NAFLD & NASH

Characteristics of hepatic fatty acid compositions in patients with nonalcoholic steatohepatitis

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Keywords
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Abbreviations
ACC, acetyl-CoA carboxylase; BMI, body mass index; ELOVL6, elongation of long-chain fatty acids family member 6; FAS, fatty acid synthase; HOMA-IR, homeostasis model assessments of insulin resistance; NAFLD, nonalcoholic fatty liver disease; NASH, nonalcoholic steatohepatitis; NAS, NAFLD activity score; PPAR, peroxisome proliferator-activated receptor; QUICKI, Quantitative Insulin Sensitivity Check Index; SCD, stearoyl-CoA desaturase; SREBP-1c, sterol regulatory element-binding protein-1c; SS, simple steatosis; T-CHO, total cholesterol; TG, triglyceride.

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Abstract
Background & Aims: Nonalcoholic fatty liver disease (NAFLD) is closely related to insulin resistance and lipid metabolism. Recent studies have suggested that the quality of fat accumulated in the liver is associated with the development of nonalcoholic steatohepatitis (NASH). In this study, we investigated the fatty acid composition in liver tissue and its association with the pathology in NAFLD patients. Methods: One hundred and three patients diagnosed with NAFLD [simple steatosis (SS): 63, NASH: 40] were examined and their hepatic fatty acids were measured using gas chromatography. In addition, relationships between the composition and composition ratios of various fatty acids and patient backgrounds, laboratory test values, histology of the liver, and expression of fat metabolism-related enzymes were investigated. Results: The C16:1n7 content, the C16:1n7/C16:0 and C18:1n9/C18:0 ratios were increased and the C18:0/C16:0 ratio was decreased in the NASH group. The C18:0/C16:0 and C18:1n9/C18:0 ratios were associated with the steatosis score in liver tissue, and the C16:1n7/C16:0 ratio was associated with the lobular inflammation score. The expressions levels of genes: SCD1, ELOVL6, SREBP1c, FAS and PPARγ were enhanced in the NASH group. In multivariate analysis, the C18:0/C16:0 ratio was the most important factor that was correlated with the steatosis score. In contrast, the C16:1n7/C16:0 ratio was correlated with lobular inflammation. Conclusion: The fatty acid composition in liver tissue and expression of genes related to fatty acid metabolism were different between the SS and NASH groups, suggesting that the acceleration of fatty acid metabolism is deeply involved in pathogenesis of NASH.

The number of patients with nonalcoholic fatty liver disease (NAFLD) has increased in Western countries and Asia, and the increase in obese people and changes in dietary life has become a major health issue (1, 2). NAFLD includes simple steatosis (SS) with a favourable prognosis and nonalcoholic steatohepatitis (NASH). NASH is considered to develop when an exacerbating factor is added to fat deposition in liver tissue, with oxidative stress, inflammatory cytokines and iron-related factor being attributed as causes of NASH (3–5). However, the detailed developmental mechanism for NASH has not been fully elucidated and no evidence-based treatment method has been established, although several drugs have been suggested to be effective (6–8). The prognosis is poor once the condition has progressed to NASH, and the incidence of liver-related death significantly increases with the progression to hepatic cirrhosis. Therefore, identifying factors that con-
tribute to the progression of SS to NASH is vitally important and a treatment method needs to be established to prevent its progression.

Previous studies have clarified that insulin resistance is closely involved in the development of NAFLD (9–11). On the other hand, it has recently been reported that the composition of fatty acids in liver tissue and the expression level of elongation of long-chain fatty acids family member 6 (ELOVL6), which regulates their composition, are factors determining insulin resistance (12), and reducing the activity of fatty acid desaturase, stearoyl-CoA desaturase 1 (SCD1), exacerbates hepatocellular disorders and liver tissue fibrosis (13). These reports have suggested an association between the development of NAFLD or NASH and the amount and composition ratios of fatty acids accumulated in the liver and the expression of enzymes regulating them. In a previous report on liver tissue fatty acids in NAFLD patients, the fatty acid composition was different from that in healthy subjects; however, the number of subjects was small and how these changes were associated with the clinical characteristics of NAFLD was not clarified (14).

Thus, in this study, we measured the fatty acid contents of liver tissue in 103 NAFLD patients, clarified the characteristics of the composition and composition ratio of these fatty acids, and investigated their association with the disease state and pathological changes. In addition, we analysed the gene expression of enzymes involved in fatty acid synthesis and degradation, which influence changes in the liver tissue fatty acid composition, and clarified their roles in the pathogenesis of NAFLD.

Materials and methods

Patients and laboratory testing

The subjects in this study were 103 patients diagnosed with NAFLD based on pathological examinations of liver tissue collected by ultrasound-guided percutaneous liver biopsies at our institution between December 1998 and September 2010. All patients were hepatitis B surface antigen (HBsAg) and hepatitis C virus antibody negative, and the volume of alcohol consumption per day was less than 20 g. A pathological evaluation was independently performed by two pathologists, and diagnoses were made based on Matteoni’s classification (15). Types 1 and 2 of this classification were defined as SS and types 3 and 4 were defined as NASH (SS: 63 patients, NASH: 40 patients). In all patients, three items of the NAFLD activity score (NAS; steatosis, lobular inflammation and hepatocellular ballooning) and fibrosis were also scored (16). In addition, 18 patients who underwent hepatectomy or autopsy for other diseases with no fibrosis or fatty changes on pathological examination of the liver or other chronic liver diseases were included as controls. The first biopsy sample was used in patients who underwent liver biopsies multiple times. All patients gave written informed consent to participate in the study in accordance with the Helsinki Declaration and this study was approved by the Regional Ethics Committee (Medical Ethics Committee of Kanazawa University, no. 829). The blood test findings of patients whose blood was collected in a fasting state on admission for liver biopsy were adopted.

Insulin resistance was evaluated based on homeostasis model assessments of insulin resistance (HOMA-IR) [(fasting serum insulin (μU/ml) \times fasting plasma glucose (mg/dl))/405] and the Quantitative Insulin Sensitivity Check Index ( QUICKI) [1/log (fasting serum insulin (μU/ml) \times fasting plasma glucose (mg/dl))/405] calculated from fasting-state blood glucose and insulin levels. In some patients (20 SS and 15 NASH patients), insulin resistance was also evaluated by performing the hyperinsulinaemic–euglycaemic clamp (17).

Fatty acid extraction

Liver specimens collected by percutaneous liver biopsy or hepatectomy were used. The wet weight of the liver specimen was measured, and fatty acids were extracted as follows: The liver specimen was placed in KOH methanol solution, combined with 100 μl of pentadecanoic acid methanol solution as an internal reference, and saponified by heating at 100°C for 30 min. After acidifying the solution with 1 N aqueous hydrochloric acid solution, fatty acids were extracted by adding hexane as a solvent, followed by methyl esterification using 14% BF3 methanol solution (P/N1022-12002, GL Sciences, Tokyo, Japan).

Measurement and analysis of liver tissue fatty acids

Extracted fatty acids were identified and quantified by gas chromatography using a Shimadzu, Kyoto, Japan Gas Chromatograph GC-2014AF/SPL and Rtx-2330 column. Chromatographs were analysed using GC solution version 2.3. (Shimadzu Corporation, Kyoto, Japan) The external reference method was employed for the identification and quantitative analysis of fatty acids using TM37Component FAME Mix 47885-U of Supelco (Sigma–Aldrich, St. Louis, MO, USA) as a reference solution. The liver tissue fatty acid content was quantified as an amount per 1 mg of wet liver tissue, and differences in the fatty acid content and composition ratio among the Control, SS and NASH groups were investigated. In this study, n-6 fatty acids were calculated by the sum of C18n2n6, 20:3n6 and 20:4n6, while n-3 fatty acids were calculated by the sum of C18n3n3 and C22:6n3. In addition, the association between physical and blood data and the pathological findings of patients with fatty acids were evaluated. To investigate the association of fatty acid-synthesizing enzymes, the substrate:product fatty acid ratio was determined, and differences among the groups and in the pathological characteristics were evaluated.
Quantitative real-time detection-PCR

We performed quantitative real-time detection (RTD)-PCR using TaqMan Universal Master Mix (PE Applied Biosystems, Foster City, CA, USA). Primer pairs and probes for SCD, ELOVL6, SREBF1, FASN, ACACA, PPARA, PPARG and GAPDH were obtained from the TaqMan assay reagent library. Total RNA was isolated from liver tissue samples using an RNA extraction kit (Micro RNA Extraction Kit; Stratagene, La Jolla, CA, USA). We reverse-transcribed 1 μg of isolated RNA to cDNA using SuperScript® II RT (Invitrogen, Carlsbad, CA, USA) according to the manufacturer’s instructions, and the resultant cDNA was amplified with appropriate TaqMan assay reagents as previously described (18).

Statistical analysis

Data are expressed as the mean ± SEM. Differences in the clinical features and amount of fatty acids among the three groups consisting of controls, patients with SS and patients with NASH were analysed for significance by Mann–Whitney’s U-test, Spearman’s rank correlation, and single and multiple regression analysis. A level of P < 0.05 was considered significant.

Table 1. Characteristics of the study population

| Variable | Control (n = 18) | SS (n = 63) | NASH (n = 40) |
|----------|-----------------|------------|--------------|
| Gender M/F | 10/8 | 37/26 | 19/21 |
| Age (years) | 62.8 ± 3.9 | 46.1 ± 1.9* | 52.2 ± 2.7* |
| Height (cm) | 160.1 ± 2.5 | 162.2 ± 1.3 | 160.5 ± 1.6 |
| Weight (kg) | 53.7 ± 2.3 | 75.6 ± 2.6* | 77.0 ± 2.9* |
| BMI (kg/m²) | 20.9 ± 0.7 | 28.7 ± 0.8* | 29.7 ± 0.8* |
| AST (IU/L) | 32.9 ± 7.2 | 35.3 ± 5.7 | 56.9 ± 4.6*, † |
| ALT (IU/L) | 32.2 ± 5.8 | 58.4 ± 11.6* | 82.0 ± 7.3*, † |
| PLT (x10⁹/mm³) | 22.6 ± 1.8 | 240.0 ± 20.9 | 20.3 ± 1.1 |
| Total Protein (g/dl) | 6.5 ± 0.3 | 7.0 ± 0.1* | 7.1 ± 0.1* |
| Albumin (g/dl) | 3.3 ± 0.2 | 4.4 ± 0.1* | 4.21 ± 0.1*, † |
| PT (%) | 77.9 ± 4.2 | 97.8 ± 1.7* | 97.2 ± 2.7* |
| HbA1c (%) | 5.8 ± 0.3 | 7.1 ± 0.2* | 7.1 ± 0.3* |
| HOMA-IR | – | 3.8 ± 0.5 | 7.2 ± 1.3*, † |
| QUICKI | – | 0.33 ± 0.0 | 0.30 ± 0.0* |
| GIR (mg/kg/min) | – | 5.9 ± 0.6 | 4.3 ± 0.3† |
| Total cholesterol (mg/dl) | 165.5 ± 11.7 | 201.2 ± 5.2* | 193.9 ± 5.7* |
| Triglycerides (mg/dl) | 90.1 ± 9.5 | 135.4 ± 9.3* | 153.6 ± 15.2* |
| HDL cholesterol (mg/dl) | 43.2 ± 4.2 | 46.1 ± 1.2 | 49.0 ± 2.2 |
| LDL cholesterol (mg/dl) | 107.9 ± 10.6 | 127.8 ± 4.9 | 115.6 ± 5.1 |

The data are expressed as the mean ± SEM. ALT, alanine aminotransferase; AST, aspartate aminotransferase; GIR, glucose infusion rate.
*P < 0.05 vs. the control.
†P < 0.05 vs. SS.

Results

Patient profiles

The backgrounds of patients in the Control, SS and NASH groups are shown in Table 1. The mean age of the patients was 50.6 years, and the male: female ratio was 66:55. No significant difference was observed in the use of medications for dyslipidaemia and diabetes between the SS and NASH groups. The body mass index (BMI), haemoglobin A1c (HbA1c) value, and total cholesterol (T-CHO) and triglyceride (TG) levels were significantly higher in the SS and NASH groups than in the Control group. Aspartate aminotransferase and alanine

Table 2. Histopathological findings of livers in the study population

| Fibrosis (0/1/2/3/4) | Control (n = 18) | SS (n = 63) | NASH (n = 40) |
|----------------------|-----------------|------------|--------------|
| 0                    | 7/52/4/0        | 1/15/11/7/6 | < 0.01      |
| 1                    | 0/30/24/9       | 0/10/15/15 | < 0.01      |
| 2                    | 6/34/23/0       | 0/8/26/6   | < 0.01      |
| 3                    | 4/21/1          | 1/17/22    | < 0.01      |

The data are expressed as 10⁻⁴ mg/mg liver, the mean ± SEM.

Lauric acid (C12:0), myristic acid (C14:0), palmitic acid (C16:0), palmitoleic acid (C16:1n7), heptadecanoic acid (C17:0), stearic acid (C18:0), oleic acid (C18:1n9), linoleic acid (C18:2n6), gondoic acid (C20:1n9), α-linolenic acid (C18:3n3), erucic acid (C22:1n9), docosadienoic acid (C22:2n6), docosahexaenoic acid (C22:6n3).

Table 3. Fatty acid composition in liver tissue of the study population

| Fatty acid composition | Control (n = 18) | SS (n = 63) | NASH (n = 40) |
|------------------------|-----------------|------------|--------------|
| C12:0                  | 0.25 ± 0.10     | 10.9 ± 2.3* | 14.4 ± 3.5* |
| C14:0                  | 2.4 ± 0.5       | 36.9 ± 5.1* | 67.2 ± 1.4* |
| C16:0                  | 54.5 ± 6.7      | 528 ± 80.3* | 928 ± 210*  |
| C16:1n7                | 5.6 ± 1.0       | 58.3 ± 10.6* | 109 ± 23.5*, † |
| C17:0                  | 3.4 ± 1.8       | 15.6 ± 2.4  | 20.3 ± 3.9  |
| C18:0                  | 33.6 ± 4.9      | 162 ± 24.3* | 210 ± 40.4* |
| C18:1n9                | 36.0 ± 4.8      | 616 ± 110*  | 1036 ± 234*|
| C18:2n6                | 36.2 ± 3.9      | 270 ± 46.5* | 387 ± 75.7* |
| C20:1n9                | 1.0 ± 0.3       | 18.1 ± 3.3* | 24.7 ± 4.4* |
| C18:3n3                | 0.4 ± 0.1       | 6.0 ± 1.0*  | 9.1 ± 1.9*  |
| C22:1n9                | 19.1 ± 2.7      | 56.3 ± 7.8* | 57.6 ± 9.5* |
| C22:6n6                | 3.06 ± 0.8      | 10.9 ± 1.5* | 10.9 ± 1.5* |
| C22:6n3                | 21.7 ± 3.7      | 54.2 ± 6.8* | 51.2 ± 6.8* |
| C18:0/C16:0            | 0.62 ± 0.02     | 0.35 ± 0.01* | 0.27 ± 0.01*, † |
| C16:1n7/C16:0 ratio    | 0.10 ± 0.01     | 0.10 ± 0.00 | 0.13 ± 0.01† |
| C18:1n9/C18:0 ratio    | 1.17 ± 0.12     | 3.43 ± 0.20* | 4.22 ± 0.19*, † |
| n-6/n-3                | 2.18 ± 0.24     | 4.21 ± 0.26* | 5.25 ± 0.38*, † |

The data are expressed as 10⁻⁴ mg/mg liver, the mean ± SEM.

Lauric acid (C12:0), myristic acid (C14:0), palmitic acid (C16:0), palmitoleic acid (C16:1n7), heptadecanoic acid (C17:0), stearic acid (C18:0), oleic acid (C18:1n9), linoleic acid (C18:2n6), gondoic acid (C20:1n9), α-linolenic acid (C18:3n3), erucic acid (C22:1n9), docosadienoic acid (C22:2n6), docosahexaenoic acid (C22:6n3).

*P < 0.05 vs. the control.
†P < 0.05 vs. SS.
aminotransferase were significantly higher, and the platelet count and albumin level were significantly lower in the NASH group than in the SS group. HOMA-IR, QUICKI and the glucose infusion rate were significantly different between the groups, with insulin resistance being significantly higher in the NASH group.

The histopathological findings of livers are shown in Table 2. The progression of steatosis, inflammation, hepatocellular disorders and fibrosis was significantly further in the NASH group than in the SS group.

Comparison of the fatty acid content of liver tissue

The fatty acids shown in Table 3 were measured in extracts from liver tissue using gas chromatography. When the fatty acid content per 1 mg of wet liver was compared, various fatty acid contents were significantly higher in the SS and NASH groups than in the control group ($P < 0.05$). In addition, the palmitoleic acid (C16:1n7) content was significantly higher in the NASH group ($P < 0.05$).

Regarding the fatty acid composition ratio, the stearic acid (C18:0)/palmitic acid (C16:0) ratio was significantly lower ($P < 0.01$) and the C16:1n7/C16:0 and oleic acid (C18:1n9)/C18:0 ratios were significantly higher in the NASH group than in the SS group ($P < 0.05$).

Fig. 1. Association between insulin resistance and the fatty acid composition ratio in liver tissue. The association between insulin resistance and changes in the fatty acid composition ratio in liver tissue was analysed using the Mann–Whitney $U$-test. (A) Patients were divided into groups with and without insulin resistance based on the Homoeostasis Model Assessment for insulin resistance (HOMA-IR) $>2.5$ as insulin-resistant. (B) Patients were divided into groups with and without insulin resistance based on the QUICKI $<0.33$ as insulin-resistant.

Fatty acid composition ratio and insulin resistance

The association between the fatty acid composition ratio in liver tissue and insulin resistance was investigated. For the indices of insulin resistance, HOMA-IR and QUICKI calculated from the fasting-state blood glucose and insulin levels were used. Firstly, patients were divided into two groups with ($>2.5$) and without ($\leq 2.5$) insulin resistance based on HOMA-IR. The C18:0/C16:0 ratio was significantly lower and that of the C18:1n9/C18:0 ratio was significantly higher in the group with insulin resistance ($p < 0.01$ and $p = 0.01$, respectively) (Fig. 1A), whereas no significant difference was noted in the C16:1n7/C16:0 ratio between the groups. Similarly, when patients were divided into two groups with ($\leq 0.33$) and without ($>0.33$) insulin resistance based on the QUICKI, the C18:0/C16:0 ratio was significantly lower and the C18:1n9/C18:0 ratio was significantly higher in the group with insulin resistance ($P < 0.01$). Differences in the fatty acid composition ratio between the SS and NASH groups were more prominent in men, while no significant difference was noted in premenopausal women (Table S1). The n-6/n-3 ratio was significantly higher in the NASH group than in the SS group ($P < 0.05$). (Table 3)
resistance ($P < 0.01$ and $P = 0.02$, respectively) (Fig. 1B), whereas the C16:1n7/C16:0 ratio showed no association with the presence or absence of insulin resistance.

**Fatty acid composition ratio and histopathological findings of the liver**

The histopathological findings of the liver with NAFLD were evaluated based on four evaluation items (three items of NAS: steatosis, lobular inflammation, hepatocellular ballooning, and liver fibrosis), and their associations with the liver tissue fatty acid composition ratio were investigated. On evaluation of the association between the NAS and fatty acid composition ratio, the C18:0/C16:0 ratio was significantly lower ($P < 0.01$) and the C18:1n9/C18:0 and C16:1n7/C16:0 ratios were significantly higher ($P < 0.01$) in the group with a 4 or lower score than in the group with a 5 or higher score, showing differences similar to those between the SS and NASH groups (Fig. 2A). Regarding fatty changes (steatosis score), various fatty acid contents significantly increased with an increase in the score. A significant decrease in the C18:0/C16:0 ratio ($P < 0.01$) and a significant increase in the C18:1n9/C18:0 ratio ($P < 0.01$) were noted in the fatty acid composition, but no association with the C16:1n7/C16:0 ratio was noted (Fig. 2B). Regarding lobular inflammation, the C18:0/C16:0 ratio significantly decreased ($P = 0.04$) and the C16:1n7/C16:0 ratio significantly increased ($P < 0.01$) with an increase in the score (Fig. 2C). Regarding hepatocellular ballooning, the C18:0/C16:0 ratio significantly decreased ($P < 0.01$) and the C16:1n7/C16:0 ratio significantly increased ($P < 0.01$) with an increase in the fibrosis score (Fig. 2E).

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**Fig. 2.** Relationship between the histopathological findings of the liver and fatty acid composition ratio. The association between the histopathological findings of the liver and fatty acid composition ratio was evaluated using the Spearman’s rank correlation coefficient. (A) NAS, (B) steatosis score, (C) lobular inflammation score, (D) hepatocellular ballooning score and (E) fibrosis score.
Expression of fatty acid metabolism-related genes

The gene expression levels of enzymes involved in fatty acid metabolism in liver tissue were investigated. Samples of 65 (SS: 35, NASH: 30) patients were subjected to RTD-PCR, and the gene expression levels of seven enzymes: SCD1, ELOVL6, fatty acid synthase (FAS), sterol regulatory element-binding protein-1c (SREBP-1c), acetyl-CoA carboxylase (ACC), peroxisome proliferator-activated receptor-α (PPARα) and PPARγ were measured. The expression levels of SCD1, ELOVL6, SREBP-1c, FAS and PPARγ were significantly higher in the NASH group than in the SS group, which confirms that the gene expression levels of enzymes involved in fatty acid metabolism were markedly different between the SS and NASH groups (Fig. 3). Thus, the associations between the gene expression levels of these enzymes and histopathological findings (steatosis, inflammation, hepatocellular ballooning and liver fibrosis) were investigated. No significant correlation was noted between the steatosis score and the expression of the fatty acid metabolism-related genes (Fig. 4A); however, a significant correlation was observed between the lobular inflammation score and SCD1 expression ($P < 0.01$), and the gene expression level rose as inflammation progressed in liver tissue (Fig. 4B). The hepatocellular ballooning score was also significantly correlated with the individual gene expression levels of SCD1, ELOVL6, SREBP-1c, FAS, ACC and PPARγ, and expression levels increased as the score rose (Fig. 4C). The fibrosis score was correlated with SREBP-1c expression, but no significant correlation with any other related genes was noted (Fig. 4D).

Finally, we performed a multiple linear regression analysis to calculate age-, sex- and BMI-adjusted coefficients between the histological scores of the liver and experimental parameters such as fatty acid composition, insulin resistance and gene expression (Table 4). In univariate analysis, the steatosis score was significantly correlated with C18:0/C16:0, C18:1n9/C18:0 and QUICKI. In multivariate analysis using these parameters, C18:0/C16:0 was the factor most associated with the steatosis score. In contrast, the inflammation score was significantly correlated with C16:1n7/C16:0, C18:0/C16:0, C18:1n9/C18:0 and SCD1 in univariate analysis and C16:1n7/C16:0 was identified to be the factor most associated with the score in multivariate analysis. The ballooning score was significantly correlated with multiple factors as shown in Table 4 and QUICKI was significantly correlated in multivariate analysis. The fibrosis score was significantly correlated with C18:0/C16:0 only.

Discussion

There have been several reports on fatty acid accumulation in liver tissue in NAFLD. Myristic acid (C14:0), palmitic acid (C16:0) and oleic acid (C18:0) were increased in NAFLD liver tissue in a mouse model (19), and decreases in ω-3-linolenic acid (C18:3n6) and arachidonic acid (20:4n6) and an increase in the ratios of n-6 and n-3 fatty acids were observed in humans, although the number of cases was small (14). Similar to these findings, the various fatty acid contents of liver tissue were increased in our NAFLD patients. In addition to these fatty acid contents, we closely investigated the fatty acid composition ratios and fatty acid-metabolizing enzymes in the liver tissue in the SS and NASH groups. Regarding the fatty acid composition ratio, significant differences were noted in the C18:0/C16:0, C18:1n9/C18:0 and C16:1n7/C16:0 ratios between the SS and NASH groups, which confirms that the composition ratio of fatty acids is closely associated with the
pathology of NASH, such as the severities of steatosis, inflammation, hepatocellular disorders, and fibrosis. To the best of our knowledge, this is the first report on the association of the liver tissue fatty acid composition ratio with the severities of liver tissue inflammation and hepatocellular disorders in NASH. The fatty acid content of liver tissue was expected to increase in patients with advanced hepatic steatosis; however, significant changes in the fatty acid composition ratios suggested that not all fatty acids homogeneously increase. Of the changes in fatty acid composition ratios observed in the SS and NASH groups, a decrease in the C18:0/C16:0 ratio and an increase in the C18:1n9/18:0 ratio (i.e. relative increases in C16:0 and C18:1n9) were associated with steatosis and insulin resistance, and an increase in the C16:1n7/16:0 ratio (i.e. a relative increase in C16:1n7) was associated with liver tissue inflammation and hepatocellular disorders. These results revealed that fatty acid components change depending on pathological differences in liver tissue in NAFLD patients.

There are two main pathways of fatty acid accumulation in the liver. The close involvement of insulin resistance in both pathways has been clarified (20, 21). The hydrolysis of fat tissue occurs in the presence of insulin resistance and increases free fatty acid inflow into the liver in one pathway. In the other, related genes, such as the SREBP-1c gene and downstream SCD1 and FAS genes, are activated in the liver in the presence of high...
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Hepatic fatty acid composition in NASH

Table 4. Multivariate correlation between histological scores, insulin resistance and genes adjusted for age, gender and BMI

|                | Steatosis score | Inflammation score | Ballooning score | Fibrosis score |
|----------------|-----------------|--------------------|------------------|----------------|
|                | Coefficient     | UA P-value         | Coefficient      | UA P-value     |
| C18:0/C16:0    | -0.610          | <0.0001            | -0.315           | 0.0022         |
| C16:1n7/C16:0  | 0.084           | 0.4071             | 0.339            | 0.0010         |
| C18:1n9/C18:0  | 0.575           | <0.0001            | 0.224            | 0.0302         |
| HOME-IR        | 0.070           | 0.5211             | 0.137            | 0.2268         |
| QUICK I        | -0.282          | 0.0108             | -0.183           | 0.1180         |
| SCD1           | 0.093           | 0.4725             | 0.266            | 0.0386         |
| ELOVL6         | 0.16            | 0.1941             | 0.161            | 0.2177         |
| SREBP-1c       | 0.104           | 0.4349             | 0.249            | 0.0591         |
| FAS            | 0.148           | 0.2543             | 0.195            | 0.1340         |
| ACC            | 0.142           | 0.2902             | 0.159            | 0.2380         |
| PPARα          | 0.131           | 0.3222             | 0.170            | 0.2005         |
| PPARγ          | 0.136           | 0.3243             | 0.155            | 0.2631         |

MA, multivariate analysis; PPARα, peroxisome proliferator-activated receptor-α; UA, univariate analysis.

blood insulin and glucose levels (22) and promote glucose uptake in the liver, enhancing the de novo synthesis of C16:0 through acetyl-CoA.

C16:0 is considered to be a toxic fatty acid for liver tissue. TGs in the liver and microsomal saturated fatty acids increased in mice fed a saturated fatty acid-enriched diet, and elevations in the activity of liver caspase-3 and transaminase levels were confirmed (23). Saturated fatty acids, such as C16:0, are not readily esterified and exhibit strong cytotoxicity in the liver (24). It is assumed that toxicity is avoided by the conversion of these saturated fatty acids to unsaturated fatty acids, such as C16:1n7 and C18:1n9, through elongation by ELOVL6 and desaturation by SCD1. As both ELOVL6 and SCD1 were controlled by SREBP-1c, their expressions are related to each other.

It has been previously reported that the expression of these genes was associated with the pathology of NASH in an animal model (25). Matsuzaka et al. have also shown that the expression level of ELOVL6 in the liver was correlated with the inflammation of liver tissue in a mouse model with NASH and was also increased in NASH patients (26). These results are consistent with our results. In this study, we evaluated the relationship between fatty acid metabolism and NASH pathology by the simultaneous examination of the fatty acid composition ratio around C16:0, fatty acid metabolic gene expression and histopathology of the liver in the same liver samples of many patients. The analysis of age-, sex- and BMI-adjusted associations between the histological scores of the liver and experimental parameters showed that a decrease in the C18:0/C16:0 ratio, an increase in the C16:1n7/16:0 ratio, and an increase in the expression of fatty acid metabolism-related genes including SCD1 and ELOVL6 correlated with inflammation or ballooning of liver tissue. Taking our results together with previous reports, fatty acid metabolism in the liver according to the development of NASH can be explained as follows.

First, a decrease in the C18:0/C16:0 ratio is because of an increase in C16:0 without an increase in the fatty acid metabolism-related genes. Next, an increase in the expression of the fatty acid metabolism-related genes including SCD1 and ELOVL6 occurs and correlates with inflammation and the ballooning of hepatocytes in liver tissue. Finally, it becomes difficult to sufficiently convert C16:0 to C18:0 by ELOVL6, and a compensatory increase in the conversion of C16:0 to C16:1n7 controlled by SCD1 occurs. Consequently, the increase in C16:1n7/C16:0 correlates with inflammation in liver tissue with the highest correlation coefficient. Therefore, our results suggest that the acceleration of overall hepatic fatty acid metabolism is more important for the pathogenesis of NASH than the expression levels of ELOVL6 in patients with NASH.

In conclusion, analysis of the liver tissue fatty acid composition and gene expression showed that an enhancement of the fatty acid metabolic pathway centring on C16:0 contributed to the progression of SS to NASH. Elucidating these changes in the metabolic pathway may lead to the development of a drug that could prevent the progression to NASH.

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Supporting information

Additional Supporting Information may be found in the online version of this article:

Table S1. Differences of fatty acid composition rates in liver tissue among male, premenopausal female, postmenopausal female.