

Original Article

Expression of Perilipin and Adipophilin in Nonalcoholic Fatty Liver Disease; Relevance to Oxidative Injury and Hepatocyte Ballooning

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Aims: Perilipin and adipophilin, PAT family proteins, play important roles in lipid metabolism. Although nonalcoholic fatty liver disease (NAFLD) is initiated by hepatocyte lipidation, little is known about the relationship between these proteins and hepatocellular injury. We investigated the expressions of perilipin and adipophilin and their relation to inflammation, fibrosis, hepatocellular ballooning, and oxidized phosphatidylcholine (oxPC) localization in human NAFLD.

Methods and Results: Liver biopsies of nonalcoholic steatohepatitis (NASH, $n=39$) or simple steatosis ($n=9$) were studied by immunohistochemical techniques using anti-perilipin, anti-adipophilin and anti-oxPC antibodies. The severity of liver damage was histologically assessed by the Brunt system and NAFLD activity score (NAS). Enlarged hepatocytes usually containing Mallory-Denk bodies were defined as ballooned. Perilipin and adipophilin were detected on the rim of lipid droplets in both NASH and simple steatosis. Perilipin was more evident in larger lipid droplets while adipophilin expression was frequent in lipid droplets of ballooned hepatocytes. The frequency of adipophilin-positive ballooned hepatocytes was correlated to inflammation ($R_s=0.72$, $p<0.0001$), fibrosis ($R_s=0.46$, $p=0.005$), NAS ($R_s=0.47$, $p=0.004$) and oxPC-positive ballooned hepatocytes ($R_s=0.35$, $p=0.033$).

Conclusions: Expression patterns of perilipin and adipophilin in NASH livers varied with the size of lipid droplets. In partiew or, adipophilin expression in ballooned hepatocytes was closely associated with oxidative damage.

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Key words; Lipid droplet, PAT family protein, Oxidized phosphatidylcholine, Nonalcoholic steatohepatitis

Introduction

Nonalcoholic fatty liver disease (NAFLD) has a

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spectrum ranging from simple steatosis to potentially progressive nonalcoholic steatohepatitis (NASH)¹⁻⁴. The widely accepted two-hit theory involves first the development of steatosis and second the occurrence of oxidative injury⁵⁻⁷. We previously demonstrated that a form of oxidized phospholipid, oxidized phosphatidylcholine (oxPC), localizes to the phospholipid monolayer surrounding fat droplets and to the endoplasmic reticulum in ballooned hepatocytes in human NASH

Table 1. Clinical Characteristics of the Patients

	Simple steatosis (n=9)	NASH (n=39)	p value
Age (years) ^a	51.0 ± 12.4	56.6 ± 13.7	0.263
Gender (F/M)	5/4	26/13	0.702
BMI (kg/m ²) ^a	24.0 ± 3.7	27.2 ± 3.8	0.024
Platelet count (/×10 ⁴ mL) ^a	24.0 ± 6.3	20.3 ± 8.8	0.236
Albumin (g/dL)	4.1 ± 0.5	4.2 ± 0.5	0.556
AST (IU/L) ^b	62 (24–249)	74 (33–332)	0.601
ALT (IU/L) ^b	93 (19–394)	106 (24–368)	0.813
Alkaline phosphatase (IU/L) ^a	185.1 ± 87.7	217.7 ± 65.9	0.215
Total bililubin (mg/dL) ^a	0.84 ± 0.27	0.94 ± 0.37	0.451
Total cholesterol (mg/dL) ^a	247.9 ± 49.8	212.5 ± 39.1	0.025
Triglyceride (mg/dL) ^b	159 (52–253)	125 (52–433)	0.931
Fasting plasma glucose (mg/dL) ^a	96.8 ± 8.8	120.8 ± 47.4	0.140
Prothrombin time INR ^a	1.00 ± 0.06	1.06 ± 0.17	0.276

BMI, body mass index; AST, aspartate aminotransferase; ALT, alanine aminotransferase; INR, international normalized ratio.

^aMean ± SD, ^bMedian (range), ^cNumber (%).

and thus may be involved in hepatocyte injury⁷).

The PAT family proteins, perilipin, adipophilin and tail-interacting protein of 47 kDa (TIP47), are amphiphilic proteins associated with the phospholipid monolayer surrounding lipid droplets which play a role in their maturation and metabolism, including hormone sensitive lipase-induced lipolysis^{8, 9}). In adipocytes, TIP47 and adipophilin are expressed on small, immature lipid droplets, while perilipin is expressed on larger, more mature lipid droplets¹⁰). Recent work demonstrates that these proteins also play a role in human NAFLD. Straub et al. reported the expression of PAT lipid droplet-associated proteins in human fatty liver¹¹). Using immunohistochemical staining supported by biological and molecular methods, they demonstrated a positive correlation between both adipophilin and perilipin expression and hepatocyte lipid droplet content, regional variation within the lobule and co-localization of perilipin and adipophilin in larger perivenular fat droplets. No difference in expression pattern was seen between NASH and simple steatosis.

In the present study, we have further examined the expression patterns of adipophilin and perilipin in the relationship to oxidized phosphatidylcholine and cellular ballooning in human NASH. Our work extends that of Straub *et al.* and unites changes in PAT protein expression with oxidative injury in the phospholipid monolayer surrounding the droplets although the mechanisms involve remain to be elucidated.

Materials and Methods

Liver Specimens

Liver biopsy specimens obtained from 39 patients with NASH and 9 patients with simple steatosis in Osaka City University Hospital from January 1998 to February 2006 were the main subjects of the present investigation. Informed consent was given by all patients, and the study was approved by the ethics committee of Osaka City University Hospital.

The presence of steatosis > 5% was confirmed in all cases. The diagnosis of NASH was based on the following: (1) histological features of steatohepatitis, including fibrosis and hepatocellular ballooning, (2) an absence of clinically significant alcohol consumption (20 g/day assessed clinically), and (3) no other identifiable causes of liver diseases, including drug-induced hepatotoxicity, hepatitis B or hepatitis C virus, autoimmune liver diseases, Wilson's disease, hemochromatosis or alpha-1-antitrypsin deficiency. The clinical characteristics and laboratory test results of the patients studied are summarized in **Table 1**. Body mass indices (BMI) were significantly ($p=0.024$) higher in patients with NASH (27.2 ± 3.8 kg/m²) than in patients with simple steatosis (24.0 ± 3.7 kg/m²).

The liver specimens were fixed in 10% buffered formalin and embedded in paraffin blocks. At least 10 consecutive sections were cut from each block. The first two sections were stained with hematoxylin-eosin and Azan-Mallory staining for histological evaluation, and the others were subjected to immunohistochemical investigations.

Conventional Histopathological Evaluations

Hematoxylin-eosin and Azan-Mallory-stained sections were observed under a light microscope, and disease severity of NAFLD/NASH was assessed according to the grading/staging system of Brunt *et al.*¹²⁾, and the NAFLD Activity Score (NAS) of Kleiner *et al.*¹³⁾.

Immunohistochemistry

Guinea pig polyclonal antibodies against adipophilin (GP40; 1:200 dilution) and perilipin (GP29; 1:500 dilution) and a mouse monoclonal antibody against adipophilin (AP125; 1:20 dilution) were purchased from Progen Biotechnik (Heidelberg, Germany) and used as primary antibodies. Oxidatively damaged hepatocytes were identified by an anti-oxPC mouse monoclonal antibody, DLH3 (1:50 dilution), which had been originally designated to recognize oxPC molecules formed in oxidized low-density lipoprotein accumulated in atherosclerotic lesions^{14,15)}.

Liver sections (5 μm thick) were deparaffinized in xylene and rehydrated through graded ethanol. The sections were heated in a microwave oven for 10 min for antigen retrieval. Endogenous peroxidase activity was quenched by incubation in 0.3% H_2O_2 containing methanol for 30 min. After washing with phosphate-buffered saline (PBS) three times, the sections were pre-incubated with 10% fetal bovine serum for 10 minutes at room temperature to block non-specific binding. The sections were then incubated with a primary antibody for 60 min at room temperature and rinsed in PBS three times. Subsequently, the sections were incubated with biotinylated anti-guinea pig (Progen) or anti-mouse antibody (Vector Laboratories, Burlingame, CA) for 30 min at room temperature, followed by conjugation with peroxidase-labeled streptavidin (Vector Laboratories) for 30 min. Enzymatic activity of peroxidase was visualized with 0.25 mg/mL 3,3'-diaminobenzidine tetrahydrochloride in the presence of 0.003% hydrogen peroxide in 0.05 M Tris-buffered saline at pH 7.4. Finally, the sections were faintly counterstained with hematoxylin, and observed under a light microscope. Human omental fatty tissue obtained at autopsy and liver tissue of chronic hepatitis B without steatosis were used as positive and negative control slides, respectively. Staining in ballooned hepatocytes in NASH livers was evaluated by scoring from 0 to 2: 0, negative; 1, ≤ 5 positive ballooned hepatocytes/10 mm^2 ; and 2, > 5 positive ballooned hepatocytes/10 mm^2 .

Double Staining

The mouse monoclonal antibody against adipophilin (AP125) and the guinea pig polyclonal anti-

body against perilipin were applied as primary antibodies. Subsequently, the sections were incubated with an Alexa Fluor 488-conjugated anti-mouse IgG antibody and an Alexa Fluor 594-conjugated anti-guinea pig IgG antibody (both from Invitrogen Molecular Probes, Eugene, OR) for 1h at room temperature, and observed under a fluorescent microscope. Images of green fluorescence (Alexa 488) and red fluorescence (Alexa 594) were separately incorporated into a personal computer and superimposed using Adobe Photoshop software (Adobe Systems Inc., San Jose, CA). A double-positive result was shown as yellow fluorescence.

OxPC and adipophilin colocalization was studied with the combination of the mouse monoclonal anti-oxPC antibody (DLH 3) and the guinea pig polyclonal anti-adipophilin antibody (GP40). The immunoreactivities of the primary antibodies on liver tissues were visualized by enzyme reaction [horseradish peroxidase (red) for oxPC and alkaline phosphatase (blue) for adipophilin]-based techniques.

Statistics

Statistical analyses were performed using Stat-View software (Abacus Concepts, Berkeley, CA). Differences between groups were tested by the Mann-Whitney *U*-test. Associations between two parameters were evaluated using the Spearman rank correlation coefficient (R_s value). *P* values < 0.05 were considered significant.

Results

Clinical and Histopathological Evaluations

All samples had more than 5% steatosis. With simple steatosis, fibrosis and hepatocellular ballooning were absent, and mild inflammatory infiltration was seen in a few cases (mean NAS 1.8 ± 1.1). In contrast, the liver tissues of NASH showed marked inflammation (mean grade 1.9 ± 0.7) with hepatocellular ballooning (defined as enlarged hepatocytes with rarefied cytoplasm and usually with Mallory-Denk bodies) and fibrosis (mean stage 2.3 ± 1.0). The NAS of all NASH samples were ≥ 3 points (mean NAS 5.2 ± 1.2). Clinically, a high inflammatory grade and NAS were related to elevated serum levels of aspartate aminotransferase (AST; $R_s = 0.32$, $p = 0.05$). Fibrosis stage scores were inversely correlated with platelet counts in peripheral blood ($R_s = -0.62$, $p = 0.0001$).

Perilipin and Adipophilin Expressions in Lipid Droplets

Consistent with previously reported expression

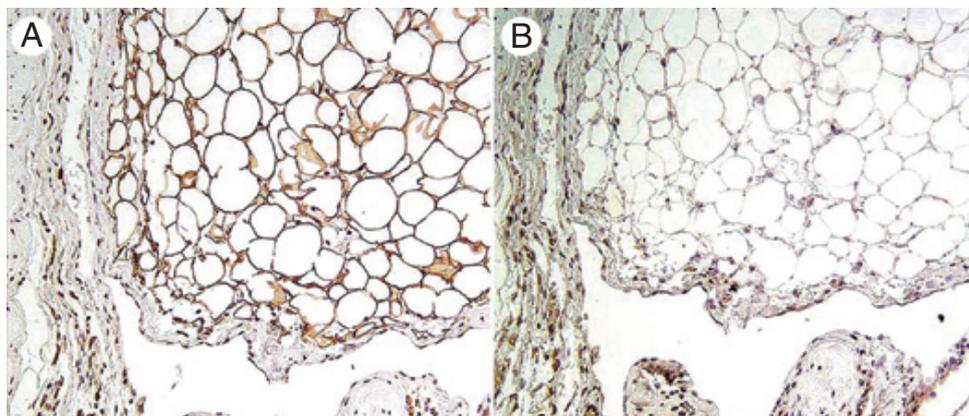


Fig. 1. Immunolocalization of perlipin (A) and adipophilin (B) in human omentum.

(A) Perlipin expression is seen on the rim of lipid droplets in mature adipocytes. (B) Adipophilin expression is not seen in mature adipocytes but in peritoneal macrophages with foamy, microvesicular, lipid droplets [Immunoperoxidase staining; original magnification, (A, B) $\times 160$].

patterns in adipose tissue⁸⁻¹⁰), perlipin was detected on the rim of lipid droplets in mature adipocytes in the omentum fatty tissue (**Fig. 1A**) while adipophilin was seen in peritoneal macrophages with foamy cytoplasmic appearance (**Fig. 1B**). The liver tissue of chronic hepatitis B without steatosis showed neither perlipin nor adipophilin expression (not shown).

Adipophilin and perlipin expressions were detected on the rim of lipid droplets in steatotic hepatocytes in both NASH and simple steatosis (**Fig. 2A, B**). Adipophilin expression was predominantly in smaller lipid droplets compared to larger perlipin-stained droplets (**Fig. 2B**) although some very large droplets were negative for both proteins and other mid-size droplets were positive for both proteins on double staining (**Fig. 2C**). Adipophilin-positive small lipid droplets were more frequently seen in NASH than simple steatosis although this did not reach significance. Ballooned hepatocytes in NASH frequently stained for adipophilin on the rim of cytoplasmic fat droplets (**Fig. 2D**). Consistent with a relationship to cellular injury, the adipophilin-positive ballooned hepatocyte score correlated to both the Brunt system [inflammatory grade ($R_s=0.72$, $p<0.0001$); fibrosis stage ($R_s=0.46$, $p=0.005$)] and NAS system ($R_s=0.47$, $p=0.004$) (**Fig. 3A-C**).

OxPC Localization and Adipophilin Expression in Ballooned Hepatocytes

As in our previous report⁷), oxPC localization was seen mainly in steatotic and ballooned hepatocytes as well as macrophages in NASH (**Fig. 4A**). The presence of oxPC-positive ballooned hepatocytes correlated with serum AST ($R_s=0.40$, $p=0.013$), higher

inflammatory grade ($R_s=0.32$, $p=0.046$) and advanced fibrosis stage ($R_s=0.31$, $p=0.056$). Consistent with these relationships to disease severity and the similar relationship seen with adipophilin-positive ballooned cells, the oxPC-positive ballooned hepatocyte scores also correlated to that of adipophilin-positive ballooned hepatocytes ($R_s=0.35$, $p=0.033$) (**Fig. 4B**).

Immunodouble staining demonstrated that many ballooned hepatocytes were positive for oxPC and/or adipophilin (**Fig. 5A, B**). These two molecules were colocalized/coexpressed in some but not all ballooned hepatocytes (**Fig. 5C, D**). Qualitatively, adipophilin-positive but oxPC-negative ballooned hepatocytes frequently appeared more severely injured than adipophilin-positive and oxPC-positive ballooned hepatocytes (**Fig. 5E**).

Discussion

As previously reported by Straub *et al.*¹¹), we found that two principal members of the PAT family of lipid droplet associated proteins (perlipin and adipophilin) are commonly expressed in human NAFLD. Consistent with their study and prior publications⁸⁻¹¹), the pattern of their expression was influenced by the size of the lipid droplets, suggesting a relationship to maturation of the lipid droplet. However, our results indicate that the severity of liver disease also appears to influence the expression of proteins and that there is an as yet unexplained relationship to cellular ballooning and to the presence of oxidized phospholipids on the rim of small droplets. Adipophilin expression was dominant in small lipid droplets, especially in ballooned hepatocytes in NASH, and the frequency of

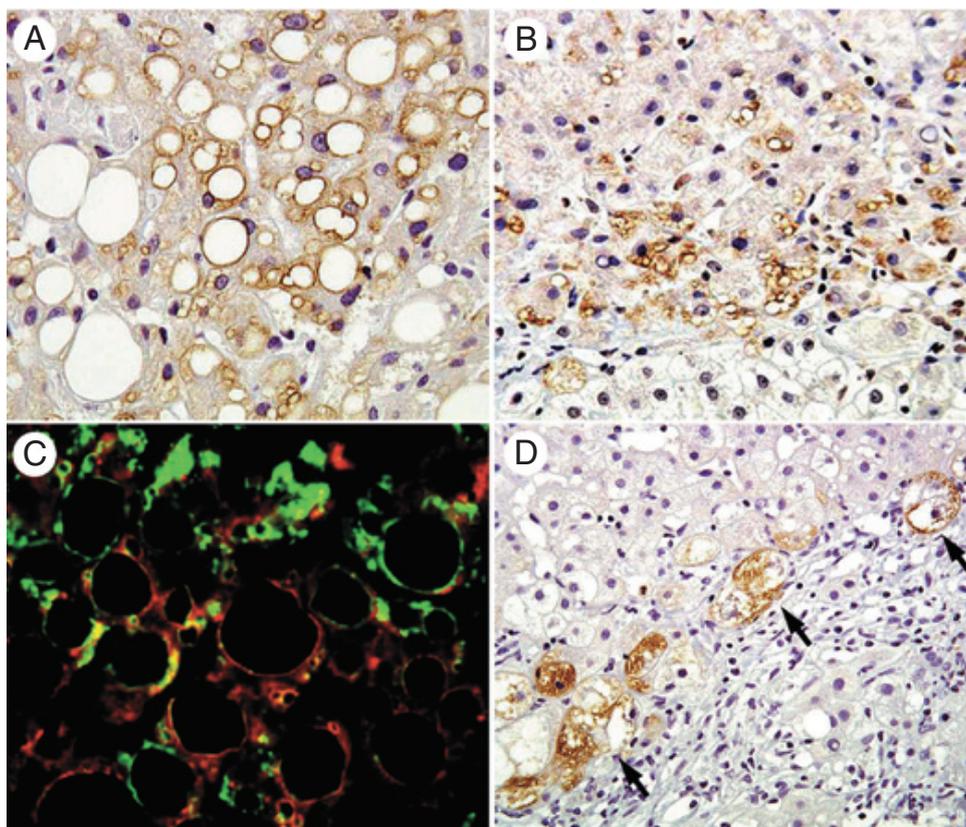


Fig. 2. Immunolocalization of perilipin (A) and adipophilin (B) in human liver tissue of NASH.

(A) Perilipin expression is seen in small to large lipid droplets in steatotic hepatocytes. (B) Adipophilin expression is clearly shown in small lipid droplets. (C) Immunofluorescent double staining demonstrates that perilipin (red fluorescent) and adipophilin (green fluorescent) expressions are not fully overlapped. (D) Adipophilin expression is prominent in ballooned hepatocytes (arrows) [Immunoperoxidase staining (A, B, and D) and immunofluorescent double staining (C); original magnification, (A-C) $\times 300$, and (D) $\times 200$].

adipophilin-positive ballooned hepatocytes correlated to the inflammatory grade, fibrosis stage, and the frequency of oxPC-positive ballooned cells.

The complex biology of hepatocyte lipid droplets, whether destined for secretion as very low-density lipoprotein or retained as storage triglyceride, begins with their formation through lipidation of the endoplasmic reticulum by microsomal triacylglycerol transfer protein^{16, 17}. Accumulation of triglyceride, the chief matrix of the lipid droplets, in hepatocytes is accelerated under diabetic or hypertriglyceridemic conditions without appropriate treatments^{18, 19}. At some early point in this process, the droplets normally become associated with proteins of the PAT family⁸⁻¹⁰. These proteins serve to regulate lipolysis of the droplet triglyceride in response to insulin and adrenergic stimulation and perhaps to anchor or move the droplets within the cell cytoskeleton²⁰. Growth of the

droplets involves fusion, which may trap PAT proteins within the enlarging droplet²¹. In adipose tissue, it is normally also associated with changes in surface PAT expression and perhaps with changes in insulin sensitivity.

Prior studies have demonstrated the expression of PAT family proteins in adipose tissue, steroidogenic cells, mammary gland tissue and in alcohol-related liver disease^{8, 22}. The potential relationships between these proteins and NAFLD have been explored experimentally. Chang *et al.*²³ showed that adipophilin-deficient mice display a 60% reduction in hepatic triglyceride and are resistant to diet-induced fatty liver. Imai *et al.*²⁴ demonstrated that a reduction in hepatic adipophilin level using an antisense oligonucleotide reverses hepatic steatosis, hyperglycemia, and insulin resistance in obese mice. In addition, breeding perilipin null alleles into Leptin knock out (db/db) mice

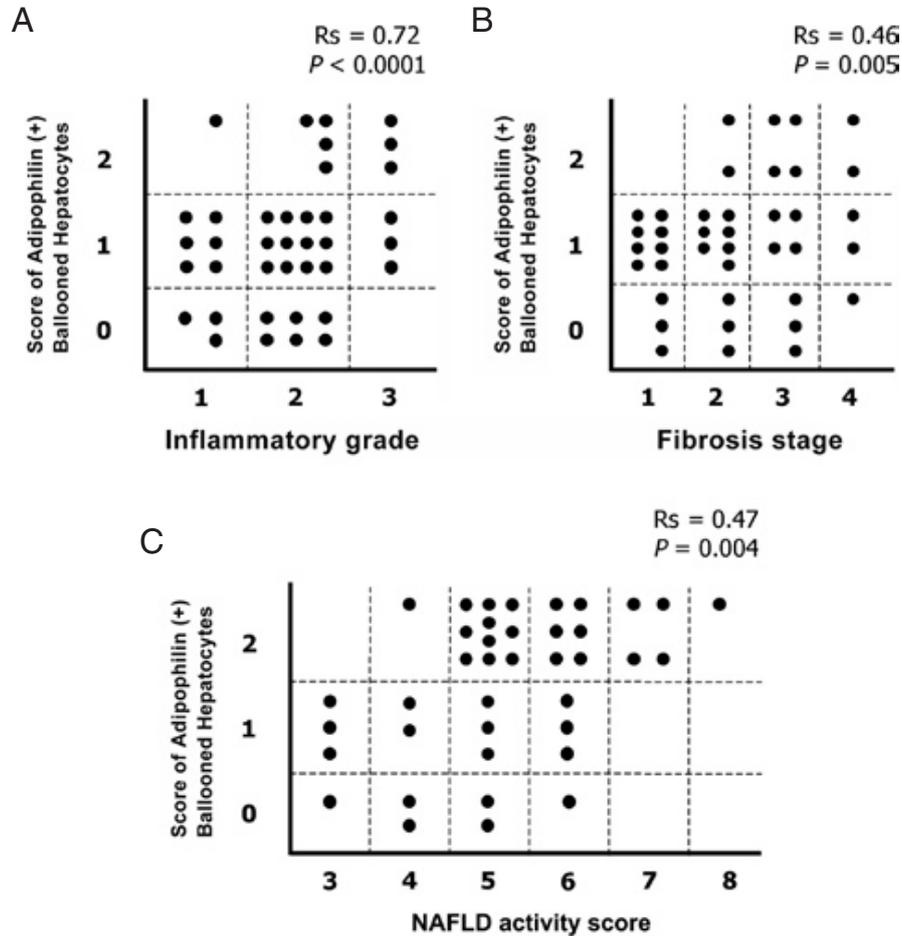


Fig. 3. Relationship between the number of adipophilin-positive ballooned hepatocytes and histopathological disease severity of NASH.

The increase in adipophilin-positive ballooned hepatocytes is associated with both high inflammatory grade (A; $R_s=0.72$, $p<0.0001$) and severe fibrosis stage (B; $R_s=0.46$, $p=0.005$). The adipophilin-positive ballooned hepatocyte score is also correlated to the NAFLD activity score (NAS) (C; $R_s=0.47$, $p=0.004$).

reverses obesity by increasing the metabolic rate of mice²⁵). Alternatively, a recent study revealed that these proteins play important roles in maintaining the size and number of hepatocyte lipid droplets²⁶). This indicates the complex nature of PAT family proteins; they are not only necessary for the formation of lipid droplets in hepatocytes, but also for stable lipid droplet metabolism, which may prevent further hepatocellular damage and consequently disease progression of NAFLD.

More recently, Straub *et al.*¹¹) reported the expression of all three members of the PAT family proteins in human NAFLD and confirmed the results of immunohistochemistry using immunoblot techniques. Their study demonstrated that adipophilin and perilipin but not TIP47 expression correlated to the degree

of steatosis. As with prior studies of non-liver tissue⁸⁻¹⁰) and as seen in the current study, different expression patterns of these proteins were seen depending on the size of the lipid droplets, suggesting a relationship with droplet maturity. TIP47 was evident in the smallest lipid droplets while adipophilin and perilipin were detected in larger droplets and were frequently colocalized. Although we did not examine TIP47, their results regarding expression patterns of adipophilin and perilipin contrasted in some respects with our results where we noted perilipin staining usually in larger droplets compared to adipophilin. The explanation for this is not clear but may relate to differences in the clinicopathological backgrounds and pathophysiological bases of NAFLD between Japanese and Western patients^{27, 28}). According to our experience of

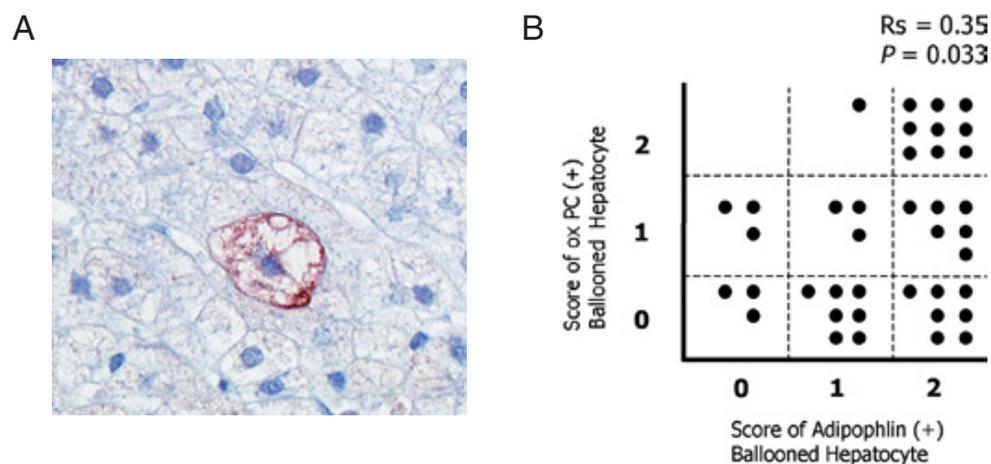


Fig. 4. OxPC localization in ballooned hepatocytes and its relation to adipophilin expression in ballooned hepatocytes.

(A) OxPC localization is seen in ballooned hepatocytes. (Immunoperoxidase staining for oxPC; original magnification, $\times 400$). (B) The score of oxPC-positive ballooned hepatocytes is correlated to that of adipophilin-positive ballooned hepatocytes ($R_s = 0.35$, $p = 0.033$).

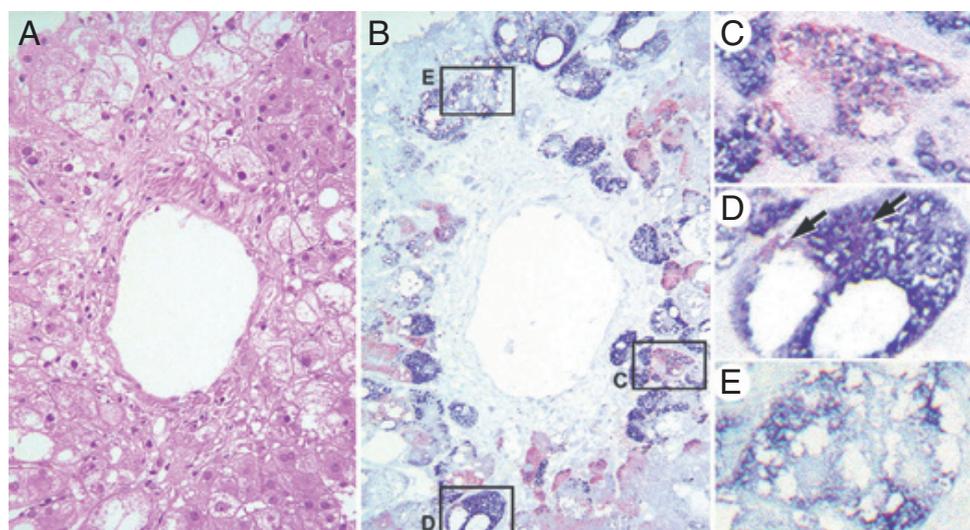


Fig. 5. The colocalization/coexpression of adipophilin and oxPC in ballooned hepatocytes.

(A) Hematoxylin-eosin-stained section of NASH liver with marked ballooning degeneration. (B) Immunohistochemical double staining for adipophilin (blue) and oxPC (red). Most ballooned hepatocytes are positive for adipophilin and/or oxPC. (C) A hepatocyte with ballooning degeneration but with preserved cytoplasmic granular appearance shows distinct double positivity. (D) In a moderately damaged hepatocyte, immunolabeling intensity is strong for adipophilin but weak for oxPC (arrows). (E) A severely damaged hepatocyte is positive for adipophilin but negative for oxPC [Original magnification, (A, B) $\times 200$ and (C-E) $\times 770$].

the comparative observation of pathologic slides of Japanese and American NAFLD cases, the histological features also seem to be slightly different between them; for example, ballooned hepatocytes tended to be larger and more lucent in American than Japanese cases. These differences might have introduced the

diversity of the immunohistochemical findings of PAT family protein expressions.

In the present study, both adipophilin-positive ballooned hepatocytes and oxPC-positive ballooned hepatocytes correlated to the degree of injury. Although their expression/localization in ballooned hepatocytes

were associated with each other, we qualitatively detected these two molecules in ballooned hepatocytes in slightly different patterns. Adipophilin expression and oxPC localization overlapped in some ballooned hepatocytes, but more severely injured hepatocytes showed only adipophilin positivity and did not express oxPC. The explanation for this is uncertain and awaits confirmation in further studies but it may represent digestive processing of oxidized lipids generated in adipophilin-positive hepatocytes. We previously demonstrated that oxPC accumulation in macrophages induces foamy cytoplasmic alteration and its intracellular processing/degradation in lysosomes¹⁵). Similar abnormal metabolic pathways related to oxidative injury may be present in damaged hepatocytes in NASH and may induce microvesicular lipid droplet formation and adipophilin expression. In the light of the importance and necessity of PAT family proteins in lipid droplet metabolism²⁶), it can be hypothesized that adipophilin expression in ballooned hepatocytes might be induced as a cellular protective reaction to prevent harmful effects from immature lipid droplets and to rescue damaged hepatocytes.

In conclusion, the present study confirmed that both perilipin and adipophilin are expressed in steatotic hepatocytes in human NASH; however, their expression patterns are dependent on the size of lipid droplets and related to the severity of disease and the degree of oxidative damage. Further studies are needed to understand the relationship between PAT family protein expression and oxidized phospholipids on the rim of lipid droplets and their relationship to cellular ballooning and disease progression.

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