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, **χ^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			

*χ^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.

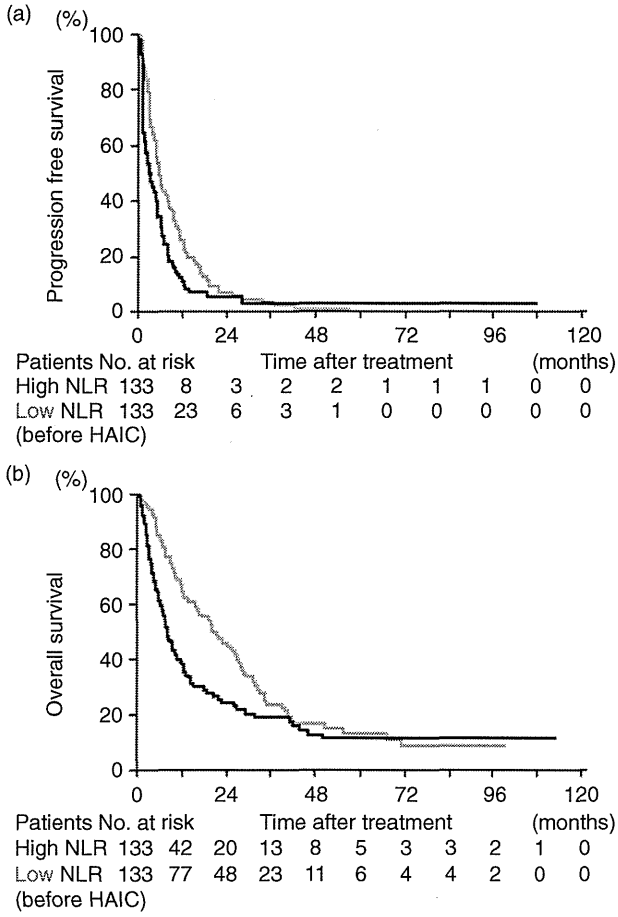

Figure 1 Kaplan–Meier plot of progression-free survival (PFS) and overall survival (OS) since commencement of HAIC according to neutrophil to lymphocyte ratio (NLR). (a) Median PFS of the patients with high NLR was 3.2 months, which was significantly worse than that of the patients with low NLR, 5.6 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.

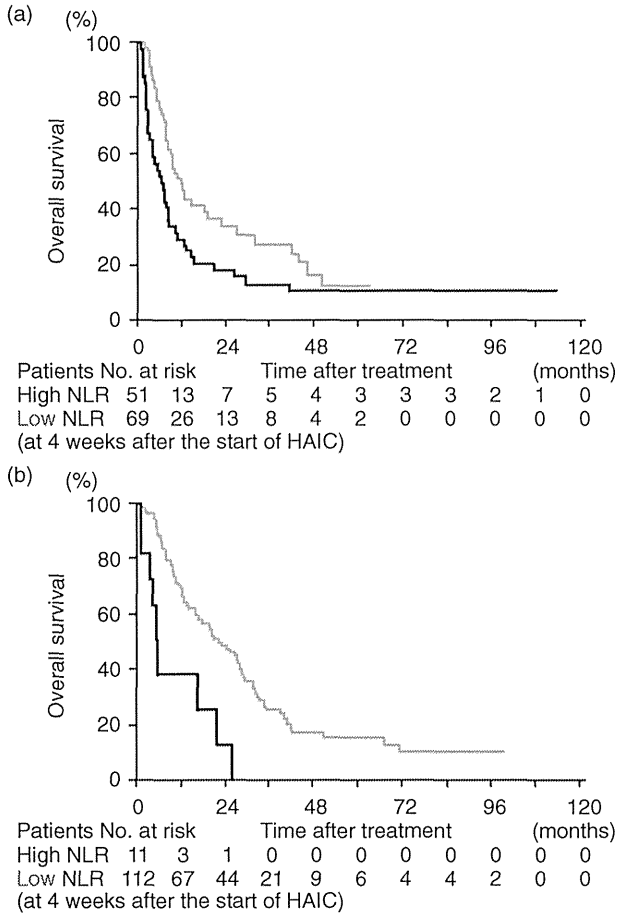

Figure 2 Kaplan–Meier plot of overall survival (OS) since commencement of hepatic arterial infusion chemotherapy (HAIC) according to neutrophil to lymphocyte ratio (NLR) at 4 weeks after the start of treatment. (a) Among the patients with high NLR before HAIC, median OS of the patients whose NLR was 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

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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 ($r = 0.227$; $P = 0.035$).

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NAFLD & NASH

Characteristics of hepatic fatty acid compositions in patients with nonalcoholic steatohepatitis

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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.

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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 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

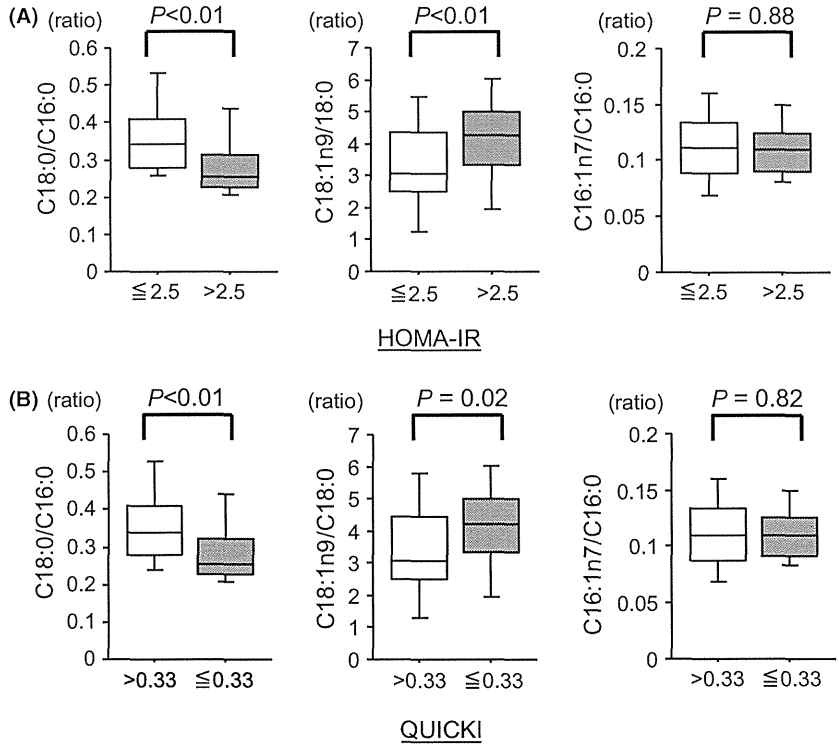

Fig. 1. Association between insulin resistance and the fatty acid composition ratio in liver tissue. The association between insulin resistance and changes in the fatty acid composition ratio in liver tissue was analysed using the Mann–Whitney *U*-test. (A) Patients were divided into groups with and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

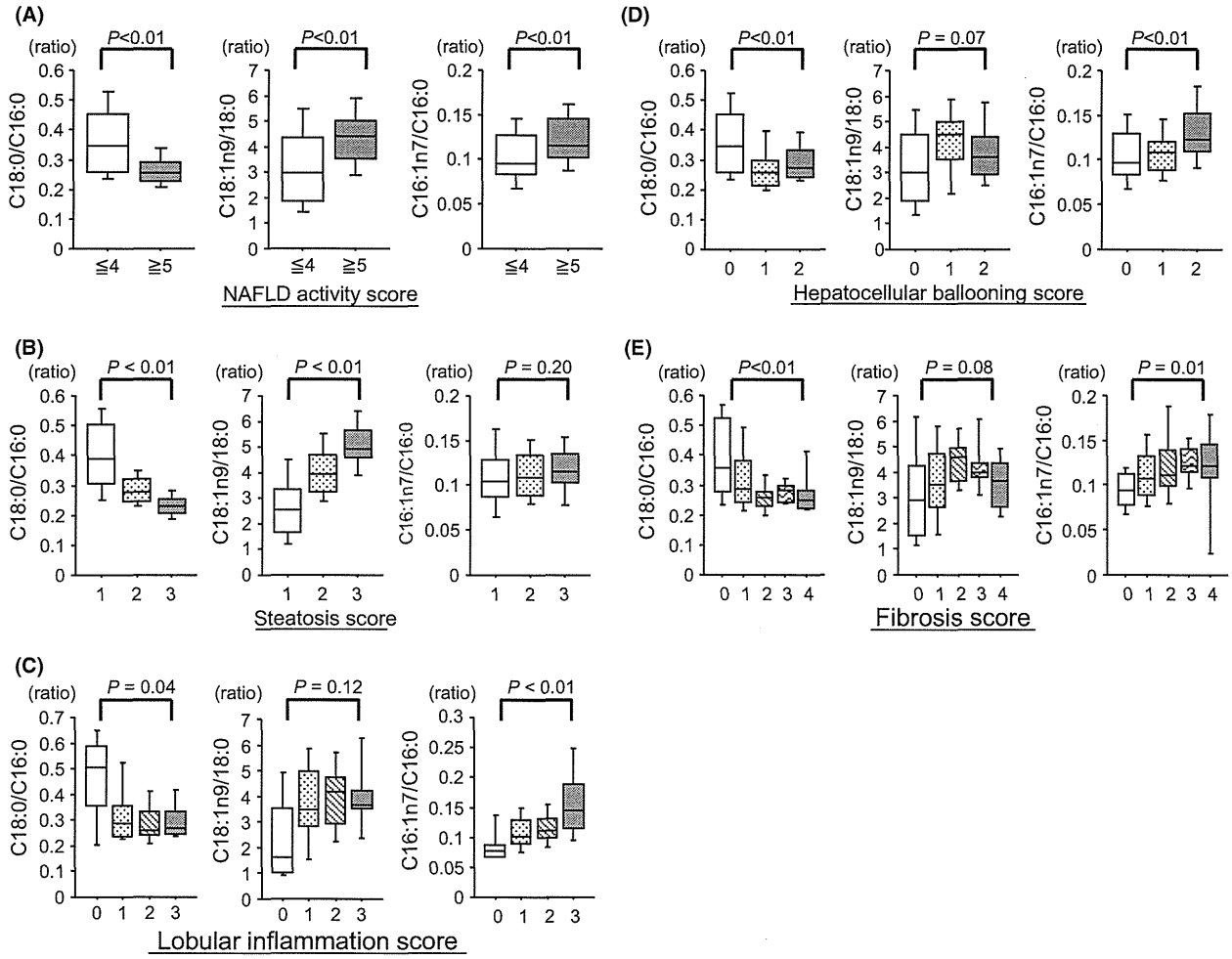

Fig. 2. Relationship between the histopathological findings of the liver and fatty acid composition ratio. The association between the histopathological findings of the liver and fatty acid composition ratio was evaluated using the Spearman's rank correlation coefficient. (A) NAS, (B) steatosis score,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

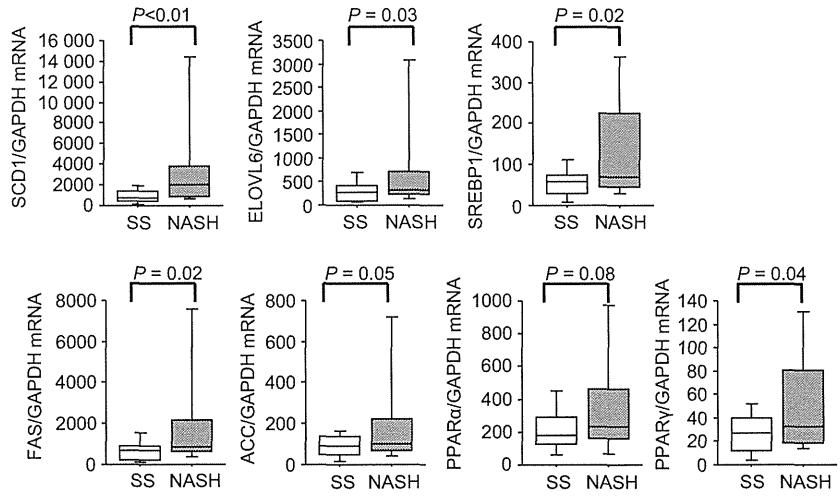

Fig. 3. Expression of fatty acid metabolism-related genes in liver tissue. In 65 patients (SS: 35, NASH: 30), the expression levels of fatty acid metabolism-related genes were measured using RT-PCR, and evaluated using the Mann–Whitney *U*-test.

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 homogenously 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

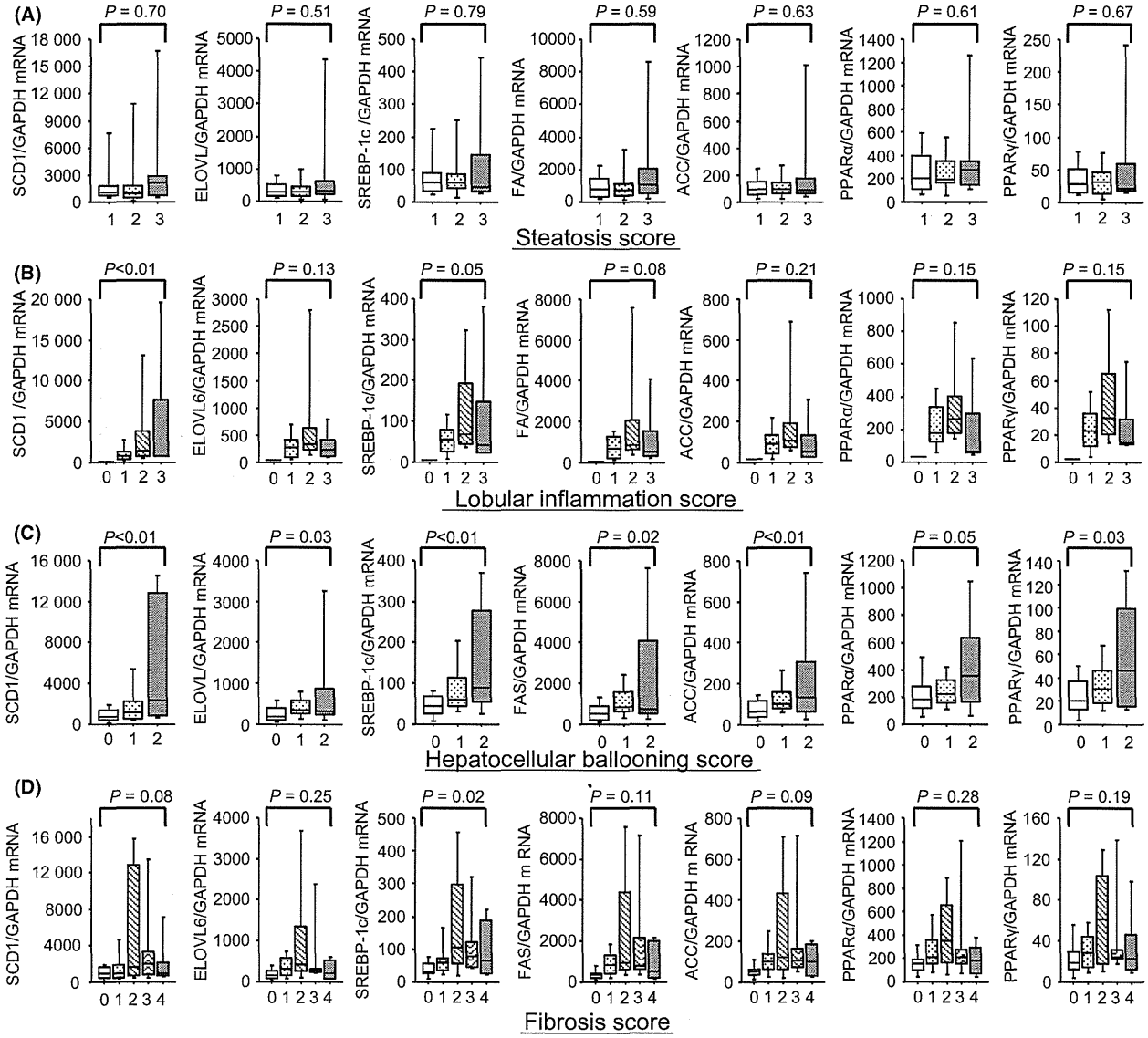

Fig. 4. Relationship between the histopathological findings of the liver and the expression levels of fatty acid metabolism-related genes. The association of the progression of the following items and expression of the fatty acid metabolism-related genes measured in liver tissue using RT-PCR was investigated: (A) steatosis score, (B) lobular inflammation score, (C) hepatocellular ballooning score and (D) fibrosis.

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	MA *P*-value	Coefficient	UA *P*-value	MA *P*-value	Coefficient	UA *P*-value	MA *P*-value	Coefficient	UA *P*-value	MA *P*-value
C18:0/C16:0	−0.610	<0.0001	0.0092	−0.315	0.0022	0.4248	−0.310	0.0016	0.3965	−0.289	0.0040	–
C16:1n7/C16:0	0.084	0.4071	–	0.339	0.0010	0.0191	0.255	0.0106	0.2753	0.141	0.1695	–
C18:1n9/C18:0	0.575	<0.0001	0.2387	0.224	0.0302	0.5603	0.243	0.0140	0.5163	0.184	0.0689	–
HOMA-IR	0.070	0.5211	–	0.137	0.2268	–	0.112	0.3083	–	−0.007	0.9485	–
QUICK I	−0.282	0.0108	0.1421	−0.183	0.1180	–	−0.271	0.0163	0.0200	−0.123	0.2901	–
SCD1	0.093	0.4725	–	0.266	0.0386	0.1785	0.321	0.0077	0.2904	0.067	0.5904	–
ELOVL6	0.16	0.1941	–	0.161	0.2177	–	0.283	0.0201	0.8737	0.037	0.7673	–
SREBP-1c	0.104	0.4349	–	0.249	0.0591	–	0.336	0.0064	0.0559	0.118	0.3534	–
FAS	0.148	0.2543	–	0.195	0.1340	–	0.320	0.0083	0.3309	0.085	0.4949	–
ACC	0.142	0.2902	–	0.159	0.2380	–	0.254	0.0441	0.1917	0.040	0.7539	–
PPARα	0.131	0.3222	–	0.170	0.2005	–	0.232	0.0637	–	0.028	0.8227	–
PPARγ	0.136	0.3243	–	0.155	0.2631	–	0.215	0.1003	–	0.030	0.8195	–

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.

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ORIGINAL INVESTIGATION

Genome-wide association study identifies a *PSMD3* variant associated with neutropenia in interferon-based therapy for chronic hepatitis C

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Abstract Cytopenia during interferon-based (IFN-based) therapy for chronic hepatitis C (CHC) often necessitates reduction of doses of drugs and premature withdrawal from therapy resulting in poor response to treatment. To identify genetic variants associated with IFN-induced neutropenia, we conducted a genome-wide association study (GWAS) in 416 Japanese CHC patients receiving IFN-based therapy. Based on the results, we selected 192 candidate single nucleotide polymorphisms (SNPs) to carry out a replication analysis in an independent set of 404 subjects. The SNP rs2305482, located in the intron region of the *PSMD3* gene on chromosome 17, showed a strong association when the results of GWAS and the replication stage were combined (OR = 2.18, $P = 3.05 \times 10^{-7}$ in the allele frequency model). Logistic regression analysis showed that rs2305482 CC and neutrophil count at baseline were independent predictive factors for IFN-induced neutropenia (OR = 2.497, $P = 0.0072$ and OR = 0.998, $P < 0.0001$, respectively). Furthermore, rs2305482 genotype was associated with the doses of pegylated interferon (PEG-IFN) that could be tolerated in hepatitis C virus genotype 1-infected patients treated with PEG-IFN plus ribavirin, but not with treatment efficacy. Our results suggest that genetic

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testing for this variant might be useful for establishing personalized drug dosing in order to minimize drug-induced adverse events.

Introduction

Chronic hepatitis C virus (HCV) infection is a significant risk factor for progressive liver fibrosis and hepatocellular carcinoma. Antiviral treatment improves the natural course in chronic hepatitis C (CHC) (George et al. 2009; Yoshida et al. 2004). Newly-developed treatments involving direct-acting antivirals (DAAs), including nonstructural (NS) 3/4A protease inhibitors have shown promising outcomes in combination with pegylated interferon (PEG-IFN) plus ribavirin (RBV) in several clinical trials. Thus, >70 % of patients infected with HCV genotype 1 are reported to achieve sustained virological responses (SVR) (Jacobson et al. 2011; Poordad et al. 2012; Zeuzem et al. 2011). Furthermore, interferon-free (IFN-free) therapies are expected to be useful especially in IFN-resistant patients and may become the standard of care in the near future. However, IFN-based regimens have been standard-of-care therapies over the last couple of decades.

IFN-based therapies are associated with various adverse effects. Cytopenia is common due to bone marrow suppression cased by IFN or DAA and hemolysis by RBV. This is particularly the case in patients with advanced hepatic fibrosis, but can sometimes also occur in those with mild fibrosis. This then often necessitates dose reduction or premature withdrawal from therapy, resulting in poor response to treatment. For instance, it was reported that rates of viral clearance were significantly reduced in patients who could not be maintained on at least 80 % of their drug doses for the duration of PEG-IFN/RBV therapy (McHutchison et al. 2002). Therefore, pre-treatment prediction of possible adverse effects in order to avoid them and undergo therapy safely is desirable.

Recent genome-wide association studies (GWASs) have identified two important host genetic variants influencing CHC treatment: (1) single nucleotide polymorphisms (SNPs) near the interleukin-28B (*IL28B*) gene, which are strongly associated with response to therapy for chronic HCV genotype 1 infection (Ge et al. 2009; Suppiah et al. 2009; Tanaka et al. 2009), and (2) SNPs in the inosine triphosphatase (*ITPA*) gene, which accurately predict RBV-induced anemia in European–American (Fellay et al. 2010) and Japanese population (Ochi et al. 2010). We validated the association between this *ITPA* genetic variant and RBV-induced anemia (Sakamoto et al. 2010), and reported that the *ITPA* genotype affects the tolerated doses of RBV and treatment response in a stratified group (Kurosaki et al. 2011; Matsuura et al. 2014). Additionally, our GWAS showed that *DDRGK1/ITPA* variants are strongly associated with IFN-induced thrombocytopenia as well as anemia during PEG-IFN/RBV therapy (Tanaka et al. 2011). Thompson et al. (2012) also reported that the *ITPA* genetic variant was associated with anemia and thrombocytopenia during PEG-IFN/RBV therapy. However they identified no genetic determinants of IFN-induced neutropenia at the level of genome-wide significance by their GWAS in populations of European Americans, African Americans and Hispanics.

Hence, to identify genetic variants associated with IFN-induced neutropenia, we conducted a GWAS in Japanese CHC patients.

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Materials and methods

Patients

From 2007 to 2012, samples for the GWAS were obtained from 416 CHC patients who were treated at 22 hospitals (liver units with hepatologists) throughout Japan. In the following stage of replication analysis, samples were collected in an independent set of 404 Japanese CHC patients. Most patients were treated with PEG-IFN-α2b (1.5 μg/kg body weight subcutaneously once a week) or PEG-IFN-α2a (180 μg once a week) plus RBV (600–1,000 mg daily according to body weight) for 48 weeks for HCV genotype 1 and 24 weeks for genotype 2. Treatment duration was extended in some patients up to 72 weeks for genotype 1 and 48 weeks for genotype 2 according to physicians' preferences. Other patients were treated with PEG-IFN-α2a or IFN monotherapy, or IFN-α2b plus RBV in standard doses of the regimens. The doses of drugs were reduced according to the recommendations on the package inserts or the clinical conditions of the individual patients. Erythropoietin or other growth factors were not given. Patients chronically infected with hepatitis B virus or human immunodeficiency virus, or with other causes of liver disease such as autoimmune hepatitis and primary biliary cirrhosis, were excluded from this study. Written informed consent was obtained from all individual participants in this study and the study protocol conformed to the ethics guidelines of the Declaration of Helsinki and was approved by the institutional ethics review committees.

Inclusion criteria of neutropenia

In the initial stage of GWAS, we defined the inclusion criteria of the case group as minimum neutrophil counts of $<750/mm^3$ at week 2 or 4 during IFN-based therapy, since the dose reduction of IFN is recommended at those levels on the package inserts. Thereafter we did it as minimum neutrophil counts of $<600/mm^3$ at week 2 or 4 in the following GWAS and the replication stages.

SNP genotyping and data cleaning

We conducted two stages of GWAS using the Affymetrix Genome-Wide Human SNP Array 6.0 (Affymetrix, Inc. Santa Clara, CA) according to the manufacturer's instructions. The cut-off value was calculated to maximize the difference, which was also close to median change. At GWAS, the average overall call rate of patients in the case and the control group reached 98.66 and 98.79 %, respectively. We then applied the following thresholds for SNP quality control (QC) in data cleaning: SNP call rate ≥95 % for all samples, minor allele frequency (MAF) ≥1 % for all samples. A total of 601,578 SNPs on autosomal chromosomes passed the QC filters and were used for association analysis. All cluster plots of SNPs showing $P < 0.0001$ in association analyses by comparing allele frequencies in both groups were checked by visual inspection and SNPs with ambiguous genotype calls were excluded. In the replication study, the genotyping of 192 candidate SNPs in an independent set of 404 Japanese HCV-infected patients was carried out using the DigiTag2 assay (Nishida et al. 2007). Successfully genotyped SNPs in the replication analysis had a >95 % call rate, and cleared Hardy–Weinberg equilibrium (HWE) $P \geq 0.001$. One SNP could not be genotyped, and hence we obtained data on 191 SNPs including rs9915252. Three SNPs, rs4794822, rs3907022, and rs3859192 located around the proteasome 26S subunits non-ATPase 3 (*PSMD3*) gene and rs8099917 near the *IL28B* gene were genotyped by TaqMan SNP Genotyping Assays (Applied Biosystems, Carlsbad, CA) following the manufacturer's protocol.

Laboratory and histological tests

Blood samples were obtained at baseline and at appropriate periods after the start of therapy and for hematologic tests, blood chemistry, and HCV RNA. Fibrosis was evaluated on a scale of 0–4 according to the METAVIR scoring system. The SVR was defined as an undetectable HCV RNA level by Roche COBAS Amplicor HCV Monitor test, v.2.0 (Roche Molecular Diagnostics, Pleasanton, CA) with a lower detection limit of 50 IU/ml or Roche COBAS AmpliPrep/COBAS TaqMan HCV assay (Roche Molecular Diagnostics, Pleasanton, CA) with a lower detection limit of 15 IU/ml 24 weeks after the completion of therapy. Serum granulocyte colony-stimulating factor (G-CSF) levels were analyzed using Human G-CSF Quantikine ELISA Kit (R&D Systems, Inc., Minneapolis, MN).

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Expression quantitative trait locus analysis

Expression quantitative trait locus analysis (eQTL) was conducted using the web-based tool, Genevar (http://www.sanger.ac.uk/resources/software/genevar) (Yang et al. 2010). We evaluated the correlations between rs2305482 genotypes and the expression of transcripts of *PSMD3* or colony-stimulating factor 3 (*CSF3*) by the Spearman's rank correlation coefficient.

Statistical analysis

In the GWAS and the replication stages, the observed association between a SNP and neutropenia induced by IFN-based therapy was assessed by the Chi square test with a two-by-two contingency table in three genetic models: the allele frequency model, the dominant-effect model and the recessive-effect model. Significance levels after Bonferroni correction for multiple testing were $P = 8.31 \times 10^{-8}$ (0.05/601,578) in the GWAS stage and $P = 2.62 \times 10^{-4}$ (0.05/191) in the replication stage. Categorical variables were compared between groups by the Chi square test, and non-categorical variables by the Student's t test or the Mann–Whitney U test. Multivariate logistic regression analysis with stepwise forward selection was performed with $P < 0.05$ in univariate analysis as the criteria for model inclusion. To evaluate the discriminatory ability of neutrophil counts at baseline to predict neutropenia during IFN-based therapy, receiver operating characteristic curve (ROC) curve analysis was conducted. Changes of serum G-CSF levels from baseline to the period with neutropenia during IFN-based therapy were compared by the repeated measure analysis of variance (ANOVA). Correlations between neutrophil counts and serum G-CSF levels were analyzed using Pearson's correlation coefficient test. $P < 0.05$ was considered significant in all tests.

Results

Genetic variants associated with IFN-induced neutropenia

We conducted two stages of GWAS by changing the terms of neutrophil counts, followed by the replication analysis (Fig. 1). The characteristics of the patients in each group for the GWAS and the replication stage are summarized in Table 1. At the first stage of GWAS (GWAS-1st), we genotyped 416 Japanese CHC patients with minimum neutrophil counts of <750/mm^3 (Case-G1, $n = 114$) and ≥1,000/mm^3 (Control-G, $n = 302$) at week 2 or 4 during IFN-based therapy. Here there may still be mixed with undesirable samples that should be removed from the case group. Therefore, we designed and carried out the second stage of GWAS (GWAS-2nd) comparing the patients with more severe neutropenia to the control group: in patients with minimum neutrophil counts of <600/mm^3 (Case-G2, $n = 50$) and ≥1,000/mm^3 (Control-G, $n = 302$) at week 2 or 4 using the same samples as used in GWAS-1st. Supplementary Fig. 1 shows a genome-wide view of the single-point association data based on allele frequencies in GWAS-1st and GWAS-2nd. No association between SNPs and IFN-induced neutropenia reached a genome-wide level of significance [Bonferroni criterion $P < 8.31 \times 10^{-8}$ (0.05/601,578)]. Therefore, we selected the candidate SNPs principally

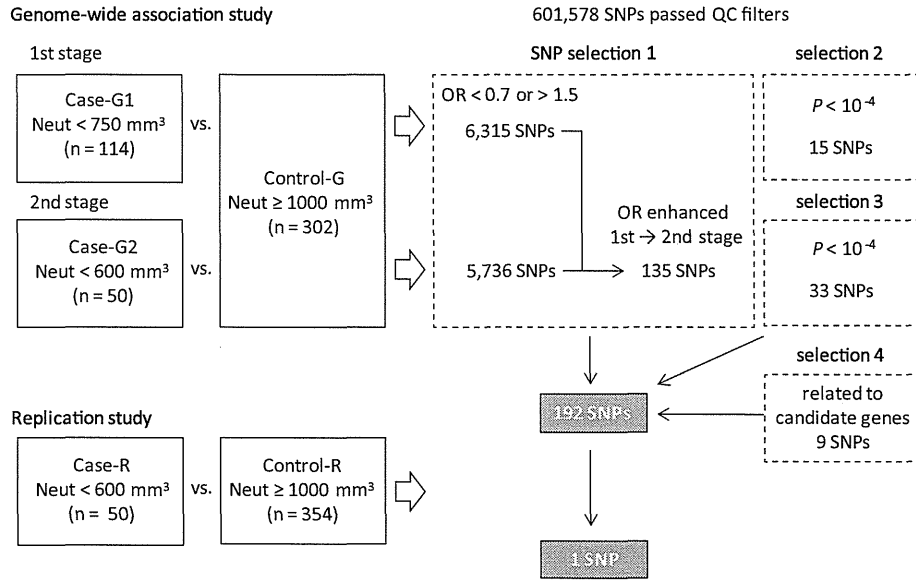

Fig. 1 Outline of the study design. *Neut* neutrophil counts, *SNP* single nucleotide polymorphism, *QC* quality control, *OR* odds ratio