Hepatology Research

Hepatology Research 2014; 44: 1234–1240 doi: 10.1111/hepr.12240

Short Communication

Potential of a no-touch pincer ablation procedure for small hepatocellular carcinoma that uses a multipolar radiofrequency ablation system: An experimental animal study

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Aim: Treatment of hepatocellular carcinoma located on the liver surface is frequently difficult because direct puncture of the tumor must be avoided during needle insertion. The aim of this study was to investigate the utility of a no-touch pincer ablation procedure that uses a multipolar radiofrequency ablation (RFA) system for a tumor located on the liver surface.

Methods: The experimental animals were three pigs, and RFA was performed with two internally cooled bipolar electrodes. Three ablative procedures were compared: linear insertion at regular 13-mm intervals (pattern 1; virtual target tumor size, <10 mm); fan-shape insertion, maximum interval 20 mm (pattern 2; virtual target tumor size, <15 mm); and 25 mm (pattern 3; virtual target tumor size, <20 mm). All electrodes were inserted at a 30-mm depth. For patterns 1 and 2, ablation was performed on three other parts of the liver, and for pattern 3, ablation was performed on two other parts.

Results: For the median transverse and longitudinal diameter to the shaft, with the pattern 1 procedure, the ablative areas were 32 mm × 30 mm, and with the pattern 2 procedure, the ablative areas were 27 mm × 30 mm with carbonization of the liver surface. In contrast, with the pattern 3 procedure, the ablative areas were 45 mm × 26 mm; however, the ablative margin did not reach the surface, and carbonization was not apparent.

Conclusion: The no-touch pincer ablation procedure (with an electrode interval of ≤20 mm) may be useful when performed with two internally cooled bipolar electrodes for small nodules that protrude from the liver surface.

Key words: bipolar, hepatocellular carcinoma, multipolar, no-touch ablation, radiofrequency ablation

INTRODUCTION

AMONG THE AVAILABLE treatment options for hepatocellular carcinoma (HCC), surgical resection is generally considered to be a local eradication method that can provide a satisfactory long-term outcome.[1-8] Recent advances in imaging procedures have led to increased detection of early-stage HCC and to improved survival due to the increased identification of patients in whom hepatic resection is possible.[9,10]

For patients who are not eligible for surgery for various reasons (e.g. lack of sufficient liver function for surgical resection), percutaneous local therapy is a viable therapeutic option. Several local ablation therapies are available, including percutaneous ethanol injection, percutaneous acetic acid injection, cryotherapy, percutaneous microwave coagulation therapy and radiofrequency ablation (RFA). In addition to surgical resection, local ablation therapies, particularly RFA, are considered to be local eradication methods for HCC that can provide good long-term outcomes.[11] Therefore, in recent years, RFA has become a widely used option for the primary treatment of small-size HCC. However, we often

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Funding: Okinaka Memorial Institute for Medical Research and Japanese Ministry of Health, Labor and Welfare.
Conflict of interest: The authors state that they have no conflicts of interest regarding the content of the article.
Author contribution: All authors had access to the data and played a role in writing this manuscript.
Received 29 May 2013; revision 26 August 2013; accepted 10 September 2013.

encounter cases of HCC that are difficult to treat with RFA as a result of tumor location, especially nodules that protrude from the liver surface. In addition, a relationship between percutaneous local approaches to HCC (including tumor biopsy) and tumor seeding has been reported previously,[12,13] and with regard to the risk of treatment-related tumor seeding, the following risk factors have been reported: tumor size, tumor location (subcapsular portion), α-fetoprotein level, tumor stage and histopathological grade.[14,15] Therefore, a no-touch approach to local therapy may be considered an ideal treatment method for HCC.

Recently, a multipolar ablation system became available. Until now, in Japan, monopolar electrodes have typically been used, and the present cases are usually treated with some technical arrangement. For example, in the case of using a multi-tined expandable electrode, after obliquely inserting the electrode to avoid direct puncture of the target tumor, the multi needles are expanded toward the target tumor via non-tumor tissue, or in the case of using an internally cooled electrode, multiple insertions are made to avoid direct puncture of the target tumor, and RFA is performed after each insertion. However, these methods do not always provide enough of a treatment effect due to the influence of uncertain treatment procedures and natural, direct puncture to a tumor is indispensable. In contrast, a multipolar ablation system that uses an internally cooled bipolar electrode can combine the use of one to three electrodes at the same treatment session. When three electrodes are used, this system can treat large tumors; however, in the case of small tumors, it is not really necessary to use three electrodes to treat the target tumor. In addition, when we used this multipolar ablation system, usually electrodes were inserted into HCC, but in theory, this system can use no-touch ablation. However, to our knowledge, there are no technical reports that describe a non-direct punctual RFA method that uses a bipolar ablation system for HCC located on the liver surface. In this experimental animal study, we assumed that a small (<20 mm) HCC nodule protruded from the liver surface, and examined proper pincer ablation methods using two internally cooled bipolar electrodes.

METHODS

Summary of experimental procedures

WE USED A bipolar RFA device (CelonPOWER System; OLYMPUS Winter & Ibe GmbH [Telto, Germany]) and two internally cooled bipolar electrodes (30-mm, 15-G, CelonProSurge; OLYMPUS Winter & Ibe GmbH). RFA was applied in the livers of three normal female domestic pigs (each pig's weight was 60 kg) under general anesthesia maintained until killing. The abdomen was opened so that the needle could be inserted under an ultrasonography (US) guide directly into the upper region of the liver where the thickness was larger than 3.5 cm. As a pig liver consists of five thin lobes, RFA sessions were performed two to three times in each liver for evaluation of the "no-touch pincer ablation procedure". After the experiments were completed, the animal was killed, and the ablated liver lobes were excised immediately. The specimen was cut in the plane of the needle tract and photographed to evaluate the shape and size of the ablated zone (white zone). The experimental protocol was approved by the Ethics Review Committee for Animal Experimentation of Toranomon Hospital.

Protocol of the no-touch pincer ablation procedure

We used a bipolar RFA device (CelonPOWER System; OLYMPUS Winter & Ibe GmbH), and all ablation procedures were performed with two internally cooled bipolar electrodes (30-mm, 15-G, CelonProSurge; OLYMPUS Winter & Ibe GmbH). Internal liquid circulation of the applicator enables the efficiency of coagulation to be increased. The delivery rate was set to 30 mL/min of saline solution at room temperature. The liquid flow was provided by a triple peristaltic pump, which is part of the system. The electrodes were operated by a power control unit working at 470 kHz and providing a maximum output power of 250 W (OLYMPUS Winter & Ibe GmbH). In this study, output power and total energy in each session were fixed at 60 W and 25 kJ, respectively, according to the dosimetry table for the bipolar RFA system (CelonPOWER System; OLYMPUS Winter & Ibe GmbH).

With regard to the ablation protocol, we performed the following three types of ablation procedure: linear insertion, at regular 13-mm intervals (pattern 1); fan-shape insertion, maximum interval of 20 mm (pattern 2); and 25 mm (pattern 3). All electrodes were inserted at a 30-mm depth from the liver surface under a US guide (Fig. 1). Each ablation procedure was performed for the following number of times: pattern 1, three sessions; pattern 2, three sessions; and pattern 3, two sessions. In this study, we assumed that the size of the virtual target tumor was less than 10 mm in pattern 1,

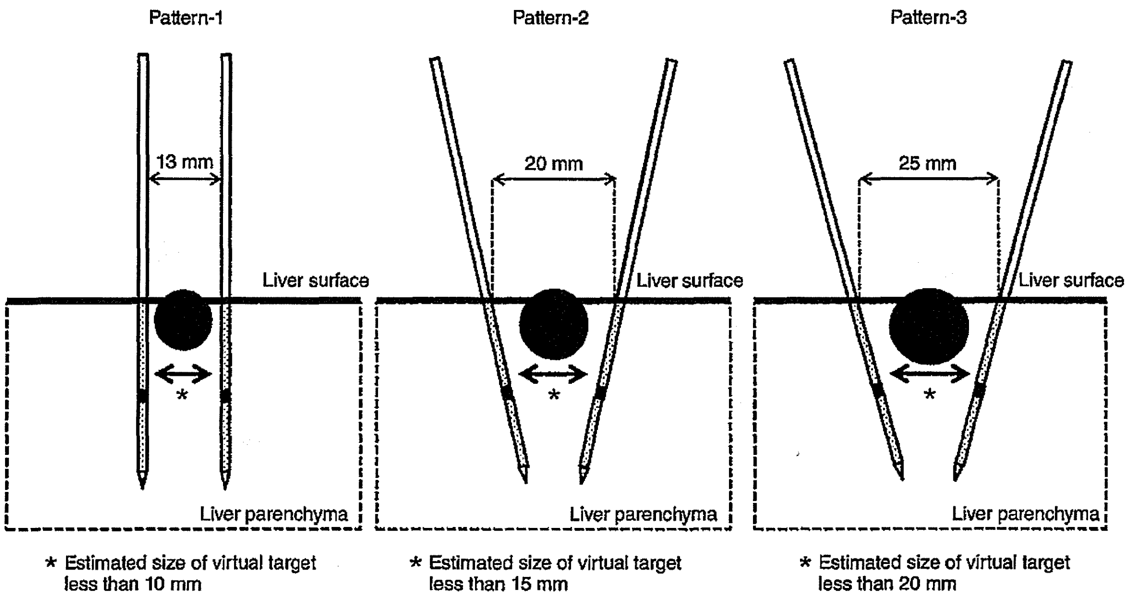


Figure 1 Protocol for a pincer ablation procedure that uses two internally cooled bipolar electrodes for a virtual nodule that protrudes from the liver surface.

less than 15 mm in pattern 2 and less than 20 mm in pattern 3.

Measurement procedure of the ablative margin

After completion of the experiments, the animal was killed and the ablated liver lobes were excised immediately. The specimen was cut in the plane of the needle tract and photographed to evaluate the shape and size of the ablated zone (white zone).

Statistical analysis

The size of the ablated zone and the duration of ablation were compared among the three groups with the Kruskal–Wallis test. All values are expressed as medians. A *P*-value of less than 0.05 denoted the presence of a statistically significant difference.

RESULTS

Features of the no-touch pincer ablation procedure

THE THREE TYPES of pincer ablation procedure applied to the pig liver were performed in the area shown in Figure 2(a).

Table 1 summarizes the features of each pincer ablation procedure for the treatment of the virtual target located on the liver surface.

In the median (range) transverse and longitudinal diameter to the shaft, ablative areas were: pattern 1, 32 (27–35) mm × 30 (30–35) mm; pattern 2, 27 (25–35) mm × 30 (30–32) mm; and pattern 3, 45 (40–50) × 26 (25–27) mm. There were no significant differences in the size of each ablative area among the three ablation procedures. However, with the pattern 3 procedure, the transverse diameter to the shaft was larger than with the other procedures, and as a result, the ablative form was flatter. On the other hand, patterns 1 and 2 acquired sufficient ablative areas that covered the liver surface with carbonization of the surface; however, with pattern 3, the ablative areas did not reach the liver surface, and carbonization of the liver surface was not apparent (Fig. 2b–d).

In addition, there were no significant differences among ablation procedures in the duration of ablative time.

DISCUSSION

WE OFTEN ENCOUNTER cases of HCC that are difficult to treat with RFA as a result of tumor location, especially nodules that protrude from the liver

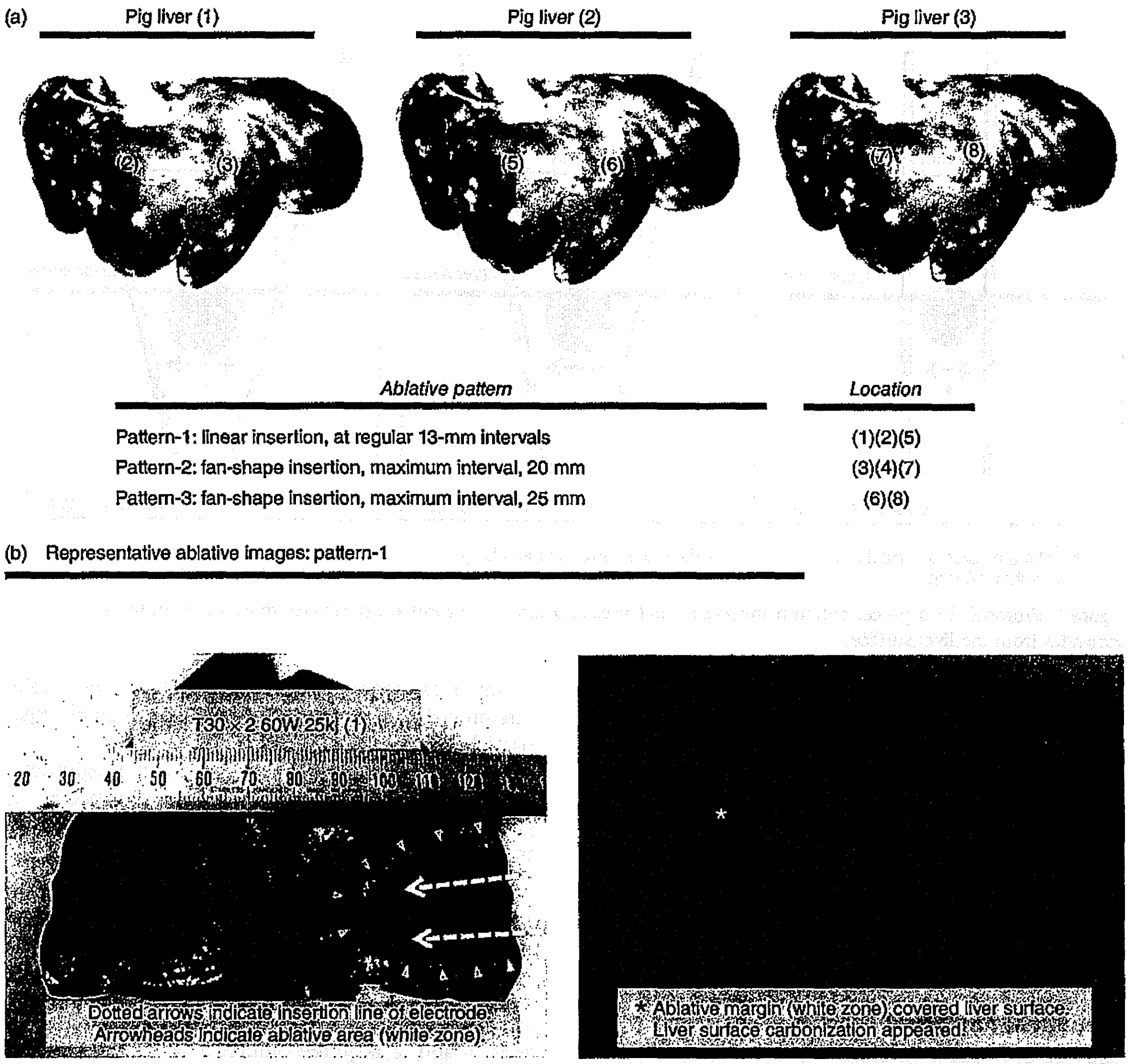


Figure 2 (a) Schema of the ablative areas of each pincer ablation procedure in the three pig livers. (b) One of the ablative shapes and the margin achieved with the pattern 1 procedure that uses two internally cooled bipolar electrodes for a virtual nodule that protrudes from the liver surface. With this pattern, we inserted the electrodes linearly (maximum interval for each electrode was 13 mm). The ablative margin covered the liver surface with carbonization of the liver surface. (c) One of the ablative shapes and the margin achieved with the pattern 2 procedure that uses two internally cooled bipolar electrodes for a virtual nodule that protrudes from the liver surface. With this pattern, we used a fan-shape insertion method (maximum interval for each electrode was 20 mm). The ablative margin covered the liver surface with carbonization of the liver surface. (d) Ablative shape and margin achieved with the Pattern 3 procedure that uses two internally cooled bipolar electrodes for a virtual nodule that protrudes from the liver surface. With this pattern, we used a fan-shape insertion method (maximum interval for each electrode was 25 mm). The ablative area close to the liver surface was larger than with the other procedures. However, the ablative margin did not cover the liver surface, and carbonization of the liver surface was not apparent.

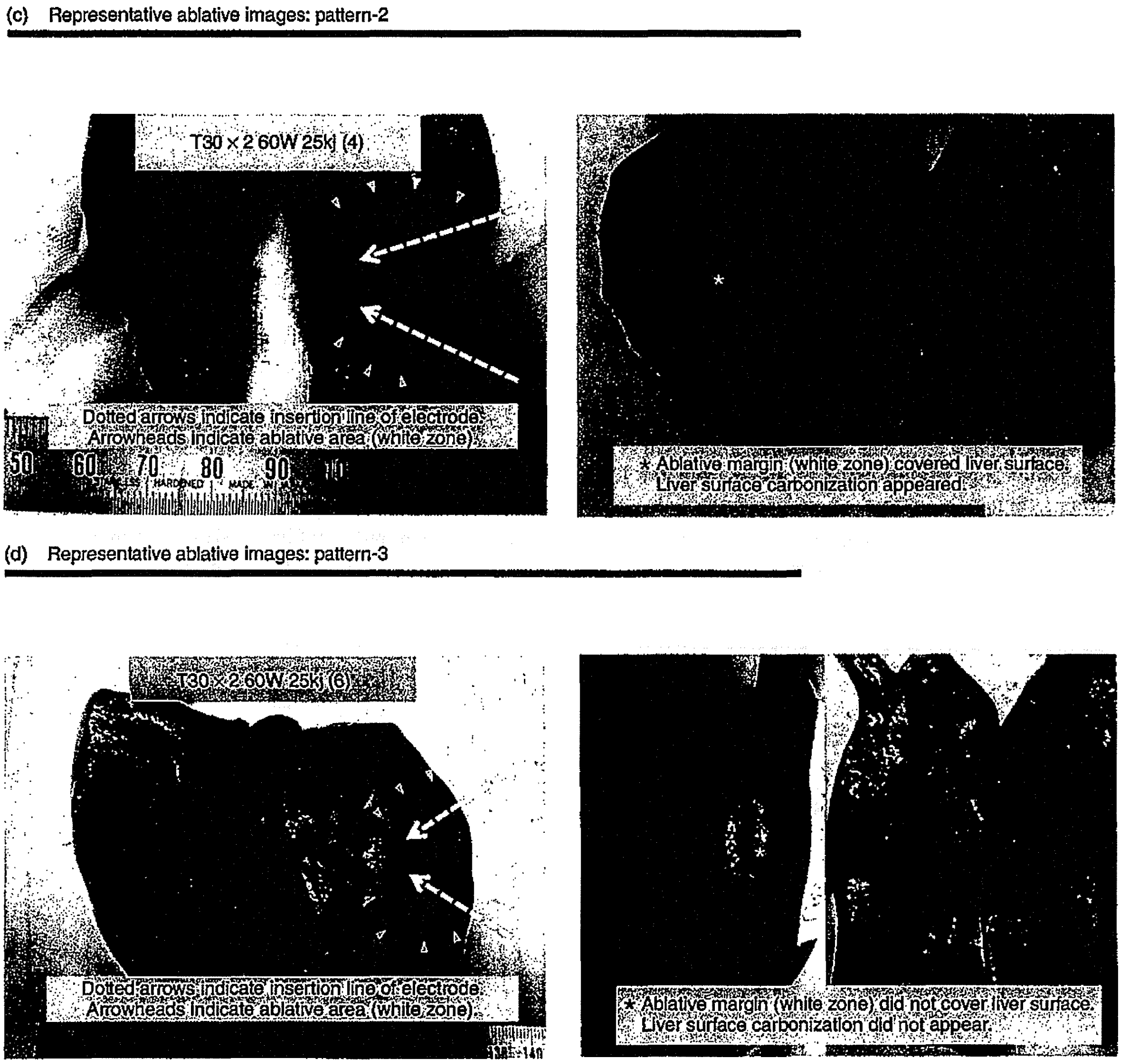


Figure 2 *Continued*

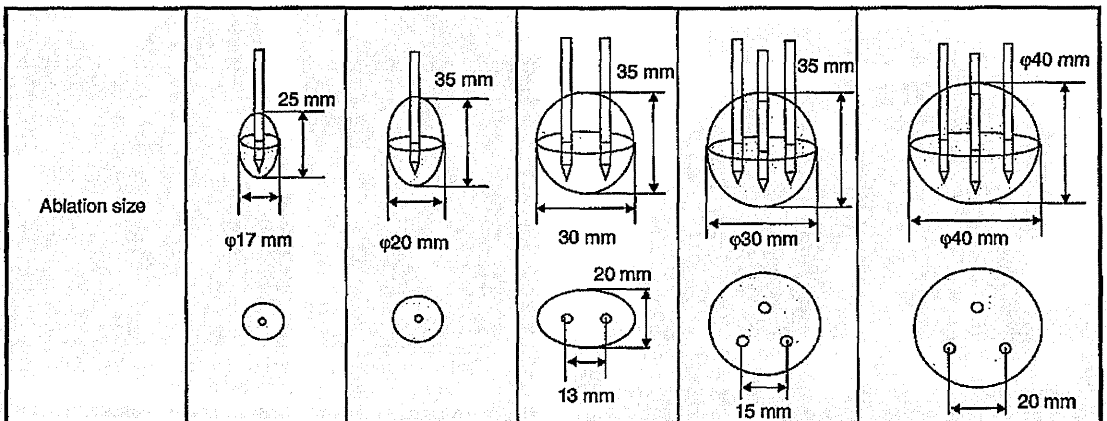
surface. In these situations, a multipolar ablation system that uses internally cooled bipolar electrodes may be suitable for treatment. With a multipolar ablation system, we can combine the use of one to three electrodes at the same treatment session, and when three electrodes are used, this system can treat a large tumor. However, in the case of small tumors (<20 mm), it is not really necessary to use three electrodes for treatment of the target tumor. However, in the dosimetry table of this bipolar system in Figure 3, which was made from previously reported early clinical data[16] and basic analysis, when two internally cooled bipolar electrodes are used (30 mm, 15-G, CelonProSurge; OLYMPUS Winter & Ibe GmbH), the recommended interval of each electrode in this system was 13 mm. With this regulation, we can treat only small tumors (<13 mm) when we perfume no-touch pincer ablation using two electrodes. Therefore, in this study we assumed a virtual target tumor with a tumor diameter less than 20 mm, and investigated the efficacy of a no-touch pincer ablation procedure and the maximum size of the tumor using two internally cooled bipolar electrodes for nodules that

Table 1 Features of each pincer ablation procedure for the treatment of the virtual target located on the liver surface

	Pattern 1			Pattern 2			Pattern 3		*P*
	1	2	3	1	2	3	1	2	
Duration	13′46″	13′16″	12′58″	14′38″	13′50″	13′30″	13′05″	12′40″	$P = 0.151$
Ablated area									
Transverse diameter, mm	27	35	32	25	27	35	45	40	$P = 0.113$
Longitudinal length, mm	35	30	30	32	30	30	27	25	$P = 0.102$
Ablated area covered liver surface	Yes	Yes	Yes	Yes	Yes	Yes	No	No	
Liver surface carbonization appeared	Yes	Yes	Yes	Yes	Yes	Yes	No	No	

protrude from the liver surface. In addition, we investigated only the fan-shape insertion method at a maximum interval of 20–25 mm. The reason for this is that in an actual RFA procedure, it is occasionally difficult to insert two electrodes in the same intercostal space for slightly large nodules that protrude from the liver surface; therefore, in this study, we examined a fan-shape ablation method that assumed two different intercostal approaches. Our results showed that with the pattern 3 treatment procedure, we could not acquire a sufficient ablative margin to the side of the liver surface. From these results, tumors of 20 mm or more may not be suitable for a no-touch pincer ablation procedure that uses two internally cooled bipolar electrodes in this bipolar system.

In contrast, with the pattern 1 and 2 treatment procedures, we acquired a sufficient ablative margin to the side of the liver surface with carbonization of the liver



Applicators	T20 × 1	T30 × 1	T30 × 2	T30 × 3	T40 × 3
Power setting	20 W	30 W	60 W	90 W	120 W
Target energy (est. time)	10 kJ (13 min)	15 kJ (13 min)	25 kJ (17 min)	35 kJ (16 min)	70 kJ (19 min)

▪ The data are based on Frericks et al., Radiology (2005) 237: 1056–1062. The reported average efficacy was ~0.5 millitre ablation volume per kilojoule.
From these data, the required energy for an ablation sphere or ellipsoid of given diameter was calculated.
▪ The application of blood flow interruption (e.g. Pringle's manoeuvre, embolization) allows for a significant reduction of the target energy.

Disclaimer: this dosimetry table does not replace the monitoring of actual ablation sizes. The ablation diameters are approximations based on statistical data; they are not guaranteed for individual clinical cases. Ablation size and shape as well as the procedure time may significantly vary due to tumor physiology and vascular structure. A deviation from the recommended applicator distances may also have an impact on the ablation dimensions.

Figure 3 Dosimetry table for the CelonPOWER system (in Japan).

surface. These results may indicate that tumors of less than 15 mm are candidates for the no-touch pincer ablation procedure that uses two internally cooled bipolar electrodes in this bipolar system.

Finally, this experimental animal study had some limitations. First, the number of animals was very small, and the target tumor was a virtual tumor. Second, an additional examination regarding a no-touch linear insertion procedure for maximum intervals of 20 mm and 25 mm for each electrode was not enforced. Third, we could not investigate the same fan-shape ablation procedure using monopolar RFA in this study, because we assumed it would be too difficult to carry out a two-step insertion method using a monopolar electrode under the influence of a first ablation for nodules that protrude from the liver surface. Fourth, we could not investigate the pathological changes in the ablative area in this study. Therefore, with only these study results, it may not be possible to draw conclusions regarding the utility of the fan-shape insertion method using a bipolar RFA device. To solve these problems, we must carry out an additional large-scale study that includes pathological examination in the near future.

Finally, to summarize the points to be noted at the time of performing the pincer ablation procedure, first, we should insert the needle carefully under US guidance, because in this procedure, measuring the distance of the needle tip from the liver surface and the two needle intervals on the liver surface correctly is the most important point.

Second, with this procedure, we should pay attention to the risk of thermal damage to the visceral peritoneum. Therefore, if possible, thermal protection using measures such as artificial ascites should be considered.

Third, in this study, we did not observe a portal or hepatic vein thrombus in the ablative area. However, this study was performed mainly in the vicinity of the liver surface, and usually this area does not include large vessels. Therefore, we need to use caution as with monopolar ablation when we ablate near large vessels.

In conclusion, the no-touch pincer ablation procedure (with an electrode interval of ≤20 mm) may be useful when performed with two internally cooled bipolar electrodes for small HCC tumors that protrude from the liver surface.

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Case Report

Transfusion-transmitted hepatitis E in a patient with myelodysplastic syndromes

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Introduction

Patients with haematological diseases occasionally exhibit liver dysfunction during treatment. This liver dysfunction can have various causes such as therapy-related drugs and hepatitis B and C infections, although the cause is unclear in some cases. It was recently reported that some patients initially diagnosed with drug-induced liver dysfunction actually had hepatitis E[1]. Several cases of transfusion-transmitted hepatitis E infections have also been reported[1,2]. In Japan, screening for hepatitis E does not appear to be performed at the initial examination of patients with acute hepatitis. This might be because hepatitis E is believed to be orally transmitted and to occur mainly in developing countries and rarely in developed countries. However, hepatitis E is a zoonotic infectious disease. Cases of regional endemic hepatitis E virus (HEV) infection have been increasing in Europe, the United States, and Japan[1].

Although HEV usually causes self-limited acute hepatitis, it sometimes progresses to a chronic infection. Most cases of chronic infection occur in patients undergoing solid organ or haematopoietic stem cell transplantation, in those receiving anti-cancer or immunosuppressant drugs, and in patients with human immunodeficiency virus infection, in whom the condition may progress to liver cirrhosis[3]. HEV RNA persisted for a long period during treatment in a patient with T-cell lymphoma[4]. Reactivation of HEV hepatitis was reported after an allogeneic haematopoietic stem cell transplant in a patient with Philadelphia chromosome-positive acute lymphoblastic leukaemia[5]. On the other hand, a low risk of HEV reactivation after haematopoietic stem cell transplantation was also reported[6]. More studies on the risk of HEV reactivation are, therefore, required.

Here, we report the case of a patient with a myelodysplastic syndrome (MDS) who developed acute hepatitis due to transfusion-transmitted HEV infection. We also review the literature on the topic.

Case report

The patient was a 70-year old Japanese man who attended our hospital for Parkinson's disease in June 2001. In July 2001, he was referred to the Haematological Department because of thrombocytopenia. Haematological examinations revealed that he had pancytopenia with a white blood cell count of 2.9×10^9/L, haemoglobin level of 9.0 g/dL, and a platelet count of 36×10^9/L. Bone marrow findings showed 8.8% myeloblasts and trilineage dysplastic features. Chromosome abnormalities with [46,XY, -10, +marker] were detected in 15 of 22 mitotic bone marrow cells. He was, therefore, diagnosed with MDS. According to the French-American-British criteria, he was classified as having refractory anaemia with excess of blasts (RAEB)-1 and was given a score of intermediate-2 according to the International Prognostic Scoring System at that time. Because he was suffering from Parkinson's disease, he received combination therapy with oral vitamin K2 (menatetrenone, 45 mg/day) and vitamin D3 (alfacalcidol, 1 μg/day)[7] instead of chemotherapy. This treatment resulted in no progression to leukemic transformation over the next 10 years. However, the pancytopenia gradually worsened, and protein anabolic steroids (metenolone, 20 mg/day) were added to the treatment in 2009. Over the next 12 months, he received repeated red cell and platelet transfusions because of anaemia and haemorrhagic symptoms. Bacterial infections often occurred during medical home care, and his Parkinson's disease worsened. On April 28th, 2011, the patient was admitted to hospital with a lung abscess and aspiration pneumonia. He had a gastrointestinal bleed after admission to hospital and the volume of blood transfusions consequently increased. Although hepatic function was within the normal range on admission, serum levels of aspartate aminotransferase (AST) and alanine aminotransferase (ALT) began to increase from May 18th, peaking at 504 and 736 IU/L, respectively, on June 8th. Although these

Blood Transfus 2014; 12: 103-6 DOI 10.2450/2013.0081-13

levels decreased transiently, they increased again from June 20th together with a rise in total bilirubin level. On June 22nd, the patient died of exacerbation of the lung abscess (Figure 1).

After the patient had died, the stocked plasma split from one of the donors of red blood cell (RBC) products given to our patient was screened for viruses before utilisation in plasma-fractionated products. The results revealed HEV RNA in the stocked plasma. We, therefore, performed complete examinations of the stocked donated blood and identified the HEV RNA-positive donor. The RBC product derived from this donor had been transfused into our patient on May 2nd. Serological examinations using the stored sera from this donor revealed an ALT level of 26 IU/L; the sera were negative for immunoglobulin (Ig)G anti-HEV and IgM anti-HEV assayed by enzyme immunoassay (IgG/IgM anti-HEV, Institute of Immunology, Tokyo, Japan) as well as hepatitis B virus (HBV) DNA and hepatitis C virus (HCV) RNA. The HEV RNA copy number quantitatively assayed with a TaqMan reverse transcription polymerase chain reaction (QIAGEN) was 1.2×10^3. These findings suggest that the blood had been donated during the "window period" of HEV infection in this donor.

We retrospectively investigated HEV RNA in our patient's stocked sera. As shown in Figure 1, HEV RNA began to rise on May 23rd, peaked on June 16th, and started to decrease on June 18th. The patient's serum from June 10th was found to be positive for IgG anti-HEV and IgM anti-HEV, whereas the sera stocked before May 30th were all negative for IgG anti-HEV and IgM anti-HEV. In addition, the results of viral examinations conducted when the patient presented with liver dysfunction were negative for HBV DNA, HCV RNA, cytomegalovirus and Epstein–Barr virus. These results strongly suggest that the patient had acquired transfusion-transmitted hepatitis E.

We then compared the sequence of the viral RNA detected from the stocked sera of the donor and patient using reverse transcription-polymerase chain reaction followed by direct sequencing. We also compared the sequence of the amplification products of open reading frame 1 (ORF1) (326 bp, nt 123-448) and ORF2 (412 bp, nt 5,987-6,398) of HEV between the patient's and donor's samples. Many different HEV sequences were determined among the sequences from the donor's sample. For precise analysis, the donor's HEV was cloned and sequenced. A total of 28 polymerase chain reaction products were obtained and seven HEV strains were isolated from the donor's stocked sera, all of which were genotype 3. The phylogenetic tree according to the ORF2 sequence is shown in Figure 2. Interestingly, the tree suggests that the donor carried two distinct phylogenies of HEV. Of these, one strain (isolate-1, in Figure 2) showed 100% sequence homology with the strain isolated from the patient's stocked serum with respect to both ORF1 and ORF2.

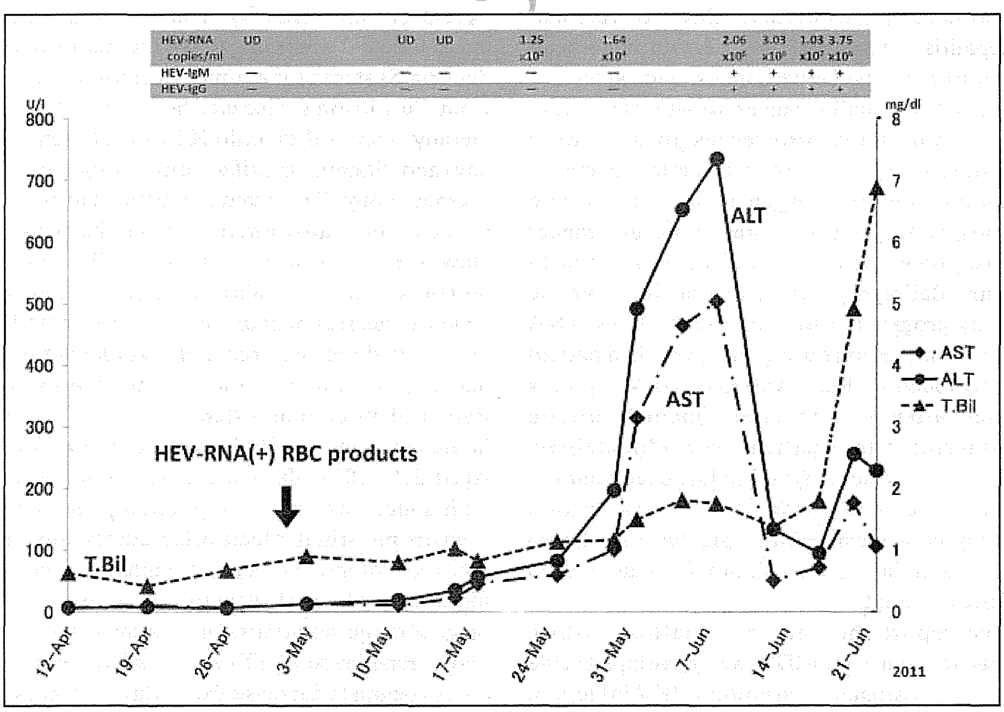

Figure 1 - Clinical course of the patient. (UD: undetectable)

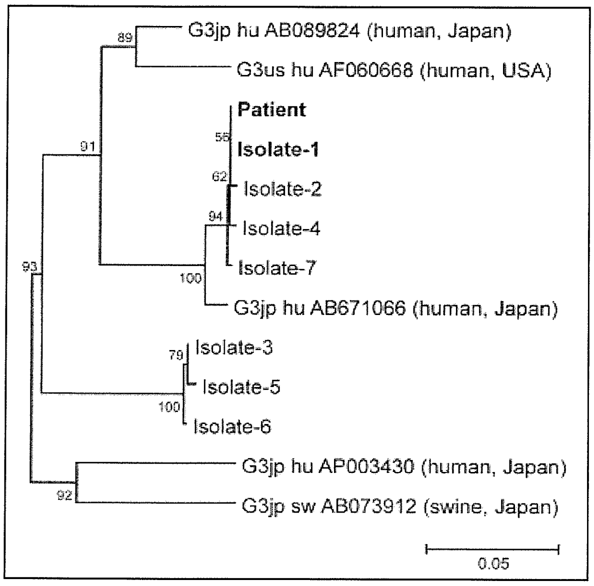

Figure 2 - Phylogenic analysis of ORF2 region Phylogenetic tree of HEV constructed by neighbor-joining method. A 100% homology is observed between the patient and isolate-1. Isolate-1 to -7: HEV clones from the blood donor.

Discussion

HEV is an RNA virus comprising approximately 7.2 kb and has four different genotypes[1]. HEV genotypes vary regionally. The epidemic form is related to genotypes 1 and 2, which cause severe acute disease and are spread via drinking water in developing countries. The regional endemic or autochthonous form is related to genotypes 3 and 4, which are zoonotic and cause mild asymptomatic disease that is spread via food in developed countries[2]. Cases of transfusion-transmitted HEV infection were recently reported in the United Kingdom, France, and Japan[8-10]. Among these, five patients including the present one had haematological diseases[4,8,11,12]. These cases comprised only male patients and the causative blood transfusion products were RBC and platelet products. The HEV genotypes were type 3 in four cases and type 4 in one case. In developed countries, most cases are reported to be of genotype 3, like the present case[2]. However, these cases are very likely to be regional endemic diseases rather than infections imported from developing countries.

Recent studies demonstrate that some cases of HEV infection were initially misdiagnosed as drug-induced liver dysfunction. A British study[13] found acute HEV infection in 13% (6/47 cases) of cases initially diagnosed as drug-induced liver dysfunction. Similarly, an American study reported HEV infection in 3% (9/318 cases) of cases initially diagnosed as drug-induced liver injury[14]. HEV RNA analysis was performed in four of these nine cases and revealed that all of the cases were caused by HEV genotype 3[14]. These data suggest that HEV infection should be included in the differential diagnosis of patients with liver dysfunction.

The prevalence rates of HEV vary among countries and even among regions within a country[15-19]. In Japan, out of 12,600 samples, 431(3.4%) were positive for IgG anti-HEV. The results of that study showed that the prevalence of IgG anti-HEV was significantly higher in eastern Japan (5.6%) than in western Japan (1.8%) ($P<0.001$), indicating marked regional variation[19]. Indeed, the infected donor involved in this case report was from Tokyo, which is in eastern Japan. Furthermore, the Hokkaido area is reported to have higher incidence of IgG anti-HEV than other eastern areas including Tokyo. Besides the high prevalence of HEV RNA[18], the incidence of HEV transmission is also high with a predominance of genotype 4, which is the viral genotype that causes severe symptoms[11,20]. Thus, HEV RNA screening of donated blood was experimentally initiated in Hokkaido in 2005.

Patients who have received many blood transfusions are reported to have a significantly higher incidence of markers of HEV infection (i.e., IgG/IgM anti-HEV and HEV RNA) than those who have received fewer blood transfusions[21]. Since HEV screening is not performed to prevent haematologically transmitted infections in developed countries, the frequency of transfusion-transmitted HEV infection might be underestimated. The present and previously reported cases indicate that any blood product, including RBC products[4,8], platelet products[11,12] and fresh-frozen plasma[10] can transmit HEV. However, the viral load required to induce transfusion-transmitted hepatitis E in recipients is unclear. Further investigation is required to clarify this point. Therefore, HEV screening for blood transfusion donors should be considered in areas in which the seroprevalence of HEV is high. However, the present case revealed that HEV can be transmitted via blood products from donors during the "window period". Moreover in two previous cases[11,12], blood products that transmitted HEV were positive for HEV RNA but negative for anti-HEV antibody. Thus, HEV RNA should be investigated at the onset of liver dysfunction in patients receiving frequent blood transfusions. Furthermore, HEV RNA screening among blood donors might be effective for the prevention of transfusion-transmitted HEV.

Keywords: hepatitis E virus, transfusion-transmitted infection, myelodysplastic syndromes.

Acknowledgements

We thank Dr. Keisuke Miyazawa (Department of Biochemistry, Tokyo Medical University) for his critical reading of our manuscript. We also thank Ms. Ayako Hirota for her assistance with preparing the manuscript.

The Authors declare no conflicts of interest.

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Arrived: 1 March 2013 - Revision accepted: 17 April 2013
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Acta Med. Okayama, 2014
Vol. 68, No. 4, pp. 243-247

Acta Medica Okayama
http://escholarship.lib.okayama-u.ac.jp/amo/

Original Article

Prevalence and Outcomes of Acute Hepatitis B in Okayama, Japan, 2006-2010

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Hepatitis B virus (HBV) is one of the major viruses causing acute hepatitis. Recently, the incidence of acute hepatitis with genotype A has been increasing in Japan. The aim of this study was to investigate acute hepatitis B (AHB) in Okayama prefecture, with special attention to HBV genotype A. AHB patients who visited one of 12 general hospitals in Okayama prefecture between 2006 and 2010 were retrospectively analyzed. Over the course of the study period, 128 patients were diagnosed with AHB. Sexual transmission was supposed in the majority of patients (78 patients, 61%), including 59 (76%) having sex with heterosexual partners. The genotypes of HBV were assessed in 90 patients (70%), of whom 27 patients were infected with genotype A, 5 with genotype B, and 58 with genotype C. The prevalence of genotype A was significantly higher among male patients (28.7%), aged 20-29 (35.6%, $p < 0.01$), among men who had sex with men (100%, $p < 0.005$), and among patients having sex with unspecified partners (44.8%, $p < 0.005$). Genotype A was not a significant factor associated with delayed HBsAg disappearance. Caution should be exercised with regard to sexually transmissible diseases in order to slow the pandemic spread of AHB due to genotype A.

Key words: acute hepatitis, hepatitis B virus

Received October 30, 2013; accepted March 26, 2014.
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Hepatitis B virus (HBV) is one of the major viruses causing acute hepatitis. In Japan, the causative viruses during the 1990s were the HBV genotypes B and C [1]. Recently, however, acute hepatitis due to HBV genotype A has been increasing in large metropolitan areas such as Tokyo or Osaka in Japan, where it is assumed to have been imported from outside Japan [2]. When adults are infected with HBV, acute hepatitis due to HBV genotypes B and C can generally be cured, while 10% of acute hepatitis cases due to HBV genotype A become chronic [3]. The incidence of chronicity is especially high in patients co-infected with HBV and human immunodeficiency virus (HIV).

Among hepatitis cases in Japan, that portion with acute viral hepatitis due to HBV genotype A was 6.0% between 1991 and 1996 but has expanded since 2000, to reach 52% in 2008 [4]. The Ministry of Health, Labor and Welfare has started an epidemiological investigation of the prevalence of acute or chronic hepatitis due to HBV genotype A, undertaken by a study group on the natural history of HBV genotype A. The aim of the present study was to investigate acute hepatitis due to HBV in Okayama prefecture, with special attention to HBV genotype A.

Materials and Methods

Patients. There are 12 general hospitals in Okayama prefecture that possess 300 or more beds for in-patient care. All patients who visited one of these hospitals and had a diagnosis of acute hepatitis B (AHB) between 2006 and 2010 were retrospectively analyzed. AHB was diagnosed by the results of elevated titer of anti-IgM HBc antibody and the exclusion of patients who may have had chronic HBV infection. Patients with drug-induced liver injury, alcoholic liver injury and autoimmune hepatitis were excluded from the study. Information regarding the cause and place of infection, HBV genotype, the positivity of the anti-HIV antibody, and the use of nucleotide analogues in the treatment of acute hepatitis, was collected from all patients. The study was performed in accordance with the Helsinki Declaration and was approved by the ethical committees of all participating institutions.

Statistical analysis. Data are expressed as means ± standard deviations. Patient characteristics were compared among groups using the Mann-Whitney U test or Kruskal-Wallis test. Factors associated with the presence of Hepatitis B surface antigen (HBsAg) 12 months after the diagnosis (*i.e.*, persistence) were analyzed by stepwise logistic regression analysis. Negativity of HBsAg was presumed by using the Kaplan-Meier method, and the results among groups were compared with the log rank test. P values < 0.05 were considered to indicate significance.

Results

Patient characteristics of the patients with acute hepatitis B. A total of 128 patients were diagnosed with AHB at the 12 participating hospitals between 2006 and 2010; the incidence of AHB was between 20 and 30 cases annually (Fig. 1). Eighty-seven patients (68%) were male, and the peak age was between 20 and 29 (45 patients, 35%). Ten patients were 60 or older. Sexual transmission had caused the viral infection in the majority of patients (78 patients, 61%), including 59 patients (76%) having sex with heterosexual partners and 4 male patients getting infected from homosexual partners. Forty-two patients got infected from a specified partner (54%), and 9 patients from non-Japanese partners (12%). Tattooing and needle stick accidents were suspected as the causes of viral infection for one and 4 patients, respectively. The places of viral infection were somewhere in Okayama prefecture for 31 patients (40%), other places than Okayama in Japan for 10 patients (13%), and Southeast Asian countries during travels for 8 patients (10%). Anti-hepatitis C virus antibody was tested for in 124 patients (97%), and 3 patients had positive results, while anti-HIV antibody was

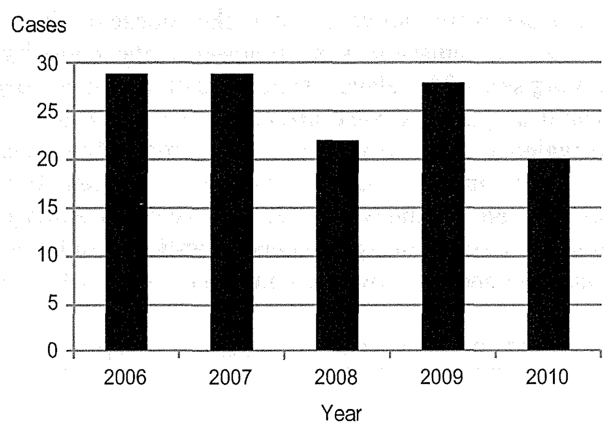

Fig. 1 Annual incidence of AHB between 2006 and 2010. The incidence of AHB was between 20 and 30 annually between 2006 and 2010.

examined in only 68 patients (53%), with positive results for 2 patients. One male patient in his 20s may have been infected by sex with an unspecified female partner. His HBV genotype was not assessed. The other was a male in his 20s who had sex with unspecified men in Fukuoka prefecture, and got infected with HBV genotype A.

Prevalence of HBV genotype A in patients with AHB. As shown in Fig. 2, the genotypes of HBV were assessed in 90 patients (70%); 27 patients were infected with HBV genotype A (21%), 5 with HBV genotype B (4%), and 58 with HBV genotype C (45%). The percentages of HBV genotype A did not change much over the 5-year study period. The prevalence of HBV genotype A was significantly higher among male patients (25 patients, 28.7%) than females (2 patients, 4.9%, $p < 0.005$), among those aged 20-29 (16 patients, 35.6%, $p < 0.01$), among men who had sex with men (4 patients, 100%, $p < 0.005$), and among patients having sex with unspecified

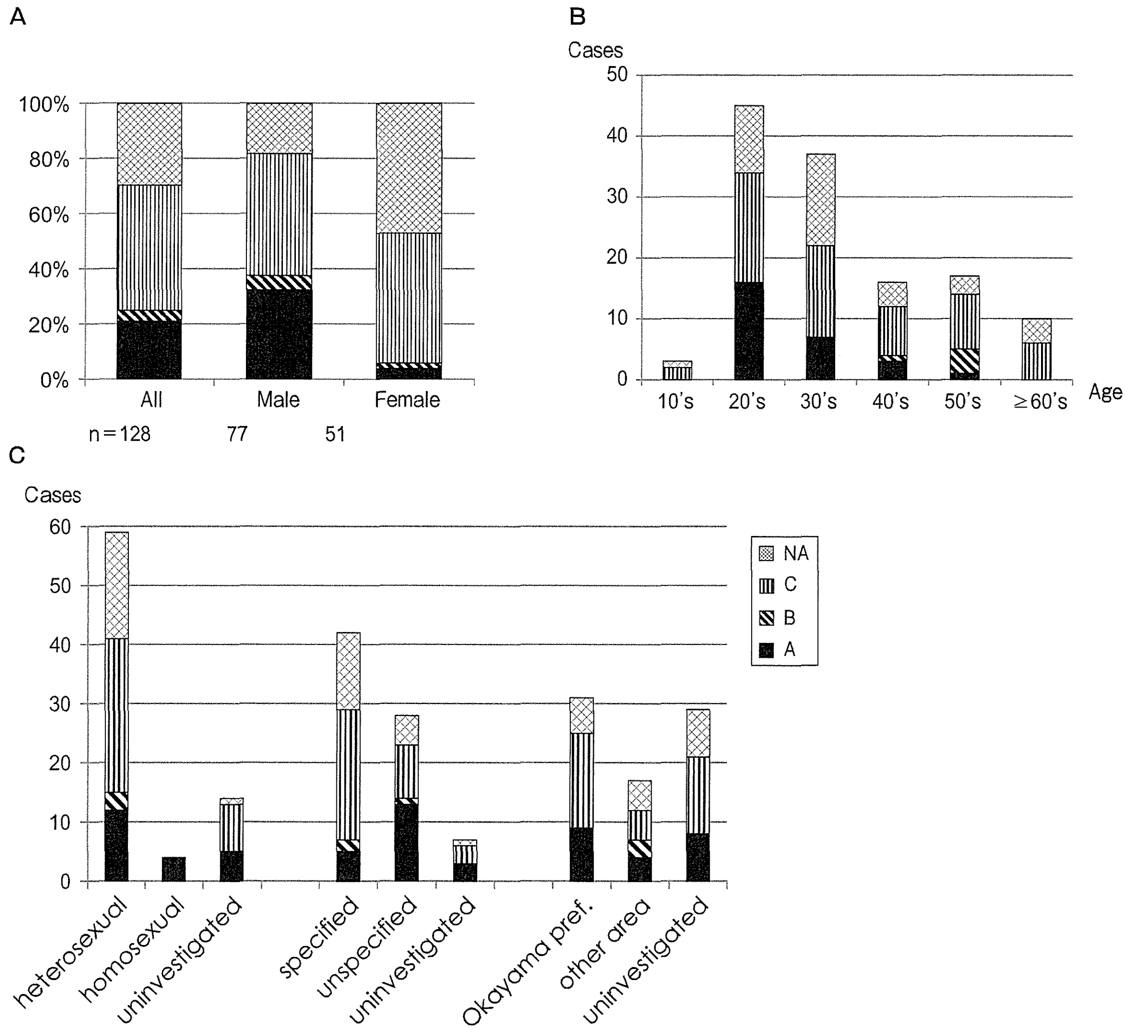

Fig. 2 Comparison of patient characteristics. HBV genotype A, black; B, slashed; C, striped; not analyzed, meshed. The prevalence of HBV genotype A was significantly higher among male patients (28.7%) than females (4.9%, $p < 0.005$, **A**), and in those aged 20-29 (35.6%, $p < 0.01$, **B**). All the patients among the men who had sex with men developed acute hepatitis with HBV genotype A (100%, $p < 0.005$, **C**), Among the patients having sex with unspecified partners and with acute hepatitis, half (44.8%) had HBV genotype A ($p < 0.005$, **C**). HBV genotype A transmission was found to occur both within and outside of Okayama prefecture.

partners (13 patients, 44.8%, $p < 0.005$). About half of the patients (9 patients) with HBV genotype A got infected in Okayama prefecture. These results suggest that HBV genotype A is already prevalent in Okayama prefecture. There were no significant differences in clinical characteristics such as total bilirubin, alanine aminotransferase, or prothrombin time among the patient groups with the different HBV genotypes.

Negativity of HBs antigen. HBsAg became negative in all cases, as shown in Fig. 3A. The median time for negative testing after the initial diagnosis was 4 months; the maximum time was 24 months. Factors associated with negativity of HBsAg within 6 months after diagnosis were investigated but no significant factor was found. Some patients with HBV genotype A achieved negative HBsAg more slowly than those with HBV genotype B or C, but this difference was not significant (Fig. 3B, $p = 0.20$).

A notification at a health center. According to the infectious diseases control law of Japan in 1999, acute hepatitis due to HBV has been classified as a category V infectious disease, with the diagnosis requiring reports to the local public health center for all cases within 1 week. Despite this requirement the number of notifications made by institutions in Okayama from 2006 to 2010 was only 36.

Discussion

We retrospectively investigated AHB cases in Okayama prefecture from 2006 to 2010, and have summarized the characteristics of the patients with HBV genotype A. Though the analysis of HBV genotype was not covered by Japanese national health insurance until 2011, 70% of HBV patients were analyzed for genotype. The most common genotype remains genotype C, but the second most common in Okayama prefecture has become genotype A, replacing genotype B.

The transmission route of genotype A HBV in recent years has included sexual transmission from a committed, non-foreign heterosexual partner as well as that from homosexual, foreign and unspecified heterosexual partners [5]. It is noteworthy that a similar pattern has appeared in Okayama prefecture, reflecting the pandemic spread of HBV genotype A to the general population from the specific groups of men who have sex with men.

As far as our investigations of AHB diagnosed within Okayama prefecture, all patients eventually achieved HBsAg negativity, with HBsAg positivity at 6 months after diagnosis found in only 21% of patients. Delayed HBsAg disappearance was observed in some patients with AHB due to HBV genotype A,

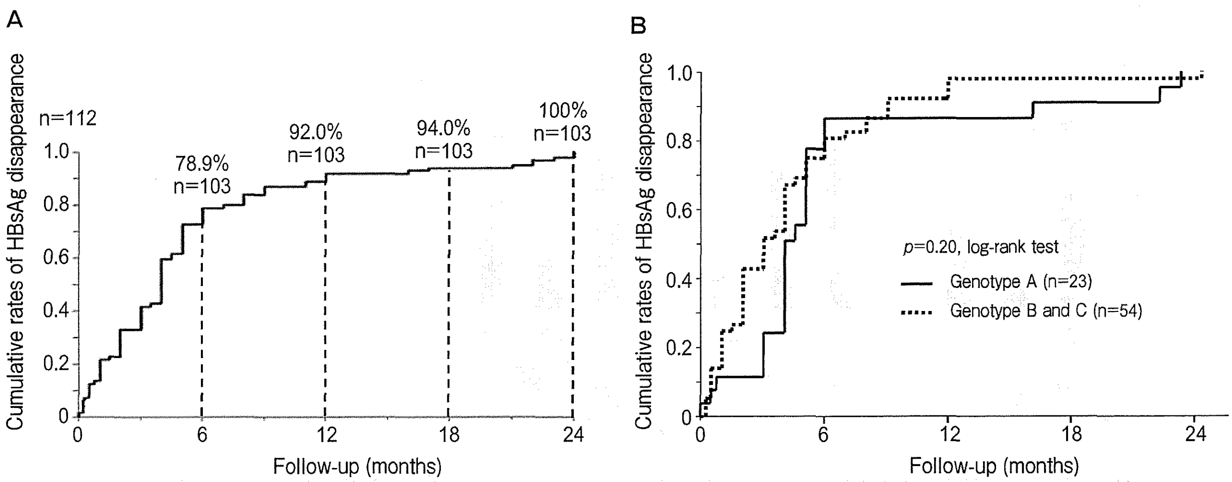

Fig. 3 Cumulative rates of serum HBsAg disappearance. **A**, shows cumulative rates of serum HBsAg disappearance. The median persistence of HBsAg was 4 months after diagnosis. Eight percent of patients retained HBsAg positivity for more than 12 months. **B**, shows the cumulative rates of HBsAg disappearance by HBV genotypes. There was no significant difference in the rates of HBsAg disappearance between the patients with HBV genotype A and others. HBV genotype A, solid line; HBV genotype B and C, dotted line.

as compared with those due to HBV genotype B or C. Though Ito *et al.* reported that AHB due to HBV genotype A was associated with viral persistence [6], our data for that trend were not statistically significant. This might be due to the small number of patients in our study and slightly insufficient follow-up.

In the circumstance of chronic persistence, reporting to a health center is especially important for national surveillance. Clinicians should take care of this responsibility.

In conclusion, a retrospective study of AHB diagnosed in Okayama between 2006 and 2010 was undertaken. The majority of patients with AHB were infected with the HBV genotype C, although it was observed that AHB due to HBV genotype A in Okayama prefecture was now being contracted through sex with heterosexual partners. Caution regarding sexually transmissible diseases is needed to prevent the pandemic spread of AHB due to HBV genotype A.

Acknowledgments. This study was conducted as part of the Japanese AHB Study Group.

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Hepatology Research

Hepatology Research 2014; 44: E229–E239 doi: 10.1111/hepr.12254

Original Article

Hepatic oxidative stress in ovariectomized transgenic mice expressing the hepatitis C virus polyprotein is augmented through suppression of adenosine monophosphate-activated protein kinase/proliferator-activated receptor gamma co-activator 1 alpha signaling

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Aim: Oxidative stress plays an important role in hepatocarcinogenesis of hepatitis C virus (HCV)-related chronic liver diseases. Despite the evidence of an increased proportion of females among elderly patients with HCV-related hepatocellular carcinoma (HCC), it remains unknown whether HCV augments hepatic oxidative stress in postmenopausal women. The aim of this study was to determine whether oxidative stress was augmented in ovariectomized (OVX) transgenic mice expressing the HCV polyprotein and to investigate its underlying mechanisms.

Methods: OVX and sham-operated female transgenic mice expressing the HCV polyprotein and non-transgenic littermates were assessed for the production of reactive oxygen species (ROS), expression of inflammatory cytokines and antioxidant potential in the liver.

Results: Compared with OVX non-transgenic mice, OVX transgenic mice showed marked hepatic steatosis and ROS production without increased induction of inflammatory cytokines, but there was no increase in ROS-detoxifying enzymes such as superoxide dismutase 2 and glutathione peroxidase 1. In accordance with these results, OVX transgenic mice showed less activation of peroxisome proliferator-activated receptor-γ co-activator-1α (PGC-1α), which is required for the induction of ROS-detoxifying enzymes, and no activation of adenosine monophosphate-activated protein kinase-α (AMPKα), which regulates the activity of PGC-1α.

Conclusion: Our study demonstrated that hepatic oxidative stress was augmented in OVX transgenic mice expressing the HCV polyprotein by attenuation of antioxidant potential through inhibition of AMPK/PGC-1α signaling. These results may account in part for the mechanisms by which HCV-infected women are at high risk for HCC development when some period has passed after menopause.

Key words: antioxidant potential, glutathione peroxidase, reactive oxygen species, superoxide dismutase

INTRODUCTION

PERSISTENT HEPATITIS C virus (HCV) infection is a major risk factor for the development of hepatocellular carcinoma (HCC) in Japan. Approximately 70% of Japanese HCC patients are currently diagnosed with HCV-associated cirrhosis or chronic hepatitis C.[1] Nevertheless, the mechanisms underlying HCV-associated hepatocarcinogenesis are incompletely understood. Notably, there is sex disparity in HCC development, that is, male sex has been demonstrated to be an independent risk factor associated with HCC development.[2–4] It is proposed that estrogen-mediated inhibition of interleukin (IL)-6 production by Kupffer cells reduces the HCC risk in females.[5] In addition, the proportion of females among elderly patients with HCV-related HCC has recently increased in Japan.[6] These results suggest that menopause may be a risk factor associated with HCC development in female patients with HCV infection.

Numerous studies have shown that oxidative stress is present in chronic hepatitis C to a greater degree than in other inflammatory disease,[7,8] and is related to

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Received 9 September 2013; revision 24 September 2013; accepted 30 September 2013.

hepatocarcinogenesis in HCV-associated chronic liver diseases.[9,10] We have previously demonstrated that transgenic mice expressing the HCV polyprotein develop liver tumors including HCC, in connection with oxidative stress induced by HCV and iron overload.[11] Interestingly, such hepatocarcinogenesis was observed only in male transgenic mice, suggesting that females are resistant to oxidative stress in these transgenic mice. On the other hand, it is reported that ovariectomy increases nicotinamide adenine dinucleotide phosphate (NADPH) oxidase activity[12] and decreases mitochondrial-reduced glutathione levels in rats.[13] However, it remains unknown how HCV affects ovariectomy-induced oxidative stress. Investigation of this issue may provide a clue for understanding why the incidence of HCC increases in elderly postmenopausal women with HCV infection. The aim of this study was to determine whether HCV proteins amplify oxidative stress induced by ovariectomy and to investigate the mechanisms underlying this.

METHODS

Animals

CONTAINING THE FULL-LENGTH polyprotein-coding region under the control of the murine albumin promoter/enhancer, the transgene pAlbSVPA-HCV has been described in detail.[14,15] Of the four transgenic lineages with evidence of RNA transcription of the full-length HCV-N open reading frame (FL-N), the FL-N/35 lineage proved capable of breeding in large numbers. There is no inflammation in the transgenic liver.[15]

Experimental design

Female FL-N/35 transgenic mice and their normal female C57BL/6 littermates were anesthetized for surgery and underwent either a bilateral ovariectomy or sham operation at the age of 4–6 weeks. We studied ovariectomized (OVX) transgenic mice ($n = 5$), sham-operated transgenic mice ($n = 5$), OVX non-transgenic mice ($n = 5$) and sham-operated non-transgenic mice ($n = 5$). These mice were fed a normal rodent diet, bred, maintained, and killed by i.p. injection of 10% pentobarbital sodium preceded by 20-h fasting at the age of 24 weeks. All experimental protocols and animal maintenance procedures used in this study were approved by the Ethics Review Committee for Animal Experimentation of Kawasaki Medical School.

Histological procedures

A portion of liver tissue was immediately snap-frozen in liquid nitrogen for determination of the hepatic triglyceride concentration. The remaining liver tissue was fixed in 4% paraformaldehyde in phosphate-buffered saline and embedded in paraffin for histological analyses. Liver sections were stained with hematoxylin–eosin.

Serum leptin concentration

The serum leptin level was measured using a Rat Leptin Elisa kit (Morinaga Institute of Biological Science, Yokohama, Japan) according to the manufacturer's instructions.

Hepatic triglyceride content

Lipids were extracted from the homogenized liver tissue by the method of Bligh and Dyer.[16] The triglyceride level was measured with a TGE-test Wako kit (Wako Pure Chemicals, Tokyo, Japan), according to the manufacturer's instructions. Protein concentrations in liver were determined by the method of Lowry *et al.*,[17] using a DC protein assay kit (Bio-Rad Laboratories, Hercules, CA, USA).

In situ detection of reactive oxygen species (ROS)

In situ ROS production in the liver was assessed by staining with dihydroethidium, as described previously.[18] In the presence of ROS, dihydroethidium (Invitrogen, Carlsbad, CA, USA) is oxidized to ethidium bromide and stains nuclei bright red by intercalating with the DNA.[19] Fluorescence intensity was quantified using National Institutes of Health image analysis software for 3 randomly selected areas of digital images for each mouse.

Hepatic iron content

Hepatic iron content was measured by atomic absorption spectrometry, as described previously,[11] and expressed as micrograms Fe per gram of tissue (wet weight).

Derivatives of reactive oxygen metabolites (dROM) and biological antioxidant potential (BAP)

The levels of dROM and BAP were measured using a Free Radical Elective Evaluator (Wismerll, Tokyo, Japan), as described previously.[20] Measurement of dROM is based on the ability of the transition metal ions to catalyze the formation of alkoxy and peroxy radicals from hydroperoxides present in serum. The results are expressed in conventional units as Carrtelli units (U.CARR), where 1 U.CARR corresponds to 0.8 mg/L H_2O_2. Measurement of BAP is based on the ability of antioxidants to reduce ferric (Fe^{3+}) ions to ferrous (Fe^{2+}) ions.

RNA isolation and real-time reverse transcription polymerase chain reaction (RT–PCR)

Total RNA was isolated using an RNeasy mini kit (QIAGEN, Hilden, Germany) and reverse-transcribed into cDNA by using a Superscript III reverse transcription kit (Invitrogen). The PCR reactions were run in the ABI Prism 7700 sequence detection system (Applied Biosystems, Foster, CA, USA). The levels of mRNA were determined using cataloged primers (Applied Biosystems) for mice (tumor necrosis factor [TNF]-α, Mm00443258_m1; IL-1β, Mm00434228_m1; IL-6, Mm00446190_m1; HAMP [gene encoding hepcidin], Mm00519025_m1; superoxide dismutase 2 [SOD2], Mm01313000_m1; glutathione peroxidase 1 [GPx1], Mm00656767_g1; and sirtuin 3 [SIRT3], Mm00452131_m1). Expression of these genes was normalized to expression of glyceraldehyde 3-phosphate dehydrogenase mRNA (GAPDH, Mm99999915_g1).

Isolation of mitochondria and nuclear fraction

Mitochondrial extraction from liver tissue was performed using a Qproteome Mitochondrial Isolation kit (QIAGEN) according to the manufacturer's instructions. The nuclear fraction from liver tissue was prepared using a Nuclear Extraction kit (Panomics, Fremont, CA, USA) according to the manufacturer's instructions.

Immunoblotting

Liver lysates and the mitochondrial and nuclear fractions from liver were separated by sodium dodecylsulfate polyacrylamide gel electrophoresis. The proteins were transferred to polyvinylidene difluoride membranes (Millipore, Bradford, MA, USA), blocked overnight at 4°C with 5% skim milk and 0.1% Tween-20 in Tris-buffered saline, and subsequently incubated for 1 h at room temperature with goat antihuman SOD2 antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA), rabbit antihuman GPx1 antibody (Abcam, Cambridge, MA, USA), rabbit antihuman SIRT3 antibody (Abcam), rabbit antihuman peroxisome proliferator-activated receptor-γ co-activator-1α (PGC-1α) antibody (Abcam), rabbit antihuman adenosine monophosphate-activated protein kinase-α (AMPKα) antibody (Cell Signaling Technology, Boston, MA, USA), rabbit antihuman phospho-AMPKα (Thr172) antibody (Cell Signaling Technology), rabbit antihuman mitochondrial heat shock protein 70 antibody (HSP70; Thermo Scientific, Rockford, IL, USA), rabbit antihuman β-actin antibody (Cell Signaling Technology) or rabbit antimouse lamin B1 antibody (Abcam). The membranes were washed and incubated with horseradish peroxidase (HRP)-conjugated donkey antigoat immunoglobulin (Ig)G (Santa Cruz Biotechnology) or HRP-conjugated donkey antirabbit IgG (GE Healthcare Life Sciences, Pittsburgh, PA, USA).

Statistical analysis

Quantitative values are expressed as mean ± standard deviation. Two groups among multiple groups were compared by the rank-based Kruskal–Wallis ANOVA test followed by Scheffé's test. The statistical significance of correlation was determined by the use of simple regression analysis. $P < 0.05$ was considered to be significant.

RESULTS

Ovariectomy enhanced hepatic steatosis in FL-N/35 transgenic mice

AS CONFIRMATION OF successful ovariectomy-induced suppression of endogenous estrogen production, the uterine weight of OVX mice was significantly decreased compared with that of sham-operated mice (Table 1). Dietary intake, bodyweight, liver weight and serum leptin levels were significantly greater in OVX mice than in sham-operated mice regardless of whether they were transgenic or non-transgenic (Table 1). Interestingly, the serum alanine aminotransferase (ALT) level was significantly higher in OVX transgenic mice than in mice in the other three groups, but the levels were comparable in OVX non-transgenic and sham-operated non-transgenic mice (Table 1). To determine why OVX transgenic mice have a higher ALT level, we investigated the liver histology of the mice in the four groups (OVX transgenic, sham-operated transgenic, OVX non-transgenic and sham-operated non-transgenic mice). In contrast to the mild to moderate degree of hepatic steatosis noted in OVX non-transgenic mice and sham-operated transgenic mice, OVX transgenic mice developed severe hepatic steatosis (Fig. 1a) without infiltration of inflammatory mononuclear cells. Hepatic triglyceride content was measured to quantify the degree of steatosis. The triglyceride content was significantly greater in OVX transgenic mice than in mice in the other three groups (Fig. 1b), which was consistent with the

Table 1 Body, liver and uterus weight and serum biochemical parameters

Body, liver, and uterus weight and serum biochemical parameters	Non-transgenic		Transgenic	
	Sham-operated	OVX	Sham-operated	OVX
Bodyweight (g)	21.5 ± 1.2	30.7 ± 4.9*	27.7 ± 4.6	34.2 ± 3.8**
Liver weight (g)	0.86 ± 0.075	1.09 ± 0.236*	0.90 ± 0.102	1.18 ± 0.156**
Ratio of liver to bodyweight	0.038 ± 0.037	0.035 ± 0.003	0.031 ± 0.002	0.034 ± 0.006
Uterus weight (g)	0.08 ± 0.01	0.01 ± 0.02*	0.09 ± 0.01	0.01 ± 0.01**
Total dietary intake (g)	337 ± 24	429 ± 13*	368 ± 28	490 ± 31**
Serum glucose (mg/dL)	222.9 ± 110.0	275.1 ± 121.4	284.0 ± 84.1	259.7 ± 108.9
Serum ALT (IU/L)	15.5 ± 6.5	30.6 ± 38.1	21.8 ± 11.4	281.2 ± 165.1***
Serum triglyceride (mg/dL)	99.9 ± 9.7	78.9 ± 10.8	98.3 ± 11.4	89.7 ± 13.3
Serum leptin (ng/mL)	0.45 ± 0.14	1.31 ± 0.31*	0.65 ± 0.22	1.60 ± 0.28**

Data are mean ± standard deviation.
*$P < 0.05$ compared with sham-operated non-transgenic mice. **$P < 0.05$ compared with sham-operated transgenic mice. ***$P < 0.01$ compared with mice in the other three groups.
ALT, alanine aminotransferase; OVX, ovariectomized.

results for hepatic steatosis. Thus, the increase in the serum ALT level in the OVX transgenic mice was thought to reflect the hepatic steatosis.

Ovariectomy increased ROS and IL-6 production in the liver

Only OVX transgenic mice showed marked hepatic steatosis, regardless of the comparable diet intake and the ratio of liver to bodyweight of OVX non-transgenic mice (Table 1). We have previously demonstrated that iron-overloaded male FL-N/35 transgenic mice expressing the HCV polyprotein develop severe hepatic steatosis through increased ROS production.[11] Therefore, we examined whether ROS production was relevant to the marked hepatic steatosis observed in the OVX transgenic mice. Ovariectomy significantly increased ROS (super-

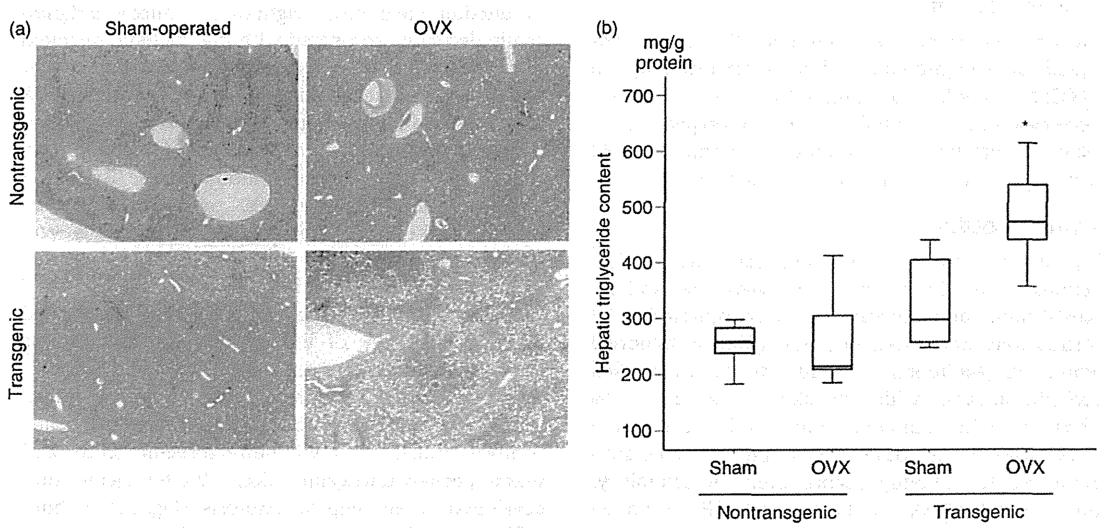

Figure 1 Hepatic steatosis and triglyceride content in sham-operated and ovariectomized (OVX) FL-N/35 transgenic and non-transgenic mice. (a) Hepatic steatosis in mice in each group (H&E, original magnification × 100). (b) Hepatic triglyceride content in mice in each group ($n = 5$). The results are shown as box plot profiles. The bottom and top edges of the boxes are the 25th and 75th percentiles, respectively. Median values are shown by the line within each box. *: $P < 0.05$ versus mice in the other three groups.