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# Serum level of adiponectin and the risk of liver cancer development in chronic Hepatitis C patients

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Obesity and metabolic syndrome are recognized risk factors for development of hepatocellular carcinoma (HCC) in patients with chronic hepatitis C (CHC). Dysregulation of adipokines, particularly the decreased secretion of adiponectin, appears to play a key role. To investigate the association between adiponectin and hepatocarcinogenesis, we conducted a large-scale retrospective cohort study. We enrolled 325 patients with CHC (146 men, 179 women; mean age 58.0 ± 10.3 years) whose serum samples were collected between January 1994 and December 2002. Subjects were divided into two groups according to their serum adiponectin levels. We evaluated the association between adiponectin level and the risk of subsequent HCC development using univariate and multivariate Cox proportional hazard regression. Because average serum adiponectin level was higher in females than males, each gender was analyzed separately. Patients with CHC had significantly higher adiponectin levels than healthy controls. During the follow-up period (mean: 9.0 years), HCC developed in 122 subjects. Unexpectedly, subjects with higher serum adiponectin levels had a higher incidence of HCC (males:  $p = 0.032$ ; females:  $p = 0.01$ ; log-rank test). Multivariate analysis revealed that a high serum adiponectin level was independently associated with HCC development (hazard ratio [HR] = 2.07;  $p = 0.031$  in females and HR = 1.82;  $p = 0.05$  in males). Isoform analysis revealed that middle- and low-molecular-weight isoforms contributed to the risk of HCC. In conclusion, Patients who had CHC with high serum adiponectin levels had a higher risk of liver cancer development. Adiponectin may thus be tumorigenic or indicate a liver disease state independently of other clinical parameters.

Hepatocellular carcinoma (HCC) is the fifth most common cancer worldwide, with an increasing incidence globally.<sup>1,2</sup> Recently, obesity and metabolic syndrome were shown in

**Key words:** hepatocellular carcinoma, carcinogenesis, chronic hepatitis C, adiponectin

**Abbreviations:** AFP: alpha fetoprotein; ALT: alanine aminotransferase; AST: aspartate aminotransferase; BMI: body mass index; CHC: chronic hepatitis C; CI: confidence interval; HCC: hepatocellular carcinoma; HCV: hepatitis C virus; HR: hazard ratio; IL-6: interleukin-6

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several epidemiologic studies to increase the risk of HCC.<sup>3-5</sup> Because the prevalence of obesity and metabolic syndrome has been increasing in both Japan and Western nations, a possible association between obesity and hepatocarcinogenesis has attracted considerable attention in recent years.

The mechanism by which obesity and metabolic syndrome promote hepatocarcinogenesis remains not fully understood. However, obesity-induced dysregulation of adipokines, cytokines secreted by adipose tissue, is considered to play a key role.<sup>6,7</sup> Adipose tissue controls the functions of other organs through the secretion of various adipokines such as leptin, adiponectin, tumor necrosis factor  $\alpha$  (TNF $\alpha$ ), interleukin-6 (IL-6), and resistin. Obesity with visceral fat accumulation increases the levels of leptin, TNF $\alpha$ , IL-6, and resistin, and decreases adiponectin levels.<sup>6,7</sup> These adipokines flow directly into the liver through the portal vein and exert a variety of effects on liver diseases.<sup>8</sup>

Adiponectin, one of the major adipokines, possesses anti-inflammatory and insulin-sensitizing properties, and levels typically decline with increasing body weight.<sup>9</sup> Hypoadiponectinemia has been implicated in the development of obesity-related morbidities such as dyslipidemia and cerebrovascular disease.<sup>10-12</sup> In addition, hypoadiponectinemia has been reported to enhance hepatic steatosis, inflammation, fibrosis,

and hepatocarcinogenesis in animal liver disease models.<sup>13–15</sup> This hypothesis is considered to be applicable to human liver disease, especially nonalcoholic steatohepatitis (NASH). Indeed, reduced adiponectin levels were found in patients with NASH and were associated with increased steatosis and necroinflammation in the liver.<sup>16</sup>

Chronic hepatitis C virus (HCV) infection is a major cause of HCC in the United States, southern European countries, and Japan.<sup>2</sup> Obesity and metabolic syndrome have been found to be associated with hepatocarcinogenesis in chronic hepatitis C (CHC) as well as in NASH,<sup>5,17</sup> and hypoadiponectinemia may be implicated in HCV-related hepatocarcinogenesis. Although some studies reported that the serum adiponectin level was associated with viral load, genotype, response to antiviral therapy, insulin resistance, and liver histology such as steatosis, inflammation, and fibrosis in CHC, such associations remain controversial.<sup>12,18–24</sup> There are also conflicting results as to whether HCV infection itself affects serum adiponectin levels.<sup>22,23</sup> Furthermore, only a few clinical studies were designed to investigate the role of adiponectin in viral hepatitis-related hepatocarcinogenesis.<sup>12,25</sup>

Based on previous reports, we hypothesized that adiponectin may have a role in ameliorating disease severity and that hypoadiponectinemia may be a risk factor for future HCC development in patients with CHC. To examine this hypothesis, we conducted a large-scale retrospective cohort study seeking to elucidate any association between serum adiponectin levels and risk of hepatocarcinogenesis in patients with CHC.

## Material and Methods

### Patients

Between January 1994 and December 2002, 1428 HCV RNA-positive patients, excluding those with (or with a history of) HCC, visited the liver clinic of the Department of Gastroenterology at the University of Tokyo Hospital. Patients whose serum samples were collected after informed consent was given were enrolled in the study. Exclusion criteria were the following: positivity for hepatitis B surface antigen, presence of infections in addition to HCV, presence of biliary disease, and ongoing interferon therapy at the time of serum collection. Patients who visited the hospital for consultation only were also excluded. Patients' history of interferon therapy and their responses to it were investigated during the follow-up period. Patients who achieved a sustained virologic response, defined by undetectable HCV-RNA at least 24 weeks after the end of therapy, were also excluded. Furthermore, we excluded patients who developed HCC within 1 year of serum collection to rule out the possibility of occult HCC. In total, 325 patients were enrolled, and the association between serum adiponectin levels at entry and the subsequent incidence of HCC was analyzed. Although no information on whether serum samples were taken under fasting conditions was available, the serum adiponectin level has been reported

to undergo no meal-related or circadian changes.<sup>26,27</sup> Therefore, we decided that these samples were appropriate for our study. All blood tests were performed at the time of serum collection. HCV RNA was measured using the Amplicor HCV assay version 1 (Roche, Tokyo, Japan) and HCV serotypes was examined using a serotyping assay (SRL, Tokyo, Japan). In patients who did not undergo liver biopsy, clinical cirrhosis was diagnosed based on the presence of clinical and laboratory features of portal hypertension (the presence of esophageal varices and/or collateral circulation at endoscopy and ultrasonography).<sup>28</sup> Control serum samples were collected from 70 age- and gender-matched healthy subjects in whom liver diseases were ruled out, recruited from the Center for Multiphasic Health Testing and Services, Mitsui Memorial Hospital. The study protocol conformed to the ethical guidelines of the 1975 Declaration of Helsinki and was approved by the ethics committee of the authors' institution.

### Follow-up and HCC diagnosis

Each subject was screened for HCC with ultrasonography at or immediately after the first visit, and those in whom HCC was detected were excluded from the study. Afterward, patients were followed-up every 3–6 months at the outpatient clinic, when blood tests including tumor markers and ultrasonography were carried out. Contrast-enhanced computed tomography was performed when HCC was suspected based on ultrasonography, and/or the serum  $\alpha$ -fetoprotein (AFP) level showed an abnormal increase. HCC was diagnosed by dynamic computed tomography, and hyperattenuation in the arterial phase with washout in the late phase was considered a definite sign of HCC. When diagnosis of HCC was ambiguous, ultrasound-guided tumor biopsy was performed and a pathologic diagnosis was made based on the Edmondson and Steiner criteria. Time to HCC occurrence was defined as the interval between the date of serum collection and the diagnosis of HCC. Patients were censored at the time of death without HCC development, the last visit when lost to follow-up, or the end of the study period. The last observation in our study was taken on January 31, 2009.

### Assay for adiponectin and high-molecular-weight adiponectin

Serum samples were stored at  $-70^{\circ}\text{C}$  until required. Adiponectin levels were measured using a commercially available enzyme-linked immunosorbent assay (ELISA) kit (R&D Systems, Minneapolis, MN) according to the manufacturer's instructions. High-molecular-weight (HMW) adiponectin levels were measured in a commercial laboratory (SRL Inc.). Middle-plus low-molecular-weight (MLMW) adiponectin levels were calculated as the difference between the levels of total adiponectin and HMW adiponectin.

Table 1. Baseline characteristics

Variable	All (n = 325)	Male (n = 146)	Female (n = 179)
Age (years) <sup>1</sup>	60 (52–65)	60 (51–66)	60 (53–65)
Platelet count ( $\times 10^3/\mu\text{l}$ ) <sup>1</sup>	147 (106–187)	148 (109–182)	144 (105–193)
Total bilirubin level (mg/dl) <sup>1</sup>	0.7 (0.5–0.9)	0.7 (0.6–0.9)	0.6 (0.5–0.8)
Serum Albumin level (g/dl) <sup>1</sup>	4.0 (3.8–4.2)	4.0 (3.8–4.2)	4.0 (3.8–4.2)
AST level (IU/l) <sup>1</sup>	53 (36–81)	54 (42–76)	52 (32–83)
ALT level (IU/l) <sup>1</sup>	59 (33–96)	65 (47–100)	51 (30.5–92.5)
AFP level ng/ml <sup>1</sup>	5.0 (3.0–11)	6.0 (3.0–11.4)	5.0 (3.5–10.5)
Prothrombin time activity (%)	85.5 (74.3–100)	85.7 (73.8–97.4)	85.1 (74.4–100)
Drinking >50 g/day, n (%)	46 (14.2)	42 (28.8)	4 (2.2)
BMI (kg/m <sup>2</sup> ) <sup>1</sup>	22.5 (20.4–24.6)	22.7 (20.9–24.6)	22.3 (20.3–24.7)
Diabetes mellitus, n (%)	38 (11.7)	23 (15.8)	15 (8.4)
HCV serotype 1, n (%)	241 (74.2)	113 (77.3)	128 (71.5)
Patients who received IFN, n (%)	49 (15.1)	21 (14.4)	28 (15.6)

<sup>1</sup>Expressed as median (25th–75th percentiles).

### Immunohistochemistry

Liver biopsy samples were fixed in 10% neutral-buffered formalin, embedded in paraffin, and then sectioned. For immunohistochemistry, liver biopsy samples were deparaffinized and incubated overnight at 4°C with antiadiponectin antibodies (Abcam, Cambridge, UK). Binding of the primary antibody was detected with antirabbit IgG antibody, followed by visualization with 3,3'-diaminobenzidine (Sigma-Aldrich, St. Louis, MO). Expression in the samples was judged as weak or strong depending on the staining intensity assessed by a single observer, blinded to the clinical data.

### Statistical analysis

Student's *t*-test was used to evaluate the differences in serum adiponectin levels between groups. Correlations between variables were analyzed using Spearman's rank correlation coefficient. A *p* value of less than 0.05 on a two-tailed test was considered significant. Cumulative HCC incidence was estimated using the Kaplan–Meier method, and the differences between groups were assessed with the log-rank test. In the analysis of risk factors for hepatocarcinogenesis, we tested the following variables obtained at the time of entry in univariate and multivariate Cox proportional hazard regression analysis: age, body mass index (BMI), heavy alcohol drinking (alcohol intake > 50 mg/day), serum albumin concentration, total bilirubin concentration, alanine aminotransferase (ALT) and aspartate aminotransferase (AST) levels, prothrombin activity, platelet count, AFP concentration, comorbidity with diabetes mellitus, and serum adiponectin level. To assess the importance of adiponectin isoforms in HCC development, we added HMW adiponectin level and MLMW adiponectin level to variables described above instead of total adiponectin, and performed multivariate Cox proportional hazard regression analysis with a step-wise selection procedure. Diagnosis of diabetes mellitus was based on medical history or a 75 g oral

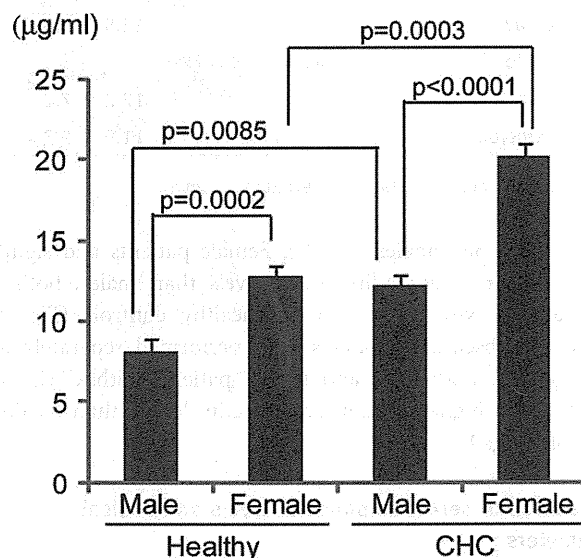


Figure 1. Serum adiponectin levels in healthy subjects (30 males, 40 females) and patients with CHC. Data are expressed as means  $\pm$  standard error of the mean (SEM).

glucose tolerance test.<sup>29</sup> Data processing and analysis were performed using S-PLUS 2000 (MathSoft, Seattle, WA) and SAS Software version 9.1 (SAS Institute, Cary, NC).

## Results

### Subject profile and serum adiponectin levels

In total, 325 subjects (146 males and 179 females; mean age:  $58.0 \pm 10.3$  years) were included in the study. Detailed demographic data are shown in Table 1. Median BMI was 22.7 for males and 22.3 for females, and diabetes mellitus was present in 15.8% of the male and 8.4% of the female subjects. The subjects diagnosed as having cirrhosis, based on liver biopsy or clinical and laboratory features, were 45 males

**Table 2.** Correlations between serum adiponectin levels and other parameters

Variables	Male			Female		
	Spearman's rho	adiponectin	<i>p</i>	Spearman's rho	adiponectin	<i>p</i>
Age	0.325		<0.0001	0.224		0.003
Platelet count	-0.189		0.023	-0.127		0.089
Total bilirubin	0.033		0.77	0.05		0.46
Albumin	-0.152		0.059	-0.077		0.27
AST	0.115		0.17	0.05		0.5
ALT	0.012		0.89	-0.001		0.98
AFP	0.028		0.77	0.087		0.27
Prothrombin time	-0.12		0.12	-0.013		0.77
BMI	-0.392		<0.0001	-0.105		0.16
<b>Diabetes mellitus</b>						
Yes <sup>1</sup>		10.8 ± 8.2	0.22		15.0 ± 11.6	0.04
No <sup>1</sup>		12.4 ± 7.7			20.6 ± 6.5	
<b>Drinking</b>						
> 50 g/day <sup>1</sup>		11.2 ± 7.5	0.25		14.4 ± 9.9	0.28
≤ 50 g/day <sup>1</sup>		12.5 ± 7.8			20.2 ± 11.8	
HCV viral load	-0.049		0.56	0.071		0.35
HCV serotype 1		12.2 ± 7.2	0.83		20.3 ± 11.5	0.77
Other serotypes		11.9 ± 9.3			19.8 ± 12.3	

<sup>1</sup>Expressed as means ± standard deviation (μg/ml).

(30.8%) and 56 females (31.2%). Female patients had significantly higher serum adiponectin levels than males, both in the patients with CHC and the healthy controls (Fig. 1). Thus, all subsequent analyses were performed separately for each gender. Both male and female patients with CHC had significantly higher serum adiponectin levels than healthy controls (Fig. 1).

#### Correlation of serum adiponectin levels and clinical parameters

The correlation between serum adiponectin levels and other clinical factors was evaluated to elucidate the clinical relevance of serum adiponectin levels in patients with CHC (Table 2). In male subjects, the serum adiponectin level was correlated positively with age and negatively with platelet count and BMI. In female subjects, the serum adiponectin level was positively correlated with age and was lower in patients with diabetes mellitus. Platelet count showed a weak negative correlation. The serum adiponectin level did not correlate with hepatitis C viral factors, such as viral load or serotype.

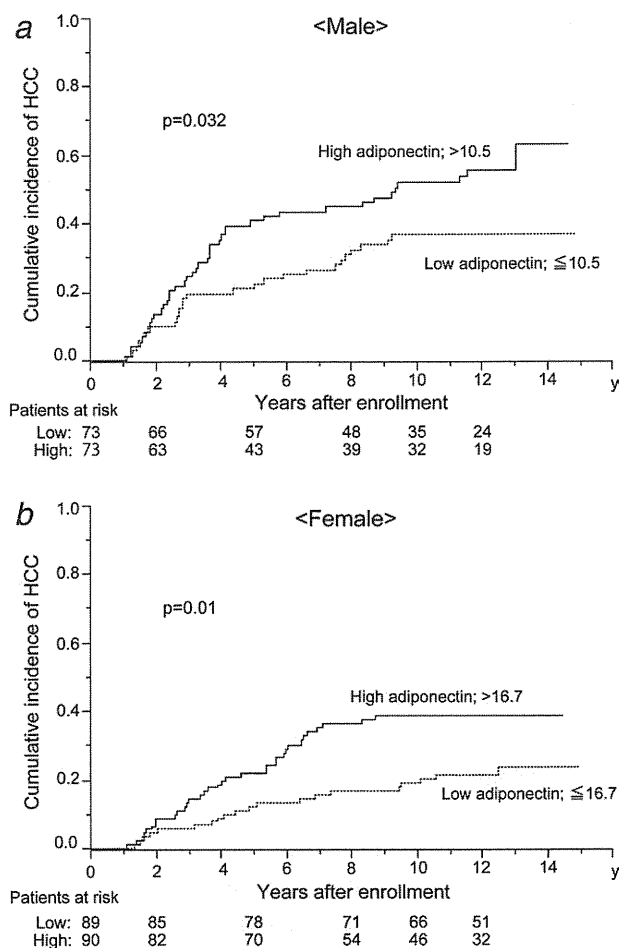
#### Incidence of HCC stratified based on serum adiponectin levels

The mean follow-up period was 9 years. During this time, 19 (13.1%) male and 17 (9.5%) female subjects were lost to follow-up. By the end of the study follow-up period, HCC had developed in 122 subjects (67 males and 55 females).

The cumulative incidence rates at 5 and 10 years were 31.5% and 42.0% (5.5% per person-year) in male and 17.3% and 29.3% (3.2% per person-year) in female subjects. Subjects were divided into two groups based on serum adiponectin levels, with the median value as the cutoff (10.5 μg/ml in male and 16.7 μg/ml in female subjects). Unexpectedly, both male and female subjects with high serum adiponectin had a significantly higher incidence of HCC (males, *p* = 0.032; females, *p* = 0.01; log-rank test; Fig. 2). In male subjects, the cumulative incidence rates at 5 and 10 years were 21.9% and 37.7% in the low, and 41.1% and 51.0% in the high adiponectin groups, respectively. In female subjects, the cumulative incidence rates at 5 and 10 years were 12.4% and 19.3% in the low, and 22.2% and 39.2% in the high adiponectin groups, respectively.

#### Risk analyses

Risk factors for HCC development were analyzed separately for each gender. In the univariate analyses, high serum adiponectin levels (> 10.5 μg/ml in males; > 16.7 μg/ml in females) was a significant risk factor for HCC in both male and female subjects (Table 3). Other significant risk factors for HCC included age, AFP level, and laboratory parameters indicative of more advanced liver disease such as serum albumin level. Heavy alcohol consumption and diabetes mellitus were significant risk factors in male subjects only, and higher BMI was a significant risk factor in female subjects only. In a multivariate proportional hazard regression analysis, a high



**Figure 2.** Cumulative incidence of HCC development stratified according to the median value of serum adiponectin for each gender: low (serum adiponectin concentration  $\leq 10.5$   $\mu\text{g/ml}$  in males,  $\leq 16.7$   $\mu\text{g/ml}$  in females) and high ( $>10.5$   $\mu\text{g/ml}$  in males,  $>16.7$   $\mu\text{g/ml}$  in females). (a) Male patients; (b) female patients.

adiponectin level was found to be an independent risk factor in female subjects, with a hazard ratio (HR) of 2.07 (95% confidence interval [CI]: 1.06–4.04;  $p = 0.031$ ; Table 3). In male subjects, a high adiponectin level was correlated with HCC development at a borderline significance level, with a HR of 1.82 (95% CI: 1.00–3.33;  $p = 0.050$ ). Age, prothrombin time, and AFP level were independent risk factors in both male and female subjects. Heavy alcohol consumption was an independent risk factor in male subjects only. BMI in females and diabetes mellitus in males were correlated with HCC at a borderline significance level, with HRs of 1.09 (95% CI: 0.99–1.19;  $p = 0.061$ ) and 1.81 (95% CI: 0.96–3.44;  $p = 0.066$ ), respectively.

The above analyses showed that both a higher BMI and higher adiponectin were risk factors for hepatocarcinogenesis, despite their negative correlation, so the relationship among BMI, adiponectin, and hepatocarcinogenesis was complex, especially in females. Thus, we investigated the contribution of adiponectin to hepatocarcinogenesis, stratified by BMI in

females. Subjects with a higher adiponectin level had a much higher incidence of HCC in the overweight group (BMI  $> 25$ ;  $p = 0.0036$ ), whereas the difference was not significant in the non-overweight group (BMI  $\leq 25$ ;  $p = 0.094$ ) (Supporting Information Figure 1). These results suggest that adiponectin might play an important role in hepatocarcinogenesis in CHC patients, especially in overweight patients.

### Immunohistochemistry

To investigate the localization of adiponectin in the liver, we performed immunohistochemistry for adiponectin using liver biopsy samples. Of the 325 patients enrolled in our study, 64 underwent a liver biopsy around the same time as serum collection. From these, 35 paraffin-embedded samples were available (F 0-2,  $n = 9$ ; F3,  $n = 10$ ; F4,  $n = 16$ ). Adiponectin was stained primarily in hepatocytes, and the staining intensity tended to be higher according to the progression of fibrosis (Supporting Information Figure 2A, B). Additionally, the serum adiponectin level was higher in patients with strong staining for adiponectin than in patients with weak staining (Supporting Information Figure 2C).

### Assessment of adiponectin isoforms

Circulating adiponectin exists in several isoforms, including low- (trimer; LMW), middle- (hexamer; MMW), and high-molecular-weight (12- to 18-mer; HMW) forms, each of which may exert distinct functions.<sup>9</sup> Recent evidence suggests that HMW adiponectin is the more biologically active form with regard to insulin sensitivity.<sup>30</sup> In addition, the ratio of HMW adiponectin to total adiponectin (HMWR) was reported to be predictive of insulin resistance, metabolic syndrome, and cardiovascular disease.<sup>31,32</sup> To investigate the composition of adiponectin isoforms, we measured serum HMW adiponectin levels in female subjects with CHC compared to healthy controls because multivariate risk analyses revealed that serum adiponectin as a risk factor was more important in women than in men. Both HMW and MLMW adiponectin levels were significantly higher in patients with CHC than in the healthy controls (Fig. 3a) and significantly correlated with total adiponectin (Spearman's  $\rho = 0.928$ ;  $p < 0.0001$ , Spearman's  $\rho = 0.985$ ;  $p < 0.0001$ , respectively), whereas HMWR was significantly lower in patients with CHC (Fig. 3b). To assess the contribution of each component to HCC development, we reanalyzed the risk factors for HCC development using HMW adiponectin level and MLMW adiponectin level instead of total adiponectin. Patients were divided into two groups based on the median value of each parameter. Whereas high HMW adiponectin ( $> 5.96$   $\mu\text{g/ml}$ ) and high MLMW adiponectin ( $> 10.6$   $\mu\text{g/ml}$ ) were significant risk factors for HCC in the univariate analysis, only the high MLMW adiponectin level retained significance in a multivariate analysis (HR: 1.96; 95% CI: 1.06–3.60;  $p = 0.029$ ; Supporting Information Table 1).

**Table 3.** Risk factors for HCC development: univariate and multivariate analyses

Variable	Univariate analyses		Multivariate analyses	
	Hazard ratio (95% CI)	<i>p</i>	Hazard ratio (95% CI)	<i>p</i>
<b>Male</b>				
Age (per year old)	1.07 (1.04–1.10)	<0.0001	1.05 (1.02–1.09)	<0.0001
Platelet count (per 10 <sup>3</sup> /μl)	0.987 (0.982–0.992)	<0.0001	0.995 (0.990–1.001)	0.13
Total bilirubin (per 0.1 mg/dl)	1.05 (1.00–1.12)	0.057	0.99 (0.92–1.07)	0.96
Serum albumin level (per 0.1 g/dl)	0.87 (0.81–0.93)	<0.0001	1.01 (0.94–1.09)	0.72
AST level (per 1 IU/l)	1.005 (1.001–1.008)	0.005	1.00 (0.99–1.02)	0.22
ALT level (per 1 IU/l)	1.002 (0.999–1.005)	0.21	0.99 (0.98–1.00)	0.41
AFP level > 10 ng/ml	3.58 (2.19–5.86)	<0.0001	2.99 (1.70–5.24)	0.0001
Prothrombin time activity (per 1%)	0.94 (0.92–0.96)	<0.0001	0.95 (0.93–0.97)	<0.0001
Drinking > 50 g/day	1.76 (1.07–2.89)	0.025	1.88 (1.04–3.37)	0.034
BMI (per 1 kg/m <sup>2</sup> )	0.97 (0.90–1.04)	0.44	1.01 (0.93–1.09)	0.76
Diabetes mellitus (yes)	1.89 (1.06–3.37)	0.029	1.81 (0.96–3.44)	0.066
Adiponectin level > 10.5 μg/ml	1.69 (1.03–2.76)	0.034	1.82 (1.00–3.33)	0.050
<b>Female</b>				
Age (per year old)	1.12 (1.07–1.16)	<0.0001	1.11 (1.06–1.17)	<0.0001
Platelet count (per 10 <sup>3</sup> /μl)	0.976 (0.969–0.982)	<0.0001	0.98 (0.97–0.99)	0.004
Total bilirubin (per 0.1 mg/dl)	1.15 (1.08–1.23)	<0.0001	0.86 (0.76–0.97)	0.015
Serum albumin level (per 0.1 g/dl)	0.82 (0.77–0.89)	<0.0001	0.94 (0.85–1.04)	0.27
AST level (per 1 IU/l)	1.008 (1.004–1.012)	<0.0001	0.99 (0.98–1.00)	0.32
ALT level (per 1 IU/l)	1.004 (1.001–1.007)	0.019	1.00 (0.99–1.01)	0.83
AFP level >10 ng/ml	10.51 (5.96–18.53)	<0.0001	4.85 (2.38–9.90)	<0.0001
Prothrombin time activity (per 1%)	0.91 (0.89–0.93)	<0.0001	0.94 (0.91–0.98)	0.018
Drinking >50 g/day	0.47 (0.17–1.30)	0.15	0.85 (0.33–2.20)	0.74
BMI (per 1 kg/m <sup>2</sup> )	1.15 (1.08–1.23)	<0.0001	1.09 (0.99–1.19)	0.061
Diabetes mellitus (yes)	1.78 (0.84–3.77)	0.13	0.84 (0.35–1.99)	0.69
Adiponectin level >16.7 μg/ml	2.02 (1.16–3.50)	0.012	2.07 (1.06–4.04)	0.031

#### Association between serum adiponectin and serum IL-6 levels

Although adiponectin has often been suggested to have anti-inflammatory properties, recent studies have revealed that adiponectin exerts pro-inflammatory effects in immune cells through nuclear factor kappa B (NF-κB) activation and subsequent secretion of IL-6 and TNFα.<sup>33,34</sup> IL-6 is one of the most important pro-inflammatory cytokines in hepatocarcinogenesis,<sup>35</sup> and we previously reported that high serum IL-6 levels were correlated with future HCC development in patients with CHC using the same subject cohort as our study.<sup>36</sup> Thus, we investigated the correlation between serum adiponectin and IL-6 levels in female subjects, but none was found (Spearman's rho = -0.018; *p* = 0.81) (Supporting Information Table 2). In addition, HWM adiponectin and MLMW adiponectin levels showed no significant correlations with serum IL-6 levels (HMW; Spearman's rho = -0.059; *p* = 0.42 and MLMW; Spearman's rho = 0.006, *p* = 0.93, respectively) (Supporting Information Table 2). Multivariate analyses of the risk factors for HCC, including total adipo-

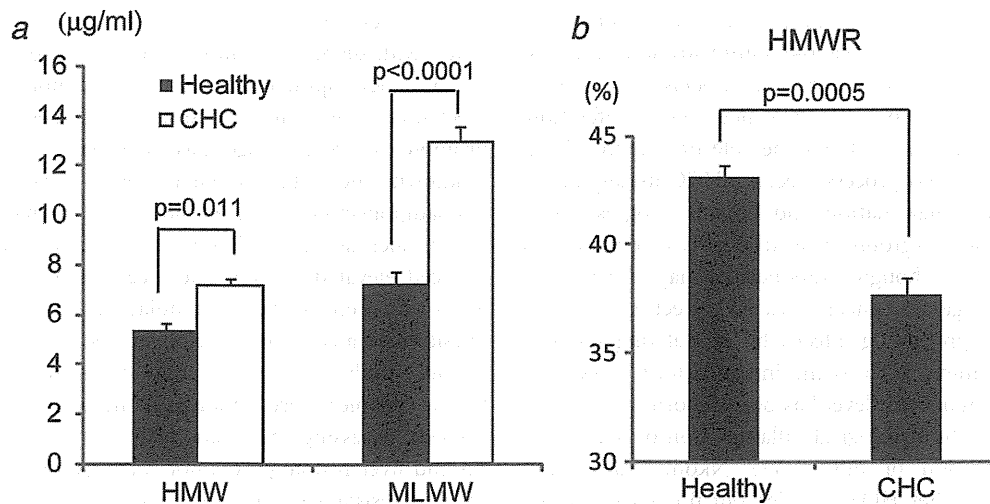
nectin and IL-6, revealed that they were independent risk factors for HCC (adiponectin > 16.7 μg/ml: HR: 2.05; 95% CI: 1.04–4.03; *p* = 0.035 and IL-6: HR: 1.49; 95% CI: 1.03–2.16 per log unit increase; *p* = 0.033) (Supporting Information Table 3). These data suggest that IL-6 is likely not a major mediator of the association of adiponectin with hepatocarcinogenesis.

#### Discussion

Adiponectin is considered to be important in metabolic syndrome, and hypoadiponectinemia has been reported to be correlated with various diseases related to metabolic syndrome.<sup>10–12</sup> However, we found that patients with CHC having high serum adiponectin levels had a higher risk of developing HCC. To our knowledge, this is the first study reporting a positive association between serum adiponectin levels and future HCC development.

Adiponectin reportedly exerts its effects through interaction with two specific receptors, AdipoR1 and AdipoR2.<sup>37</sup> AdipoR1 is expressed in skeletal muscle and other tissues,





**Figure 3.** Assessment of adiponectin isoforms in healthy female subjects and patients with CHC. (a) Serum HMW and MLMW adiponectin levels; (b) ratio of HMW adiponectin to total adiponectin (HMWR). Data are expressed as means  $\pm$  standard error of the mean (SEM).

whereas AdipoR2 is expressed primarily in the liver. AdipoR1 activates the AMP kinase (AMPK) pathway and AdipoR2 the peroxisome proliferator-activated receptor alpha (PPAR $\alpha$ ) pathway to increase insulin sensitivity and decrease inflammation.<sup>30</sup> However, recent studies have revealed that different forms of adiponectin may exert distinct functions. For example, HMW adiponectin is considered to play a crucial role in insulin sensitization, whereas MLMW adiponectin transverse the blood–brain barrier and activates AMPK in the hypothalamus, resulting in promotion of food intake.<sup>38</sup> Hui *et al.*<sup>39</sup> reported that serum MMW adiponectin levels were elevated in patients with chronic hepatitis B and declined markedly after antiviral therapy, particularly in patients with a virological response. We showed that both HMW adiponectin and MLMW adiponectin levels were elevated in patients with CHC, and a higher MLMW adiponectin level was an independent risk factor for HCC development. These findings suggest that an elevated MMW or LMW adiponectin level may represent a particular liver disease state, independently of other clinical parameters. However, both the HMW and MLMW adiponectin levels showed strong positive correlations with the total adiponectin level, so measuring the total adiponectin level may be sufficient for assessing the association between adiponectin and hepatocarcinogenesis.

Several studies have reported that serum adiponectin levels in patients with advanced liver fibrosis were elevated.<sup>39</sup> Because adiponectin is largely metabolized by the liver,<sup>40</sup> serum adiponectin concentration may increase due to decreased hepatic degradation. Serum adiponectin may represent a surrogate marker of liver fibrosis, as it was negatively correlated with platelet count in our study. However, the multivariate analysis revealed that high serum adiponectin was an independent risk factor of hepatocarcinogenesis, particularly in female subjects. A recent cross-sectional study reported that high serum adiponectin was independently correlated with HCC in patients with chronic hepatitis B.<sup>25</sup>

Additionally, our immunohistochemical analysis revealed that adiponectin accumulated in the fibrotic liver, consistent with a previous report.<sup>39</sup> These data suggest that adiponectin, after accumulation in the fibrotic liver, may possess tumorigenic functions.

Adiponectin is often considered to have anti-inflammatory properties. However, elevated plasma adiponectin levels have recently been reported in several diseases associated with inflammation, such as arthritis,<sup>41</sup> preeclampsia<sup>42</sup> and end-stage renal disease.<sup>43</sup> Furthermore, high serum adiponectin was a significant predictor of progression of chronic kidney disease.<sup>44</sup> In the rodent liver injury model, adiponectin was induced by ischemia–reperfusion and exerted a harmful effect on the liver under certain circumstances.<sup>45</sup> In our study, serum adiponectin level was elevated in patients with CHC as compared to healthy controls, which is consistent with a previous report.<sup>23</sup> On the other hand, serum adiponectin was significantly lower in patients with NASH compared to healthy subjects (Nakagawa H, unpublished observation). Thus, adiponectin may play different roles in inflammatory or infectious diseases and in metabolic diseases, including NASH. We speculate that while hypoadiponectinemia may be the initiator in the pathogenesis of NASH, adiponectin is elevated in CHC due to fibrosis progression and subsequently modulates disease progression. No significant correlation between adiponectin and IL-6 levels in serum was found, although other inflammatory factors (such as TNF $\alpha$ ) may contribute to the link between adiponectin and hepatocarcinogenesis. An alternative explanation for the association of high adiponectin levels with HCC development could be adiponectin resistance caused by downregulation of adiponectin receptor. However, a causal relationship between adiponectin and hepatocarcinogenesis was not evaluated here, so further study will be needed.

Recently, two studies described a protective role of adiponectin in HCC progression using HCC cell lines cultured *in*

*vitro* and an *in vivo* xenograft model, and these results may conflict with ours.<sup>46,47</sup> However, the administration of adiponectin to cancer cell lines or xenograft models can examine the direct effects of adiponectin on cancer cell proliferation, apoptosis, and metastasis, but not the role of adiponectin in the entire carcinogenesis process. Because HCC usually develops after chronic inflammation and fibrosis progression in CHC, the status of background liver disease is very important in carcinogenesis. Although adiponectin may have some protective effects against cancer cells, adiponectin may have potentially tumor-promoting effects, by modulating the surrounding environment, such as the inflammatory process. In fact, the serum adiponectin level has been reported to be positively correlated with histological inflammation of the liver.<sup>19</sup>

During preparation of this article, Nkontchou *et al.*<sup>48</sup> reported that the higher HOMA index but not serum adiponectin level was a risk factor for HCC development in cirrhotic patients with hepatitis C in univariate and multivariate analyses. A major difference in the study design between their study and ours is that all patients are diagnosed as cirrhosis by liver biopsy in their study, whereas patients clinically diagnosed as cirrhosis were about one-third of subjects in our study. Thus, the different results between two studies suggest that adiponectin may be a surrogate marker of severity of liver disease or play some roles in the progression of chronic hepatitis. On the other hand, the correlations of serum adiponectin levels and clinical parameters were similar in the two studies. The serum adiponectin level is higher in females than in males, while incidence of HCC is significantly higher in males than in females; thus, a gender-stratified analysis would show results similar to ours, at least when unadjusted. Besides, from the results of their study and ours, we can at least conclude that hypoadiponectinemia is not a major reason why obesity promotes hepatocarcinogenesis in patients with CHC.

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## Altered composition of fatty acids exacerbates hepatotumorigenesis during activation of the phosphatidylinositol 3-kinase pathway

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**Background & Aims:** Some clinical findings have suggested that systemic metabolic disorders accelerate *in vivo* tumor progression. Deregulation of the phosphatidylinositol 3-kinase (PI3K)/Akt pathway is implicated in both metabolic dysfunction and carcinogenesis in humans; however, it remains unknown whether the altered metabolic status caused by abnormal activation of the pathway is linked to the protumorigenic effect.

**Methods:** We established hepatocyte-specific *Pik3ca* transgenic (Tg) mice harboring N1068fs\*4 mutation.

**Results:** The Tg mice exhibited hepatic steatosis and tumor development. PPAR $\gamma$ -dependent lipogenesis was accelerated in the Tg liver, and the abnormal profile of accumulated fatty acid (FA) composition was observed in the tumors of Tg livers. In addition, the Akt/mTOR pathway was highly activated in the tumors, and in turn, the expression of tumor suppressor genes including *Pten*, *Xpo4*, and *Dlc1* decreased. Interestingly, we found that the suppression of those genes and the enhanced *in vitro* colony formation were induced in the immortalized hepatocytes by the treatment with oleic acid (OA), which is one of the FAs that accumulated in tumors.

**Conclusions:** Our data suggest that the unusual FA accumulation has a possible role in promoting *in vivo* hepato-tumorigenesis under constitutive activation of the PI3K pathway. The *Pik3ca* Tg mice might help to elucidate molecular mechanisms by which metabolic dysfunction contributes to *in vivo* tumor progression.

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### Introduction

Accumulating clinical evidence suggests that systemic metabolic disorders including obesity and insulin resistance can affect or even promote *in vivo* tumor progression [1–4]. Some studies have outlined the impact of fat-enriched diets in the development of hepatocellular carcinoma (HCC) [5–7]. However, the mechanistic insights regarding metabolites or cellular signaling responsible for the development of HCC in altered metabolic states remain unknown.

The phosphatidylinositol 3-kinase (PI3K)/Akt signaling pathway is involved in various cellular processes including cell metabolism, growth, and survival [8,9]. The altered expression and mutation of PI3K/Akt-related signaling components have been detected in some human cancers [10]. In particular, the *PIK3CA* gene encoding p110 $\alpha$ , which is a catalytic subunit of PI3K, has somatic mutations in some carcinomas [11]. Additionally, a mutation in its kinase domain has been reported in HCC and gastric cancer [12]. These findings indicate that deregulated PI3K activity plays certain roles in oncogenesis in humans [11,13]. PI3K signaling is antagonized by phosphatase and tensin homolog deleted on chromosome 10 (PTEN) phosphatase [14]. The expression of PTEN is decreased or absent in approximately half of HCC patients [15], and hepatocyte-specific *Pten* knockout

Keywords: Hepatocellular carcinoma; Fatty acids; NAFLD; Tumor suppressor genes.

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Abbreviations: PI3K, phosphatidylinositol 3-kinase; Tg, transgenic; FA, fatty acid; OA, oleic acid; HCC, hepatocellular carcinoma; PTEN, phosphatase and tensin homolog deleted on chromosome 10; FBS, fetal bovine serum; Erk, extracellular signal-regulated kinase; WT, wild type; PA, palmitic acid; H&E, hematoxylin and eosin; NASH, non-alcoholic steatohepatitis.



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mice develop steatohepatitis and HCC [16]. These findings indicate that PTEN is a tumor suppressor in the liver [17]. Although recent reports have suggested unique functions of PTEN that are independent of the PI3K-Akt axis [18–20], it is unknown whether the phenotype in *Pten*-deficient mice is due to PI3K-dependent or PI3K-independent processes.

To address the pathological consequences caused by the abnormal activation of PI3K pathway *in vivo*, we generated liver-specific *Pik3ca* transgenic (Tg) mice. In this study, we proposed that abnormal fat composition, as observed in the *Pik3ca* Tg liver, is a mechanism by which metabolic deregulation is linked to *in vivo* tumor progression.

## Materials and methods

### Generation of *Pik3ca* Tg mice

The *Pik3ca* Tg mice were generated as described previously [21]. Briefly, Myc-tagged mouse *Pik3ca* cDNA (N1068fs\*4) was cloned into the p2335A-1 vector (provided by Drs. Palmiter and Chisari) [22,23]. The microinjection was conducted by the Research Laboratory for Molecular Genetics, Yamagata University. Founder BDF1 mice (F0) were backcrossed with C57BL/6Jcl mice (CLEA Japan, Japan), and F5 mice were analyzed. The primers for genotyping were 5'-ATGGAACAGAACTCATCTCT-3' and 5'-GGGTGACACTTACGAAAT-3'. All procedures involving animals were performed in accordance with protocols approved by the institutional committee for animal research at the University of Tokyo and complied with the Guide for the Care and Use of Laboratory Animals.

### Cell cultures, viruses, and treatment with fatty acids

Lentiviral short hairpin RNA vectors were purchased from Open Biosystems (Huntsville, AL, USA). BNL-CL2 cells were infected with the virus according to the manufacturer's protocol and selected by puromycin. BNL-CL2 cells were incubated with either 50  $\mu\text{mol/L}$  fatty acids or ethanol (mock) for 12 h in the absence of fetal bovine serum (FBS) in some experiments.

### Antibodies and primers

The primers for quantitative RT-PCR are shown in Supplementary Table 1. Antibodies against phospho-Akt (Ser473 and Thr308), Akt, phospho-extracellular signal-regulated kinase (Erk) 1/2 (Thr202/Tyr204), Erk1/2, phospho-TSC2, phospho-S6K, TSC2, S6K, and SREBP1 were obtained from Cell Signaling Technology (Danvers, MA, USA). The anti-PTEN antibody was purchased from Neomarkers Inc. (Fremont, CA, USA). The anti-TFIIID antibody was purchased from Upstate Biotechnology Inc. (Lake Placid, NY, USA). For immunohistochemistry, the anti-phospho-Akt (Ser473) antibody and anti-Myc antibodies (Cell Signaling Technology) were used. The immunoblot data were quantified using Multi Gauge ver. 3.1 software (Fuji Film Corp., Tokyo, Japan).

### Triacylglycerol content, serum alanine aminotransferase (ALT) levels, and FA composition

Triacylglycerols were extracted from the liver with chloroform-methanol (2:1, v/v), and the levels were determined by the GK-GPO method (Wako, Tokyo, Japan). Serum samples for ALT measurement were collected after a 16-h starvation (SRL, Tokyo, Japan). Fatty acids were extracted from frozen liver samples, and the composition was analyzed by gas chromatography (Kotobiken Medical Laboratories, Inc., Tokyo, Japan).

### Glucose tolerance tests

Glucose was intraperitoneally injected into 8-week-old mice fasting for 16 h (1.5 mg of glucose/g body weight). Glucose concentration was measured using the FreeStyle FREEDOM Blood Glucose Monitoring System (Nipro, Tokyo, Japan) at 0, 15, 30, 60, 90, and 120 min after injection.

### Oxidative stress evaluation

The measurement of hydrogen peroxide concentrations was performed by the Colorimetric Hydrogen Peroxide Kit (Assay Designs, Inc., Ann Arbor, MI, USA). Thiobarbituric acid reactive substances (TBARS) were measured by the TBARS Assay Kit (ZeptoMetrix, Buffalo, NY, USA).

### Immunohistochemistry

Antigen retrieval on paraffin sections was performed by the acetylation method. Proteins were visualized using the standard 3,3'-diaminobenzidine protocol.

### Soft agar assay

The lower layer of 0.5% agar in media was placed in a 35-mm dish. Cells ( $2.5 \times 10^4$ ) were suspended in the upper layer of 0.3% agar. Colonies ( $>25 \mu\text{m}$  in diameter) were counted after 14 days. Oleic acid (OA) (50  $\mu\text{mol/L}$ ) or ethanol was added to the upper layer in some experiments.

### Statistics

All results are indicated as means  $\pm$  SE. Statistics were performed by Student's *t*-test or ANOVA followed by Fisher's PLSD *post-hoc* test. *p*-Values  $<0.05$  were considered statistically significant.

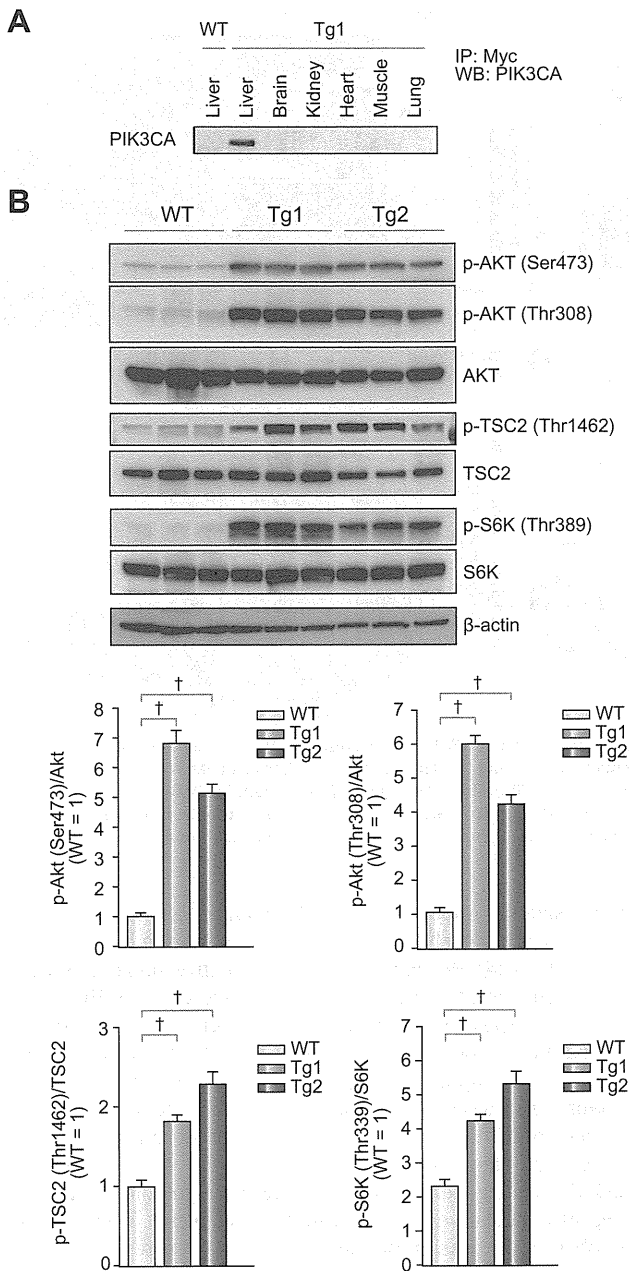
## Results

### Generation of hepatocyte-specific *Pik3ca* Tg mice

We established 2 independent lines of hepatocyte-specific Tg mice (*Pik3ca* Tg mice) harboring an "N1068fs\*4" mutation in the kinase domain [12]. Myc-tagged mutant *Pik3ca* was designed to be expressed under the albumin promoter (Supplementary Fig. 1), and the liver-specific expression of the transgene was confirmed as shown in Fig. 1A. To assess the *in vivo* effect of the *Pik3ca* N1068fs\*4 transgene, we analyzed the activity of molecules downstream of PIK3CA including Akt, TSC2, and S6K via immunoblotting. The phosphorylation of Akt, TSC2, and S6K was clearly increased both in the two lines of Tg livers, but not in the wild-type (WT) livers (Fig. 1B).

### Constitutive activation of *Pik3ca* leads to fat accumulation in the liver

Both lines of *Pik3ca* Tg mice survived, and no difference in total body weight was observed between *Pik3ca* Tg and WT mice at 4 or 24 weeks of age (data not shown). The *Pik3ca* Tg2 mice exhibited better glucose tolerance than WT mice at 8 weeks (Supplementary Fig. 2). The ratio of liver weight to body weight was significantly increased in the *Pik3ca* Tg mice compared to that of WT mice (Fig. 2A). The livers of 4 week-old *Pik3ca* Tg mice appeared slightly enlarged and light-colored, and they exhibited obvious fatty changes by 24 weeks (Fig. 2B). The Tg livers contained a greater volume of triacylglycerol than WT (Fig. 2C). The results of Western blotting revealed that Tg2 mice exhibited a relatively low activation of Akt and S6K as compared to Tg1 (Fig. 1B); however, hepatic triacylglycerol levels were clearly increased in the two lines Tg mice (Fig. 2C). Indeed, even Tg2 mice demonstrated an obvious fatty change in their livers by 24 weeks (Fig. 2B and D). These findings indicated that the constitutive expression of the *Pik3ca* N1068fs\*4 transgene has a potential to establish *in vivo* hepatic steatosis. In addition, we found



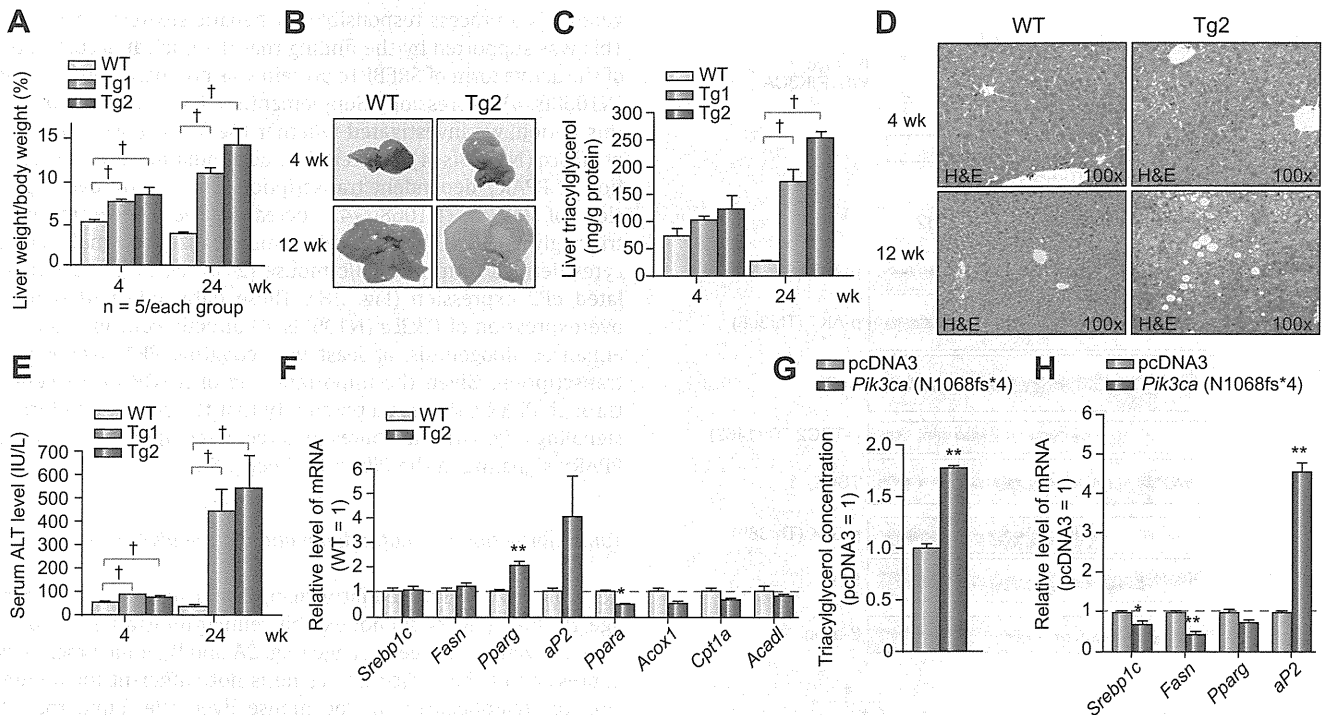
**Fig. 1. Establishment of *Pik3ca* Tg mice.** (A) Liver-specific expression of the mutant PIK3CA (N1068fs\*4). (B) Immunoblots and quantification of the ratios of phosphorylated-Akt, TSC2, and S6K levels to total protein levels ( $p < 0.05$ , ANOVA; post hoc test with WT).

that ALT levels in the *Pik3ca* Tg mice were higher than those of WT mice (Fig. 2E), suggesting the coexistence of liver damage. Next, we examined how the *Pik3ca* Tg liver induced unusual lipid accumulation. Because lipogenesis is mainly mediated by two major transcription factors, PPAR $\gamma$  and SREBP1C [24,25], we measured their expression levels and their target genes in Tg2 mice livers and observed the upregulation of PPAR $\gamma$  and its target *aP2* but not of SREBP1C or its target *FASN* (Fig. 2F). Given the previous finding that activated PI3K signaling can induce steatosis through PPAR $\gamma$  [26], we speculated that PPAR $\gamma$ -dependent lipo-

genesis is a process responsible for hepatic steatosis in Tg mice. This was supported by the finding that the nuclear accumulation of the active form of SREBP1c protein was not increased by *Pik3ca* (N1068fs\*4) expression (Supplementary Fig. 3). To emphasize this notion, we investigated whether the *in vitro* overexpression of *Pik3ca* (N1068fs\*4) induced lipid accumulation and the activation of PPAR $\gamma$ -dependent transcription. The *in vitro* overexpression of *Pik3ca* (N1068fs\*4) increased the concentration of triacylglycerol in BNL-CL2 cells, immortalized normal hepatocytes derived from a BALB/c mouse [27] (Fig. 2G), and upregulated *aP2* expression (Fig. 2H). These data indicated that the overexpression of *Pik3ca* (N1068fs\*4) directly contributes to the enhanced lipogenesis, at least via activating PPAR $\gamma$ -dependent transcription. Given the important role of mTOR in lipogenesis through PPAR $\gamma$ , there is a possibility that the activation of mTOR signaling (Fig. 1B) contributes to deregulated lipogenesis through PPAR $\gamma$  signaling in the *Pik3ca* Tg liver [26].

*Tumor formation without inflammation in the *Pik3ca* Tg mice*

Regardless of the marked fatty changes and suggested liver damage, *Pik3ca* Tg livers did not exhibit cellular infiltration or fibrotic change even at 52 weeks of age (Fig. 3A and B), which means the expression of the *Pik3ca* transgene is not sufficient for progression to steatohepatitis in the mouse liver. We found that the inflammatory cytokine IL-1 $\alpha$  and Fas ligand were highly expressed in the *Pik3ca* Tg liver than WT (Supplementary Fig. 4). Given the previous findings that these factors can be responsible for liver damage [28,29], the abnormal upregulation of IL-1 $\alpha$  and Fas ligand in Tg livers may explain a part of the mechanisms of liver damage, whereas the entire molecular process inducing them remains unknown. Notably, macroscopic hepatic tumors developed in 94% of Tg1 mice (30/32) and 100% of Tg2 mice (11/11) at 52 weeks of age (Fig. 3C, left). Most of the tumors were hepatocellular adenomas containing abundant lipid droplets (Fig. 3C, right). Some tumors had rough surfaces and irregular shapes with necrosis and hemorrhaging (Fig. 3D, left) and microscopically demonstrated characteristics of HCC such as enlarged and hyperchromatic nuclei and trabecular patterns (Fig. 3D, right). HCC tissues did not always exhibit lipid accumulation as shown in Fig. 3D. As the *Pik3ca* Tg mice aged, hepatic tumors became increased in number and size, whereas no WT littermates developed any tumors (Fig. 3E). These data clearly indicate that the *in vivo* constitutive expression of *Pik3ca* (N1068fs\*4) leads to hepatic tumor development. To assess the functional activity of PIK3CA (N1068fs\*4) for tumorigenesis, we examined the *in vitro* transforming ability using BNL-CL2 cells. Remarkably, *Pik3ca* (N1068fs\*4) expression did not stimulate colony formation of BNL-CL2 cells (Supplementary Fig. 5). In addition, we analyzed the phosphorylation level of Akt by the *in vitro* overexpression of *Pik3ca* genes including wild type, H1047R, or N1068fs\*4 in 293T cells. The overexpression of *Pik3ca* (H1047R) possessing *in vitro* transforming capacity [13] resulted in strong phosphorylation of Akt, as previously reported (Supplementary Fig. 6) [30]. Conversely, the overexpression of *Pik3ca* (wild type) without any transforming capacity [13] resulted in lower phosphorylation of Akt. The mutant PIK3CA (N1068fs\*4) induced phosphorylation of Akt, but the level was comparable to that of wild type, and less than that of H1047R (Supplementary Fig. 6). These findings suggested that *Pik3ca* (N1068fs\*4), as compared to H1047R, has less capacity for activating Akt and little



**Fig. 2. Steatosis in the *Pik3ca* Tg liver.** (A) Increased liver weight in *Pik3ca* Tg mice. (N = 5/group; <sup>†</sup>*p* < 0.05, ANOVA; post hoc test with WT). (B) Representative liver images of WT and *Pik3ca* Tg mice. (C) High concentrations of intrahepatic triacylglycerol in the Tg mice (N > 5/group; <sup>†</sup>*p* < 0.05, ANOVA; post hoc test with WT). (D) H&E staining of livers from WT and *Pik3ca* Tg mice at 4 weeks (top) and 24 weeks (bottom) of age. (E) Higher serum ALT levels in the Tg mice (N = 5/group; <sup>†</sup>*p* < 0.05, ANOVA; post hoc test with WT). (F) The expression of fat metabolism genes in the 4-week-old liver (N = 3–4/group; <sup>\*</sup>*p* < 0.05, <sup>\*\*</sup>*p* < 0.01, Student's *t*-test). (G) Cellular triacylglycerol levels and (H) the expression of lipogenesis-related genes in BNL-CL2 cells stably expressing *Pik3ca* (N1068fs\*4) (N = 3/group; <sup>\*</sup>*p* < 0.05, <sup>\*\*</sup>*p* < 0.01, Student's *t*-test).

oncogenic activity in itself [13] and that there might be unknown factors promoting *in vivo* tumorigenesis in the *Pik3ca* Tg liver.

*Downregulation of tumor suppressor genes in tumors derived from Pik3ca Tg livers*

To further assess the related cellular signaling for tumorigenesis in the *Pik3ca* liver, we evaluated the activation of Akt, S6K, and Erk among the WT liver, non-tumor Tg liver, and tumor tissues from 52-week-old mice (Fig. 4A). Tumor tissues exhibited significantly enhanced activation of Akt compared to the Akt activation in non-tumor background or WT livers. We observed stronger phosphorylation of Akt in the non-tumor Tg liver than in WT livers, but the difference was not statistically significant as determined by ANOVA. Furthermore, the immunohistochemistry for phospho-Akt did not demonstrate clear differences between non-tumor livers and WT tissues. In contrast, the expression of *Myc-Pik3ca* was sustained in the non-tumor liver at 52 weeks (Supplementary Fig. 7). Those findings suggest the possibility that continuous activation of Akt induced by overexpressed *Pik3ca* is important for tumor formation in the Tg livers [31], whereas it remains unknown why Akt phosphorylation was attenuated in the non-tumor liver at 52 weeks despite the sustained expression of *Pik3ca* (Fig. 4A and Supplementary Fig. 7). In addition, the phosphorylation of S6K and Erk tended to be higher in Tg livers than in WT livers (Fig. 4A), but the difference became attenuated at 52 weeks compared to that at 4 weeks (Figs. 1B and 4A and Supplementary Fig. 8). These data do not exclude the possible role of these molecules in tumorigenesis in Tg livers but at least may

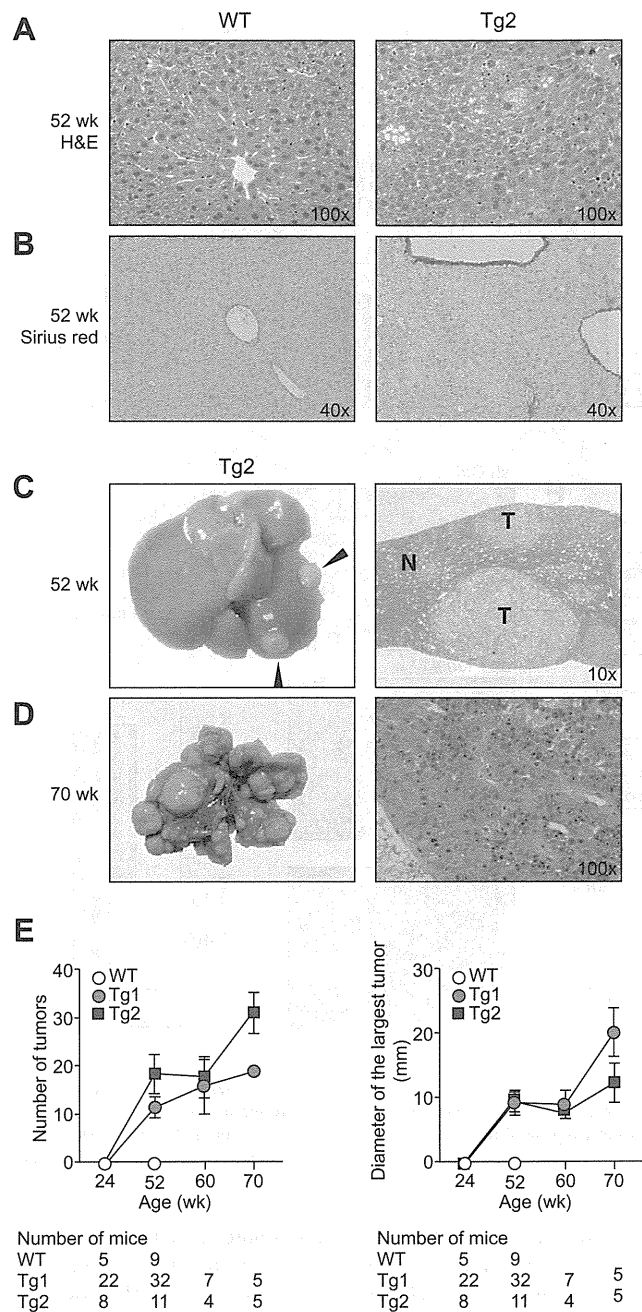
emphasize the importance of Akt activation. Next, we examined the expression levels of genes involved in murine hepatotumorigenesis [32–34]. We observed decreased expression of four tumor suppressor genes, *Pten*, AT-rich interactive domain 5B (*Arid5b*), exportin 4 (*Xpo4*), and deleted in liver cancer 1 (*Dlc1*), in the tumor compared to the non-tumor background of *Pik3ca* Tg livers (Fig. 4B and Supplementary Fig. 9). PTEN protein levels were downregulated (Fig. 4C). To address whether the downregulation of *Pten* contributes to the tumorigenic activity in liver cells, we established *Pten*-depleted BNL-CL2 cells (Fig. 4D). *Pten*-depleted BNL-CL2 cells generated significantly more colonies in soft agar (Fig. 4E), indicative of enhanced tumorigenicity. These findings emphasize the possibility that the decreased expression of tumor suppressor genes has a certain role in tumorigenesis in the *Pik3ca* Tg liver. Importantly, the *in vitro* overexpression of mutant *Pik3ca* (N1068fs\*4) only suppressed *Arid5b* expression but did not decrease the expression of *Pten*, *Xpo4*, or *Dlc1* in BNL-CL2 cells, indicating that certain additional mechanisms repressed their expression (Supplementary Fig. 10). Although several reports suggested a relationship between oxidative stress and hepatocarcinogenesis [35], the levels of hydrogen peroxide and lipid peroxidation were comparable between Tg and WT livers (Supplementary Fig. 11).

*Tumors contain higher concentrations of OAs and palmitic acids (PAs) compared to the background tissues in the Pik3ca Tg liver*

Recent intensive research has shed light into the significance of fatty acid (FA) as a potent biological stimulator of intracellular



# Research Article



**Fig. 3. Liver tumors in the *Pik3ca* Tg mice.** (A) H&E and (B) Sirius red staining of livers at 52 weeks. (C) Macroscopic view (left) of the representative liver adenomas (arrowheads) at 52 weeks of age. H&E staining of an adenoma (T) and adjacent parenchyma (N) (right). (D) Tumors in *Pik3ca* Tg mice at 70 weeks (left). H&E staining of HCC (right). (E) The number (left) and size (right) of hepatic tumors. The number of mice examined is shown below the graphs.

signaling [36]. Interestingly, unsaturated FAs inhibit *Pten* expression via microRNA-21 in hepatoma [7,37,38], and the overexpression of a FA receptor (FFAR2) transformed the 3T3 fibroblasts [39], suggesting the possible relationship between FA and tumorigenesis. In the *Pik3ca* Tg liver, the tumor tissues contained higher concentrations of FAs than the non-tumor background tissues (Fig. 5A). The difference in total FA levels was largely due to

the increase in levels of OA (C18:1n9) and PA (C16:0) in the tumors (Fig. 5B and C, Supplementary Fig. 12 and Table 2).

*OA has the potential to repress the expression of tumor suppressors and enhance colony formation in vitro*

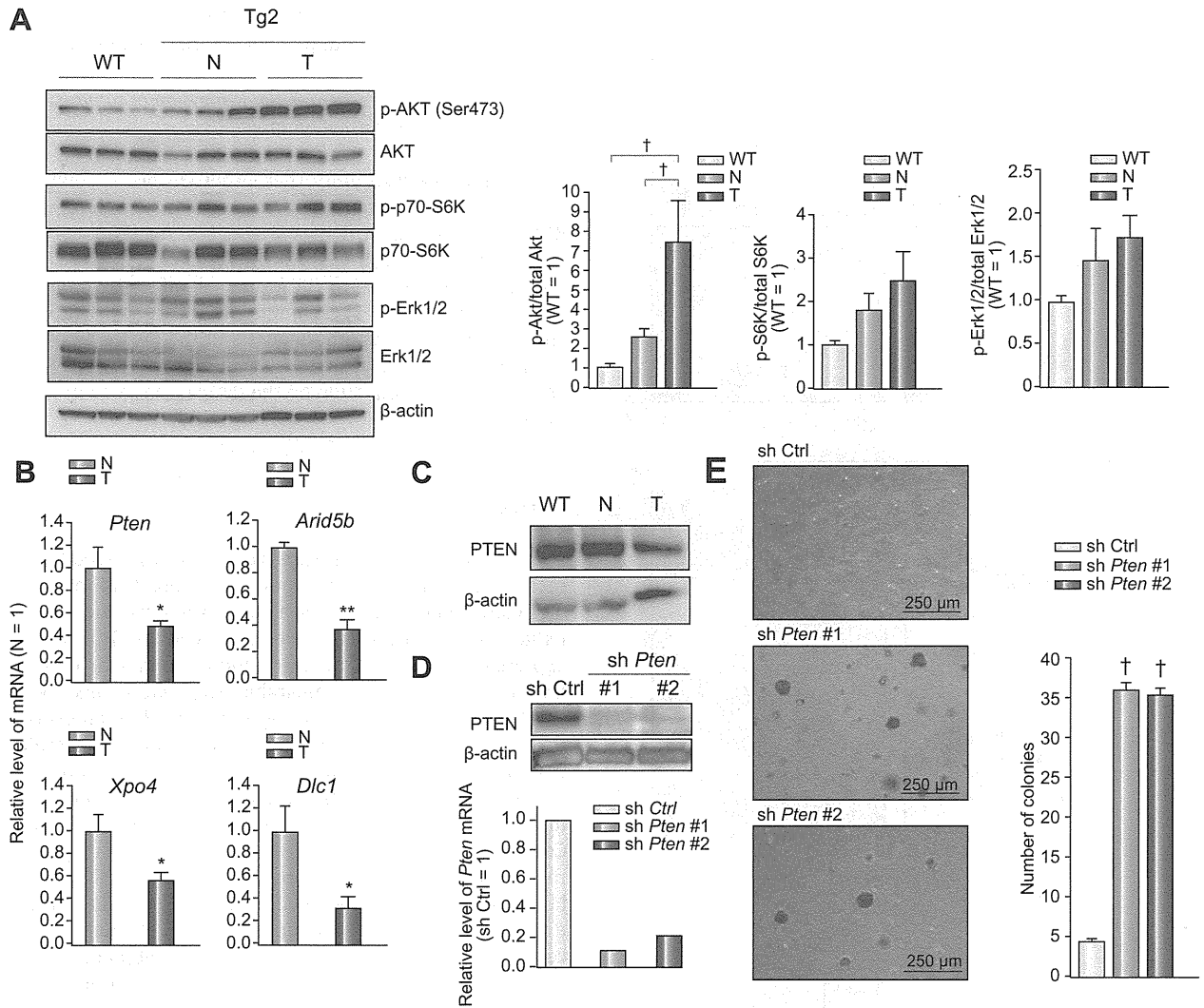
To examine the possibility that either OA or PA downregulates the expression of tumor suppressors including *Pten*, we treated BNL-CL2 cells with OA or PA. OA, but not PA, repressed the expression of *Pten*, *Arid5b*, *Xpo4*, and *Dlc1* (Fig. 6A). Moreover, BNL-CL2 cells exposed to OA formed significantly more colonies in soft agar (Fig. 6B). These findings indicate that OA potentially enhances the *in vivo* tumorigenesis in the *Pik3ca* Tg liver. As an example, it is likely that decreased PTEN expression could enhance the Akt activation by the *Pik3ca* transgene in Tg-derived tumors (Fig. 1B).

## Discussion

Hepatocyte-specific overexpression of *Pik3ca* (N1068fs\*4) leads to steatosis and hepatic tumor formation. This mutation was originally isolated in human HCC and gastric cancers [12], but its functional analysis has never been reported. The *in vitro* overexpression of this mutant clearly induced Akt activation, but the level of activation was comparable with that of *Pik3ca* wild type and lower than that of the oncogenic H1047R mutant, suggesting that the *Pik3ca* Tg mice provide a model for studying effects of PIK3CA overexpression rather than a gain-of-function of PIK3CA. Furthermore, the N1068fs\*4 mutation was not sufficient for cellular transformation *in vitro*, different from *Pik3ca* H1047R [40]. Considering results from a previous report suggesting the pivotal role of Akt activation in cell transformation by PIK3CA mutation [13], the activation level of Akt induced by *Pik3ca* (N1068fs\*4) expression should not be sufficient for the cell-transforming process. These data indicated that the development of hepatic tumors in Tg mice might not be always a direct effect of *Pik3ca* (N1068fs\*4) but instead promoted by other *in vivo* protumorigenic factors.

We focused on FA as an additional protumorigenic factor contributing to *in vivo* hepato-tumorigenesis in Tg mice, based on recent research on their oncogenic capacity [39]. Previous studies reported that OA inhibits *PTEN* expression via the upregulation of microRNA-21 through an mTOR/NF- $\kappa$ B-dependent mechanism [37,38] and also that exposure to OA increases tumor growth in xenografts [7]. Here, we demonstrated the correlation between OA accumulation and downregulation of other tumor suppressors, whereas the entire molecular mechanism remains to be elucidated. At least, there is a possibility that, in the Tg-derived tumors, OA accumulation enhanced the Akt activation by the *Pik3ca* transgene, which phosphorylates Akt less strongly than other oncogenic mutants *in vitro* (Fig. 1B, Supplementary Figs. 6 and 13).

Lipogenesis is mainly mediated by two major transcription factors, PPAR $\gamma$  and SREBP1C [24,25]. Hepatocyte-specific *Pten* KO mice exhibited increased expression of both PPAR $\gamma$  and SREBP1c in the liver, whereas only PPAR $\gamma$  was highly expressed in the *Pik3ca* Tg liver [16]. Our *in vitro* data suggested that the PI3K signaling is upstream of the activation of PPAR $\gamma$  in hepatocytes. A recent study shows that levels of PPAR $\gamma$  as well as SREBP1c mRNA are higher in the livers of patients with steatosis



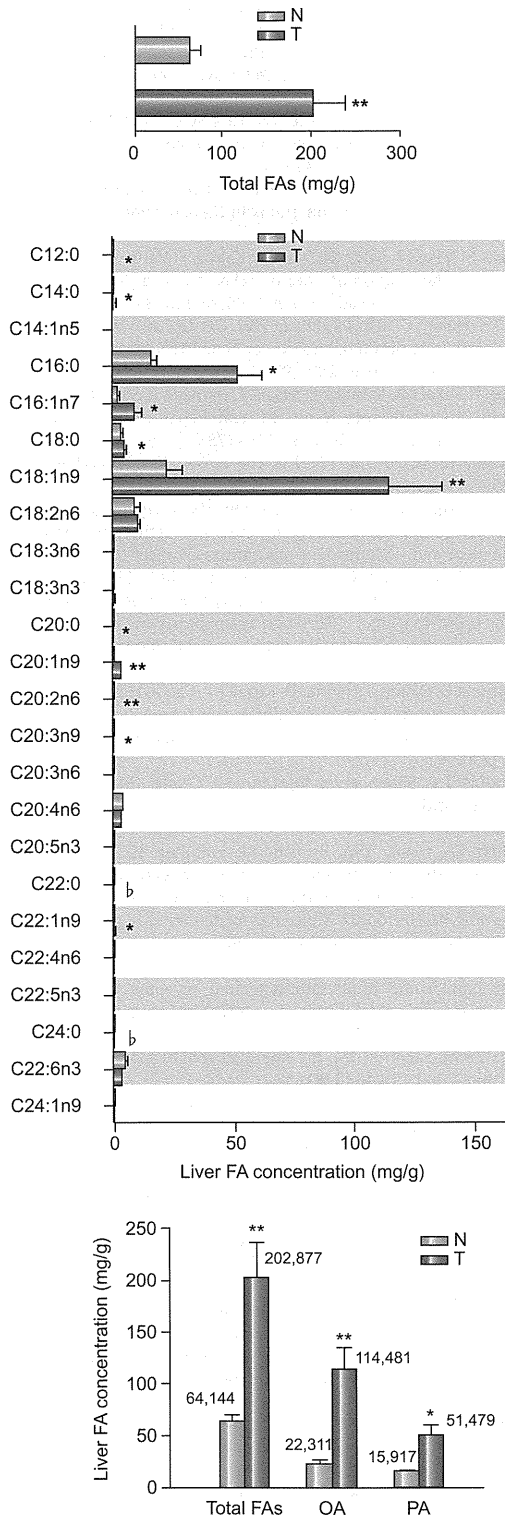
**Fig. 4. *Pten* downregulation in the *Pik3ca* Tg liver.** (A) Immunoblots and quantification of liver homogenates at 52 weeks ( $\dagger p < 0.05$ , ANOVA; post hoc test with WT). (B) The decreased expression of *Pten*, *Arid5b*, *Xpo4*, and *Dlc1* mRNA in the *Pik3ca* Tg liver tumors (T) relative to their expression in background liver tissues (N) (N = 5/group; \* $p < 0.05$ , \*\* $p < 0.01$ , Student's *t*-test). (C) Representative images of immunoblots of liver tissues from the littermates at 52 weeks. (D) Knockdown of *Pten* in BNL-CL2 cells confirmed at the protein (top) and mRNA (bottom) levels. (E) Both lines of *Pten*-depleted BNL-CL2 cells (sh*Pten* #1 and #2) formed more colonies in soft agar (N = 3/group;  $\dagger p < 0.05$ , ANOVA; post hoc test with control cells (shCtrl)).

or steatohepatitis, suggesting that the activity of PPAR $\gamma$  is implicated in the abnormal lipid accumulation in human livers [41] (Supplementary Fig. 13).

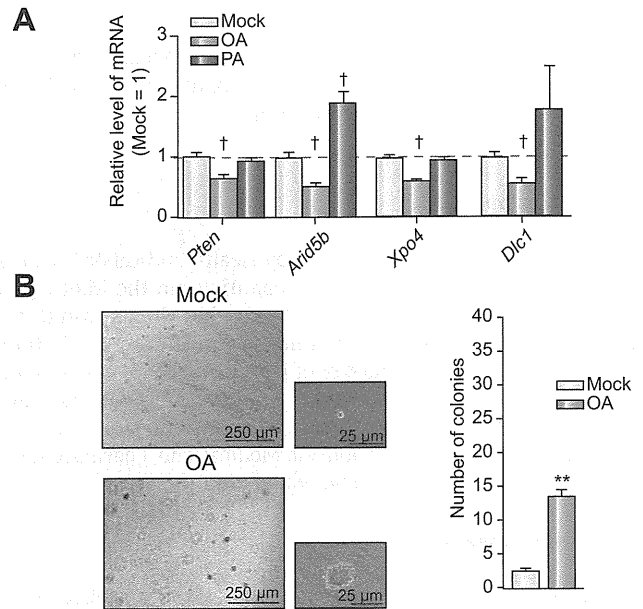
Unlike the hepatocyte-specific *Pten* KO mice [16], cellular infiltration and fibrosis were not observed in the *Pik3ca* Tg liver. One explanation is the possibility that *Pten* deficiency induces certain pathological mechanisms independently of PI3K-Akt activation, as previously reported for mammary tumorigenesis [18–20,42–45]. Indeed, although genetic changes in PTEN result in potent Akt phosphorylation, *in vivo* studies have suggested that they show distinct phenotypes [42]. The conditional knockout of PTEN enhanced tumorigenesis in the mammary gland [43]; however, transgenic mice expressing constitutively active Akt in the mammary gland did not show tumor formation [44]. PTEN directly associates with p53, thereby increasing its stability, protein level, and transcriptional activity [18,19]. PTEN induces apoptosis and cell cycle arrest through PI3K/Akt-independent pathways [20]. PTEN also has important roles in integrin signal-

ing and has the ability to dephosphorylate focal adhesion kinase, reducing cell adhesion and enhancing migration [46]. These findings support an alternative mechanism of PTEN-mediated tumorigenesis independent on PI3K/Akt pathway. As a second reason for the difference from *Pten* KO mice, it is possible that PI3K catalytic beta has a distinct role with PIK3CA in the phenotype of *Pten* deficiency [47].

The discrepancy between the scarce inflammatory levels in the *Pik3ca* Tg liver and the strong increase in serum ALT levels indicative of severe liver injury is to be solved in the near future. We found that inflammatory cytokine IL-1 $\alpha$  and Fas ligand were more highly expressed in the *Pik3ca* Tg liver than in the WT liver (Supplementary Fig. 4). Taking into account reports demonstrating that these factors can lead to liver damage [28,29], it can be suggested that their abnormal upregulation in Tg livers is in part responsible for liver damage, whereas the entire molecular process inducing them remains unknown (Supplementary Fig. 13).



**Fig. 5. The total FA composition in the *Pik3ca* Tg liver tissues and tumors.** (A) The levels of FAs in the tumor (T) and non-tumor background tissue (N) in *Pik3ca* Tg mice at 52 weeks (N = 4/group; \*\**p* < 0.01, Student's *t*-test). (B) FA composition in background (N) and tumor tissues (T) (N = 4/group; statistically increased FA levels in the tumors are shown with asterisks (\**p* < 0.05, \*\**p* < 0.01) and significantly decreased levels are shown with flat ( $\mu$ , *p* < 0.05), Student's *t*-test). (C) The concentration of total FAs, OA, and PA in background (N) and tumor tissues (T) (N = 4/group; \**p* < 0.05, \*\**p* < 0.01, Student's *t*-test).



**Fig. 6. OA enhances the colony-forming activity of immortalized hepatocytes.** (A) OA but not PA decreased *Pten*, *Arid5b*, *Xpo4*, and *Dlc1* mRNA *in vitro* (N = 3/group; †*p* < 0.05, ANOVA; post hoc test with Mock group). (B) Colony formation assay of BNL-CL2 cells with or without 50  $\mu$ mol/L OA in 10% or 0.5% FBS media (N = 3/group; \*\**p* < 0.01, Student's *t*-test).

Mechanisms involved in the pathogenesis of non-alcoholic steatohepatitis (NASH) remain unclear, but the “two-hit theory” is widely accepted [48]. That is, in the first hit, insulin-resistance is followed by lipid accumulation in the liver, and the second hit, possibly involving inflammatory cytokines or oxidative stress, results in hepatic injury and fibrosis. It has been reported that ROS has certain roles in *in vivo* carcinogenesis [35], and the concentration of ROS is upregulated in the liver suffering NASH or NASH-derived HCC [49]. Regardless of the obvious fatty liver, our model mice have not shown impaired glucose tolerance. The concentration of ROS in the *Pik3ca* Tg mice was comparable with that of WT mice (Supplementary Fig. 11), which can be partly explained by the lower expression of fat-oxidative genes (Fig. 2F) and lack of inflammatory cell infiltration. These findings indicate that *Pik3ca* Tg mice do not always mimic the entire pathological mechanisms causing NASH, while they might be useful as a prototype to determine which pathological processes are required for the progression from the fatty liver to NASH. In addition, given the low rate of HCC development in these mice, they can be potentially useful for discovering tumor-promoting factors in hepatic steatosis. For example, although it was unlikely that ROS is involved in the initiation of hepatic tumor in the *Pik3ca* Tg liver, we can examine the pathological significance of ROS in tumor progression as well as hepatitis induction by applying the *Pik3ca* Tg liver to the condition producing high levels of ROS.

Recent clinical findings have advocated the relationship between volume of visceral fat and tumor progression [1–4]. While there is no direct molecular evidence to address the notion that abnormal body fat accumulation accelerates tumor growth, our data might provide new insights into the mechanisms of the “lipotoxicity-related” tumorigenesis. Future researches are needed to unravel how OA affects gene expression.

**Conflict of interest**

The authors who have taken part in this study declared that they do not have anything to disclose regarding funding or conflict of interest with respect to this manuscript.

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**Supplementary data**

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jhep.2011.03.025.

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