

conducted using logistic regression. The cumulative incidence curve was determined using the Kaplan–Meier method and differences between groups were assessed by the log-rank test. For all methods, the level of significance was set at  $p < 0.05$ . Multivariate analysis of the risk of HCC was carried out using the Cox proportional hazard model. Statistical analyses were performed using the Statistical Package for the Social Sciences software version 11.0 (SPSS, Chicago, IL, USA). In Study 1, age, sex, platelet count, and total bilirubin levels were identified as independent factors for the development of HCC; therefore, these factors were selected for the propensity-matched control study (Study 2) in which 59 patients from the PegIFN $\alpha$ -2a group were included.

## Results

### Study 1

We analyzed the factors involved in the development of HCC in patients who received 90  $\mu$ g PegIFN $\alpha$ -2a weekly or biweekly for more than a year. The incidence of HCC did not differ significantly between the groups treated with PegIFN $\alpha$ -2a weekly and biweekly (34 of 512 vs. 15 of 82, respectively). As shown in Table 2, univariate analysis revealed statistically significant differences in the pre-treatment parameters including age, sex, fibrosis of the liver, platelet count, albumin level, and total bilirubin, between patients who developed HCC and those who did not. Endoscopy was carried out in 375 patients, and esophageal varices were noted in 31 of them. The incidence of HCC was higher in patients with esophageal varices than in those without varices [29.0 % (9 of 31) vs. 6.4 % (22 of 344)]. Assessment of on-treatment factors by univariate analysis revealed statistically significant differences in serum ALT, AFP, and HCV RNA levels 24 weeks after the start of PegIFN $\alpha$ -2a maintenance treatment (Table 2).

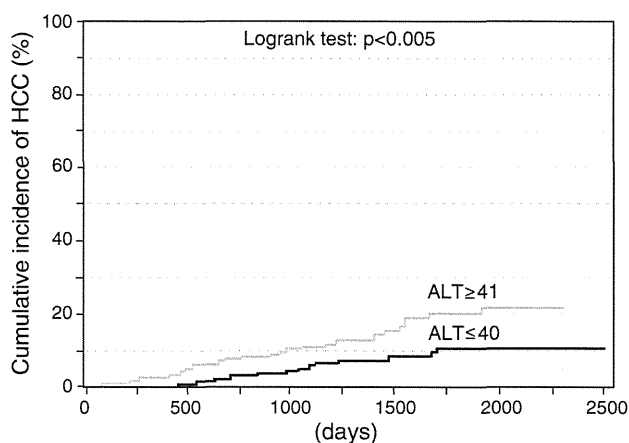
Multivariate analysis including pretreatment parameters revealed that age, sex, fibrosis of the liver, platelet count, and total bilirubin were independent risk factors for HCC development (Table 3). Multivariate analysis including on-treatment parameters identified ALT levels of  $\geq 41$  IU/L and AFP levels of  $\geq 10$  ng/L 24 weeks after the start of the PegIFN $\alpha$ -2a therapy as independent risk factors for HCC development (Table 3).

The incidence of HCC was significantly lower in patients with ALT levels of  $\leq 40$  IU/L than in those with ALT levels of  $\geq 41$  IU/L 24 weeks after the start of observation (Fig. 2). The incidence of HCC was also significantly lower in patients with AFP concentrations of  $< 10$  ng/mL at 24 weeks after the start of observation than in those with AFP concentrations of

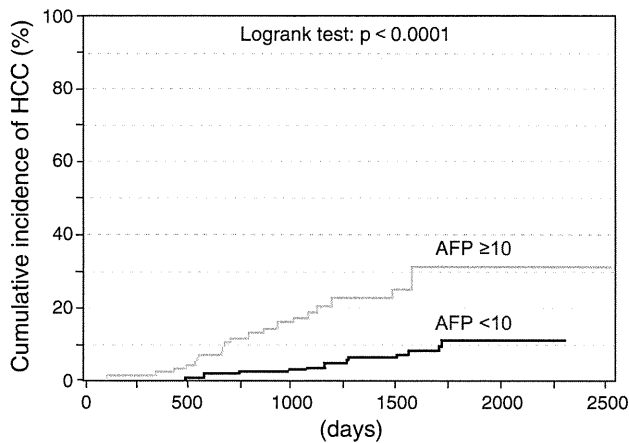
$\geq 10$  ng/mL (Fig. 3). The dose of PegIFN $\alpha$ -2a was reduced to 45  $\mu$ g in 16 patients because of neutropenia and thrombocytopenia. In addition, PegIFN $\alpha$ -2a was discontinued in 18 patients because of adverse events, including depression (7 patients), interstitial pneumonitis (3 patients), thrombocytopenia (3 patients), neutropenia (1 patient), itching (1 patient), and ascites (3 patients). No statistically significant differences were found between the patients with reduced dosage or treatment interruption and those without treatment modifications with respect to overall survival, HCC incidence, ascites formation, variceal bleeding, hepatic encephalopathy, and 2-point increases in the Child-Pugh score. No patients underwent liver transplantation.

**Table 3** Independent risk factors for HCC development in patients treated with 90  $\mu$ g PegIFN $\alpha$ -2a weekly or bi-weekly, evaluated by multivariate analysis (logistic regression analysis)

	Multivariate analysis		
	Odds ratio	95 % Confidence interval (CI)	<i>p</i>
Age (years) (every 5 years)	2.24	1.76–9.33	<0.005
Sex (male/female)	3.16	1.56–10.7	<0.005
Fibrosis (F3, 4/F0, 1, 2)	1.69	1.18–5.2	<0.01
Platelet count ( $< 120 \times 10^3/\mu$ L vs. $\geq 120 \times 10^3/\mu$ L)	3.24	1.44–27.6	<0.01
Total bilirubin (mg/dL)	1.59	1.09–2.58	<0.05
ALT (at 24 weeks) ( $\geq 41$ vs. $< 40$ IU/L)	2.49	1.51–8.28	<0.05
AFP (at 24 weeks) ( $\geq 10$ vs. $< 10$ ng/L)	3.78	1.92–11.8	<0.01



**Fig. 2** Comparison of HCC rates in patients administered with PegIFN $\alpha$ -2a ( $n = 594$ ) with respect to alanine aminotransferase (ALT) levels 24 weeks after the start of therapy. *Black line* patients with ALT  $\geq 41$  IU/L in the first 24 weeks, *gray line* patients with ALT  $\leq 40$  IU/L in the first 24 weeks



**Fig. 3** Comparison of HCC rates in patients administered PegIFN $\alpha$ -2a ( $n = 594$ ) with respect to alpha-fetoprotein (AFP) levels in the first 24 weeks after the start of therapy. *Black line* patients with AFP  $\geq 10$  ng/mL at 24 weeks, *gray line* patients with AFP  $< 10$  ng/mL at 24 weeks

**Study 2**

We compared the incidence of HCC between 59 patients in the control group and the same number of patients in the PegIFN $\alpha$ -2a group using the matched-pair test. The backgrounds of the patients are shown in Table 4. The PegIFN $\alpha$ -2a group had higher rates of advanced fibrosis (F3 and F4) and active inflammation (A2 and A3). No other differences were found between the two groups, except for the white blood cell count (Table 4).

Development of HCC was observed in 2 patients in the PegIFN $\alpha$ -2a group and 8 in the control group. The incidence of HCC was compared between the two groups, using the Kaplan–Meier method. The incidence of HCC in the PegIFN $\alpha$ -2a group was significantly lower than that in the control group (log-rank test,  $p = 0.0187$ ; Fig. 4). Among the patients with advanced fibrosis of the liver (F3 and F4), those in the PegIFN $\alpha$ -2a group had a lower incidence of HCC than those in the control group. The independent risk factors for the development of HCC were analyzed using the stepwise Cox proportional hazard model. Only PegIFN $\alpha$ -2a administration and age were identified as independent risk factors for the development of HCC (Table 5).

**Discussion**

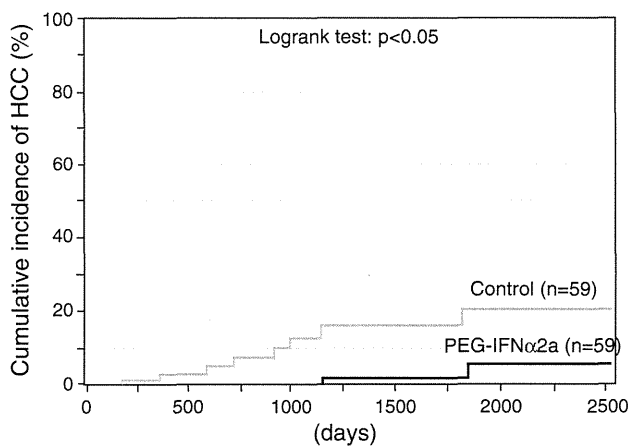
The number of HCC cases resulting from HCV infection continues to increase worldwide [19]. To date, IFN therapy is the most effective preventive measure against HCC in patients with chronic hepatitis C; furthermore, the

**Table 4** Backgrounds of the patients in the propensity-matched control study (PegIFN $\alpha$ -2a group,  $n = 59$ ; control group,  $n = 59$ )

	PegIFN $\alpha$ -2a group ( $n = 59$ )	Control group ( $n = 59$ )	$p$ value
Age (years)	60.5 $\pm$ 13.0	63.3 $\pm$ 10.5	n.s.
Gender (male/female)	24/35	25/34	n.s.
BMI	22.9 $\pm$ 3.6	22.9 $\pm$ 3.4	n.s.
Genotype (1/2)	49/10	46/13	n.s.
History of excess alcohol consumption (60 g/day; yes/no)	10/49	4/55	n.s.
Fibrosis (F0, 1, 2/F3, 4)	37/22	43/16	$< 0.05$
Development of HCC (F0–2/F3, 4)	1/1	1/7	n.s.
Inflammatory activity (A0,1/A2, 3)	19/40	30/29	$< 0.05$
Diabetes mellitus (no/yes)	57/2	56/3	n.s.
LDL cholesterol (mg/dL)	95.3 $\pm$ 23.8	117.0 $\pm$ 4.2	n.s.
White blood cell count (/mm <sup>3</sup> )	4,260 $\pm$ 1,239	5,193 $\pm$ 2,078	$< 0.05$
Red blood cell count ( $\times 10^{-4}$ / $\mu$ L)	430 $\pm$ 57.8	441 $\pm$ 44.9	n.s.
Hemoglobin (g/dL)	13.6 $\pm$ 1.5	13.6 $\pm$ 1.9	n.s.
Platelet count ( $\times 10^{-3}$ / $\mu$ L)	14.5 $\pm$ 5.7	15.8 $\pm$ 5.7	n.s.
Albumin (g/dL)	4.1 $\pm$ 0.5	4.1 $\pm$ 0.4	n.s.
Total bilirubin (mg/dL)	0.7 $\pm$ 0.5	0.9 $\pm$ 0.7	n.s.
AST (IU/L)	58.3 $\pm$ 47.7	49.7 $\pm$ 26.6	n.s.
ALT (IU/L)	63.6 $\pm$ 68.7	58.0 $\pm$ 39.2	n.s.
Gamma-GTP (IU/L)	78.3 $\pm$ 81.3	55.3 $\pm$ 75.1	n.s.
Baseline alpha-fetoprotein (AFP) (ng/L)	7.2 (4.3–14.2)	7.7 (3.9–13.8)	n.s.
Baseline HCV RNA level (KIU/mL)	1,230 (24–3,870)	1,024 (38–3,110)	n.s.

incidence of HCC is reduced in patients who achieve an SVR to IFN [6–9] Therefore, achieving an SVR is the most effective approach for reducing the risk of developing HCC. In Japan, the incidence of HCC is elevated in older patients with hepatitis C. Corroborating this finding, the results of a Japanese study show a higher risk of HCC in patients aged 65 years and more [10]. Therefore, prevention of HCC in aged patients is an important challenge.

In the present multicenter, cooperative, retrospective study conducted in Japan, the incidence of HCC was reduced in patients who received 90  $\mu$ g PegIFN $\alpha$ -2a weekly or biweekly and had AFP values of  $< 10$  ng/mL and ALT values of  $\leq 40$  IU/L 24 weeks after the start of the treatment. The results of the matched case–control study of the PegIFN $\alpha$ -2a group and the non-IFN control group show that the incidence of HCC was significantly lower in the PegIFN $\alpha$ -2a group than in the control group, especially in patients with advanced fibrosis of the liver (F3 and F4). However, there could have been a selection bias between



**Fig. 4** Comparison of HCC rates between the long-term PegIFN $\alpha$ -2a administration group ( $n = 59$ ) and non-administration group ( $n = 59$ ) in the propensity-matched control study (Kaplan–Meier log-rank test,  $p = 0.019$ )

**Table 5** Risk factors for HCC in the propensity-matched control study (Cox proportional hazard model)

Variables	Risk ratio	95 % CI	$p$ value
PegIFN versus control	0.17	0.03–0.75	<0.05
Age (every 1 year)	1.12	1.02–1.25	<0.05
Fibrosis (F3, 4 vs. F0, 1, 2)	1.70	0.75–4.16	n.s.
Platelet count (every $10 \times 10^3/\mu\text{L}$ )	0.89	0.73–1.09	n.s.
Albumin (every 1.0 g/dL)	0.80	0.10–6.68	n.s.
On-treatment AFP (<10 vs. $\geq 10$ ng/L)	4.07	0.59–40.12	n.s.

the PegIFN $\alpha$ -2a group and the control group (patients who did not agree to receive IFN treatment), because this was a retrospective and non-randomized study. However, concordant with the findings of the HALT-C study [14], the present results show that PegIFN $\alpha$ -2a inhibits the development of HCC in patients with advanced fibrosis of the liver.

Recent studies show that polymorphisms in the host *IL28B* gene are important factors in the response to PegIFN $\alpha$  and ribavirin combination therapy [20, 21]. However, the mechanism of *IL28B* involvement in the response to PegIFN $\alpha$  and ribavirin has not been elucidated completely. A recent report has shown that *IL28B* is a significant factor in the development of HCC as well as in the response to IFN therapy [22]. Further studies are warranted to analyze the relationship between *IL28B* and inhibition of the development of HCC by PegIFN $\alpha$  in chronic hepatitis C.

Risk factors for the development of HCC have been discussed previously. Increased intrahepatic fat is involved in the development of HCC in chronic hepatitis C patients [23, 24]. In addition, diabetes-associated fat disorder [25,

26], hepatic iron overload [27], advanced fibrosis, older age, and fatty deposits in the liver are risk factors for HCC development [4]. Therefore, it is important to establish strategies to mitigate these risk factors to prevent the development of HCC and thus improve the outcomes of hepatitis C patients.

IFN therapy after HCC treatment is reported to inhibit the recurrence of tumors [28, 29], and a meta-analysis has revealed a trend toward inhibition of the recurrence of HCC [30, 31]. The prevention of HCC is an important issue that needs to be addressed to improve the survival of chronic hepatitis C patients. The findings of the present study and the HALT-C trial [14] indicate the effectiveness of long-term administration of maintenance IFN for preventing the development of HCC in chronic hepatitis C patients without an SVR. Improvement in ALT levels is also known to be an important predictor for the prevention of HCC [32]. A low AFP value during IFN administration is also recognized as a significant indicator of a lower risk of HCC [33, 34]. Recently, Osaki et al. [35] reported that a decrease of serum AFP during treatment with IFN was associated with a reduced incidence of HCC. Taking these findings and our own together, we conclude that maintenance administration of low-dose PegIFN $\alpha$ -2a weekly or biweekly to non-SVR patients with chronic hepatitis C decreases the incidence of HCC, especially in patients whose serum ALT and AFP levels are within the normal range 24 weeks after the start of treatment. The preventive effects of IFN against the development of HCC without elimination of the virus may be associated with its anticarcinogenic effects [16, 35]; however, the precise mechanism should be investigated.

The limitations of the present study are that it is retrospective and multicentric; therefore, potentially there may have been a selection bias. However, the reduction of the rate of development of HCC by maintenance administration of PegIFN $\alpha$ -2a in the patients in whom serum ALT and AFP levels were within the normal ranges 24 weeks after the start of treatment may be attributable to the anticarcinogenic effects of IFN without elimination of the virus.

## Conclusion

The incidence of HCC was lower in non-SVR patients with chronic hepatitis C who were administered with maintenance low-dose PegIFN $\alpha$ -2a; especially in those whose serum ALT and AFP levels were within the normal ranges 24 weeks after the start of treatment.

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**Conflict of interest** Namiki Izumi received lecture fees from Chugai Co. and MSD Co. in 2011.

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今月の主題 肝硬変update  
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肝硬変の診断法

## 腹腔鏡・肝生検の位置づけ

長谷部千登美 細木 卓明 関谷 千尋

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# 腹腔鏡・肝生検の位置づけ

長谷部 千登美・細木 卓明・関谷 千尋

## ポイント

- ★肝硬変の正確な診断のために、腹腔鏡の意義は大きい。
- ★腹腔鏡診断は、特に初期の肝硬変の診断において、画像診断よりも信頼性が高い。
- ★腹腔鏡診断は、肝生検組織診断に比べて進行度が高く診断されることが多い。

腹腔鏡検査は、腹壁を通して腹腔鏡を挿入し、肝表面の凹凸や色調などを観察し、肉眼的な肝表面像から肝疾患の病態診断をつける手段である。また、同時に直視下に肝生検を行い、病理組織診断に用いられる肝組織を採取することにも重要な意義がある。

本検査は、肝表面の所見を直接観察しうるので、肝硬変の特徴である再生結節の有無を正確に診断することが可能であり、ごく細い組織のみから判断しなければいけない肝生検組織診断に比べ、肝硬変の診断という点に関しては信頼性の高い検査である。

しかし、腹腔鏡は侵襲の大きな検査であるため、各種の画像検査や血液検査によって肝予備能・肝線維化の程度がほぼ推測しうようになった近年においては、診断の目的のみで行われることは少なくなりつつある。本稿では、腹腔鏡検査の意義と肝硬変診療における位置づけに

焦点をしばって概説する。

## 腹腔鏡検査の方法

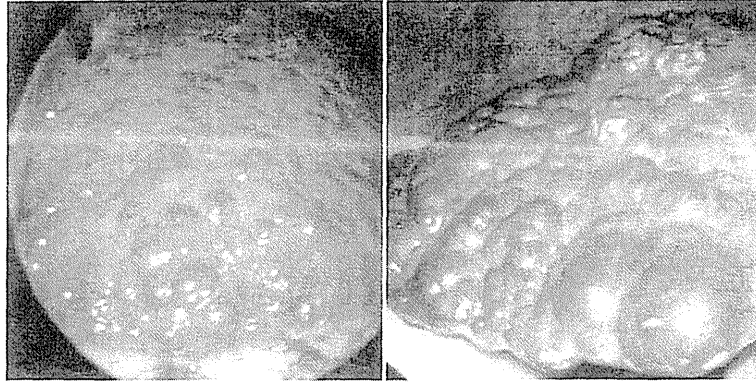
腹腔鏡検査は、二酸化炭素などの気体を腹腔内に注入(気腹)し、そのスペースに腹腔鏡を挿入して肝表面の観察を行う。観察可能な肝表面は、外側区・内側区・S5/8の腹側表面などである。多くの場合、肝表面の観察に引き続いて、直視下に生検を行う。腹腔鏡下肝生検によく用いられるシルバーマン針は外径が約2 mmと太いが、スポンゼルなどを用いて確実に止血することができるので、肝硬変症例に対しても比較的安全に組織を採取することができる。

## 腹腔鏡所見の見方： 各種肝硬変における特徴

肝硬変の診断においては、増生した線維により肝実質部が分断されて生じる結節の所見を確認することが、最も重要な診断根拠となる。結節の存在診断に際しては、色素撒布法を併用して肝表面の陥凹部を強調して観察すると、微細な結節の診断が可能となる<sup>1)</sup>。

結節の性状は、肝硬変の原因疾患によって大きな違いがみられる<sup>2)</sup>。

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【図 1】 B 型肝硬変の腹腔鏡所見



【図 2】 C 型肝硬変の腹腔鏡所見

- ・ **B 型肝硬変**：比較的大きく，隆起の強い結節が観察されることが多い(図 1)。
- ・ **C 型肝硬変**：肝表面の凹凸の程度に部位差を伴うことが多く，やや小さめで隆起の弱い結節が不規則な分布で見られることが多い(図 2)。
- ・ **アルコール性肝硬変**：非常に微細な結節が生じることが特徴的で，一見すると肝表面が平坦に見えることもある。
- ・ **自己免疫性肝炎**：広範な区域性の壊死の結果，粗大な陥凹が形成されて，いわゆる癆痕肝の所見を呈することが多い。  
このような疾患ごとの肝表面像の特徴は，生検組織所見で捉えることは難しく，腹腔鏡検査の大きな特徴といえる。

### 腹腔鏡検査と画像検査の比較

肝硬変の診断に際しては，実際には腹部 CT や超音波検査などの画像検査が参考にされる場合が多い。いずれの画像においても，肝全体の变形や，腫大・萎縮の程度，表面の凹凸，脾腫，腹水や側副血行路の存在などから肝硬変の診断が下される。これらの所見は進行した肝硬変症例ではしばしば認められるが，比較的初期の肝硬変においてははっきりしないことが多い。したがって，初期肝硬変の診断法としては明らかに腹腔鏡検査が優れている。

近年では，超音波を応用した肝硬度測定(Fibroscan<sup>®</sup> 3)，real-time tissue elastography<sup>4)</sup> などの方法も実用化されており，今後は肝硬変



の診断に対する画像診断の意義が徐々に大きくなるのが期待される。

## 腹腔鏡検査と肝生検組織検査の比較

肝生検組織で小葉構造の乱れや結節形成を確認することは、確実な肝硬変の診断手段であるが、腹腔鏡所見と生検組織所見との乖離もしばしば経験される<sup>5)</sup>。これは、組織診断がごく細い肝組織を用いてなされるので、採取された部位によるサンプリングエラーが生じるためと考えられる。

例えば、比較的大きな結節が形成されるB型肝硬変などの例では、結節全体を生検組織で捉えることは不可能であり、標本中にみられる線維化の形態や程度によって推測するしか診断の方法がない。また、C型慢性肝炎では炎症や線維化の程度に部位差を伴うという特徴があるため、サンプリングエラーが発生しやすいともいわれている。

腹腔鏡では、小葉改築や初期の結節の所見が部分的にあるだけでも明らかに観察しうるので、生検組織所見よりも進行度が高く診断される症例が多い。従来の検討では、腹腔鏡下肝生検でF3と診断された症例の腹腔鏡的診断をみると、B型肝炎では36%、C型肝炎では29%が、腹腔鏡的には肝硬変と診断されていた<sup>5)</sup>。さらに、腹腔鏡を用いないエコー下肝生検においては、腹腔鏡で採取されるよりもさらに細かい組織での判断になるため、線維化の進行度判定ではより軽く診断される可能性が高くなる。

このように、肝硬変の病態を確実に診断するには、腹腔鏡に色素撒布法を併用して、肝線維化の程度を正確に把握することが理想的である。エコー下生検のみで診断する場合には、進行度が実際よりも軽く診断される可能性があることを念頭に置き、血液生化学所見や各種画像

所見と併せて慎重に診断する意識をもつことが大変重要である。

## 腹腔鏡検査の合併症と問題点

これまで述べてきたとおり、腹腔鏡検査は、肝硬変を的確に診断するうえで大変有用な検査であり、肝疾患の診断にあたって不可欠とされていた時代もある。しかし、疼痛・気腹による苦痛など被検者に与える負担が大きいこと、血管や腹腔内臓器損傷などの合併症のリスクが高いこと、手間と時間がかかり医療経済的な面から回避される傾向になったことなどから、積極的に行う施設が減少してきた。もちろん、各種画像診断や血液検査の発展によって腹腔鏡を行わなくても肝硬変の病態診断が可能になった影響も大きい。

被検者の立場で考えると、苦痛やリスクをなるべく軽減して確実な診断が得られるのが好ましいわけで、診断のみの目的で行われる腹腔鏡が徐々に減っていることはやむをえないともいえる。ただ、肝硬変の病態を正しく診断するうえで、腹腔鏡で得られる肝表面所見の確認が大変有用であるという点を理解したうえで、肝生検によるunder-diagnosisの可能性も念頭に置き診療を進めることが重要であろう。

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## Original Article

## Augmented hepatic Toll-like receptors by fatty acids trigger the pro-inflammatory state of non-alcoholic fatty liver disease in mice

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**Aim:** There is considerable evidence that intestinal microbiota are involved in the development of metabolic syndromes and, consequently, with the development of non-alcoholic fatty liver disease (NAFLD). Toll-like receptors (TLRs) are essential for the recognition of microbiota. However, the induction mechanism of TLR signals through the gut-liver axis for triggering the development of non-alcoholic steatohepatitis (NASH) or NAFLD remains unclear. In this study, we investigated the role of palmitic acid (PA) in triggering the development of a pro-inflammatory state of NAFLD.

**Methods:** Non-alcoholic fatty liver disease was induced in mice fed a high fat diet (HFD). The mice were killed and the expression of TLRs, tumor necrosis factor (TNF), interleukin (IL)-1 $\beta$ , and phospho-interleukin-1 receptor-associated kinase 1 in the liver and small intestine were assessed. In addition, primary hepatocytes and Kupffer cells were treated with PA,

and the direct effects of PA on TLRs induction by these cells were evaluated.

**Results:** The expression of inflammatory cytokines such as TNF, IL-1 $\beta$ , and TLR-2, -4, -5, and -9 was increased in the liver, but decreased in the small intestine of HFD-fed mice *in vivo*. In addition, the expression of TLRs in primary hepatocytes and Kupffer cells was increased by treatment with PA.

**Conclusion:** In the development of the pro-inflammatory state of NAFLD, PA triggers the expression of TLRs, which contribute to the induction of inflammatory cytokines through TLR signals by intestinal microbiota.

**Key words:** fatty acids, gut-liver axis, non-alcoholic fatty liver disease, pro-inflammatory state, Toll-like receptor

## INTRODUCTION

NON-ALCOHOLIC FATTY LIVER disease (NAFLD) is a form of steatosis with or without inflammation of the liver, and it is not related to excessive alcohol intake. NAFLD includes both simple steatosis and non-alcoholic steatohepatitis (NASH), the latter developing further into cirrhosis and hepatocellular carcinoma.<sup>1</sup> NAFLD is one of the most common liver diseases world-

wide and is considered to be related to obesity, insulin resistance, and metabolic syndrome.<sup>2</sup>

A two-hit theory has been proposed to explain the pathogenesis of NASH.<sup>3</sup> First, simple steatosis is induced by obesity and insulin resistance. Second, NASH develops by several hits, including adipocytokines, iron, and bacterial endotoxins/lipopolysaccharide (LPS) derived from gram-negative bacteria.<sup>4-6</sup>

Toll-like receptors (TLRs) recognize pathogen- and endogenous damage-associated molecular patterns and activate nuclear factor- $\kappa$ B (NF- $\kappa$ B), which induces pro-inflammatory cytokines/chemokines and type I interferon through phosphorylation of interleukin-1 receptor-associated kinase 1 (IRAK1) and IRAK4.<sup>7</sup> Therefore, TLRs may play important roles in the activation of innate immunity. Among TLRs, TLR2, TLR4, TLR5, and

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TLR9 were identified as bacterial recognition receptors capable of recognizing lipopeptide, LPS, flagellin, and CpG-DNA, respectively.<sup>8</sup> Recently it was reported that TLR signal pathways, the ligands of which are bacterial components, play an important role in the pathogenesis of alcoholic liver disease and NASH.<sup>9</sup> In particular, the association between TLR4 signal pathways and the development of NASH was investigated.<sup>6,10,11</sup> More recently, Miura *et al.* reported decreased levels of steatohepatitis and liver fibrosis in TLR9 knockout mice compared with those in wild-type mice in a choline-deficient amino acid-defined (CDAA) diet-induced NASH model.<sup>12</sup> In contrast, in TLR2-deficient mice fed a methionine- and choline-deficient (MCD) diet, an increased level of liver injury was noted, suggesting a potential protective role of TLR2 in fatty liver.<sup>6</sup>

An increasing proportion of the general population suffers from obesity, which is an emerging global problem along with its related disorders such as metabolic syndrome. Much recent evidence shows that microbiota are associated with these conditions.<sup>13–19</sup> In the intestine, TLRs are typically expressed in the epithelial cells and are involved in the production of immunoglobulin A (IgA), maintenance of tight junctions, proliferation of epithelial cells, and expression of antimicrobial peptides.<sup>20</sup> TLR5, which specifically recognizes flagellin, is involved in promoting the pathophysiology of inflammatory bowel disease.<sup>21</sup> While the above reports suggest that intestinal TLRs play an important role in innate immunity of the gut, the association between their role in the small intestine and that in the development of NASH remains unclear.

The present study was based on hypernutrition and obesity and evaluated the significance of TLRs and their signaling in the liver and small intestine using a high-fat diet (HFD)-induced NAFLD mouse model. In addition, a gut-sterilized mouse model treated with antibiotics was used to confirm whether there is an association between intestinal microbiota and TLR expression.

## METHODS

### Animal studies

**I**N THE HFD group, 8-week-old male C57BL/6J mice (Charles River Japan, Tokyo, Japan) were fed a HFD containing 60% triglycerides with oleic acid (OA), palmitic acid (PA), and stearic acid (Table 1) (F2HFD2; Oriental Yeast Company, Tokyo, Japan). Control mice were fed a diet containing 5% triglycerides (MF; Oriental Yeast Company). All mice were maintained under controlled conditions (22 °C; humidity, 50–60%, 12-h

**Table 1** Composition of fatty acids in control diet and high fat diet

	Control diet (%)	High fat diet (%)
Oleic acid	No detect	30
Palmitic acid	No detect	25
Stearic acid	0.22	16
Palmitoleic acid	0.05	2.0
Myristic acid	0.03	1.5

light/dark cycle) with food and water *ad libitum*. Mice from both groups were killed at 4, 8, and 16 weeks for blood and tissue collection. These animals were fasted for 10-h before blood and tissue collection. After each mouse was anesthetized with diethyl ether and weighed, blood was collected by a cardiac puncture and subsequently assayed for biochemical parameters. The liver and small intestine were dissected, weighed, and frozen in liquid nitrogen. These samples were used later for histological and polymerase chain reaction (PCR) analysis. All experiments were performed in accordance with the rules and guidelines of the Animal Experiment Committee of Asahikawa Medical University.

### Isolation and primary culture of hepatocytes and Kupffer cells

Mouse hepatocytes and Kupffer cells were isolated using a modified collagenase perfusion method.<sup>22</sup> Briefly, the liver was perfused via the portal vein with Ca<sup>2+</sup> and Mg<sup>2+</sup> free Hank's balanced salt solution (HBSS(-)) at 39 °C for 5 min at 10 mL/min, followed by HBSS(+) for 5 min at 10 mL/min supplemented with 0.05% collagenase (Wako, Tokyo, Japan). The liver was then removed, fragmented and vortexed for a few seconds. After filtration with mesh, the cell suspension was centrifuged at 500 rpm for 1 min. The cells in the pellet were minced twice and used as primary hepatocytes for culture in William's E medium with epidermal growth factor (5 µg), insulin (5 mg), L-glutamine, penicillin, streptomycin, and 10% fetal bovine serum (FBS). The supernatant was centrifuged at 500 rpm for 1 min at three to four times to remove any remaining hepatocytes. The supernatant was then minced twice and cultured in Dulbecco's modified Eagle's medium with penicillin, streptomycin, and 10% FBS. After 60 min, adhesion cells were used as Kupffer cells.

### PA treatment

Isolated hepatocytes and Kupffer cells were treated with PA. PA complexed with 1% bovine serum albumin

(BSA) was added to the medium to attain final concentrations of 10  $\mu$ M and 100  $\mu$ M over 24 h.

### Fat droplet evaluation

4,4-difluoro-1,3,5,7,8-pentamethyl-4-bora-3a,4a-diazas-indacene (BODIPY 493/503, Invitrogen, Carlsbad, CA, USA) was added as a lipid probe overnight to the culture medium and fluorescent images were observed.

### Biochemical analyses

Serum alanine aminotransferase (ALT) and free fatty acids were measured using the Automatic Analyzer 7180 (Hitachi High-Technologies Corporation, Tokyo, Japan).

### Histopathological evaluation

Samples of remaining liver tissue were fixed in 10% formalin buffer, embedded in paraffin, sectioned, and stained with hematoxylin and eosin (H&E).

### RNA isolation and first strand complementary DNA synthesis

Total RNA was isolated from the liver, small intestine, primary hepatocytes, and Kupffer cells using QIAGEN RNeasy Mini Kit (QIAGEN, Hilden, Germany). RNA was reverse-transcribed by RETROscript using Random decamers (Ambion, Austin, TX, USA). Detailed methods were performed according to the manufacturers' instructions.

### Primer pairs of TLR-related molecules

Mouse 18srRNA was used as an endogenous amplification control. The use of this universally expressed house-keeping gene allows for correction of variations in the efficiency of RNA extraction and reverse transcription. TaqMan assays were used for specific primer and probe sets on TLR2, TLR4, TLR5, TLR9, tumor necrosis factor (TNF), interleukin (IL)-1 $\beta$ , and 18srRNA (Applied Biosystems, Foster City, CA, USA).

### Quantitative real-time PCR

The expression of TLR2, TLR4, TLR5, TLR9, IL-1 $\beta$ , and TNF in mouse liver, small intestine, primary hepatocytes, and Kupffer cells was evaluated by quantitative real-time PCR (qPCR) (7300 Real-time PCR system; Applied Biosystems). In this method, all reactions were run in 96-well plates with a total volume of 20  $\mu$ L. The reaction mixture consisted of 10  $\mu$ L TaqMan Universal PCR Master mix, 1  $\mu$ L 18srRNA, 1  $\mu$ L primer, 5  $\mu$ L RNAase free water, and 3  $\mu$ L complementary DNA. The

PCR reaction involved the following steps: (i) 50°C for 2 min to prevent carryover of DNA, (ii) 95°C for 10 min to activate polymerase, and (iii) 40 cycles each of 95°C for 15 s, 60°C for 15 s, and 72°C for 45 s. qPCR data were analyzed by the comparative CT method.

### Immunohistochemistry/ immunocytochemistry

Immunohistochemistry using F4/80 as a macrophage marker was performed on cryostatally sectioned liver and staining was performed by immunofluorescence. The sections were fixed in 2% paraformaldehyde for 10 min and washed three times with PBS for 5 min. Furthermore, sections for F4/80 were blocked with 3% BSA/PBS for 1 h at room temperature, followed by incubation with monoclonal antibody against F4/80 (Abcam, Cambridge, MA, USA) 1:100 diluted in 3% BSA/PBS for 1 h at room temperature. After washing, F4/80 slides were incubated with 1:200 diluted Alexa Fluor 488 goat anti-rat IgG (Invitrogen) for 1 h at room temperature and washed.

Immunocytochemical staining was also performed using the immunofluorescence method. After the chamber slides in which primary Kupffer cells had been cultured were washed twice with PBS for 5 min, primary Kupffer cells were fixed in 2% paraformaldehyde for 20 min and washed twice with PBS for 5 min. Primary Kupffer cells were then incubated with 0.1% Triton X-100 in PBS for 2 min to permeabilize the membranes and washed twice with PBS. The slides for phospho-interleukin-1 receptor-associated kinase1 (pIRAK1; Abcam) were blocked with 3% BSA/PBS for 1 h at room temperature, followed by incubation with monoclonal antibody against pIRAK1 diluted 1:500 in 3% BSA/PBS for 1 h at room temperature. After washing, the slides were incubated with 1:500 diluted Alexa Fluor 594 goat anti-rat IgG (Invitrogen) for 1 h at room temperature and washed.

### Western blotting analysis

Protein expression of pIRAK1, the key mediator in the TLR signaling pathway,<sup>23</sup> in the liver (30  $\mu$ g), small intestine (30  $\mu$ g) was studied by Western blot analysis. Protein concentrations were measured by the Bradford method using the Pierce BCA Protein Assay kit (Thermo Scientific, Rockford, IL, USA) according to the manufacturer's instructions. Separation of 30  $\mu$ g of protein was then performed by 12% Mini PROTEAN TGX Precast Gels (Bio-Rad, Hercules, CA, USA). After electrophoresis, proteins were transferred to nitrocellulose membranes (Amersham Life Science Piscataway, NJ, USA),

blocked in 5% skim milk, and 0.2% Tween20 in PBS (PBS-T) for 1 h at room temperature, reacted overnight at 4°C with either rabbit polyclonal anti-pIRAK1 (Abcam) or  $\beta$ -actin (BD Biosciences) as a control, washed with 0.2% PBS-T, reacted with secondary antibody horseradish peroxidase-conjugated anti-rabbit IgG and anti-mouse IgG (RD, Minneapolis, MN, USA) for 1 h, and washed with PBS-T. After reaction with horseradish peroxidase-conjugated anti-rabbit and anti-mouse IgG, immune complexes were visualized by Super Signal West Pico Chemoluminescent Substrate (Thermo Scientific) according to the manufacturer's suggested procedure. pIRAK1 was analyzed by Image J software under the area, which compensated for  $\beta$ -actin.

### Statistical analysis

The results are expressed as mean  $\pm$  standard error, with the two groups being analyzed by Student's *t*-test and datasets involving more than two groups being analyzed by analysis of variance (ANOVA). *P*-values of <0.05 were considered statistically significant.

### Gut sterilization

Mice were treated with ampicillin (1 g/L, Sigma-Aldrich, St. Louis, MO, USA), neomycin (1 g/L, Sigma), metronidazole (1 g/L, Sigma), and vancomycin (500 mg/L, Sigma) in drinking water for 8 weeks.<sup>24</sup> This treatment was followed by feeding with HFD for further 8 weeks.

## RESULTS

### Fatty liver in HFD-fed mice

AT 16 WEEKS, body weight and serum ALT levels were significantly higher in the HFD-fed mice (group F) than in those fed the control diet (group C) (body weight: C, 41.6 g; F, 51.0 g; serum ALT: C, 34 IU/L; F, 180 IU/L, Fig. 1a,b). Histopathological liver findings from group F demonstrated the absence of fat droplets at 4 weeks (Fig. 1c). However, the deposition of micronodular fat droplets in the centrilobular zone (Fig. 1d) was observed at 8 weeks and macronodular fat droplets and ballooning degeneration (Fig. 1e) were observed at 16 weeks, without any obvious infiltration of inflammatory cells. F4/80 staining for macrophage markers did not demonstrate an increased number of Kupffer cells (Fig. 1f,g).

### Upregulation of cytokines in the fatty liver of HFD-fed mice

Histopathological examination of livers from group F demonstrated no obvious infiltration of inflammatory

cells, while mRNA levels of the inflammatory cytokines IL-1 $\beta$  and TNF were significantly higher at 16 weeks (Fig. 2a,b).

### Upregulation of TLRs in the fatty liver of HFD-fed mice

To confirm whether TLRs expression contributes to the induction of the abovementioned cytokines, we analyzed the mRNA of TLR2, TLR4, TLR5, and TLR9 that recognize bacterial components in the liver. The expression of these TLRs in the liver was not different between the two groups at 4 and 8 weeks, but at 16 weeks, this was significantly higher in the F group than in the C group (Fig. 2c–f). Western blot analysis also demonstrated that the expression of pIRAK1 in the liver was significantly upregulated in the F group compared with that in the C group at 16 weeks (Fig. 2g). These findings suggest that TLR upregulation contributes to the induction of cytokines and that the TLR signal pathway is genetically enhanced in simple steatosis in the absence of inflammation.

### Downregulation of TLRs and cytokines in the small intestine of HFD-fed mice

The expression of TLRs that recognize bacterial components was significantly upregulated in the NAFLD liver. Because liver injury has a connection with exposure to bacterial components of intestinal origin, we then examined the small intestine of the NAFLD model mice. The mRNA expression of small intestinal TLR2, TLR4, TLR5, and TLR9 was not significantly different between the two groups at 4 and 8 weeks. Histopathological examination of the small intestine revealed no difference between the groups at 16 weeks, but, mRNA expression of all four TLRs was significantly lower in the F group than in the C group at 16 weeks (Fig. 3a–d). Expression of IL-1 $\beta$  and TNF was also downregulated at 16 weeks (Fig. 3e,f). Moreover, pIRAK1 expression was also significantly decreased in the F group compared with that in the C group at 16 weeks (Fig. 3g). These findings indicate that the TLR signal pathway is genetically attenuated in the NAFLD small intestine.

### Antibiotic treatment improved steatosis and TLRs expression in the liver of HFD-fed mice

Small intestinal bacterial overgrowth (SIBO) was reported to coexist with NASH,<sup>14,25</sup> and the following factors can predispose to SIBO: morbid obesity,<sup>26</sup> aging,<sup>27</sup> concurrent use of proton pump inhibitors,<sup>26</sup> and abnormal small intestinal motility.<sup>29</sup> Therefore, we hypothesized that attenuation of TLR signal

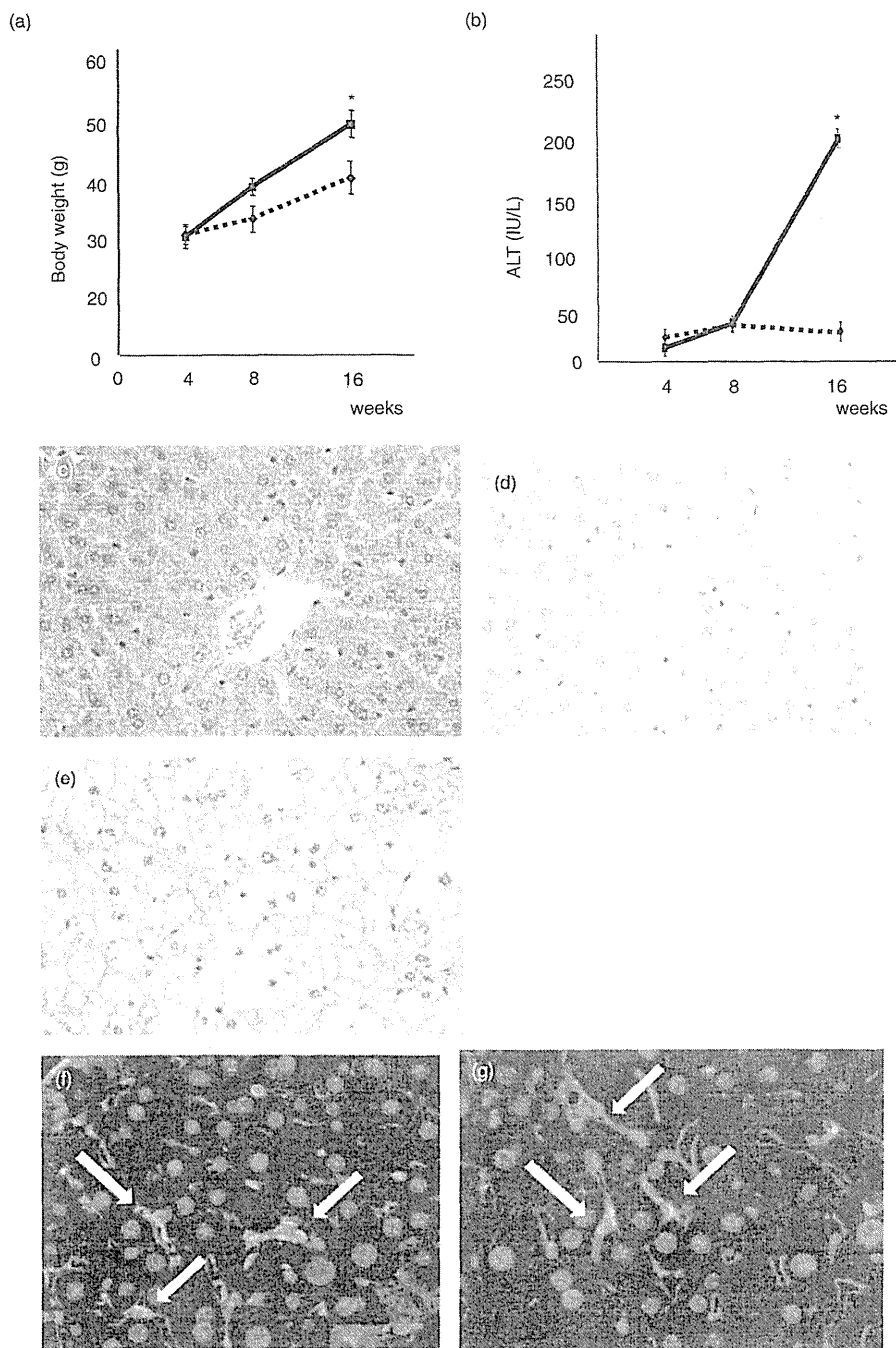


Figure 1 Body weight, serum alanine aminotransferase (ALT), and histopathological findings in high fat diet (HFD)-fed and control mice. Body weight (a) and serum ALT levels (b) were significantly higher in HFD-fed mice (F) than in controls at 16 weeks. Histopathological liver findings in F mice at 4, 8, and 16 weeks with H&E staining ( $\times 400$ , 4 weeks [c]; 8 weeks [d]; 16 weeks [e]). Micronodular fat droplets deposited in hepatocytes in the centrilobular zone at 8 weeks (d). Macronodular fat droplets and ballooning degeneration were marked at 16 weeks, but no obvious infiltration of inflammatory cells was observed (e). F4/80 staining for macrophage marker did not demonstrate any increase in Kupffer cells (control (f); 16 weeks (g); white arrows indicate Kupffer cells,  $\times 400$ ). (\* $P < 0.05$ ).

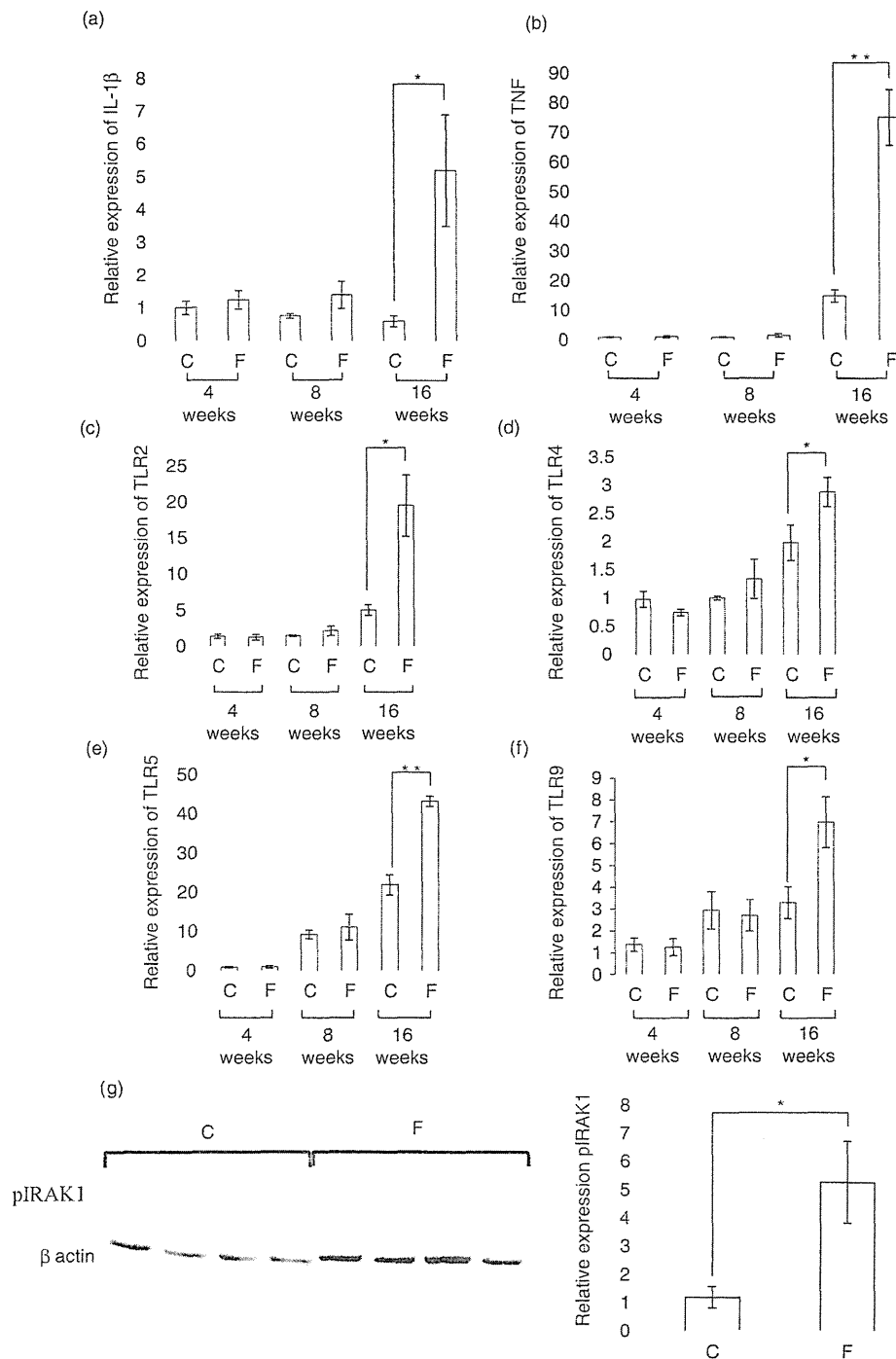


Figure 2 mRNA expression of inflammatory cytokines and Toll-like receptors in the liver of high fat diet (HFD)-fed and control mice. mRNA expression of interleukin (IL)-1 $\beta$  (a), and tumor necrosis factor (TNF) (b) was significantly higher in the liver of HFD-fed mice (F) than in that of controls (C) at 16 weeks. The expression of TLR2 (c), TLR4 (d), TLR5 (e), and TLR9 (f) mRNA was significantly higher in the liver in group F than in group C at 16 weeks. Western blot analysis demonstrated a higher expression of pIRAK1 in the liver in group F than in group C at 16 weeks (g). (\* $P < 0.05$ , \*\* $P < 0.01$ ).

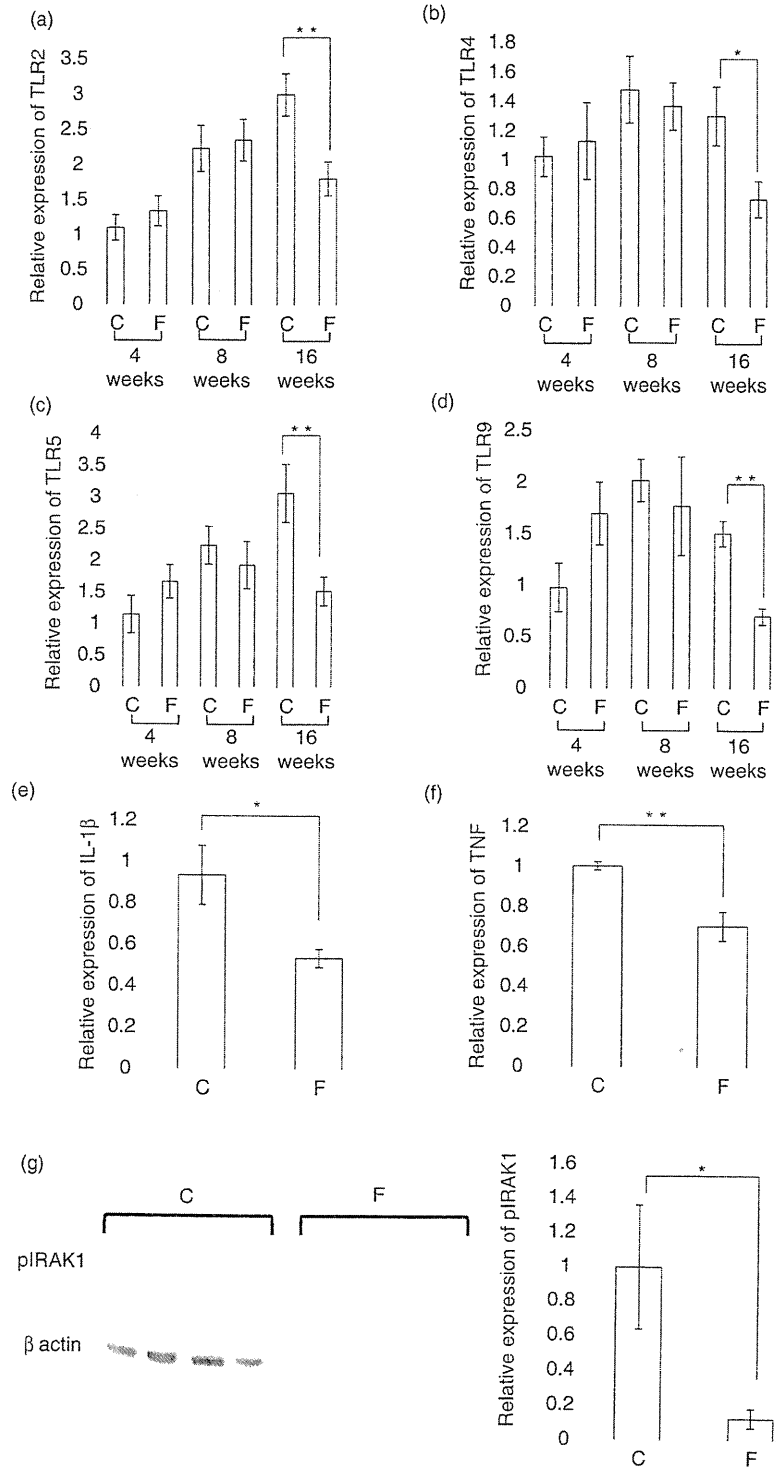


Figure 3 mRNA expression of inflammatory cytokines and Toll-like receptors in the small intestine of high fat diet (HFD)-fed and control mice. mRNA expression of TLR2 (a), TLR4 (b), TLR5 (c), and TLR9 (d) in the small intestine was significantly lower in HFD-fed mice (F) than in control mice (C) at 16 weeks. mRNA expression of IL-1β (e) and TNF (f) in the small intestine was also lower in group F than in group C at 16 weeks. Western blot analysis demonstrated a lower expression of phospho-interleukin-1 receptor-associated kinase1 (pIRAK1) in the small intestine in group F than in group C at 16 weeks (g). (\* $P < 0.05$ , \*\* $P < 0.01$ ).



pathways may induce immunotolerance, altered levels of microbiota, and bacterial overgrowth in the NAFLD small intestine. To investigate whether intestinal microbiota contribute to TLR expression, we eliminated them by treatment with non-absorbable broad-spectrum antibiotics.<sup>24</sup>

Body weight, serum ALT levels, and serum free fatty acids levels were significantly decreased in mice fed HFD and administered antibiotics (FA) compared with that in the mice fed HFD and water only (FC) (body weight: control diet and water only (CC), 28.9 g; control diet and antibiotics (CA), 28.6 g; FA, 34.7 g; FC, 51.9 g; serum ALT: CC, 19.5 IU/L; CA, 23.4 IU/L; FA, 34.7 IU/L; FC, 147.6 IU/L; serum free fatty acids: CC, 677.1  $\mu$ Eq/L; CA, 818.7  $\mu$ Eq/L; FA, 635.6  $\mu$ Eq/L; FC, 962.7  $\mu$ Eq/L; Fig. 4a–c). Histopathological findings from the livers of FC mice demonstrated the deposition of macronodular fat droplets in the centrilobular area and ballooning degeneration of hepatocytes. In contrast, FA mice showed a marked decrease in steatosis compared with the FC mice (Fig. 4d–g). The expression of TLRs (Fig. 5a–d) and inflammatory cytokines (Fig. 5e,f) was also significantly lower in the liver of FA mice than in that of FC mice. These data indicate associations among intestinal microbiota, TLR expression, and fatty acid metabolism.

#### Antibiotic treatment did not alter TLRs expression in the small intestine of HFD-fed mice

In the small intestine of mice fed the control diet, the expression of TLR2, TLR4, TLR5, and TLR9 was downregulated by antibiotic treatment (Fig. 6). However, contrary to expectations, the expression in HFD-fed mice did not alter (Fig. 6). These data suggest that microbiota contribute to TLRs expression in the small intestine of mice fed the control diet but not in that of mice fed HFD.

#### PA upregulated TLR2, TLR4, TLR5, and TLR9 expression in primary Kupffer cells

Because both serum free fatty acids and TLR expression were coincidentally suppressed in the intestinal bacterial eradication model, we examined whether fatty acids would alter TLR expression. First, we investigated TLR expression in primary Kupffer cells.

To determine the effects of fatty acids on TLR expression in Kupffer cells, PA was added to primary Kupffer cells for 24 h, where it induced the deposition of fat droplets (Fig. 7a,b). The mRNA expression of TLR2, TLR4, TLR5, and TLR9 was significantly higher in

primary Kupffer cells treated with 10  $\mu$ M PA for 24 h than in control cells (Fig. 7c).

Immunocytochemistry demonstrated that the expression of pIRAK1 was strongly positive in primary Kupffer cells with fat deposits (Fig. 7d). mRNA expression in IL-1 $\beta$  was not significantly different, but the expression of TNF was significantly higher in primary Kupffer cells exposed to 10  $\mu$ M PA for 24 h than in controls (Fig. 7e,f). These findings indicate that PA may enhance the TLR signal pathway in Kupffer cells.

#### PA upregulated TLR4 and TLR9 expression in primary hepatocytes

Second, we examined TLR expression in primary hepatocytes treated with 100  $\mu$ M PA for 24 h, which induced the deposition of fat droplets in these cells (Fig. 8a,b). mRNA expression of TLR4 and TLR9, but not that of TLR2 and TLR5 was significantly upregulated in primary hepatocytes treated with 100  $\mu$ M PA for 24 h (Fig. 8c). The expression of IL-1 $\beta$  and TNF was significantly upregulated in primary hepatocytes treated with 100  $\mu$ M PA for 24 h (Fig. 8d,e). These findings suggest that PA can, at least in part, enhance TLR signal pathways in hepatocytes.

## DISCUSSION

MICE FED THE MCD diet demonstrated steatosis, macrophages accumulation, and clustering of neutrophils in the liver. Consequently, the expression of TLR4 and TNF- $\alpha$  was increased; however, the destruction of Kupffer cells prevented an increase in TLR4 expression,<sup>10</sup> indicating that increased expression levels had contributed to infiltration of inflammatory cells. It was reported that fatty liver in NASH resulted in increased liver injury and inflammation following intra-peritoneal LPS injection in an MCD diet-induced NASH mouse model, suggesting that the MCD diet-induced NASH liver is sensitive to the TLR4 ligand LPS.<sup>6</sup> In our present model, mice fed HFD for 16 weeks developed steatosis with no histological evidence of inflammation and fibrosis, which is known as simple steatosis. However, the expression of inflammatory cytokines was upregulated, and our findings established that this was the mechanism by which TLR signal pathways were upregulated in the NAFLD liver prior to the development of steatohepatitis. Moreover, F4/80 staining revealed that this upregulation was not affected by an altered number of Kupffer cells but by changes in their activity in regard to inflammatory cytokine production. Our findings show that simple steatosis prior to NASH

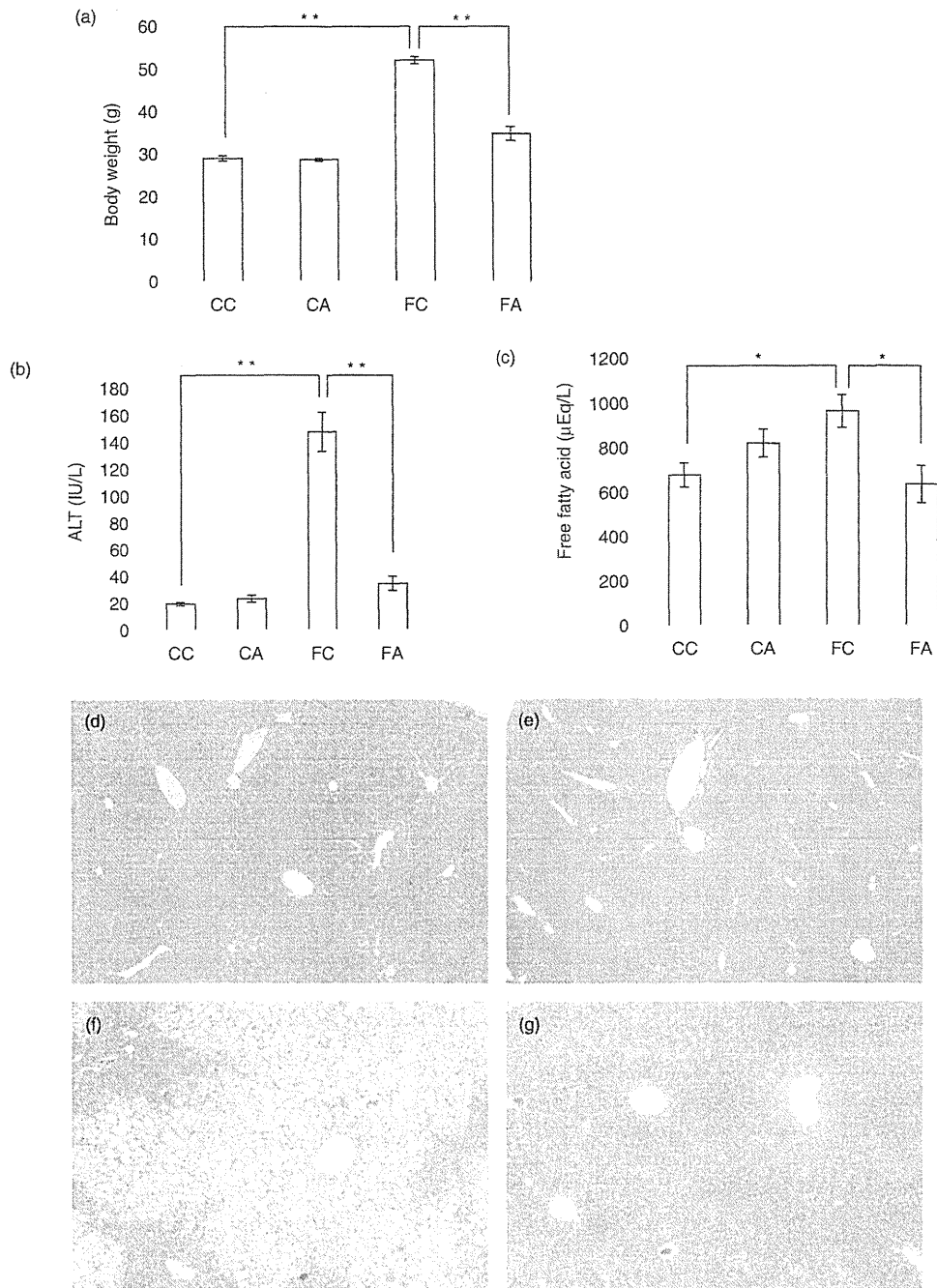


Figure 4 Body weight, serum alanine aminotransferase (ALT) levels, serum free fatty acids, and histopathological findings in high fat diet (HFD)-fed and control mice with or without antibiotic administration. CC, control diet and water only; CA, control diet and antibiotics; FC, HFD and water only; FA, HFD and antibiotics. Body weight was significantly higher in FC mice than in FA mice (a). Serum ALT levels (b) and serum free fatty acids levels (c) were also suppressed in FA mice compared with those in FC mice. Histopathological liver findings for CC (d), CA (e), FC (f), and FA (g) mice with H&E staining ( $\times 100$ ) demonstrated macro-nodular fat droplets and ballooning degeneration in the liver in FC mice, whereas steatosis was obviously suppressed in FA mice. (\* $P < 0.05$ , \*\* $P < 0.01$ ).

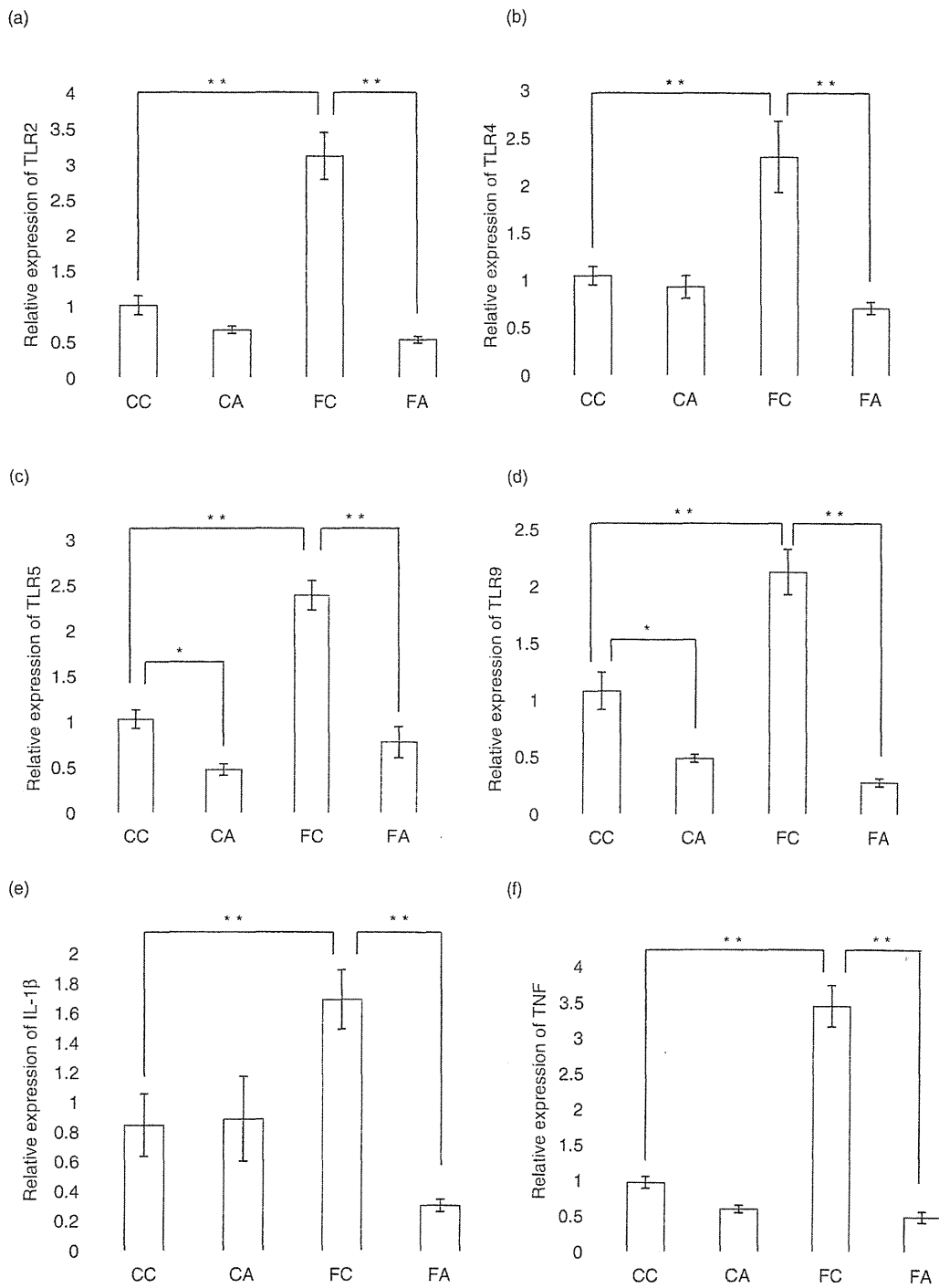


Figure 5 mRNA expression of Toll-like receptors and inflammatory cytokines in the liver in high fat diet (HFD)-fed and control mice with or without antibiotic administration. Expression of TLR2 (a), TLR4 (b), TLR5 (c), and TLR9 (d) was suppressed more in the liver of FA mice than in the liver of FC mice. Expression of IL-1 $\beta$  (e) and tumor necrosis factor (TNF) (f) was also suppressed. (\* $P < 0.05$ , \*\* $P < 0.01$ ).

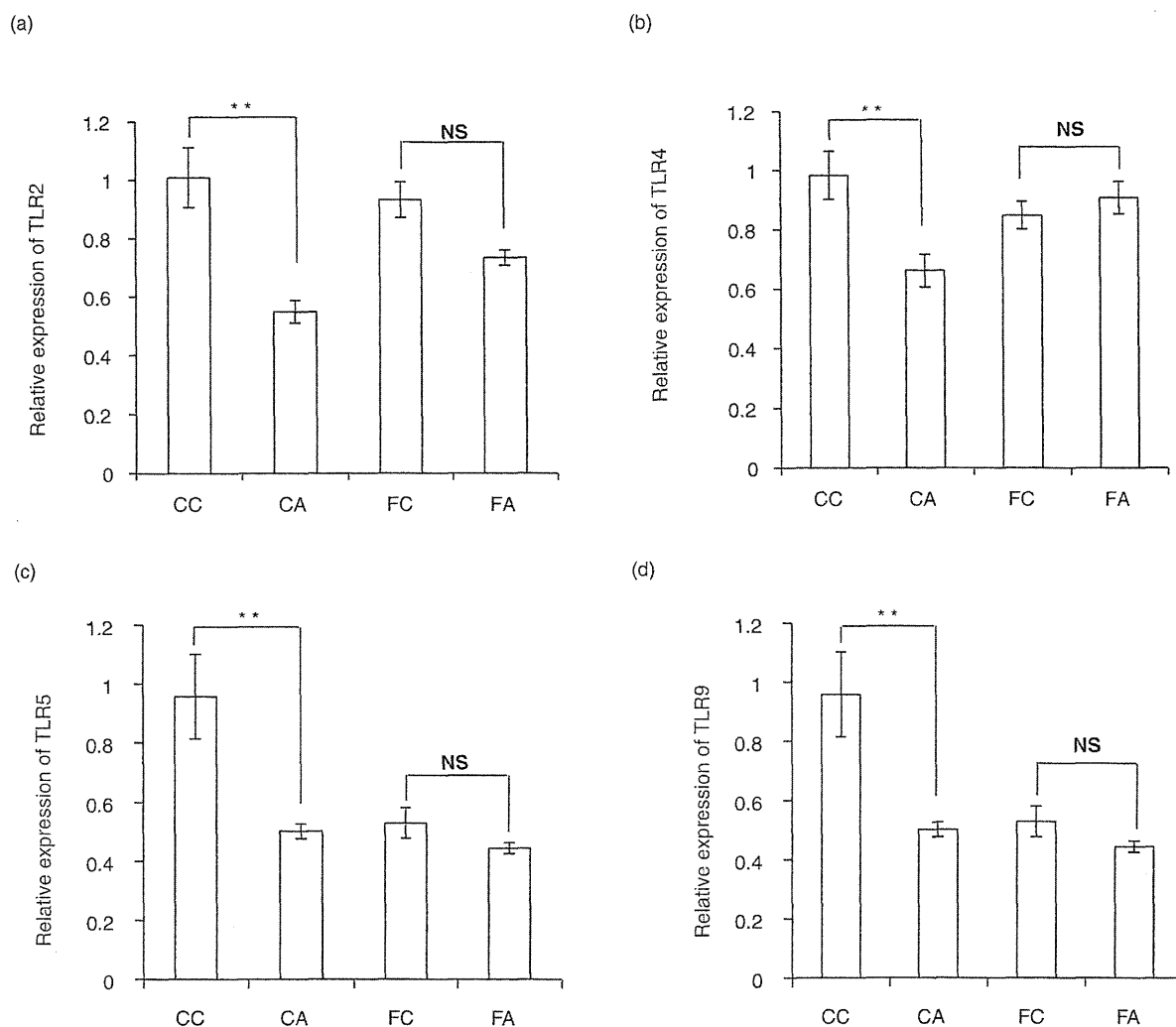


Figure 6 mRNA expression of Toll-like receptors in the small intestine of high fat diet (HFD)-fed and control mice with or without antibiotic administration. Toll-like receptors (TLRs) expression was not altered by antibiotic treatment in non-alcoholic fatty liver disease (NAFLD) small intestine (TLR2 (a), TLR4 (b), TLR5 (c), and TLR9 (d)). (\* $P < 0.05$ , \*\* $P < 0.01$ ) (NS, not significant).

demonstrates upregulation of TLR signal pathways and may be more sensitive to the ligands of intestinal microbial components. Bertola *et al.* reported that the expression of TLRs and certain cytokines/chemokines in genes was upregulated in biopsy specimens of morbidly obese patients with histologically normal liver or severe steatosis with or without NASH,<sup>30</sup> and our findings from this animal study concur with this.

Although many studies have examined the association between TLR signal pathways and the pathogenesis of NASH, these reports focused mainly on hepatocytes,

Kupffer cells, and hepatic stellate cells.<sup>6,16,12,31</sup> However, small intestinal microbiota are the cause of liver damage in NASH, and to our knowledge, this is the first report to investigate the expression of intestinal TLR signal pathways in NAFLD model and to reveal a discrepancy in TLRs expression in the gut-liver axis. The microbial TLR signal pathway and downstream cytokines were downregulated in the small intestine of NAFLD model and this downregulation may contribute to imbalance of the immune system and, consequently, alter the microflora component ratio and induce SIBO with NAFLD.