**Table 3** Tumor response

| Response to HAIC† | All, *n* (%) | Child-Pugh class[b] | | Response to sorafenib | | | | Progression pattern[c] | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | A | B or C | PR | SD | PD | NE | IHG§§ | NIH¶¶ | NEH[a] |
| CR‡ | 0 (0) | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| PR§ | 8 (29.6) | 1 | 7 | 0 | 4 | 3 | 1 | 3 | 0 | 1 |
| SD¶ | 9 (33.3) | 5 | 4 | 1 | 5 | 3 | 0 | 7 | 2 | 0 |
| PD†† | 9 (33.3) | 3 | 6 | 0 | 4 | 4 | 1 | 6 | 2 | 0 |
| NE‡‡ | 1 (3.7) | 0 | 1 | 1 | 0 | 0 | 0 | 1 | 0 | 0 |
| Total | 27 (100) | 9 | 18 | 2 | 13 | 10 | 2 | 17 | 4 | 1 |

†HAIC: hepatic arterial infusion chemotherapy.†, ‡, §, ¶, ††, ‡‡, §§ and ¶¶.
‡CR: complete response.
§PR: partial response.
¶SD: stable disease.
††PD: progressive disease.
‡‡NE: not evaluable.
§§IHG: intrahepatic growth.
¶¶NIH: new intrahepatic lesion.
[a]NEH: new extrahepatic lesion.
[b]At decision-making of HAIC.
[c]At termination of sorafenib therapy.

hematological toxicity, particularly neutropenia and thrombocytopenia, was high. One of the possible causes of these toxicities was pre-existing pancytopenia derived from liver cirrhosis in most patients, and another was the concurrent administration of IFN added to 5-FU and CDDP.[15] All of the patients recovered immediately after the end of treatment and no additional complications were noted. Moreover, the frequencies of leukocytopenia, neutropenia and thrombocytopenia observed in this study (74.1%, 77.8% and 88.9%, respectively) were very similar to those of patients who were not pretreated by sorafenib and underwent HAIC with the same protocol, including 5-FU/cisplatin/IFN (75.4%, 77.2% and 89.5%, respectively),[15] which suggested that prior administration of sorafenib did not have an additional impact on hematological toxicities. With regard to non-hematological toxicities, most of them were less frequent than those in a previous report,[15] and there were no unexpected adverse reactions. These favorable results may be derived from newly available drugs such as a second-generation 5-hydroxytryptamine 3 receptor antagonist and neurokinin-1-receptor antagonist or active supportive therapy. These findings suggested that HAIC was considered tolerable even for those patients who were previously administered sorafenib.

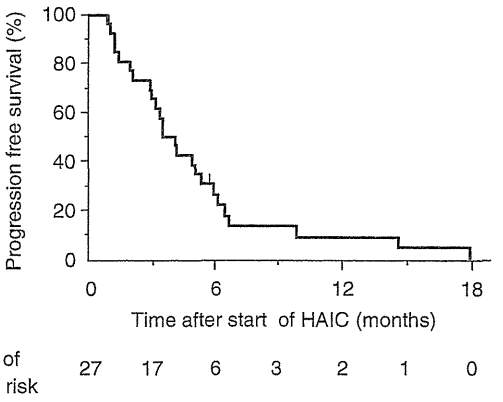


**Figure 1** Kaplan–Meier plot of progression-free survival (PFS) since commencement of hepatic arterial infusion chemotherapy (HAIC). Median PFS was 4.0 months.

The response rate obtained in the present study (29.6%) appears to be low compared with that of previous reports.[14,15] Although it is difficult to compare the response rates among studies, possible reasons include variation in patients' hepatic function, the criteria used to evaluate responses, the effect of previous administration of sorafenib, and the relatively small number of patients. In addition, the proportion of patients with extrahepatic lesions may have been a meaningful factor because it was higher (44.4%) in this study than that of the previous study (0–14%)[14,15] and the response rate was reported to be lower in patients with HCC having extrahepatic metastases than in those without.[27] We could not identify any significant

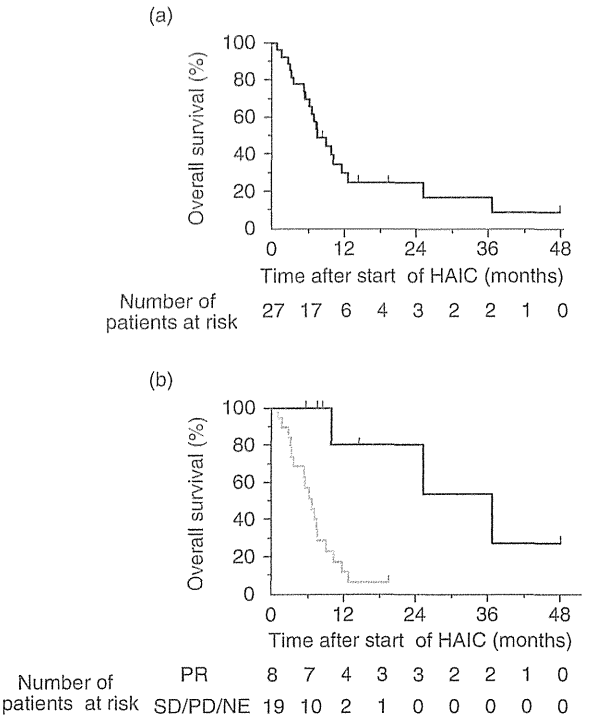


**Figure 2** Kaplan–Meier plot of overall survival since commencement of hepatic arterial infusion chemotherapy (HAIC): (a) all patients and (b) according to response to HAIC. The median survival time (MST) of all patients was 7.6 months, and the MST of patients who achieved partial response (PR) were 36.7 months (black line), which was significantly better than that of the patients with stable disease (SD)/progressive disease (PD)/ not evaluable (NE), namely, 6.6 months (gray line) ($P < 0.01$).

predictive markers for the response to HAIC in this study, and further investigation is needed to examine the factors affecting the response rate of HAIC, and to select the appropriate population to receive HAIC after sorafenib therapy.

Another interesting finding of the present study was that half of our patients were categorized as Child–Pugh class B, and no correlation was observed between the response to HAIC and Child–Pugh classification. Although certain molecular targeted agents are currently being tested for sorafenib-refractory patients with HCC, the objectives in most of these trials are restricted to patients with good hepatic function. Other reports have described systemic chemotherapy by combination of gemcitabine and oxaliplatin is potentially safe for patients with Child–Pugh class B[28] and useful in sorafenib-refractory patients with HCC.[29] The results of the present study suggest that HAIC may be also considered as one of treatment procedures for patients with Child–Pugh class B after sorafenib therapy.

The present study has several limitations, including its retrospective nature, the small number of patients, the lack of controls, and single-institution subsets. A prospective trial with a larger number of patients in proper design is needed to confirm our findings.

In conclusion, HAIC has good feasibility and moderate antitumor activity and is a useful treatment option for patients with advanced HCC after failure of sorafenib therapy.

## ACKNOWLEDGMENTS

NONE.

## CONFLICTS OF INTEREST

NONE TO DECLARE.

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## SUPPORTING INFORMATION

ADDITIONAL SUPPORTING INFORMATION may be found in the online version of this article at the publisher's web-site:

**Figure S1.** Kaplan–Meier plot of overall survival since commencement of hepatic arterial infusion chemotherapy according to progression pattern. Patient prognosis did not differ among intrahepatic growth (IHG) group (black line), new intrahepatic lesion (NIH) group (gray line), and new extrahepatic lesion and/or vascular invasion (NEH) group (dashed line).
**Table S1.** Predictive marker for response to hepatic arterial infusion chemotherapy.

Hepatology Research

*Hepatology Research* 2014 doi: 10.1111/hepr.12436

*Original Article*

# Blood neutrophil to lymphocyte ratio as a predictor in patients with advanced hepatocellular carcinoma treated with hepatic arterial infusion chemotherapy

Takeshi Terashima, Tatsuya Yamashita, Noriho Iida, Taro Yamashita, Hidetoshi Nakagawa, Kuniaki Arai, Kazuya Kitamura, Takashi Kagaya, Yoshio Sakai, Eishiro Mizukoshi, Masao Honda and Shuichi Kaneko

*Department of Gastroenterology, Kanazawa University Hospital, Kanazawa, Ishikawa, Japan*

*Aim:* Inflammation plays a critical role in cancer. The aim of the present study was to investigate the impact of neutrophil to lymphocyte ratio (NLR) on patients with advanced hepatocellular carcinoma (HCC) treated with hepatic arterial infusion chemotherapy (HAIC).

*Methods:* We retrospectively evaluated 266 patients with advanced HCC treated with HAIC between March 2003 and December 2012. NLR was calculated from the differential leukocyte count by dividing the absolute neutrophil count by the absolute lymphocyte count.

*Results:* The cut-off level of NLR was set as the median value of 2.87 among all patients in this study. The objective response rate in the patients with low NLR was 37.6%, which was significantly better than that of the patients with high NLR (21.1%; $P < 0.01$). Multivariate analysis revealed that low NLR remained associated with the response to HAIC ($P = 0.024$). Median progression-free survival and median overall survival in patients with high NLR were 3.2 and 8.0 months, respectively, which were significantly shorter than that of the patients with low NLR (5.6 and 20.7 months; $P < 0.01$ and $P < 0.01$, respectively). High NLR was an independent unfavorable prognostic factor in multivariate analysis. The patient outcome was stratified more clearly by NLR calculated after HAIC added to calculations before HAIC. Serum platelet-derived growth factor-BB level was positively correlated with NLR.

*Conclusion:* Results suggest that NLR is a useful predictor in patients with advanced HCC treated with HAIC. These findings may be useful in determining treatment strategies or in designing clinical chemotherapy trials in future.

**Key words:** hepatic arterial infusion chemotherapy, hepatocellular carcinoma, neutrophil lymphocyte ratio, predictive factor, prognostic factor

## INTRODUCTION

HEPATOCELLULAR CARCINOMA (HCC) is the third leading cause of cancer death and remains a worldwide health concern because the incidence of HCC continues to increase globally.[1] A variety of new techniques of imaging modalities have enabled the detection of HCC at early stages, and advances of various therapeutic procedures have improved the curability of patients with HCC.[2] Despite those recent advances in diagnostic and therapeutic technologies, the prognosis of patients with HCC remains poor due to impaired liver function and frequent recurrence of HCC.[3]

Although sorafenib has been established as the standard of care for advanced HCC,[4] its efficacy and tolerability are limited.[5] As an alternative therapy to sorafenib, hepatic arterial infusion chemotherapy (HAIC) has been conducted in Asia, including Japan, and it has been reported as a promising treatment procedure.[6,7] However, application of HAIC and its predictive and prognostic markers have not been fully established.

Inflammation plays a critical role in the development and progression of various cancers.[8] Inflammation caused by extrinsic factors including a variety of infectious agents and environmental toxins, as well as intrinsic factors including active oncogenes, reactive

*Correspondence: Dr Tatsuya Yamashita, Department of Gastroenterology, Kanazawa University Hospital, 13-1 Takara-machi, Kanazawa, Ishikawa 920-8641, Japan. Email: ytatsuya@m-kanazawa.jp*

*Conflicts of interest:* None to declare.

*Received 1 September 2014; revision 3 October 2014; accepted 11 October 2014.*

oxygen species and necrosis existing in the cancer tissues, promote various processes of cancer initiation and progression, such as mutation, proliferation, immortalization, invasiveness, angiogenesis, epithelial–mesenchymal transition and immunosuppression.[9] Additionally, the release of inflammation-related substances is closely related to symptoms such as loss of bodyweight, fatigue and appetite loss among cancer patients. Therefore, inflammation-induced cancer progression and cachectic patient status affect quality of life and patient outcomes.[10] The inflammation-related markers such as absolute white blood cell count, C-reactive protein (CRP), neutrophil to lymphocyte ratio (NLR), platelet to lymphocyte ratio and cytokines have been suggested to be associated with outcomes of patients with various malignancies[11] including at an early or intermediate disease stage of HCC.[12–16] However, whether these markers can serve as biomarkers of treatment efficacies and patient outcome in more advanced stages of HCC remains unclear.

The objectives of the present study were to investigate the correlation between NLR and patient characteristics in advanced HCC patients. We also analyzed the impact of NLR on the treatment efficacies as well as the outcome of patients with advanced HCC treated with HAIC. Moreover, to assess inflammatory molecules associated with NLR, serum level of cytokines and growth factors were measured. This approach provides useful information in determining treatment strategies for patients with advanced HCC.

## METHODS

### Patients

THE SUBJECTS IN this study were patients treated with HAIC at the Kanazawa University Hospital between March 2003 and December 2012 for advanced HCC with vascular invasion and/or intrahepatic multiple lesions considered unsuitable for surgical resection, locoregional therapy and transarterial chemoembolization. All patients underwent dynamic computed tomography (CT) or dynamic magnetic resonance imaging (MRI) to diagnose HCC and assess the extent of cancer. Additionally, HCC was diagnosed according to the guidelines of the American Association for the Study of Liver Disease.[17] Patients with extrahepatic lesions were also considered eligible for HAIC if their extrahepatic lesions were mild; intrahepatic lesions were considered to be prognostic factors. Other inclusion criteria were Eastern Cooperative Oncology Group performance status (ECOG PS) of 2 or less, appropriate major organ functions, including bone marrow, kidney, cardiac functions and hepatic function (Child–Pugh A or B), and no clinical symptoms or signs of sepsis.

### HAIC

The technique for implantation of the reservoir system has been thoroughly described elsewhere.[18] Catheters were induced through the right femoral artery and angiography from the celiac artery was first performed to localize the HCC and evaluate the intrahepatic and extrahepatic vascularization. Then, we inserted a catheter with a side opening into the gastroduodenal artery, positioning the side opening in the common hepatic artery by an image-guided procedure. The gastroduodenal artery, right gastric artery and other arteries that were suspected to nourish the gastroduodenal region were embolized as much as possible to prevent the gastrointestinal mucositis. The other end of the catheter was connected to the injection port subcutaneously implanted in the right lower abdomen. Finally, we confirmed blood flow redistribution.

Hepatic arterial infusion chemotherapy was conducted approximately 5 days after the reservoir was implanted. The treatment protocol was as follows: all patients received 5-fluorouracil (FU) (330 mg/m$^2$ per day) administrated continuously for 24 h from day 1 to day 5 and day 8 to day 12, and either interferon (IFN)-α-2b or pegylated (PEG) IFN-α-2b used at the treating physician's discretion. PEG IFN-α-2b (1.0 μg/kg) was administrated s.c. on days 1, 8, 15 and 22, and IFN-α-2b ($3 \times 10^6$ U) was administrated i.m. thrice weekly. Some patients underwent cisplatin administration (20 mg/m$^2$ per day) into the hepatic artery for 10 min prior to 5-FU. A treatment cycle consisted of 28 days of drug administration, followed by a 14-day rest period. The treatment was repeated until tumor progression or unacceptable toxicity was observed, or until the patient refused the treatment. The treatment protocol was approved by the ethics Committee of Kanazawa University, and informed consent for participation in the study was obtained from each subject and conformed to the guidelines of the 1975 Declaration of Helsinki.

### Data collection

We reviewed the medical records of the patients, and collected demographic, clinical and laboratory data, including patient age, sex, ECOG PS, history of viral infection, hepatic reserve (Child–Pugh score), imaging data (vascular invasion and extrahepatic lesion) and tumor marker analyses. We collected laboratory data on complete blood count and CRP. The NLR was calculated

from the differential leukocyte count by dividing the absolute neutrophil count by the absolute lymphocyte count. We used the laboratory data obtained within 7 days prior to day 1 of treatment in this study. We also collected NLR values at 4 weeks after the treatment began to evaluate the impact of the NLR trend on patient outcomes. Cytokine and chemokine profiling was obtained as described below:[19] after venous blood was centrifuged at 1580 *g* for 10 min at 4°C, serum fractions were obtained and stored at −20°C until used. Serum levels of various cytokines and chemokines were measured using the Bio-Plex Protein Array System (Bio-Rad, Richmond, CA, USA) according to the manufacturer's protocol. Briefly, frozen serum samples were thawed at room temperature, diluted 1:4 in sample diluents, and 50 μL aliquots of diluted sample were added in duplicate to the wells of 96-well microtiter plates containing the coated beads for a validated panel of human cytokines and chemokines according to the manufacturer's instructions. The following 20 cytokines and chemokines were targeted: epidermal growth factor (EGF), basic fibroblast growth factor, hepatocyte growth factor, IFN-γ, interleukin (IL)-2, IL-4, tumor necrosis factor-α (TNF-α), IL-6, IL-8, IL-10, IL-5, IFN γ-induced protein (IP)-10, monokine induced by IFN-γ (MIG), platelet-derived growth factor (PDGF)-BB, transforming growth factor (TGF)-β, TGF-α, vascular endothelial growth factor (VEGF), stem cell factor, IL-12 and stromal cell-derived factor 1. Nine standards (range, 0.5–32 000 pg/mL) were used to generate calibration curves for each cytokine. Data acquisition and analysis were performed using Bio-Plex Manager software version 4.1.1 (Bio-Rad).

### Evaluation of antitumor effect

The efficacy of HAIC was assessed every 4–6 weeks by dynamic CT or dynamic MRI during the treatment period. The response to chemotherapy was assessed by treating physicians according to the Response Evaluation Criteria in Solid Tumors version 1.1.[20] An objective response rate was defined as the sum of complete response rate and partial response rate.

### Statistical analysis

We compared patient backgrounds according to NLR and patient demographics using the $\chi^2$-test for categorical variables when appropriate. Student's *t*-test and Mann–Whitney *U*-test were used for continuous variables. We set the cut-off level of continuous variables as the median value among all patients in this study. We divided the patients into two groups according to NLR before and after treatment, respectively, and compared the response to HAIC and patient outcome between groups. The $\chi^2$-test was also used to evaluate the relation between NLR and the response to HAIC in univariate analysis. Logistic regression analysis was used for multivariate analysis. Progression-free survival (PFS) was calculated from the first day of HAIC until the date of radiological progression, death or the last day of the follow-up period. Overall survival (OS) was calculated from the first day of HAIC until the date of death or the last day of the follow-up period. To compare PFS and OS between groups, the cumulative survival proportions were calculated using the Kaplan–Meier method, and any differences were evaluated using the log–rank test. Only variables that achieved statistical significance in the univariate analysis were subsequently evaluated in the multivariate analysis using Cox's proportional hazards regression model. Linear regression was used to explore the relationship between cytokine or chemokine profiling and NLR. A *P*-value of less than 0.05 was considered statistically significant. All statistical analyses were performed using the SPSS statistical software program package (SPSS, Chicago, IL, USA).

## RESULTS

### Patients characteristics stratified by NLR

WE RETROSPECTIVELY LISTED 267 consecutive patients who met the above-described criteria and reviewed their medical records. The information regarding the differential leukocyte count could not be obtained in one patient, and then the remaining 266 patients were analyzed. One hundred and thirty-three (50.0%) of 266 patients had NLR higher than 2.87, the median value among all patients before treatment. Patient demographic characteristics are summarized in Table 1. Patients with high NLR had a significantly worse performance status than those with low NLR ($P = 0.020$). With regard to tumor status, vascular invasion and extrahepatic dissemination were observed more often in the patients with high NLR (57.1% and 27.8%, respectively) than in those with low NLR (39.8% and 18.0%, respectively), and des-γ-carboxyprothrombin (DCP) was higher in the group with high NLR (median, 1286 mAU/mL) than in the one with low NLR (median, 214 mAU/mL). Sorafenib was administrated as prior treatment before HAIC in 25 patients (9.4%) and as subsequent therapy after HAIC in 26 patients (9.8%). The proportion of the patients receiving sorafenib before HAIC was similar between the two groups, whereas the proportion of the patients

**Table 1** Clinical characteristic of the patients according to NLR

| | All ($n = 266$) | High NLR ($n = 133$) | Low NLR ($n = 133$) | $P$ |
|---|---|---|---|---|
| Age, years | | | | <0.01* |
| Mean ± SD | 66.3 ± 9.1 | 64.7 ± 9.9 | 68.0 ± 7.8 | |
| Sex, *n* (%) | | | | 0.30** |
| Male | 209 (78.6) | 108 (81.2) | 101 (75.9) | |
| ECOG PS, *n* (%) | | | | 0.020** |
| 0 | 220 (82.7) | 103 (77.4) | 117 (88.0) | |
| 1 | 41 (15.4) | 25 (18.8) | 16 (12.0) | |
| 2 | 5 (1.9) | 5 (3.8) | 0 | |
| Sorafenib before HAIC | | | | 0.83** |
| Present | 25 (9.4) | 12 (9.0) | 13 (9.8) | |
| Sorafenib after HAIC | | | | 0.013** |
| Present | 26 (9.8) | 19 (14.3) | 7 (5.3) | |
| HBs antigen, *n* (%) | | | | 0.27** |
| Positive | 70 (26.3) | 39 (29.3) | 31 (23.3) | |
| HCV antibody, *n* (%) | | | | <0.01** |
| Positive | 146 (54.9) | 57 (42.9) | 89 (66.9) | |
| Child–Pugh score, *n* (%) | | | | 0.34** |
| 5–6 | 134 (50.4) | 61 (45.9) | 73 (54.9) | |
| 7 | 55 (20.7) | 30 (22.6) | 25 (18.8) | |
| 8–9 | 77 (28.9) | 42 (31.6) | 35 (26.3) | |
| Vascular invasion, *n* (%) | | | | <0.01** |
| Positive | 129 (48.5) | 76 (57.1) | 53 (39.8) | |
| Extrahepatic lesion, *n* (%) | | | | 0.058** |
| Positive | 61 (22.9) | 37 (27.8) | 24 (18.0) | |
| CRP, mg/dL | | | | <0.01* |
| Mean ± SD | 1,9 ± 3.0 | 2.8 ± 3.8 | 0.9 ± 1.2 | |
| AFP, ng/mL | | | | 0.41*** |
| Median, range | 241.5, <10–1 637 200 | 312.5, <10–745 900 | 119.5, <10–1 637 200 | |
| DCP, mAU/mL | | | | <0.01*** |
| Median, range | 567, <10–1 208 000 | 1 286, <10–1 208 000 | 214, <10–326 300 | |

*Student's *t*-test, **$\chi^2$-test, ***Mann–Whitney *U*-test.
AFP, α-fetoprotein; CRP, C-reactive protein; DCP, des-γ-carboxyprothrombin; ECOG PS, Eastern Cooperative Oncology Group performance status; HBs antigen, hepatitis B surface antigen; HCV antibody, hepatitis C virus antibody; NLR, neutrophil to lymphocyte ratio; SD, standard deviation.

receiving sorafenib after HAIC was higher in the group with high NLR (14.3%) than in the one with low NLR (5.3%) ($P = 0.013$).

## Treatment

The data collection cut-off was 20 April, 2014. The median follow-up period was 11.4 months (range, 0.3–127.6). At the time of the analysis, 212 patients (79.7%) had died. A total of 715 courses were administrated to 266 patients, with a median number of two (range, 0–13). All but 18 patients including 12 patients (9.0%) in the high NLR group and six (4.5%) in the low NLR group completed at least one course of HAIC.

Of the 266 patients, IFN-α-2b and PEG IFN-α-2b was used in 131 patients (49.2%) and 135 patients (50.8%), respectively. The response to HAIC and the patient outcomes were similar between the different IFN groups. Cisplatin was administrated in 186 patients (69.9%). Although response to HAIC had a tendency to be better in patients in the cisplatin group than those of the patients without cisplatin, there was no significant differences of the treatment efficacies.

## Response to HAIC and PFS stratified by pretreatment NLR

Of the 266 patients, 15 patients could not receive radiological assessment because of worsened general condition, hepatic failure or loss to follow up, and the remaining 251 were assessable for response to treatment. The tumor responses to HAIC are shown in

**Table 2** Tumor responses according to NLR

| Response* to HAIC | All ($n = 266$) | High NLR ($n = 133$) | Low NLR ($n = 133$) |
|---|---|---|---|
| CR | 16 (6.0) | 3 (2.3) | 13 (9.8) |
| PR | 62 (23.3) | 25 (18.8) | 37 (27.8) |
| SD | 83 (31.2) | 40 (30.1) | 43 (32.3) |
| PD | 90 (33.8) | 55 (41.4) | 35 (26.3) |
| NE | 15 (5.6) | 10 (7.5) | 5 (3.8) |
| Objective response rate | 29.3% | 21.1% | 37.6% |
| | $P < 0.01$** | | |

*RECIST version 1.1, **$\chi^2$-test.
Data are presented as *n* (%).
CR, complete response; HAIC, hepatic arterial infusion chemotherapy; NE, not evaluated; NLR, neutrophil to lymphocyte ratio; PD, progressive disease; PR, partial response; SD, stable disease.

Table 2. The objective response rate was 37.6% in patients with low NLR, which was significantly better than that of the patients with high NLR (21.1%; $P < 0.01$). Multivariate logistic regression analysis revealed that low NLR (hazard ratio [HR], 1.918; $P = 0.024$) as well as vascular invasion (HR, 1.874; $P = 0.029$) and extrahepatic lesion (HR, 2.723; $P = 0.012$) remained independently associated with the response to HAIC (Table 3).

The median PFS of all patients was 4.5 months. The PFS of patients with high NLR was shorter than that of the patients with low NLR, and the median PFS of the patients with high NLR was 3.2 months, which was significantly worse than that of the patients with low NLR of 5.6 months (Fig. 1a). The following nine of the

**Table 3** Pretreatment factors affecting objective response

| | | *n* | ORR (%) | Univariate *P** | Hazard ratio (95% CI) | Multivariate *P*** |
|---|---|---|---|---|---|---|
| NLR | <2.87 | 133 | 37.6 | <0.01 | 1.918 (1.092–3.369) | 0.024 |
| | ≥2.87 | 133 | 21.1 | | | |
| Age, years | ≥67 | 136 | 31.6 | 0.40 | | |
| | <67 | 130 | 26.9 | | | |
| Sex | Male | 209 | 29.7 | 0.81 | | |
| | Female | 57 | 28.1 | | | |
| ECOG PS | 0 | 220 | 32.3 | 0.051 | | |
| | 1 | 41 | 17.1 | | | |
| | 2 | 5 | 0 | | | |
| Prior treatment of sorafenib | Absence | 241 | 29.5 | 0.88 | | |
| | Presence | 25 | 28.0 | | | |
| HBs antigen | Positive | 70 | 32.9 | 0.45 | | |
| | Negative | 196 | 28.1 | | | |
| HCV antibody | Positive | 146 | 31.5 | 0.39 | | |
| | Negative | 120 | 26.7 | | | |
| Child–Pugh score | 5–6 | 134 | 35.8 | 0.054 | | |
| | 7 | 55 | 25.5 | | | |
| | 8–9 | 77 | 20.8 | | | |
| Vascular invasion | Absence | 137 | 36.5 | <0.01 | 1.874 (1.067–3.292) | 0.029 |
| | Presence | 129 | 21.7 | | | |
| Extrahepatic lesion | Absence | 205 | 33.7 | <0.01 | 2.723 (1.250–5.932) | 0.012 |
| | Presence | 61 | 14.8 | | | |
| CRP, mg/dL | <0.8 | 127 | 33.9 | 0.11 | | |
| | ≥0.8 | 136 | 25.0 | | | |
| AFP, ng/mL | <235.5 | 133 | 31.6 | 0.42 | | |
| | ≥235.5 | 133 | 27.1 | | | |
| DCP, mAU/mL | <567 | 133 | 33.8 | 0.11 | | |
| | ≥567 | 133 | 24.8 | | | |

*$\chi^2$-Test, **logistic regression analysis.
AFP, α-fetoprotein; CI, confidence interval; CRP, C-reactive protein; DCP, des-γ-carboxyprothrombin; ECOG PS, Eastern Cooperative Oncology Group performance status; HBs antigen, hepatitis B surface antigen; HCV antibody, hepatitis C virus antibody; NLR, neutrophil to lymphocyte ratio; ORR, objective response rate.

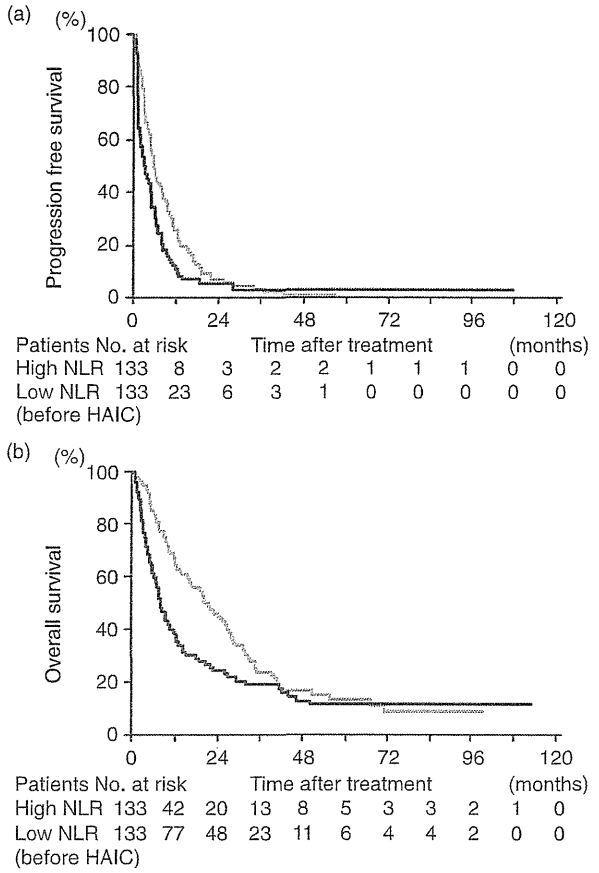


**Figure 1** Kaplan–Meier plot of progression-free survival (PFS) and overall survival (OS) since commencement of HAIC according to neutrophil to lymphocyte ratio (NLR). (a) Median PFS of the patients with high NLR was 3.2 months, which was significantly worse than that of the patients with low NLR, 5.6 months ($P < 0.01$). (b) Median OS of the patients with high NLR was 8.0 months, which was significantly worse than that of the patients with low NLR, 20.7 months ($P < 0.01$). —, High NLR; —, Low NLR.

12 pretreatment variables were significantly associated with the PFS times in univariate analyses: ECOG PS ($P < 0.01$), hepatitis B surface antigen (HBsAg; $P < 0.01$), hepatitis C virus antibody ($P = 0.044$), vascular invasion ($P < 0.01$), extrahepatic lesion ($P < 0.01$), CRP ($P < 0.01$), α-fetoprotein (AFP) ($P < 0.01$) and DCP ($P < 0.01$) as well as NLR. Pretreatment high NLR was an independent unfavorable factor for PFS (HR, 1.363; $P = 0.044$) as well as ECOG PS 1 and 2 (HR compared with ECOG PS, 1.585; $P = 0.019$ and 3.301; $P = 0.025$, respectively), HBsAg positive (HR, 1.687; $P < 0.01$), extrahepatic lesion (HR, 1.500; $P = 0.025$) and AFP of 235.5 ng/mL or more (HR, 1.580; $P < 0.01$) in Cox's proportional hazards regression model (Table 4).

## Patient outcome stratified by pretreatment NLR

The median OS of all patients was 12.6 months. The OS in the patients with high NLR was shorter than that of the patients with low NLR ($P < 0.01$), and the median OS in the patients with high NLR was 8.0 months, which was significantly worse than that of the patients with low NLR (20.7 months) (Fig. 1b). The following eight of the 12 pretreatment variables were significantly associated with the OS in univariate analyses: ECOG PS ($P < 0.01$), Child–Pugh score ($P < 0.01$), vascular invasion ($P < 0.01$), extrahepatic lesion ($P < 0.01$), CRP ($P < 0.01$), AFP ($P < 0.01$) and DCP ($P < 0.01$) as well as NLR. Pretreatment high NLR was an independent unfavorable factor for OS (HR, 1.492; $P < 0.01$) as well as ECOG PS 1 and 2 (HR compared with ECOG PS 0, 1.597; $P = 0.034$ and 3.825; $P = 0.013$, respectively), Child–Pugh score 8 or 9 (HR compared with Child–Pugh score 5 or 6, 1.454; $P = 0.036$), extrahepatic lesion (HR, 1.677; $P < 0.01$), CRP of 0.8 or more (HR, 1.406; $P = 0.031$) and AFP of 235.5 or more (HR, 1.702; $P < 0.01$) in Cox's proportional hazards regression model (Table 5).

## Patient outcome according to trend of NLR

We obtained the NLR value at 4 weeks after the start of HAIC in 243 patients. Of the patients with high NLR before HAIC ($n = 120$), NLR was low at 4 weeks after the start of HAIC (High-Low) in 69 patients (57.5%). The median PFS in the patients with High-Low was 4.9 months, which was significantly better than that of the patients with high NLR at 4 weeks after the start of HAIC (High-High), 2.0 months ($P = 0.030$). The median OS in the patients with High-Low was 11.5 months, which was significantly better than that of the patients with High-High, 6.1 months ($P < 0.01$) (Fig. 2a). In contrast, of the patients with low NLR before HAIC ($n = 123$), NLR was high at 4 weeks after the start of HAIC (Low-High) in 11 (8.9%) patients. The median PFS in the patients with Low-High was 2.0 months, which was significantly worse than that of the patients with low NLR at 4 weeks after the start of HAIC (Low-Low), 6.0 months ($P < 0.01$). The median OS in the patients with Low-High was 5.5 months, which was significantly worse than that of the patients with Low-Low, 22.6 months ($P < 0.01$) (Fig. 2b).

**Table 4** Pretreatment factors affecting progression-free survival

| | | *n* | mPFS (months) | Univariate *P** | Hazard ratio (95% CI) | Multivariate *P*** |
|---|---|---|---|---|---|---|
| NLR | ≥2.87 | 133 | 3.2 | <0.01 | 1.363 (1.008–1.843) | 0.044 |
| | <2.87 | 133 | 5.6 | | | |
| Age, years | <67 | 130 | 4.0 | 0.46 | | |
| | ≥67 | 136 | 5.2 | | | |
| Sex | Male | 209 | 4.5 | 0.31 | | |
| | Female | 57 | 5.1 | | | |
| ECOG PS | 2 | 5 | 0.9 | <0.01 | 3.301 (1.165–9.355) | 0.025 |
| | 1 | 41 | 2.7 | | 1.585 (1.079–2.330) | 0.019 |
| | 0 | 220 | 4.9 | | | |
| Prior treatment of sorafenib | Absence | 241 | 4.5 | 0.95 | | |
| | Presence | 25 | 4.8 | | | |
| HBs antigen | Positive | 70 | 2.5 | <0.01 | 1.687 (1.163–2.447) | <0.01 |
| | Negative | 196 | 5.5 | | | |
| HCV antibody | Negative | 120 | 3.1 | 0.044 | 0.841 (0.596–1.188) | 0.33 |
| | Positive | 146 | 5.5 | | | |
| Child–Pugh score | 8–9 | 77 | 3.2 | 0.099 | | |
| | 7 | 55 | 4.5 | | | |
| | 5–6 | 134 | 5.1 | | | |
| Vascular invasion | Presence | 129 | 2.7 | <0.01 | 1.191 (0.876–1.619) | 0.27 |
| | Absence | 137 | 6.2 | | | |
| Extrahepatic lesion | Presence | 61 | 2.8 | <0.01 | 1.500 (1.053–2.138) | 0.025 |
| | Absence | 205 | 5.5 | | | |
| CRP, mg/dL | ≥0.8 | 136 | 2.8 | <0.01 | 1.293 (0.952–1.758) | 0.10 |
| | <0.8 | 127 | 6.2 | | | |
| AFP, ng/mL | ≥235.5 | 133 | 2.8 | <0.01 | 1.580 (1.162–2.148) | <0.01 |
| | <235.5 | 133 | 6.2 | | | |
| DCP, mAU/mL | ≥567 | 133 | 3.2 | <0.01 | 1.203 (0.873–1.659) | 0.26 |
| | <567 | 133 | 5.6 | | | |

*Log–rank test, **Cox's proportional hazards regression model.
AFP, α-fetoprotein; CI, confidence interval; CRP, C-reactive protein; DCP, des-γ-carboxyprothrombin; ECOG PS, Eastern Cooperative Oncology Group performance status; HBs antigen, hepatitis B surface antigen; HCV antibody, hepatitis C virus antibody; mPFS, median progression-free survival time; NLR, neutrophil to lymphocyte ratio.

### Correlation between cytokine or chemokine profiling and NLR

Data of cytokine and chemokine profiling were obtained in 86 patients. We investigated the association between the value of cytokine or chemokine and NLR to analyze the mechanisms of NLR to cancer biology. Results are shown in Table 6. Serum PDGF-BB concentration had a significant positive correlation with NLR ($r = 0.227$; $P = 0.035$) (Fig. S1). No other cytokine or chemokine was correlated with NLR.

## DISCUSSION

THE FIRST AIM of this study was to investigate the correlation between NLR and patient characteristics in advanced HCC. Some reports have suggested that NLR is correlated with tumor biology in unselected cohorts of patients with HCC.[21] Our analysis also demonstrated the corresponding results in patients with HCC at an advanced stage. Moreover, it was newly clarified that NLR had a strong relation with ECOG PS, which was an important factor reflecting a variety of complications of liver cirrhosis or tumor-related symptoms.[22]

The most important insight of our study was that NLR was correlated with the treatment efficacies presented as response to HAIC or PFS as well as patient outcome given that this is the largest cohort of patients with advanced HCC treated with HAIC, to the best of our knowledge. Our results should be interpreted with caution because of the bias introduced by the differences of patient characteristics observed between the

**Table 5** Pretreatment factors affecting overall survival

| | | *n* | mOS (months) | Univariate *P** | Hazard ratio (95% CI) | Multivariate *P*** |
|---|---|---|---|---|---|---|
| NLR | ≥2.87 | 133 | 8.0 | <0.01 | 1.492 (1.106–2.012) | <0.01 |
| | <2.87 | 133 | 20.7 | | | |
| Age, years | <67 | 130 | 9.9 | 0.18 | | |
| | ≥67 | 136 | 17.7 | | | |
| Sex | Female | 57 | 10.7 | 0.091 | | |
| | Male | 209 | 13.6 | | | |
| ECOG PS | 2 | 5 | 2.4 | <0.01 | 3.825 (1.329–11.009) | 0.013 |
| | 1 | 41 | 7.3 | | 1.597 (1.035–2.463) | 0.034 |
| | 0 | 220 | 14.5 | | | |
| Prior treatment of sorafenib | Presence | 25 | 11.6 | 0.77 | | |
| | Absence | 241 | 13.1 | | | |
| HBs antigen | Positive | 70 | 8.4 | 0.095 | | |
| | Negative | 196 | 15.4 | | | |
| HCV antibody | Negative | 120 | 10.7 | 0.096 | | |
| | Positive | 146 | 16.6 | | | |
| Child–Pugh score | 8–9 | 77 | 6.9 | <0.01 | 1.454 (1.024–2.064) | 0.036 |
| | 7 | 55 | 13.7 | | 0.942 (0.621–1.429) | 0.78 |
| | 5–6 | 134 | 16.6 | | | |
| Vascular invasion | Presence | 129 | 8.2 | <0.01 | 1.138 (0.819–1.582) | 0.44 |
| | Absence | 137 | 19.6 | | | |
| Extrahepatic lesion | Presence | 61 | 6.5 | <0.01 | 1.677 (1.144–2.458) | <0.01 |
| | Absence | 205 | 16.6 | | | |
| CRP, mg/dL | ≥0.8 | 136 | 8.7 | <0.01 | 1.406 (1.031–1.917) | 0.031 |
| | <0.8 | 127 | 22.6 | | | |
| AFP, ng/mL | ≥235.5 | 133 | 8.7 | <0.01 | 1.702 (1.228–2.359) | <0.01 |
| | <235.5 | 133 | 21.8 | | | |
| DCP, mAU/mL | ≥567 | 133 | 9.0 | <0.01 | 1.123 (0.808–1.568) | 0.49 |
| | <567 | 133 | 20.7 | | | |

*Log–rank test, **Cox's proportional hazards regression model.
AFP, α-fetoprotein; CI, confidence interval; CRP, C-reactive protein; DCP, des-γ-carboxyprothrombin; ECOG PS, Eastern Cooperative Oncology Group performance status; HBs antigen, hepatitis B surface antigen; HCV antibody, hepatitis C virus antibody; mOS, median overall survival time; NLR, neutrophil to lymphocyte ratio.

high NLR group and low NLR group. However, our results suggested that NLR was a predictor of response to HAIC in multivariate analysis independent of ECOG PS, hepatic reserve and tumor-related factors in this study. CRP was suggested as a prognostic marker for patients with HCC treated with sorafenib;[23] however, it remains unclear whether such factors can predict antitumor effects of sorafenib or the prognosis of patients with advanced HCC. NLR may be a stronger predictor than CRP of both of antitumor effects and prognosis of patients with advanced HCC treated with HAIC. The differential leukocyte count is an inexpensive and routinely measured marker in daily clinical practice and, therefore, NLR is a simple and easily available marker for the selection of suitable patients to undergo HAIC.

Another interesting point of the present study was that the cumulative survival curve was stratified according to trend of NLR before and after HAIC. The antitumor effect was evaluated generally by radiological findings and the trends of tumor markers, such as AFP or DCP in HCC.[24] However, these modalities have disadvantages such as complications, cost of measurements and lack of universality because the evaluation was often difficult to interpret.[25] Further, tumor markers were not elevated in one-third of the patients with HCC.[17] Our findings suggested that NLR, a simple and economical marker derived from routinely available blood tests, was helpful in evaluating the efficacy of HAIC or predicting the outcomes of the patients with advanced HCC by following its trend.

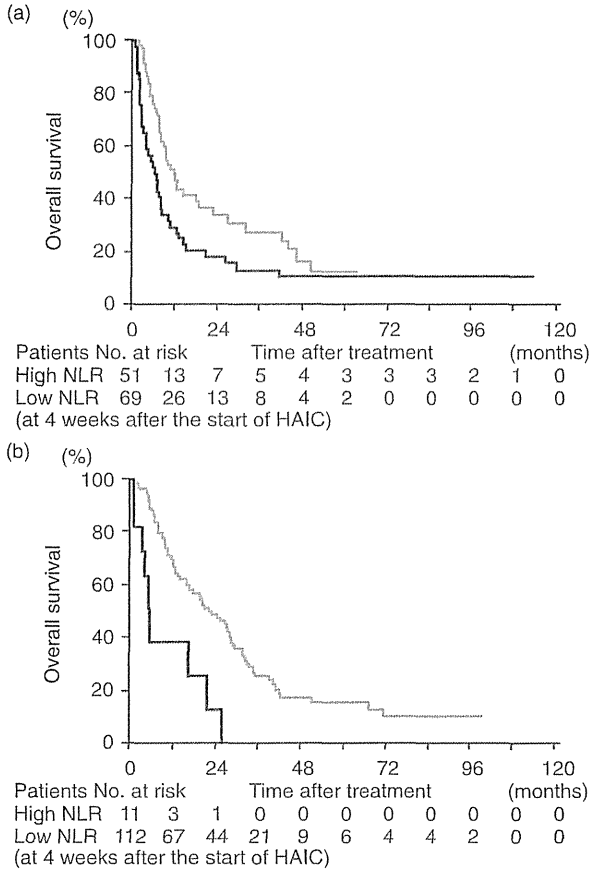


**Figure 2** Kaplan–Meier plot of overall survival (OS) since commencement of hepatic arterial infusion chemotherapy (HAIC) according to neutrophil to lymphocyte ratio (NLR) at 4 weeks after the start of treatment. (a) Among the patients with high NLR before HAIC, median OS of the patients whose NLR was reduced (High-Low) was 11.5 months, which was significantly better than that of the patients with remaining high NLR (High-High), 6.1 months ($P < 0.01$). (b) Among the patients with low NLR before HAIC, median OS of the patients whose NLR was elevated (Low-High) was 5.5 months, which was significantly worse than that of the patients with remaining low NLR (Low-Low), 22.6 months ($P < 0.01$).

Finally, our findings indicated that PDGF-BB was a candidate of mediators for NLR, reflecting tumor biology and response to HAIC. It was reported that activated neutrophils stimulate the growth and progression of the cancer cells by releasing growth factors such as PDGF-BB.[26] It has been shown that PDGF-BB also promotes angiogenesis and subsequent vascular invasion[27] and may reduce the sensitivity to cytotoxic agents in HCC.[28] Some reports stated that the serum level of PDGF-BB correlated with the efficacy of treatments for HCC,[27,29] and should be paid more attention when considering treatment of patients with HCC.

The present study has several limitations. For instance, the study was retrospective in nature and it was conducted at a single center. Therefore, further study is needed to validate our findings.

In conclusion, high NLR was strongly correlated with poor general condition and advanced tumor progression in patients with advanced HCC. NLR can act as a predictive and prognostic factor for patients with advanced HCC treated with HAIC. The trends of NLR after treatment of HAIC strongly reflected the patient outcomes in this study. Our findings can be useful in determining treatment strategies or in designing future clinical chemotherapy trials of advanced HCC.

## ACKNOWLEDGMENTS

THE AUTHORS THANK Masayo Baba for data collection and Tadashi Toyama for statistical advice.

**Table 6** Association between cytokine or chemokine and NLR

| | *r* | *P** |
|---|---|---|
| EGF | 0.001 | 0.99 |
| FGF | 0.141 | 0.20 |
| HGF | 0.011 | 0.92 |
| IFN-γ | 0.132 | 0.23 |
| IL-2 | 0.103 | 0.35 |
| IL-4 | 0.161 | 0.14 |
| TNF-α | 0.124 | 0.26 |
| IL-6 | 0.159 | 0.15 |
| IL-8 | −0.080 | 0.47 |
| IL-10 | 0.121 | 0.27 |
| IL-5 | −0.035 | 0.75 |
| IP10 | −0.089 | 0.42 |
| MIG | −0.112 | 0.31 |
| PDGF-BB | 0.227 | 0.035 |
| TGF-β | 0.000 | 1.00 |
| TGF-α | −0.041 | 0.71 |
| VEGF | −0.102 | 0.35 |
| SCF | −0.088 | 0.42 |
| IL-12 | 0.040 | 0.71 |
| SDF-1 | −0.077 | 0.48 |

*Linear regression.
EGF, epidermal growth factor; FGF, fibroblast growth factor; HGF, hepatocyte growth factor; IFN, interferon; IL, interleukin; IP, interferon-γ-induced protein, MIG, monokine induced by interferon-γ; PDGF, platelet-derived growth factor; SCF, stem cell factor; SDF, stromal cell-derived factor; TGF, transforming growth factor; TNF, tumor necrosis factor; VEGF, vascular endothelial growth factor.

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## SUPPORTING INFORMATION

ADDITIONAL SUPPORTING INFORMATION may be found in the online version of this article at the publisher's website:

**Figure S1** Relationship between overall survival and platelet-derived growth factor (PDGF)-BB. PDGF-BB concentration had significant positive correlation with neutrophil to lymphocyte ratio (NLR) on the basis of weighted linear regression ($r = 0.227$; $P = 0.035$).

Liver INTERNATIONAL

Liver International ISSN 1478-3223

NAFLD & NASH

# Characteristics of hepatic fatty acid compositions in patients with nonalcoholic steatohepatitis

Kazutoshi Yamada[1], Eishiro Mizukoshi[1], Hajime Sunagozaka[1], Kuniaki Arai[1], Tatsuya Yamashita[1], Yumie Takeshita[2], Hirofumi Misu[2], Toshinari Takamura[2], Seiko Kitamura[3], Yoh Zen[3], Yasuni Nakanuma[4], Masao Honda[1] and Shuichi Kaneko[1]

1 Department of Gastroenterology, Graduate School of Medicine, Kanazawa University, Kanazawa, Japan
2 Department of Disease Control and Homeostasis, Graduate School of Medicine, Kanazawa University, Kanazawa, Japan
3 Division of Pathology, Kanazawa University Hospital, Kanazawa, Japan
4 Department of Human Pathology, Graduate School of Medicine, Kanazawa University, Kanazawa, Japan

**Keywords**
fatty acid metabolism – insulin resistance – palmitic acid – toxic lipid

**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, homoeostasis 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.

**Correspondence**
Shuichi Kaneko, Department of Gastroenterology, Graduate School of Medicine, Kanazawa University, Kanazawa, Ishikawa 920-8641, Japan
Tel: +81 76 265 2230
Fax: +81 76 234 4250
e-mail: skaneko@m-kanazawa.jp

Received 9 January 2014
Accepted 3 September 2014

DOI:10.1111/liv.12685
Liver Int. 2015; 35: 582–590

## 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 homoeostasis model assessments of insulin resistance (HOMA-IR) [fasting serum insulin (μU/ml) × fasting plasma glucose (mg/dl)/405] and the Quantitative Insulin Sensitivity Check Index (QUICKI) [1/log (fasting serum insulin (μU/ml) × 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 C18:3n3 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 (×10⁴/mm³) | 22.6 ± 1.8 | 24.0 ± 0.9 | 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

| | SS | NASH | *P*-value |
|---|---|---|---|
| Fibrosis (0/1/2/3/4) | 7/52/4/0/0 | 1/15/11/7/6 | < 0.01 |
| Steatosis (0/1/2/3) | 0/30/24/9 | 0/10/15/15 | < 0.01 |
| Lobular inflammation (0/1/2/3) | 6/34/23/0 | 0/8/26/6 | < 0.01 |
| Hepatocellular ballooning (0/1/2) | 41/21/1 | 1/17/22 | < 0.01 |

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

| | 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* |
| C22:2n6 | 3.08 ± 0.6 | 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 ratio | 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 than in the SS 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 ($P < 0.01$). 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)

### 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 (≦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 (≦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

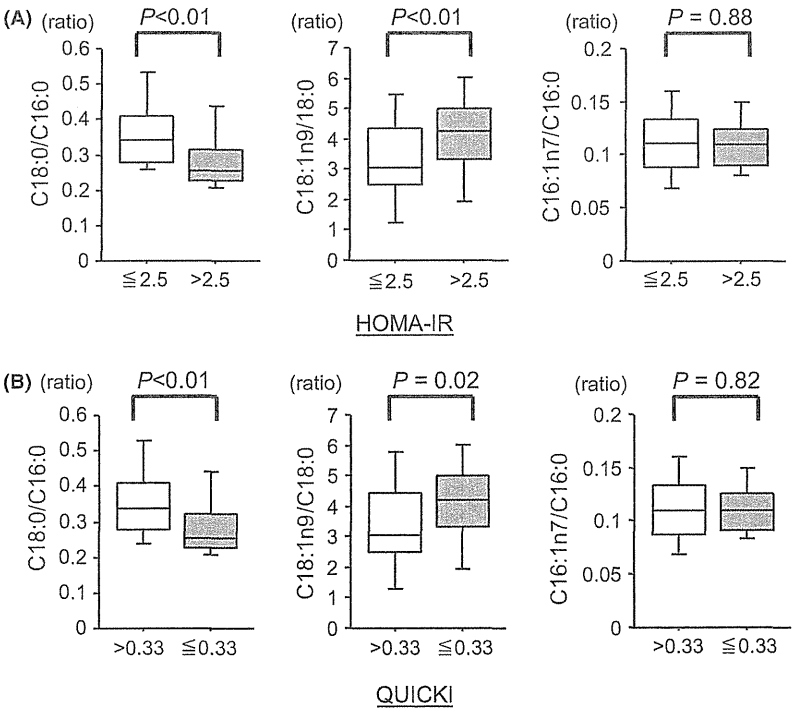


**Fig. 1.** Association between insulin resistance and the fatty acid composition ratio in liver tissue. The association between insulin resistance and changes in the fatty acid composition ratio in liver tissue was analysed using the Mann–Whitney *U*-test. (A) Patients were divided into groups with 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.

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 score (Fig. 2D). Similarly, 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).

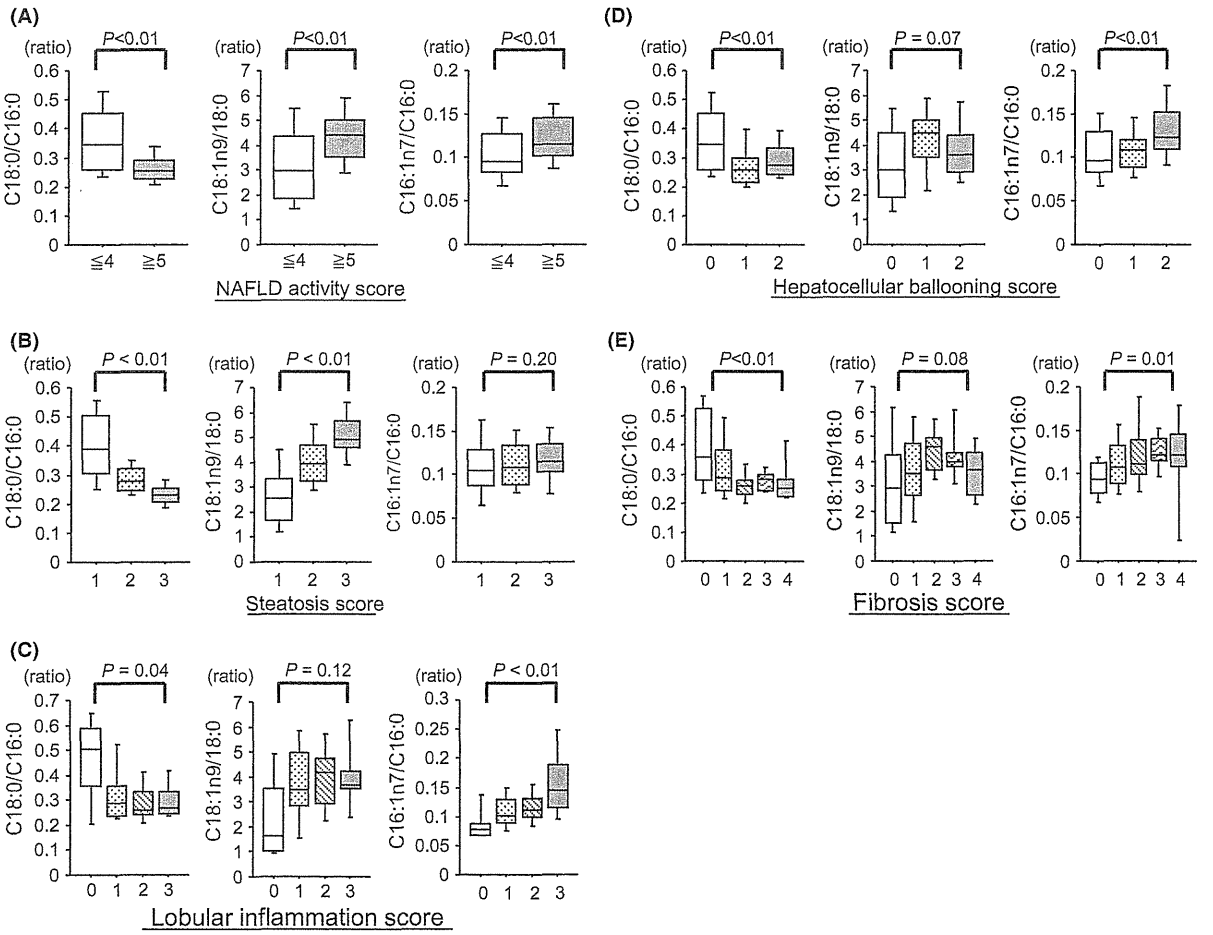


**Fig. 2.** Relationship between the histopathological findings of the liver and fatty acid composition ratio. The association between the histopathological findings of the liver and fatty acid composition ratio was evaluated using the Spearman's rank correlation coefficient. (A) NAS, (B) steatosis score, (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 γ-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

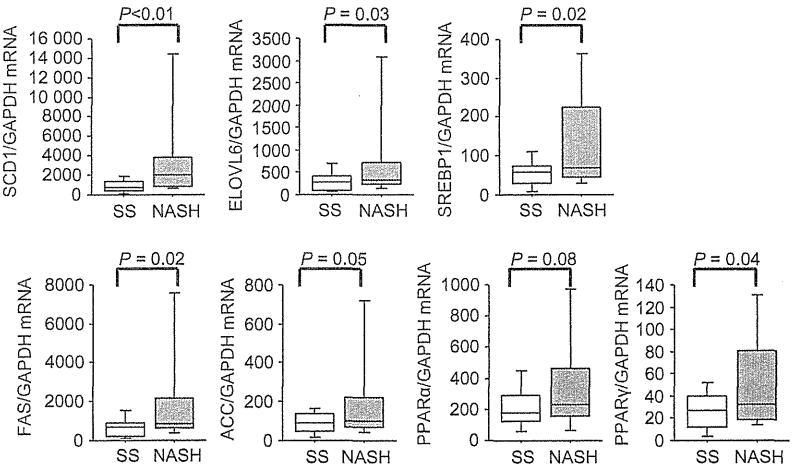


**Fig. 3.** Expression of fatty acid metabolism-related genes in liver tissue. In 65 patients (SS: 35, NASH: 30), the expression levels of fatty acid metabolism-related genes were measured using RT-PCR, and evaluated using the Mann–Whitney *U*-test.