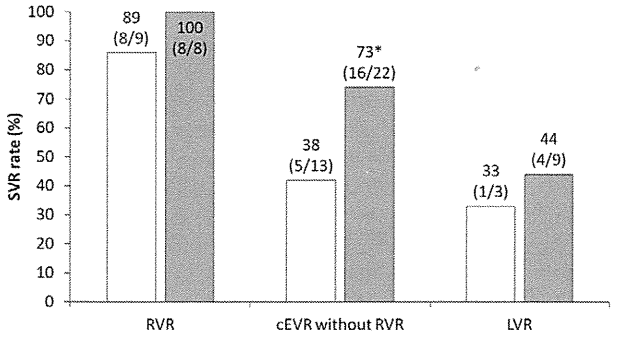

Figure 3 Sustained virological response (SVR) rates according to virological response in re-treatment and treatment duration in patients with genotype 1. □, Patients treated for 48 weeks; ▩, patients treated for 72 weeks. RVR, rapid virological response; cEVR, complete early virological response; LVR, late virological response. *$P < 0.05$; compared to 48 weeks of treatment.

genotype and antiviral effect of re-treatment because of their small number in this study. In this study, only one patient with the minor allele of IL-28B and NR in previous treatment could start and continue with the increased dose of PEG IFN (from 1.37 μg/kg in the previous treatment to 1.79 μg/kg in re-treatment) and ribavirin (from 10.3 mg/kg per day in the previous treatment to 11.1 mg/kg per day in re-treatment) and attained SVR by extended treatment. If the drug adherence does not improve, patients with the minor allele of IL-28B who show NR in the previous treatment should be treated with new drugs.

The next question is how the patients should be re-treated in order to attain SVR on re-treatment. In this study, the patients with a low serum HCV RNA level (<5 $\log_{10}$ IU/mL) at the start of re-treatment showed a significant rate of cure on re-treatment, and this is almost the same result as that previously reported.[16,17] In this study, the two patients with NR in the previous treatment and with less than 5 $\log_{10}$ IU/mL of HCV RNA level (20 KIU/mL and 52 KIU/mL of HCV RNA) at the start of re-treatment attained SVR. On the other hand, even if the previous treatment response was a relapse, the SVR rates were 58% (25/43) among the patients with 5 $\log_{10}$ IU/mL or more of HCV RNA. Because the HCV RNA level changed after the antiviral treatment, it is important to not miss the timing of when the HCV RNA level is low.

With respect to treatment duration among patients with HCV RNA negativiation during re-treatment, 72 weeks of treatment significantly increased the SVR rate compared to 48 weeks. This result was almost the same as that of the REPEAT study.[16] In our present study, the SVR rate among the patients with c-EVR but not RVR in re-treatment was significantly high by 72 weeks of treatment. On the other hand, the SVR rates among the

Table 4 Factors associated with a sustained virological response in re-treatment with PEG IFN plus ribavirin in patients with genotype 2

Factor		SVR	Non-SVR	*P*-value
No. of patients		17	10	
Age (years)		57.7 ± 8.8	63.7 ± 5.1	0.06
Sex: male/female		7/10	8/2	0.11
Serum HCV RNA (log IU/mL)		5.4 ± 1.4	6.1 ± 0.8	0.15
Serum HCV RNA: <5 log/≥5 log		5/11	1/9	0.35
White blood cells (/mm^3)		5049 ± 1355	4171 ± 910	0.10
Neutrophils (/mm^3)		2556 ± 1064	1999 ± 404	0.24
Hemoglobin (g/dL)		14.1 ± 1.3	13.8 ± 1.6	0.51
Platelets (×10^4/mm^3)		17.9 ± 5.4	14.8 ± 4.3	0.17
ALT (IU/L)		38 ± 19	48 ± 47	0.71
IL-28B SNP: TT/TG		6/2	4/2	1.00
ITPA SNP: CC/CA		5/1	4/0	1.00
PEG IFN: α-2a/α-2b		4/13	2/8	1.00
PEG IFN dose (μg/kg per week)	α-2a	3.23 ± 0.34	2.24 ± 2.25	1.00
	α-2b	1.32 ± 0.28	1.18 ± 0.23	0.21
Ribavirin dose (mg/kg per day)		10.4 ± 2.21	10.1 ± 1.31	0.44
1st treatment virological response	RVR/non-RVR	4/13	3/7	1.00

ALT, alanine aminotransferase; HCV, hepatitis C virus; IFN, interferon; IL, interleukin; ISDR, IFN-sensitivity determining region; PEG, pegylated; RVR, rapid virological response; SNP, single nucleotide polymorphism; SVR, sustained virological response.

patients with RVR in re-treatment were similar between the patients with 48 weeks and 72 weeks of treatment. Thus, patients with c-EVR but not RVR in re-treatment should be re-treated for a longer period. In order to attain better SVR, extended treatment duration is generally recommended for patients with on-treatment LVR, whereas standard treatment duration is considered to be sufficient for patients with on-treatment c-EVR. However, the present study revealed that, even if patients achieved c-EVR on re-treatment, 72 weeks of treatment seems to be better than 48 weeks for treatment-experienced patients. The majority of naïve patients showing on-treatment c-EVR could eradicate HCV with 48 weeks of treatment while some could not. In a treatment-experienced setting, patients who are able to respond early but not eradicate HCV would be selected, and therefore extended treatment may be needed.

With genotype 2, the SVR rate was relatively high (63%). The patients who could not attain SVR in re-treatment (two patients) showed NR in the previous treatment. Thus, the patients with genotype 2 and showing NR in previous treatment seemed to be difficult to treat and could be treated with other drugs. Among the patients with RVR in re-treatment, the SVR rates were similar among those with RVR in re-treatment between 24 weeks and 48 weeks of treatment. The effectiveness of extended treatment for the patients with genotype 2 in re-treatment could not be demonstrated because of their small number in this study. Further investigation is needed to clarify this.

In conclusion, this study shows that the efficacy of re-treatment for genotype 1 patients who failed to show SVR to previous treatment with PEG IFN plus ribavirin could be predicted from the previous treatment response and a low HCV RNA level at the start of re-treatment. Re-treatment for 72 weeks led to clinical improvement for genotype 1 patients with c-EVR and without RVR on re-treatment.

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Review Article

Treatment strategies for hepatocellular carcinoma in Japan

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The main methods of treatment for hepatocellular carcinoma (HCC) in Japan are hepatic resection, radiofrequency ablation (RFA) and transcatheter arterial chemoembolization (TACE). Meticulous follow up is then undertaken to check for recurrence, which is treated using repeated RFA or TACE. Hepatic arterial infusion chemotherapy has been introduced as treatment for advanced HCC, and the molecular-targeted drug sorafenib is also now available. Rigorous medical care using these treatment methods and early diagnosis mean that the prognosis for HCC in Japan is the best in the world. This paper reviews the treatment strategies for HCC in Japan.

Key words: hepatocellular carcinoma, treatment algorithm, treatment strategies

INTRODUCTION

TREATMENT FOR HEPATOCELLULAR carcinoma (HCC) is peculiar in that, unlike other solid carcinomas, the treatment methods must be selected in consideration of the underlying clinical condition of the liver. A wide range of treatment methods is available, including hepatectomy, liver transplant, radiofrequency ablation (RFA), transcatheter arterial chemoembolization (TACE), sorafenib therapy, hepatic arterial infusion chemotherapy (HAIC) and radiotherapy. These treatment methods can also be used in combination. This paper reviews the treatment strategies for HCC in Japan.

CHOICE OF TREATMENT METHOD

MANY CASES OF HCC arise from liver cirrhosis, and are associated with deterioration in liver function. This means that in addition to cancer stage, hepatic reserve is also an important prognostic factor. This balance must be taken into account when choosing between different types of treatment. In Japan, the Japan Society of Hepatology issued consensus-based HCC treatment guidelines in 2010, which include a HCC treatment algorithm that offers the closest method of selecting treatment to current clinical practice (Fig. 1).[1]

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In this algorithm, the treatment method is guided by five factors: extrahepatic lesions; hepatic reserve (Child–Pugh class); vascular invasion; number of tumors; and tumor diameter. This algorithm was prepared on the basis of another algorithm compiled in evidence-based clinical practice guidelines for HCC – the Japan Society of Hepatology 2009 update[2] – and reflects the consensus reached among HCC treatment specialists in Japan. This algorithm is somewhat complex, listing multiple methods of treatment with the addition of numerous comments, but reflects the current Japanese choices of treatment for HCC almost in their entirety.[1]

This treatment algorithm was basically prepared for the treatment of primary HCC, but also provides a reference for recurrent HCC, for which the treatment method is determined by taking into account the time to recurrence, type of recurrence, anticipated tumor malignancy according to tumor markers and pathology, age at recurrence, degree of deterioration in liver function between primary occurrence and recurrence, and the adverse effects of initial treatment.

HEPATIC RESECTION

ALONG WITH LIVER transplantation, this offers the most radical treatment, but the degree of surgical invasiveness, complications and the deterioration of hepatic reserve after resection must be taken into account.

Hepatic resection procedures include partial resection, subsegmental resection, segmental resection, two-segment resection, extended two-segment resection and three-segment resection. As HCC frequently metastasizes

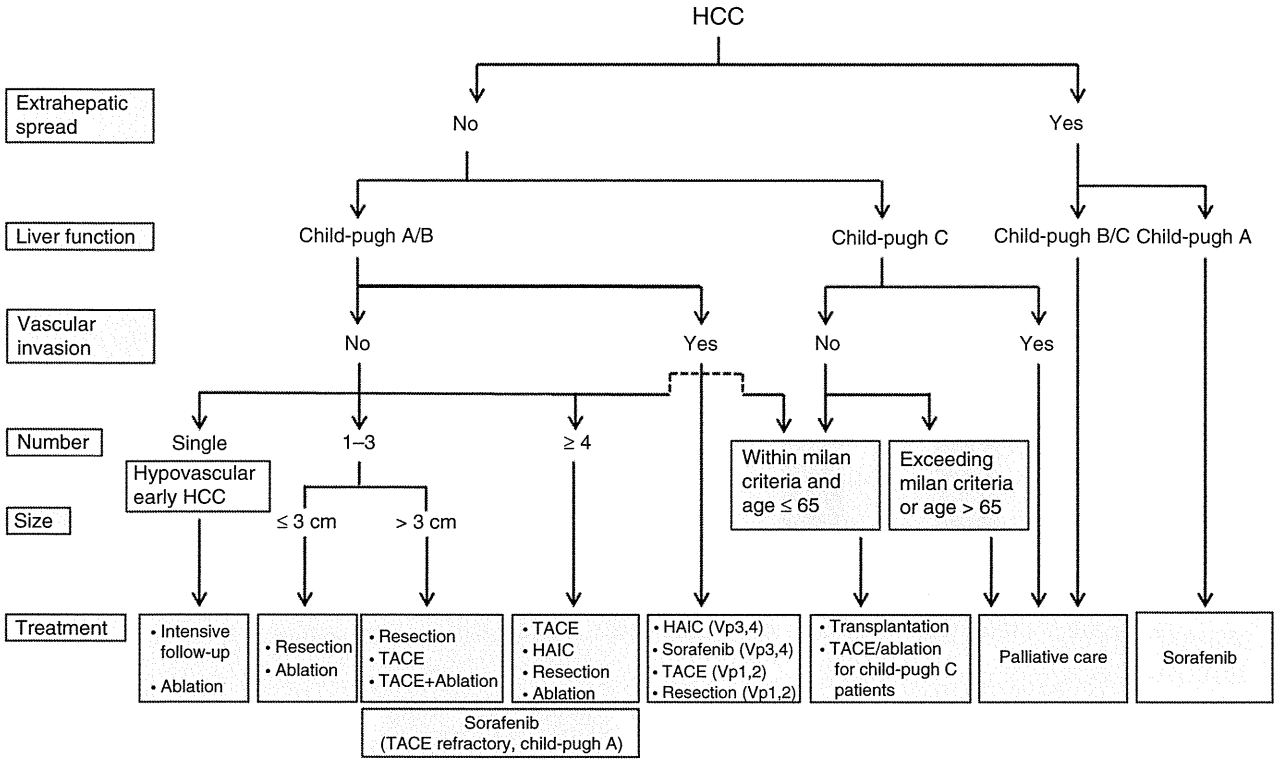

Figure 1 Consensus-based treatment algorithm for HCC proposed by Japan Society of Hepatology 2009 revised in 2010 (modified from ref. [1]). HAIC, hepatic arterial infusion chemotherapy; HCC, hepatocellular carcinoma; TACE, transcatheter arterial chemoembolization.

within the liver via the portal vein, anatomical resection of the entire portal segment where the cancer is located increases the curative nature of the procedure, and anatomical resection is therefore commonly performed provided hepatic reserve is sufficient. The standard procedure is to inject dye under guidance of ultrasonography (USG) into the portal vein in the segment containing the cancer, and to perform systematic subsegmental resection to remove all areas stained by the dye.[3,4]

It is important to evaluate hepatic reserve prior to hepatic resection, and the permissible extent of resection is considered on the basis of presence or absence of ascites, jaundice and the indocyanine green (ICG) retention rate at 15 min when determining the type of resection procedure.[5] If necessary, technetium-99*m* diethylenetriamine pentaacetic acid galactosyl human serum albumin single photon emission computed tomography (CT) is used to evaluate patients who cannot be adequately evaluated by means of an ICG load test.[6,7]

According to the report of the 18th follow-up survey of primary liver cancer in Japan, hepatic resection was performed in 31.7% of all cases of HCC, with operative mortality of 1.4% (Fig. 2).[9] Three-, 5- and 10-year survival rates after hepatic resection were 69.5%, 54.2% and 29.0%, respectively.[9]

As a recent trend in surgery, minimally invasive resection methods such as laparoscopic hepatectomy[10–12] and robot surgery[13] have been developed for some cases of HCC. Percutaneous isolated hepatic perfusion chemotherapy following debulking hepatectomy is reportedly useful in treating patients with severe advanced HCC with tumor thrombus of major vessels.[14]

LIVER TRANSPLANTATION

LIVER TRANSPLANTATION IS the best treatment method for removing metastatic foci in the liver together with the cirrhotic liver from which the cancer develops. In Japan, living-donor liver transplantation has been covered by health insurance since January 2004.

According to reports published up to the end of 2009, almost all liver transplantations for HCC in Japan

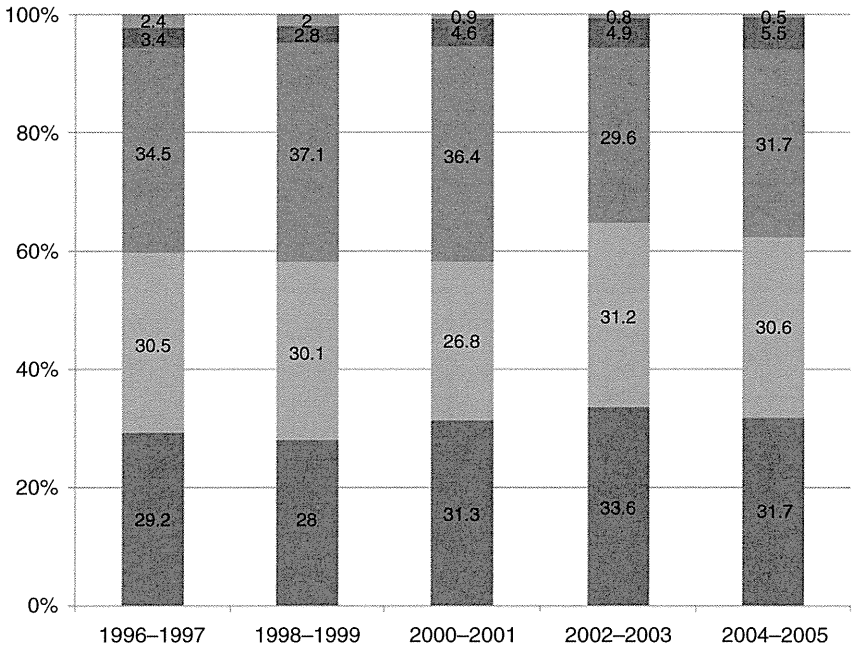

Figure 2 Changes in treatment methods for primary hepatocellular carcinoma in Japan between 1996 and 2005 (modified from ref. [8]). ■, Others; ■, chemotherapy; ■, transcatheter arterial chemoembolization; ■, ablation; ■, resection.

involved living donors, with 1131 transplantations from living donors and seven from deceased donors.[15] As liver transplantations are taken from living donors, indications for liver transplantation in Japan only cover those patients who meet the Milan criteria (≤3 tumors with tumor diameter ≤3 cm or a single tumor ≤5 cm in diameter), but whose hepatic reserve has deteriorated severely (Child–Pugh class C),[1,2] meaning that liver transplantations are regarded very differently in comparison with other countries where the majority of transplantations are from deceased donors.[15]

However, because most liver transplantations are from living donors, issues of the appropriate distribution of liver grafts and waiting times involved in transplantations from deceased donors are almost non-existent. Recently, tumor markers have also been included in the criteria, and attempts are being made to extend indications beyond those of the Milan criteria.[16,17] In addition, donors are restricted to close relatives. As a result, blood groups are frequently mismatched, although in almost all cases this can be managed by the preoperative administration of anti-CD20 antibodies and plasmapheresis.[18]

According to a report by the Japanese Liver Transplantation Society, 1-, 3-, 5- and 10-year survival rates following liver transplantation from a living donor were 84.4%, 73.9%, 68.5% and 58.8%, respectively.[15]

The Act on Organ Transplantation was revised in July 2010 to enable organ donation with the family's permission even if the donor's own intentions had not been made clear, and since then the number of liver transplants from deceased donors has gradually been increasing.

LOCAL ABLATION THERAPY

LOCAL ABLATION THERAPY constitutes the main medical therapy for HCC in Japan. According to the report of the 18th follow-up survey, local ablation therapies were used in 30.6% of cases, administrated percutaneously in approximately 90% of those cases. RFA was used in 72.1% of cases (Fig. 2).[9]

Radiofrequency ablation has been covered by health insurance in Japan since April 2004, and its efficacy has been demonstrated in several subsequent randomized comparative trials,[19–22] making this the first choice in percutaneous local therapy today.[2] Percutaneous ethanol injection therapy, the therapy previously used, is still performed in rare cases for sites where insertion of an electrode for RFA is regarded as dangerous.

Indications for RFA are generally considered to be three or less tumors with a tumor diameter of 3 cm or less, with Child–Pugh class A or B liver function, no uncontrollable ascites and no hemorrhagic tendencies. In practice, commonly used criteria comprise platelet count of 50 000/μL or more, prothrombin time of 50% or more and serum bilirubin of 3 mg/dL or less. For

tumors more than 3 cm in diameter, TACE is frequently performed first, followed by additional RFA.[8]

According to the report of the 18th follow-up survey, 1-, 3- and 5-year survival rates for RFA were 95.0%, 76.7% and 56.3%, respectively.[9]

Radiofrequency ablation is usually performed percutaneously; however, this method can be adapted by performing RFA laparoscopically for lesions on the liver surface or touching neighboring organs such as the intestines or diaphragm,[23] and can also be carried out with artificial pleural effusion for lesions under the diaphragm or when the lungs intrude on the puncture route.[24,25] Artificial ascites can also be used to prevent perforation of the digestive tract for lesions touching the intestines,[24–28] and an endoscopic nasobiliary drainage tube can be used to cool the bile duct before treatment when the lesion is close to the bile duct and the latter is at risk of damage.[24,29] For lesions in which the tumor boundaries are not clearly demarcated and that are difficult to visualize under b-mode USG, or when performing additional treatment to secure ablative margins around the target lesion, treatment can be assisted using contrast USG using Sonazoid[24,30,31] or a real-time virtual sonography system that synchronizes image data from or multidetector-row computed tomography with the position of the USG probe, and simultaneously displays the USG images and virtual images from CT data.[32]

TACE

TRANSCATHETER ARTERIAL CHEMOEMBOLIZATION is widely used in Japan to treat HCC.[9] Usually, an adequate amount of emulsion containing oil-based contrast agent Lipiodol and anticancer agents is injected through a catheter then the selected arteries are embolized by embolic agents. Formerly, the embolic agents used in Japan were the absorbent gelatin sponge materials Gelfoam or Spongel treated to create fine fragments, but Gelpart porous gelatin granules were approved for health insurance coverage in 2006 and are now in common use.

Superselective TACE is generally used in Japan to minimize damage to non-tumorous areas by using a microcatheter to embolize only the cancerous subsegment.[33–35] Epirubicin and cisplatin are commonly used as anticancer agents, and miriplatin, a new platinum drug, came into use in 2010.[36,37]

Indications for TACE are wide-ranging, and the procedure is generally performed in patients with hypervascular HCC who are not indicated for surgery or local therapy for reasons such as multiple bilobar HCC, liver dysfunction, old age or comorbidity, and in whom the first branch from the main portal vein is not occluded. In practice, this technique is commonly indicated for patients who are Child–Pugh class A or B with multiple tumors with a diameter of 3 cm or more or with four or more HCC (Fig. 1).[1,2]

According to the report of the 18th follow-up survey, 3-, 5- and 10-year survival rates for TACE (including chemolipiodolization) used to treat HCC were all poor, at 43.2%, 24.1% and 6.6%, respectively.[9] These outcomes are due to the inclusion of patients in poor condition with hepatic reserve or tumor stage that contraindicates hepatic resection or RFA. The same Japanese follow-up survey of outcomes for TACE as initial therapy for Child–Pugh class A patients with a single tumor found that 1-, 3- and 5-year survival rates were good, at 93%, 73% and 52%, respectively.[35,38]

Transcatheter arterial chemoembolization is performed as initial treatment in 31.7% of cases,[9] but is the most frequently used treatment for recurrence, and it is no exaggeration to say that most HCC patients undergo this therapy at some point (Fig. 2). TACE is periodically repeated in Europe and the USA, but this situation rarely arises in Japan. When one to three intrahepatic lesions are present, TACE is followed by additional RFA with the aim of improving local control. With the advent of sorafenib, definitions of TACE failure/refractory HCC have now been proposed to prevent liver dysfunction from decreasing after excursively repeating TACE and to maintain opportunities to administrate sorafenib.[1]

MOLECULAR-TARGETED DRUGS

SORAFENIB WAS APPROVED as a molecular-targeted drug for the treatment of HCC in Japan from May 2009. This agent was approved based on the results of two randomized control trials from outside of Japan[39,40] and a phase I clinical trial carried out in Japan.[41] However, studies continued after sorafenib entered the market due to a lack of experience with administration in Japan. A safety alert was initially issued due to early deaths resulting from liver failure and hepatic encephalopathy, but it has since been used correctly. The median survival period in Japan is 11.0 months and the response rate is 4%, almost the same outcomes as those of the SHARP trial, but reports to date have shown a tendency for a greater number of side-effects, including hand–foot skin reaction, diarrhea, hypertension, loss of appetite and fatigue.[42]

Sorafenib is used to treat Child–Pugh class A patients who have extrahepatic lesions or multiple intrahepatic

lesions who are unable to undergo TACE or HAIC, and patients with vascular invasion.[1]

Measures taken in Japan to reduce side-effects include a low initial dose of 400 mg/day,[42] but drug effectiveness at half dose has yet to be fully investigated. Sorafenib has also not been compared with HAIC, which was already being performed in Japan, and there is debate on its positioning in the treatment of advanced intrahepatic cancer. A study is currently underway to verify the effects of combining sorafenib therapy and HAIC.

HAIC

HEPATIC ARTERIAL INFUSION chemotherapy has been used in Japan for some time to treat intrahepatic advanced HCC that is not expected to respond to other existing treatment methods. According to the report of the 18th follow-up survey, chemotherapy is used in approximately 5% of cases of primary HCC, and is administrated arterially in 87% of cases (Fig. 2).[9] HAIC enables high-concentration anticancer agents to be administrated directly into the carcinoma, and is also used as a treatment method to keep systemic concentrations of anticancer agent low due to the first-pass effect, with the aim of reducing systemic side-effects. There is little evidence for the efficacy of this approach, with randomized control trials showing no effect in improving survival prognosis. In addition, the therapeutic regimen has not been standardized, and the treatment is associated with many side-effects including hematological toxicities (neutropenia and thrombopenia) and non-hematological toxicities (nausea, vomiting, peptic ulcers, reservoir infection, catheter dislocation and vasculitis along injection site).

In general, HAIC is indicated for patients with multiple intrahepatic lesions or vascular invasion who are excluded from the indications for TACE and other existing treatments or for whom these are not expected to be effective, other than Child–Pugh class C patients with severe liver dysfunction.[1]

In Japan, the main forms used are interferon-combined 5-fluorouracil (5-FU) HAIC,[39,40,43–45] low-dose cisplatin-combined 5-FU HAIC[43,46–48] and HAIC with cisplatin alone.[43,49] All of these have a response rate of approximately 30–40%, and the addition of more curative therapy is known to dramatically improve prognosis in responders. Use of a subcutaneous implantable HAIC reservoir enables HAIC to be administrated in outpatient clinics.[44,45] In terms of side-effects, attention must be paid not only to the side-effects of the anticancer agents used in treatment, but also to complications such as catheter displacement, reservoir infection and peptic ulcer that are specific to hepatic arterial infusion, and the management techniques affect treatment response.[45]

RADIOTHERAPY

RADIOTHERAPY IS ANOTHER treatment option. According to the report of the 18th follow-up survey, this treatment is administrated to only 1.5% of cases,[9] but reports in recent years have described the efficacy of stereotactic radiotherapy, which enables selective irradiation of the tumor alone while avoiding the background liver (which has a low tolerance for radiation), and of intensity-modulated radiotherapy,[50] as well as of good outcomes from particle beam therapies such as proton-beam and carbon-beam therapy.[51,52]

PREVENTION OF RECURRENCE

HEPATOCELLULAR CARCINOMA HAS two mechanisms of recurrence – multicentric carcinogenesis and intrahepatic metastasis – and a high annual recurrence rate of 20–30% even after treatment.[53] Aiming for long-term survival is thus impossible without suppressing this recurrence, even if curative treatment is performed. If the underlying condition is viral hepatitis, interferon therapy is administrated proactively with the aim of viral elimination in the case of hepatitis C, whereas the nucleoside analog entecavir is given for hepatitis B. Even if this cannot be administrated, alanine transferase levels are kept as low as possible and hepatitis proactively suppressed by means of glycyrrhizin, ursodeoxycholic acid, phlebotomy or low-dose long-term interferon therapy, and branched-chain amino acids are administrated and nutritional management implemented with the aim of preventing reduced hepatic reserve at the time of recurrence.

CONCLUSION

IN ADDITION TO the so-called three major cancer treatments of surgery, chemotherapy and radiotherapy, methods of treatment for HCC also include RFA, TACE and liver transplantation. These treatment methods are all major interventions that depend on therapeutic techniques, and it must be understood that treatment procedures vary greatly not only between Japan, Europe and the USA, but also between

institutions within a single country. The good outcomes for HCC seen in Japan[9] compared with those in Europe[54] and the USA[55] are the result of the meticulous medical care for HCC that has been practiced in Japan.

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ARTICLE

The effects of ezetimibe on non-alcoholic fatty liver disease and glucose metabolism: a randomised controlled trial

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Abstract
Aims/hypothesis The cholesterol absorption inhibitor ezetimibe has been shown to ameliorate non-alcoholic fatty liver disease (NAFLD) pathology in a single-armed clinical study and in experimental animal models. In this study, we investigated the efficacy of ezetimibe on NAFLD pathology in an open-label randomised controlled clinical trial.
Methods We had planned to enrol 80 patients in the trial, as we had estimated that, with this sample size, the study would have 90% power. The study intervention and enrolment were discontinued because of the higher proportion of adverse events (significant elevation in HbA_{1c}) in the ezetimibe group than in the control group. Thirty-two patients with NAFLD were enrolled and randomised (allocation by computer program). Ezetimibe (10 mg/day) was given to 17 patients with NAFLD for 6 months. The primary endpoint was change in serum aminotransferase level. Secondary outcomes were change in liver histology (12 control and 16 ezetimibe patients), insulin sensitivity including a hyperinsulinaemic–euglycaemic clamp study (ten control and 13 ezetimibe patients) and hepatic fatty acid composition (six control and nine ezetimibe patients). Hepatic gene expression profiling was completed in 15 patients using an Affymetrix gene chip. Patients and the physician in charge knew to which group the patient had been allocated, but people carrying out measurements or examinations were blinded to group.
Results Serum total cholesterol was significantly decreased in the ezetimibe group. The fibrosis stage and ballooning score were also significantly improved with ezetimibe treatment. However, ezetimibe treatment significantly increased HbA_{1c} and was associated with a significant increase in hepatic long-chain fatty acids. Hepatic gene expression analysis showed coordinate downregulation of genes involved in skeletal muscle development and cell adhesion molecules in the ezetimibe treatment group, suggesting a suppression of stellate cell development into myofibroblasts. Genes involved in the L-carnitine pathway were coordinately downregulated by ezetimibe treatment and those in the steroid metabolism pathway upregulated, suggestive of impaired oxidation of long-chain fatty acids.
Conclusions/interpretation Ezetimibe improved hepatic fibrosis but increased hepatic long-chain fatty acids and HbA_{1c} in patients with NAFLD. These findings shed light on previously unrecognised actions of ezetimibe that should be examined further in future studies.
Trial registration University Hospital Medical Information Network (UMIN) Clinical Trials Registry UMIN000005250.
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Keywords Ezetimibe · Fatty acid · Gene expression · Non-alcoholic fatty liver disease

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Abbreviations

ALT	Alanine aminotransferase
H-IR	Hepatic insulin resistance index
hsCRP	High-sensitivity C-reactive protein
ICG15	Indocyanine green retention rate at 15 min after venous administration
LXR	Liver-X-receptor
MCR	Glucose metabolic clearance rate
miR	MicroRNA
NAFLD	Non-alcoholic fatty liver disease
NAS	NAFLD activity score
NASH	Non-alcoholic steatohepatitis
NPC1L1	Niemann–Pick C1-like 1
PAI-1	Plasminogen activator inhibitor-1
RLP-C	Remnant-like particle cholesterol
sdLDL	Small dense LDL
SREBP	Sterol regulatory element binding protein
QUICKI	Quantitative insulin sensitivity check index

Introduction

Multiple metabolic disorders, such as diabetes [1], insulin resistance and dyslipidaemia [2], are associated with non-alcoholic fatty liver disease (NAFLD), ranging from simple fatty liver to non-alcoholic steatohepatitis (NASH). Steatosis of the liver is closely associated with insulin resistance. However, the toxic lipids are not intrahepatic triacylglycerols but, rather, it is non-esterified cholesterol [3, 4] and some NEFA [5] that contribute to inflammation and insulin resistance in hepatocytes.

The level of cholesterol is tightly regulated by endogenous synthesis in the liver and dietary absorption/biliary reabsorption in the small intestine. Niemann–Pick C1-like 1 (NPC1L1) plays a pivotal role in cholesterol incorporation in enterocytes [6]. Ezetimibe, a potent inhibitor of cholesterol absorption, inhibits NPC1L1-dependent cholesterol transport at the brush border of the intestine and the liver [6]. This suggests that ezetimibe ameliorates toxic-lipid-induced inflammation and insulin resistance by inhibiting cholesterol absorption. Indeed, ezetimibe improves liver steatosis and insulin resistance in mice [7] and Zucker obese fatty rats [8], although the beneficial effects of ezetimibe are observed only when the animals are fed a high-fat diet. Ezetimibe can also ameliorate liver pathology in patients with NAFLD [9, 10]; however, these studies lack a control group, which precludes meaningful conclusions as liver pathology can improve over the natural course of the disease or with tight glycaemic control in some NAFLD patients [1]. In the present study, we investigated the efficacy of ezetimibe treatment in patients with NAFLD for 6 months in an open-label randomised control study by examining liver pathology, as well as hepatic enzymes, glucose metabolism, hepatic fatty acid composition and hepatic gene expression profiles.

Methods

Patient selection Study staff recruited participants from outpatients at Kanazawa University Hospital, Ishikawa, Japan. Patients were recruited from April 2008 to August 2010, with follow-up visits during the 6 months thereafter. The study lasted from April 2008 to February 2011.

The inclusion criterion was a biopsy consistent with the diagnosis of NAFLD. Exclusion criteria included hepatic virus infections (hepatitis C virus [HCV] RNA–PCR-positive, hepatitis B and C, cytomegalovirus and Epstein–Barr virus), autoimmune hepatitis, primary biliary cirrhosis, sclerosing cholangitis, haemochromatosis, α_1-antitrypsin deficiency, Wilson's disease, history of parenteral nutrition and use of drugs known to induce steatosis (e.g. valproate, amiodarone and prednisone) or hepatic injury caused by substance abuse and/or the current or past consumption of more than 20 g of alcohol daily. None of the patients had any clinical evidence of hepatic decompensation, such as hepatic encephalopathy, ascites, variceal bleeding or an elevated serum bilirubin level more than twofold the upper normal limit.

A random allocation sequence was computer-generated elsewhere and assigned participants in a 1:1 ratio to treatment with ezetimibe or to the control group. All patients and responsible guardians underwent an hour of nutritional counselling by an experienced dietitian before starting the 6 month treatment period. The experienced dietitians were unaware of the study assignments. In addition, all patients were given a standard energy diet (125.5 kJ/kg per day; carbohydrate 50–60%, fat 20–30%, protein 15–20%) and exercise (5–6 metabolic equivalent estimations for 30 min daily) counselling before the study. Patients remained on stable doses of medications for the duration of the study. The patients in the ezetimibe group received generic ezetimibe (10 mg/day; Zetia, [Merck, Whitehouse Station, NJ, USA]) for 6 months.

The study was conducted with the approval of the Ethics Committee of Kanazawa University Hospital, Ishikawa, Japan, in accordance with the Declaration of Helsinki. Written informed consent was obtained from all individuals before enrolment. This trial is registered with the University Hospital Medical Information Network (UMIN) (Clinical Trials Registry, no. UMIN000005250).

Primary and secondary outcomes The primary endpoint was change in serum alanine aminotransferase (ALT) level at month 6 from baseline. Secondary outcomes included changes in the histological findings for NAFLD, hepatic gene expression profiling, fatty acid compositions of plasma and liver biopsy samples, lipid profiles, insulin resistance and

anthropometric measures, as well as assessment of ezetimibe safety. We had planned to enrol 80 patients in the trial, as we had estimated that with this sample size, the study would have 90% power at an α (two-tailed) value of 0.05 showing a 50% decrease of serum ALT values with 6 months of pioglitazone therapy on the basis of a previous study [11]. At the time of adverse event analyses, 32 of the targeted 80 patients had been randomly assigned and were included in the safety analyses.

Data collection Clinical information, including age, sex and body measurements, was obtained for each patient. Venous blood samples were obtained after the patients had fasted overnight (12 h) and were used to evaluate blood chemistry. Insulin resistance was estimated by HOMA-IR, calculated as [fasting insulin (pmol/l) × fasting glucose (mmol/l)]/22.5 [12] and insulin sensitivity was estimated as the quantitative insulin sensitivity check index (QUICKI)[13]. The adipose tissue insulin resistance index (adipose IR) was calculated as fasting NEFA (mmol/l)×fasting insulin (pmol/l) [14–16]. The indocyanine green retention rate at 15 min after venous administration (ICG15) was assessed using standard laboratory techniques before and after treatment. Serum fatty acids were measured with a gas chromatograph (Shimizu GC 17A, Kypto, Japan) at SRL (Tokyo, Japan).

Evaluation of insulin sensitivity derived from an OGTT After an overnight fast (10–12 h), a 75 g OGTT was performed at 08:30 hours. The OGTT-derived index of beta cell function, the insulinogenic index, computed as the suprabasal serum insulin increment divided by the corresponding plasma glucose increment in the first 30 min (ΔI30/ΔG30) [15, 17, 18] was calculated. From the OGTT data, the Matsuda index [19] was calculated. The hepatic insulin resistance index (H-IR) was calculated as the product of the total AUCs for glucose and insulin during the first 30 min of the OGTT (glucose 0–30 [AUC] [mmol/l]×insulin 0–30 [AUC] [pmol/l]). Skeletal muscle insulin sensitivity can be calculated as the rate of decline in plasma glucose concentration divided by plasma insulin concentration, as follows. Muscle insulin sensitivity index=dG/dt/mean plasma insulin concentration, where dG/dt is the rate of decline in plasma glucose concentration and is calculated as the slope of the least square fit to the decline in plasma glucose concentration from peak to nadir [20]. See the electronic supplementary material (ESM) for further details.

Evaluation of insulin sensitivity derived from the euglycaemic insulin clamp Insulin sensitivity in 23 of the 31 patients (10 control and 13 ezetimibe patients) was also evaluated in a hyperinsulinaemic–euglycaemic clamp study [21]. Patients did not receive any medication on the morning of the examination. At ~09:00 hours, after an overnight fast of at least 10 h, an intravenous catheter was placed in an antecubital vein in each individual for infusion, while a second catheter was placed in the contralateral hand for blood sampling. The euglycaemic–hyperinsulinaemic clamp technique was performed using an artificial pancreas (model STG-22; Nikkiso, Tokyo, Japan), as described previously [22]. See ESM for further details. The mean glucose metabolic clearance rate (MCR) in healthy individuals (n=9; age, 26.60± 2.9 years; body mass index, 22.3±2.1 kg/m^2) was 13.5± 3.4 mg kg^{-1} min^{-1} [2].

Liver biopsy pathology A single pathologist, who was blinded to the clinical information and the order in which the biopsies were obtained, analysed all biopsies twice and at separate times. The sections were cut from a paraffin block and stained with haematoxylin and eosin, Azan–Mallory and silver reticulin impregnation. The biopsied tissues were scored for steatosis (from 0 to 3), stage (from 1 to 4) and grade (from 1 to 3) as described [2], according to the standard criteria for grading and staging of NASH proposed by Brunt et al [23]. The NAFLD activity score (NAS) was calculated as the unweighted sum of the scores for steatosis (0–3), lobular inflammation (0–3) and ballooning (0–2), as reported by Kleiner et al [24].

Gene expression analysis of liver biopsied samples Gene expression profiling was performed in samples from nine patients in the ezetimibe group and six in the control group. Liver tissue RNA was isolated using the RNeasy Mini kit (QIAGEN, Tokyo, Japan) according to the manufacturer's instructions. See ESM for further details. Data files (CEL) were obtained using the GeneChip Operating Software 1.4 (Affymetrix). Genechip data analysis was performed using BRB-Array Tools (http://linus.nci.nih.gov/BRB-ArrayTools.html). The data were log-transformed ($\log_{10}$), normalised and centred. To identify genetic variants, paired t tests were performed to define p values <0.05 and fold change>1.5. Pathway analysis was performed using MetaCore (GeneGo, St Joseph, MI, USA). Functional ontology enrichment analysis was performed to compare the gene ontology (GO) process distribution of differentially expressed genes ($p < 0.01$).

Fatty acid composition of liver Aliquots (0.2 mg) of liver samples snap-frozen by liquid nitrogen were homogenised in 1 ml normal NaCl solution (NaCl 154 mmol/l). Briefly, fatty acids were extracted by using pentadecanoic acid, and saponified with alkaline reagent (0.5 mmol/l KOH/ CH_3OH). The fatty acid methyl esters were analysed in a gas chromatograph (Shimadzu GC-2014 AF/SPL; Shimadzu Corporation, Kyoto, Japan) equipped with a flame ionisation detector and an auto injector. See ESM for further details. Mass spectra were analysed using GC solution (v. 2.3) software (Shimadzu Corporation, Kyoto, Japan, www.shimadzu.com). The changes in hepatic fatty acid composition are expressed as 10^{-4} mg/mg liver.

Statistical analysis Data are expressed as mean ± one standard error, unless indicated otherwise. The Statistical Package for the Social Sciences (SPSS; version 11.0; Chicago, IL, USA) was used for the statistical analyses. For univariate comparisons between the patient groups, Student's t test or Mann–Whitney's U test was used, as appropriate, followed by the Bonferroni multiple-comparison test. A value of $p < 0.05$ was considered to indicate statistical significance.

Results

Enrolment and discontinuation The data and safety monitoring board recommended that the study intervention and enrolment be discontinued because of the higher proportion of adverse events (significant elevation in HbA_{1c}) in the ezetimibe group than in the control group. At the time of adverse event analyses, 32 of the targeted 80 patients had been randomly assigned and were included in the safety analyses. In our open-label trial, 32 patients with NAFLD were enrolled. They were randomised to treatment with ezetimibe ($n=17$) or a control ($n=15$) with no significant clinical differences in variables between the groups. Of the 32 randomly assigned patients, 31 had completed the 6 month intervention period; one patient dropped out of the study. One case in the control group withdrew consent after randomisation and before intervention (ESM Fig. 1). The patient who withdrew was excluded from analysis because he did not start his course of treatment. Two analyses were conducted in the remaining patients. In the intention-to-treat analysis (ESM Tables 1 and 2), measures that were missing for participants who discontinued the study were replaced with baseline measures. In the second analysis, the only data included were from participants who completed the study to the end of the 6 month follow-up period. We performed a completed case analysis because there were few dropouts unrelated to baseline values or to their response.

Patient characteristics The 31 study patients (mean age 52.7±2.1 years; mean BMI 29.2±1.0) included 14 randomised to the control group and 17 to the ezetimibe group (ESM Table 3).

At baseline, the characteristics of patients in the ezetimibe and control groups were comparable except for the waist circumference ($p=0.085$) and the Matsuda index ($p=0.060$). The histological features of the liver are summarised in Table 1. At baseline, neither the severity of the individual histological features nor the proportion of patients distributed in the three NAS categories was significantly different between the two groups. All 31 participants agreed to complete the follow-up venous blood samples including OGTT. The ICG15 was conducted in 24 patients (ten control and 14 ezetimibe patients).

Changes in laboratory variables The primary study outcome, serum alanine aminotransferase levels, did not change after ezetimibe treatment (Table 2).

After 6 months of ezetimibe treatment, systolic blood pressure, HbA_{1c}, glycated albumin, and lathosterol were significantly increased, while total cholesterol levels, campesterol, sitosterol and ferritin were significantly decreased. In contrast, body weight, BMI, fasting plasma glucose, plasma γ-glutamyltransferase, triacylglycerols, HDL-cholesterol, small dense LDL (sdLDL), remnant-like particle cholesterol (RLP-C), type IV collagen 7 s levels, NEFA, total bile acid, high-sensitivity C-reactive protein (hsCRP), adiponectin, TNF-α, plasminogen activator inhibitor-1 (PAI-1), 8-isoprostanes and ICG15 did not change after ezetimibe treatment (Table 2). Adipose IR tended to increase in the ezetimibe group (from 88.1±25.5 to 107.5±25.5, $p=0.070$), but not in the control group.

When changes in the groups were compared, the ezetimibe group, but not the control group, had a significant decrease in total cholesterol (ezetimibe, −0.49±0.19 vs control, 0.06±0.14 mmol/l; $p=0.037$), whereas the ezetimibe group, but not control group, showed a significant elevation in HbA_{1c} (ezetimibe, 0.46±0.12% [4.95±1.28 mmol/mol] vs control, 0.08±0.13% [0.78±1.46 mmol/mol]; $p=0.041$). Also, there were significant differences between the groups in cholesterol and HbA_{1c} levels at 6 months. The multiple-comparison

Table 1 Histological characteristics of the livers of patients who completed the study at baseline and 6 months

Variable	Control		p^a	Ezetimibe		p^a	p^b
	Before	After		Before	After		
Steatosis	1.42±0.15	1.17±0.17	0.082	1.56±0.18	1.31±0.15	0.300	0.989
Stage	1.71±0.40	1.71±0.39	1.000	1.75±0.28	1.53±0.26	0.048	0.163
Grade	0.88±0.28	0.79±0.26	0.339	0.84±0.21	0.72±0.15	0.362	0.628
Acinar inflammation	0.88±0.20	0.83±0.20	0.674	1.00±0.13	0.97±0.13	0.751	0.060
Portal inflammation	0.67±0.19	0.71±0.13	0.795	0.44±0.16	0.56±0.16	0.333	0.941
Ballooning	0.58±0.23	0.58±0.23	1.000	0.69±0.20	0.41±0.15	0.045	0.677
NAFLD activity score	3.25±0.53	2.82±0.59	0.139	3.71±0.50	3.06±0.45	0.185	0.705

Data are expressed as the means ± SE

[a] p value for the intergroup comparison (baseline vs 6 month)

[b] p value for the intergroup comparison (changes from baseline between groups)

Table 2 Laboratory values, insulin sensitivity and insulin resistance derived from the euglycaemic insulin clamps and OGTTs of patients who completed the study at baseline and 6 months

Variable	Control			Ezetimibe			
	Before	After	p^a	Before	After	p^a	p^b
Male/female	9/5			11/6			0.232
Age (years)	55.5±3.0			50.4±2.9			
Body weight (kg)	74.4±6.2	73.0±5.6	0.144	81.5±4.6	80.1±4.2	0.367	0.983
BMI (kg/m^2)	27.7±1.7	27.3±1.5	0.172	30.5±1.2	30.0±1.1	0.383	0.999
Waist circumference (cm)	93.1±2.7	92.6±3.4	0.709	99.9±2.5	100.0±2.6	0.956	0.713
Systolic blood pressure (mmHg)	125.2±3.9	126.4±4.9	0.771	124.0±2.4	130.7±2.8	0.048	0.269
Fasting plasma glucose (mmol/l)	7.15±0.63	6.52±0.40	0.240	6.62±0.30	6.87±0.34	0.411	0.131
HbA_{1c} (%)	5.9±0.2	6.0±0.2	0.603	6.1±0.2	6.5±0.2	0.001	0.041
HbA_{1c} (mmol/mol)	40.8±2.2	41.6±2.6	0.603	43.0±2.6	48.0±2.3	0.001	0.041
Hepaplastin test (%)	115.9±5.8	117.1±6.4	0.624	113.7±4.6	111.8±3.7	0.583	0.459
Glycated albumin (%)	15.9±0.8	16.2±1.0	0.397	15.7±0.5	16.8±0.5	0.014	0.196
Serum aspartate aminotransferase (μkat/l)	31.1±4.4	30.3±3.0	0.780	41.8±6.7	33.7±4.1	0.252	0.365
Serum ALT (μkat/l)	37.9±6.8	38.0±4.5	0.978	53.2±8.6	49.3±6.5	0.683	0.723
Plasma γ-glutamyltransferase (μkat/l)	74.9±27.8	65.8±19.5	0.345	71.4±23.4	60.5±16.1	0.220	0.892
Total cholesterol (mmol/l)	5.14±0.21	5.20±0.18	0.672	5.14±0.20	4.65±0.17	0.024	0.037
Triacylglycerols (mmol/l)	1.34±0.12	1.17±0.12	0.105	1.43±0.11	1.46±0.13	0.857	0.303
HDL-C (mmol/l)	1.40±0.08	1.45±0.06	0.914	1.36±0.08	1.36±0.06	0.942	0.903
sdLDL (mmol/l)	0.52±0.07	0.54±0.07	0.782	0.61±0.10	0.50±0.06	0.201	0.251
RLP-C (mmol/l)	0.13±0.01	0.11±0.01	0.163	0.12±0.01	0.11±0.01	0.601	0.365
Lathosterol×10^{-3} (μmol/l)	2.27±0.43	2.85±0.52	0.001	3.52±0.52	5.01±0.67	<0.001	0.018
Campesterol×10^{-3} (μmol/l)	4.32±0.65	6.20±0.68	0.004	3.78±0.42	2.49±0.30	0.007	<0.001
Sitosterol×10^{-3} (μmol/l)	3.04±0.47	3.89±0.39	0.079	2.73±0.28	1.81±0.19	0.004	0.002
Ferritin (pmol/l)	412.1±85.6	235.3±47.0	0.009	395.7±81.3	247.8±56.8	0.005	0.689
Type IV collagen 7 s (μg/l)	4.52±0.48	4.42±0.45	0.622	4.23±0.23	4.33±0.20	0.592	0.465
NEFA (mmol/l)	0.50±0.09	0.63±0.06	0.160	0.51±0.05	0.57±0.03	0.835	0.447
Total bile acid (μmol/l)	12.5±8.0	8.8±5.2	0.214	5.0±0.7	4.8±1.3	0.893	0.267
hsCRP×10^{-3} (μg/ml)	0.12±0.02	0.09±0.02	0.050	0.14±0.04	0.13±0.04	0.886	0.767
Adiponectin (μg/ml)	4.0±0.5	4.6±0.8	0.114	3.0±0.6	3.3±0.6	0.299	0.670
TNF-α ×10^{-5} (pmol/ml)	10.4±2.3	15.6±8.1	0.094	8.1±0.6	30.0±12.7	0.183	0.084
Leptin×10^{-3} (μg/l)	8.1±1.0	9.7±1.3	0.044	10.8±1.4	12.4±1.5	0.085	0.982
PAI-1 (pmol/l)	400.0±44.2	436.5±44.2	0.401	550.0±71.2	488.5±67.3	0.217	0.136
8-Isoprostanes (pmol/mmol creatinine)	76.9±14.3	57.0±8.0	0.147	56.5±6.6	68.0±7.7	0.092	0.031
ICG15 (%)	8.7±2.4	8.5±2.0	0.662	7.7±1.7	7.7±1.5	0.984	0.796
HOMA-IR	10.1±6.5	5.0±2.1	0.471	9.5±2.6	9.3±2.2	0.839	0.479
QUICKI	0.32±0.01	0.33±0.01	0.443	0.30±0.01	0.30±0.01	0.984	0.019
Adipose IR	55.8±15.5	78.8±31.7	0.441	88.1±25.5	107.5±25.5	0.070	0.099
Insulinogenic index	0.43±0.09	0.53±0.11	0.307	0.41±0.08	0.35±0.09	0.501	0.765
H-IR×10^6	1.82±0.46	2.29±0.44	0.568	2.29±0.33	2.66±0.41	0.221	0.796
Matsuda index	3.03±0.45	3.35±0.49	0.368	1.99±0.28	2.01±0.29	0.895	0.013
Muscle insulin sensitivity	0.039±0.006	0.058±0.016	0.210	0.036±0.005	0.034±0.004	0.560	0.067
MCR	4.86±0.50	4.36±0.45	0.174	4.70±0.31	4.80±0.35	0.827	0.352

Data are expressed as means ± SE

[a] p value for the intergroup comparison (baseline vs 6 month)

[b] p value for the intergroup comparison (changes from baseline between groups)

HDL-C, HDL-cholesterol

Table 3 Signalling pathway gene expression changes in the ezetimibe group

Pathway	Gene symbol	Gene name	Affy ID	Up or down	Function
Development_skeletal muscle development	*VEGFA*	Vascular endothelial growth factor A	210512_s_at	Down	Angiogenesis
	ACTA2	Actin, α2, smooth muscle, aorta1	200974_at	Down	Cytoskeleton and cell attachment
	TCF3	Transcription factor 3	209153_s_at	Down	Differentiation
	TTN	Titin	1557994_at	Down	Abundant protein of striated muscle
	TPM2	Tropomyosin 2	204083_s_at	Down	Actin filament binding protein
	MYH11	Myosin, heavy chain 11, smooth muscle	201496_x_at	Down	Smooth muscle myosin
Immune response_phagocytosis	*FYB*	FYN-binding protein	205285_s_at	Up	Platelet activation and IL2 expression
	FCGR3A	Fc fragment of IgG, low affinity IIIA	204006_s_at	Up	ADCC and phagocytosis
	LCP2	Lymphocyte cytosolic protein 2	244251_at	Up	T cell antigen receptor mediated signalling
	CLEC7A	C-type lectin domain family 7, member A	221698_s_at	Up	T cell proliferation
	MSR1	Macrophage scavenger receptor 1	214770_at	Up	Macrophage-associated processes
	FCGR2A	Fc fragment of IgG, low affinity IIA	1565673_at	Up	Promotes phagocytosis
	PRKCB	Protein kinase C, β	209685_s_at	Up	B cell activation, apoptosis induction
	PLCB4	Phospholipase C, β4	240728_at	Up	Inflammation, cell growth, signalling and death
Cell adhesion_integrin priming	*GNA12*	G protein α12	221737_at	Down	Cytoskeletal rearrangement
	ITGB3	Integrin, β3	204628_s_at	Down	Ubiquitously expressed adhesion molecules
	PIK3R2	Phosphoinositide-3-kinase, regulatory subunit 2	229392_s_at	Down	Diverse range of cell functions
Cell adhesion_cadherins	*PTPRF*	Protein tyrosine phosphatase, receptor type, F	200636_s_at	Down	Cell adhesion receptor
	BTRC	β-Transducin repeat containing E3 ubiquitin protein ligase	222374_at	Down	Substrate recognition component of a SCF E3 ubiquitin-protein ligase complex
	CDHR2	Cadherin-related family member 2	220186_s_at	Down	Contact inhibition at the lateral surface of epithelial cells
	SKI	V-ski sarcoma viral oncogene homologue	229265_at	Down	Repressor of TGF-β signalling
	MLLT4	Myeloid/lymphoid or mixed-lineage leukaemia	214939_x_at	Down	Belongs to an adhesion system
	VLDLR	Very low density lipoprotein receptor	209822_s_at	Down	Binds VLDL and transports it into cells by endocytosis
O-Hexadecanoyl-L-carnitine pathway	*TUBB2B*	Tubulin, β 2B class IIB	209372_x_at	Down	Major component of microtubules
	TUBB2A	Tubulin, β2A class IIA	209372_x_at	Down	Major component of microtubules
	PLCE1	Phospholipase C, epsilon 1	205112_at	Down	Hydrolyses phospholipids into fatty acids and other lipophilic molecules
	CPT1B	Carnitine palmitoyltransferase 1B (muscle)	210070_s_at	Down	Rate-controlling enzyme of the long-chain fatty acid β-oxidation pathway
	CPT1A	Carnitine palmitoyltransferase 1A (liver)	203634_s_at	Down	Carnitine-dependent transport across the mitochondrial inner membrane
	NR1H4	Nuclear receptor subfamily 1, group H, member 4	243800_at	Down	Involved in bile acid synthesis and transport.
GalNAcbeta1-3Gal pathway	*PLCB4*	Phospholipase C, β4	240728_at	Up	Formation of inositol 1,4,5-trisphosphate and diacylglycerol
Steroid metabolism_cholesterol biosynthesis	*CYP51A1*	Cytochrome P450, family 51, subfamily A, polypeptide 1	216607_s_at	Up	Transforms lanosterol
	SREBF2	Sterol regulatory element binding transcription factor 2	242748_at	Up	Transcriptional activator required for lipid homeostasis
	SQLE	Squalene epoxidase	209218_at	Up	Catalyses the first oxygenation step in sterol biosynthesis

Table 3 (continued)

Pathway	Gene symbol	Gene name	Affy ID	Up or down	Function
	SC5DL	Sterol-C5-desaturase-like	215064_at	Up	Catalyses the conversion of lathosterol into 7-dehydrocholesterol
	HMGCS1	3-Hydroxy-3-methylglutaryl-CoA synthase 1	205822_s_at	Up	Condenses acetyl-CoA with acetoacetyl-CoA to form HMG-CoA

Bonferroni test revealed highly significant differences in the changes in total cholesterol ($p = 0.037$) and HbA_{1c} ($p = 0.040$) between the ezetimibe and control groups.

Increased concentrations of the cholesterol synthesis markers lathosterol (ezetimibe, 1.49±0.32 nmol/l vs control, 0.58±0.14 nmol/l; $p = 0.018$) and decreased concentrations of the cholesterol absorption markers campesterol (ezetimibe, −1.28±0.41 nmol/l vs control, 1.88±0.54 nmol/l, $p = 0.000$) and sitosterol (ezetimibe, −0.91±0.27 nmol/l vs control, 0.85± 0.45 nmol/l; $p = 0.002$) were observed on treatment. The ezetimibe group had an increase, whereas the control group had a decrease, in the level of 8-isoprostanes (ezetimibe, 11.6± 6.4 pmol/mmol creatinine vs control, −19.9±12.9 pmol/mmol creatinine; $p = 0.031$).

When changes between groups were compared, the ezetimibe group had a greater decrease in the Matsuda index (ezetimibe=−0.78±0.57 vs control=−1.35±0.55, $p = 0.013$), QUICKI (ezetimibe= −0.02±0.01 vs control=0.03±0.0, $p = 0.019$), and muscle insulin sensitivity (ezetimibe= −0.002±0.004 vs control=0.019±0.014, $p = 0.067$) than the control group.

Changes in liver histology Twenty-eight of 31 participants, 16 in the ezetimibe group and 12 in the control group, agreed to complete the follow-up and undergo a liver biopsy at 6 months, allowing for complete case analysis of the data (Table 1). After 6 months, the changes in staging score (from 1.75±0.28 to 1.53±0.26) and ballooning score (from 0.69± 0.20 to 0.41±0.15) were significantly improved in the ezetimibe group compared with the control group, whereas the scores of steatosis, lobular inflammation and NAS were not significantly changed in either group. The degree of all of these histological features was not significantly different between the two groups (Table 1).

Serial changes in liver gene with ezetimibe treatment Gene expression profiling was conducted in samples from nine patients in the ezetimibe group and six in the control group (ESM Table 4). In the ezetimibe group, 434 genes were upregulated and 410 genes downregulated, while in the control group, 643 genes were upregulated and 367 genes downregulated. Pathway analysis of the process network of differentially expressed genes showed coordinate downregulation of genes involved in skeletal muscle development and cell adhesion molecules in the ezetimibe group, suggesting a suppression of stellate cell development into myofibroblasts (Table 3). In addition, ezetimibe activated the immune response pathway. In contrast, genes involved in skeletal muscle development were upregulated and those in the immune response downregulated in the control group (Table 4). Pathway analysis of the metabolic network also revealed decreased L-carnitine pathway and increased steroid metabolism with ezetimibe treatment, but decreased CoA biosynthesis and increased glycerol 3-phosphate pathway in the control group (ESM Fig. 2).

Changes in plasma fatty acid composition and fatty acid composition extracted from liver tissue The changes in plasma fatty acid composition are shown in Table 5. Compared with baseline levels, only eicosatrienoic acid was significantly increased in the ezetimibe group.

Fatty acid composition in extracted liver tissue was available for 16 NAFLD patients treated with ezetimibe and 12 controls (Table 6). Ezetimibe treatment for 6 months significantly and markedly increased hepatic lauric, myristic, palmitic, palmitoleic, margaric and stearic acids compared with the control group. The changes in hepatic fatty acid composition did not correlate with the changes in serum fatty acid composition before and after ezetimibe treatment (ESM Table 5).

Discussion

This is the first report of the efficacy of ezetimibe treatment on liver pathology in patients with NAFLD in an open-label randomised controlled trial. Treatment with 10 mg/day ezetimibe for 6 months did not alter the primary study outcome, serum aminotransferase levels. Ezetimibe significantly decreased serum cholesterol levels and cholesterol absorption markers as expected, whereas, in contrast to previous reports, ezetimibe treatment did not decrease serum levels of triacylglycerol. Our initial hypothesis was that ezetimibe treatment ameliorates liver pathology by inhibiting the absorption of toxic lipids such as oxidised cholesterol and palmitate. In our animal model, cholesterol feeding to mice increased not

Table 4 Signalling pathway gene expression changes in the control group

Pathway	Gene symbol	Gene name	Affy ID	Up or down	Function
Muscle contraction	*MYH11*	Myosin, heavy chain 11, smooth muscle	201497_x_at	Up	Smooth muscle myosin
	CALM1	Calmodulin 1	241619_at	Up	Ion channels and other proteins by Ca^{2+}
	KCNJ15	Potassium inwardly-rectifying channel, subfamily J, member 15	211806_s_at	Up	Integral membrane protein, inward-rectifier type potassium channel
	SRI	Sorcin	208920_at	Up	Modulates excitation–contraction coupling in the heart
	ACTA2	Actin, α2, smooth muscle, aorta	215787_at	Up	Cell motility, structure and integrity
	TTN	Titin	1557994_at	Up	Abundant protein of striated muscle
	EDNRA	Endothelin receptor type A	204463_s_at	Up	Receptor for endothelin-1
	TPM2	Tropomyosin 2	204083_s_at	Up	Actin filament binding protein
	CRYAB	Crystallin, αB	209283_at	Up	Transparency and refractive index of the lens
Development_skeletal muscle development	*GTF2IRD1*	GTF2I repeat domain containing 1	218412_s_at	Up	Transcription regulator involved in cell-cycle progression, skeletal muscle differentiation
	ADAM12	ADAM metallopeptidase domain 12	213790_at	Up	Skeletal muscle regeneration
	MAP1B	Microtubule-associated protein 1B	226084_at	Up	Facilitates tyrosination of α-tubulin in neuronal microtubules
	MYOM1	Myomesin 1	205610_at	Up	Major component of the vertebrate myofibrillar M band
Cell cycle_G1-S growth factor regulation	*DACH1*	Dachshund homologue 1	205472_s_at	Up	Transcription factor that is involved in regulation of organogenesis
	FOXN3	Forkhead box N3	229652_s_at	Up	Transcriptional repressor, DNA damage-inducible cell cycle arrests
	TGFB2	Transforming growth factor, β2	228121_at	Up	Suppressive effects on interleukin-2 dependent T cell growth
	PIK3CD	Phosphatidylinositol-4,5-bisphosphate 3-kinase, catalytic subunit delta	203879_at	Up	Generate PIP3, recruiting PH domain-containing proteins to the membrane
	EGFR	Epidermal growth factor receptor	1565484_x_at	Up	Antagonist of EGF action
	CCNA2	Cyclin A2	203418_at	Up	Control of the cell cycle at the G1/S and the G2/M transitions
	AKT3	v-Akt murine thymoma viral oncogene homologue 3	219393_s_at	Up	Metabolism, proliferation, cell survival, growth and angiogenesis
	PRKD1	Protein kinase D1	205880_at	Up	Converts transient DAG signals into prolonged physiological effects
Regulation of metabolism_	*INSR*	Insulin receptor	226450_at	Down	Pleiotropic actions of insulin
Bile acid regulation of lipid metabolism and	*SLC27A5*	Solute carrier family 27, member 5	219733_s_at	Down	Bile acid metabolism
Negative FXR-dependent regulation of bile acids concentration	*MBTPS2*	Membrane-bound transcription factor peptidase	1554604_at	Down	Intramembrane proteolysis of SREBPs
	PIK3R3	Phosphoinositide-3-kinase, regulatory subunit 3	202743_at	Down	During insulin stimulation, it also binds to IRS-1
	MTTP	Microsomal triacylglycerol transfer protein	205675_at	Down	Catalyses the transport of triglyceride, cholesteryl ester, and phospholipid
	PPARA	Peroxisome proliferator-activated receptor α	226978_at	Down	Ligand-activated transcription factor
	CYP7A1	Cytochrome P450, family 7, subfamily A	207406_at	Down	Catalyses cholesterol catabolism and bile acid biosynthesis
	FOXA3	Forkhead box A3	228463_at	Down	Transcription factor

Table 4 (continued)

Pathway	Gene symbol	Gene name	Affy ID	Up or down	Function
Immune response_phagosome in antigen presentation	*HLA-B*	Major histocompatibility complex, class I, B	211911_x_at	Down	Foreign antigens to the immune system
	CD14	CD14 molecule	201743_at	Down	Mediates the innate immune response to bacterial lipopolysaccharide
	LBP	Lipopolysaccharide binding protein	211652_s_at	Down	Binds to the lipid A moiety of bacterial lipopolysaccharides
	CTSS	Cathepsin S	202901_x_at	Down	Thiol protease
	DERL1	Derlin 1	222543_at	Down	Functional component of endoplasmic reticulum-associated degradation
	CFL2	Cofilin 2	224352_s_at	Down	Reversibly controls actin polymerisation and depolymerisation
	PAK1	p21 protein (Cdc42/Rac)-activated kinase 1	230100_x_at	Down	Activated kinase acts on a variety of targets
Vitamin, mediator and cofactor	*SLC1A2*	Solute carrier family 1, member 2	1558009_at	Down	Transports L-glutamate and also L- and D-aspartate
Metabolism_CoA biosynthesis and transport	*PANK3*	Pantothenate kinase 3	218433_at	Down	Physiological regulation of the intracellular CoA concentration
	PANK1	Pantothenate kinase 1	226649_at	Down	Physiological regulation of the intracellular CoA concentration
	VNN1	Vanin 1	205844_at	Down	Membrane-associated proteins
	ACSL5	Acyl-CoA synthetase long-chain family member 5	222592_s_at	Down	Synthesis of cellular lipids and degradation via β-oxidation
	ACOT1	Acyl-CoA thioesterase 1	202982_s_at	Down	Catalyses the hydrolysis of acyl-CoAs to the NEFA and coenzyme A
	ACOT2	Acyl-CoA thioesterase 2	202982_s_at	Down	Catalyses the hydrolysis of acyl-CoAs to the NEFA and coenzyme A
	ENPP1	Ectonucleotide pyrophosphatase/ phosphodiesterase 1	229088_at	Down	Involved primarily in ATP hydrolysis at the plasma membrane
Phatidic acid pathway	*GPR63*	G protein-coupled receptor 63	220993_s_at	Up	Orphan receptor. May play a role in brain function
2-Oleoyl-glycerol_3-phosphate pathway	*LPAR1*	Lysophosphatidic acid receptor 1	204037_at	Up	Receptor for LPA, a mediator of diverse cellular activities

only cholesterol but also triacylglycerols in the liver, and upregulated the gene for sterol regulatory element binding protein (SREBP)-1c that governs fatty acid synthesis [3], probably via activation of liver-X-receptor (LXR) in the liver [25]. Therefore, in experimental models of high-cholesterol-diet-induced steatohepatitis, ezetimibe ameliorated liver steatosis by reducing cholesterol-induced activation of LXR and SREBP-1c [26, 27]. In the present study, however, treatment with ezetimibe unexpectedly ameliorated liver fibrosis staging and ballooning scores without significantly changing hepatic steatosis and insulin resistance.

One possible explanation for the improvement of hepatic fibrosis by ezetimibe treatment may be related to the direct effect of cholesterol on hepatic fibrogenesis. The cholesterol molecule affects membrane organisation and structure, which are critical determinants of membrane bilayer permeability and fluidity [28]. Altered cholesterol metabolism has several toxic effects on hepatocytes, resident macrophages, Kupffer cells and hepatic stellate cells, which promote NASH through diverse mechanisms. Hepatic stellate cells, in particular, are responsible for liver fibrosis in NASH. It has recently been reported that intracellular cholesterol accumulation directly activates hepatic stellated cells through a toll-like receptor-4-dependent pathway and triggers hepatic fibrosis [29]. These effects might be more evident in humans because, unlike rodents, where NPC1L1 is primarily expressed in the intestine, in humans *NPC1L1* mRNA is highly expressed both in the small intestine and liver. Therefore, ezetimibe is estimated to inhibit not only dietary and biliary cholesterol absorption through the small intestine, but also reabsorption of biliary cholesterol in the liver [30, 31]. Thus, ezetimibe may inhibit liver fibrosis by ameliorating

Table 5 Changes in plasma fatty acid composition

Fatty acid	Control		p^a	Ezetimibe		p^a	p^b
	Before	After		Before	After		
C12:0 (lauric acid)	1.9±0.5	1.2±0.2	0.177	2.3±0.6	2.1±0.5	0.753	0.301
C14:0 (myristic acid)	24.9±2.5	23.6±2.9	0.575	27.1±2.8	29.5±3.7	0.441	0.352
C16:0 (palmitic acid)	698.0±24.7	690.0±38.2	0.827	714.3±32.5	717.0±36.2	0.991	0.893
C16:1n-7 (palmitoleic acid)	68.6±6.5	72.5±9.6	0.643	62.4±5.0	69.9±6.2	0.219	0.721
C17:0 (margaric acid)	NE	NE		NE	NE		
C18:0 (stearic acid)	203.3±9.4	196.7±6.9	0.488	207.2±7.7	211.0±9.9	0.854	0.571
C18:1n-9 (oleic acid)	560.2±31.3	556.4±30.3	0.914	547.3±23.9	578.8±32.1	0.475	0.550
C18:2n-6 (linoleic acid)	745.8±26.3	750.6±34.4	0.910	735.8±34.2	713.5±31.4	0.558	0.629
C18:3n-6 (γ-linolenic acid)	9.8±1.3	9.2±1.0	0.506	9.8±0.9	11.1±1.5	0.402	0.300
C18:3n-3 (α-linolenic acid)	21.7±1.6	20.1±1.4	0.285	23.0±2.2	21.6±1.5	0.507	0.924
C20:0n-6 (arachidic acid)	7.0±0.4	6.9±0.3	0.671	7.2±0.2	7.0±0.3	0.410	0.642
C20:1n-9 (eicosenoic acid)	4.8±0.3	4.8±0.4	0.323	4.3±0.2	4.2±0.3	0.831	0.343
C20:2n-6 (eicosadienoic acid)	6.1±0.4	6.1±0.3	0.899	5.6±0.2	5.7±0.3	0.774	0.770
C20:3n-6 (dihomo-γ-linolenic acid)	36.6±3.0	37.3±2.8	0.784	36.5±2.4	40.6±3.7	0.247	0.438
C20:3n-9 (eicosatrienoic acid)	2.5±0.4	2.4±0.4	0.941	1.9±0.2	2.7±0.5	0.034	0.079
C20:4n-6 (arachidonic acid)	135.7±8.4	138.8±6.0	0.689	143.8±11.1	151.1±11.0	0.538	0.787
C20:5n-3 (eicosapentaenoic acid)	67.0±9.0	71.3±9.3	0.640	64.4±7.2	59.1±5.7	0.385	0.369
C22:0 (behenic acid)	16.6±0.8	18.3±1.0	0.035	17.1±0.8	17.9±1.3	0.623	0.468
C22:1n-9 (erucic acid)	1.6±0.1	1.3±0.1	0.066	1.3±0.1	1.3±0.1	0.914	0.170
C22:2n-6 (docosadienoic acid)	NE	NE		NE	NE		
C22:4n-6 (docosatetraenoic acid)	3.9±0.2	4.2±0.2	0.252	4.4±0.3	4.9±0.6	0.262	0.689
C22:5n-3 (docosapentaenoic acid)	20.0±1.4	20.7±1.7	0.657	20.7±1.7	21.5±1.7	0.839	0.887
C22:6n-3 (docosahexaenoic acid)	128.7±9.8	138.6±9.3	0.231	126.5±10.0	128.3±10.8	0.936	0.456
C24:1 (nervonic acid)	35.4±2.2	36.1±2.1	0.656	31.6±1.8	30.3±1.9	0.275	0.263

Data are expressed as means ± SE

[a] p value for the intergroup comparison (baseline vs 6 month)

[b] p value for the intergroup comparison (changes from baseline between groups)

NE, not estimated

cholesterol-induced activation of hepatic stellate cells in patients with NAFLD. This hypothesis was well supported by the hepatic gene expression profile induced by ezetimibe administration. Ezetimibe treatment coordinately downregulated genes involved in skeletal muscle development and cell adhesion molecules, suggesting that ezetimibe suppressed stellate cell development into myofibroblasts and thereby inhibited fibrogenesis.

Another important finding of the present study was that treatment with ezetimibe significantly deteriorated glycaemic control. Ezetimibe therapy also altered the hepatic profile of fatty acid components by significantly increasing hepatic levels of lauric, myristic, palmitic, palmitoleic, margaric, stearic, oleic and linoleic acids. Experimentally, palmitate induces interleukin-8 [32], endoplasmic reticulum stress, and c-Jun amino-terminal kinase activation and promotes apoptosis in the liver [5, 33, 34]. Lipid-induced oxidative stress and inflammation are closely related to insulin resistance [3, 5], which could be relevant to the ezetimibe-induced deterioration of glucose homeostasis. Indeed, urinary excretion of 8-isoprostanes was significantly increased in the ezetimibe group compared with the control, and showed significant negative correlation with insulin sensitivity indices such as the Matsuda index and QUICKI in the present study (ESM Table 6). Moreover, hepatic gene expression in the ezetimibe group showed coordinated upregulation of genes involved in the immune response compared with those in the control group, suggestive of oxidative stress caused by ezetimibe treatment.

Pathway analysis of the metabolic network showed unique metabolic changes in the ezetimibe group compared with the control group. In the control group, genes involved in the CoA-biosynthesis pathway were coordinately downregulated, and those in the glycerol-3 phosphate pathway coordinately upregulated, suggesting activated triacylglycerols biosynthesis. In the ezetimibe group,