxynucleotidyl transferase-mediated uridine 5′-triphosphate-biotin nick-end labeling (TUNEL) staining. Additionally, the effect of GalNAc-T3 on cell proliferation was further studied by MIB-1 staining using control and GalNAc-T3 RNAi S2-013 cells (Figure 5b). MIB-1 recognizes the Ki-67 nuclear antigen, which is associated with cell proliferation and is found throughout the cell cycle (G_1, S, G_2 and M phases) but not in resting (G_0) cells (Cattoretti *et al.*, 1992). The number of MIB-1-positive cells among GalNAc-T3-depleted cells was significantly reduced. To investigate the mechanism by which GalNAc-T3 induces cell growth/survival, the activities of extracellular signal-regulated kinases 1 and 2, Akt and pro-survival nuclear factor-κB were assessed. Suppression of GalNAc-T3 did not change the phosphorylation levels of any of these molecules linking proliferation and apoptosis (data not shown).

Identification of GNAT1 as a substrate protein of GalNAc-T3

To identify target candidates of GalNAc-T3, we identified differently expressed proteins in the membrane fractions of stable control and GalNAc-T3 RNAi S2-013 cells by silver staining sodium dodecyl sulfate–PAGE gels. *O*-glycosylated proteins generally attach to membrane and cell surfaces (Spiro, 2002; Jensen, 2006); thus, isolating subcellular fractions is useful for enriching membrane and membrane-associated proteins. Carbohydrate structural changes may occur as a result of alterations in the levels of glycosyltransferases and glycosylation can alter the charge, conformation and stability of proteins, and thereby induce heterogeneous protein profiles as a consequence of production of