と様々であるが，とくに過去の治療における再燃例では，再治療効果が高い．一方，AASLDのpractice guideline[1]では，過去に十分なPEG-IFN・RBV併用療法が行われた再燃・無効例に対しては，PEG-IFN・RBV併用療法の再投与は推奨されていない．しかし最近，過去のPEG-IFN・RBV併用療法の再燃・無効例であっても，PEG-IFN・RBVの再投与により12週でHCV RNAの陰性化が達成されれば，48～72週投与により48%～68%のSVR率が得られたとの報告がある[45)~52)]．再治療により12週陰性化が得られる症例を治療前に同定することは現時点では困難であることや，プロテアーゼ阻害薬の開発状況を考慮すると依然議論の余地はあるが，以下のconsensus statementが採用された．

Consensus Statement 13:

過去のIFN療法の再燃・無効例であっても，PEG-IFN・RBV併用療法の再治療で，HCV RNAの12週陰性化が達成されれば，ウイルス学的著効が期待できる．(Level 2b，Grade B)

2．従来型IFNあるいはPEG-IFN単独療法の位置づけ

我が国では，1992年にC型慢性肝炎に対するIFN単独治療が開始され，その臨床的効果が多数報告されている．しかし現在では，1b・高ウイルス量例に対する標準治療はPEG-IFN・RBV併用療法となり，HCV排除を目的とする単独療法の適応は，一部の患者に限定されている．IFNあるいはPEG-IFN単独治療の対象として，1．低ウイルス症例，2．リバビリン併用困難例(慢性腎不全など)，3．急性肝炎例，4．肝癌根治例，5．維持療法（線維化進展例や抗ウイルス療法抵抗例）が挙げられている[53)]．

アンサーパッドにおいて，「初回治療例において，低ウイルス量の患者にふさわしい治療は？」と質問したところ，PEG-IFNまたはIFN単独療法は60%に支持されたが，最初からIFN・RBVの併用療法を行うとする意見が37%に見られた．欧米のガイドラインではPEG-IFN・RBV併用療法のみを推奨しているが，我が国では初回の低ウイルス量例にはPEG-IFNあるいはIFN単独療法も一定の評価を受けていることが示された．

急性HCV感染は，70%程度が慢性感染に移行するため，治療介入が必要である．急性C型肝炎患者で持続感染への移行が疑われる症例では，発症後12週から24週以内にIFN6MIU週3回あるいはPEG-IFN週1回12～24週間の単独治療が推奨される．しかし，リバビリンの併用が治療効果を向上した成績がなく，単独療法で十分な有効性が期待できる[54)]．

わが国ではIFNの発がん抑制効果について多くの臨床的検討が行われてきた[55)~57)]．肝硬変例を対象とするメタアナリシス解析ではIFN投与により発癌率が低下することが確認されている．また線維化進展例（F3-F4）においてもIFN投与にてHCVが排除されると発癌率が低下し予後改善効果があることも報告されている[16)]．我が国の成績では，60歳以上の患者への少量長期IFN単独治療は，ALTおよびAFPの低下を誘導し非治療群と比較して肝発癌を抑制することが示されている[56)]．さらに，我が国を含めて複数の施設から肝癌根治療例に対するIFNの発癌抑制効果が報告されている[57)]．特に，IFN投与群では二次再発，三次再発が低下することも注目すべき効果である[58)]．一方，欧米で実施された前向きランダム化試験（HALT-C）ではPEG-IFN・RBV無反応例に対するPEG-IFN少量長期投与が肝疾患の進展を阻止しなかったことが示された[59)]．すなわちIFN維持療法に関する欧米と我が国の臨床成績が相反する結果が示された．この理由として，我が国の治療対象が高齢であり，かつ肝発癌率が高いことが治療介入による効果の差になっていることが推測され，今後明らかにすべき課題である．

今回，SVRが期待できない場合でも，「IFN長期投与はALT値の低下が見られれば，肝発癌抑制や生命予後の改善効果を期待できるか？」という質問に対し，89%の同意が得られた．

Consensus Statement 14:

肝癌根治例では生命予後延長効果を期待したIFN投与を推奨する．(Level 1，Grade A)

Consensus Statement 15:

SVRが期待できない場合でも，IFN長期投与はALT値の低下が見られれば，肝発癌抑制や生命予後の改善効果が期待できる．(Level 2a，Grade B)

3．治療方針のコンセンサス

C型肝炎に対する治療方針として，C型肝炎の治療目標，抗ウイルス療法の治療適応，Peg-IFN・RBV併用療法(薬剤投与量との関係)，ウイルス排除不能例・肝硬変例の治療，新規抗ウイルス剤の各項目について，下記のようにまとめた．

治療目標

治療目標の第一は，HCV RNA排除による肝炎治癒であり，IFN治療によってウイルス排除が得られた場合

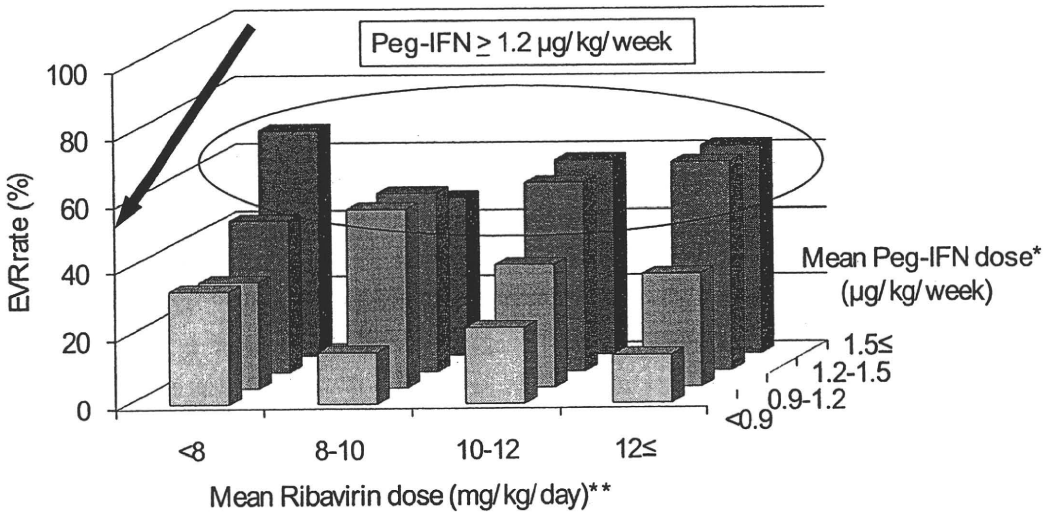

Fig. 3 C-EVR rate according to PEG-IFN alfa-2b and ribavirin doses during 12 weeks after start of therapy. *, p<0.0001; Peg-IFN **, p=0.34; Ribavirin (Mantel-Haentzel chi-square test). The c-EVR rates were 54% and 56% for patients who received more than 1.5 μg/kg/week and 1.2-1.5 μg/kg/week of Peg-IFN alfa-2b and declined to an average rate of 38% in patients given 0.9-1.2 μg/kg/week of Peg-IFN alfa-2b, to an average rate of 22% in patients given less than 0.9 μg/kg/week of Peg-IFN alfa-2b. [61]

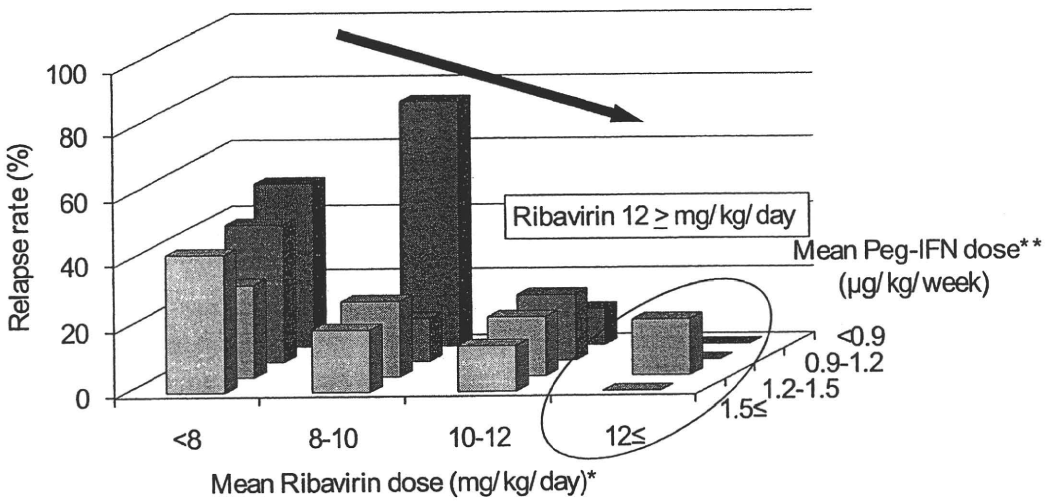

Fig. 4 Relapse rate according to Peg-IFN alfa-2b and ribavirin doses during treatment of patients who completed treatment. *, p=0.0001; Ribavirin **, p=0.15; Peg-IFN (Mantel-Haentzel chi-square test). The relapse rate was 60% in patients receiving less than 6 mg/kg/day of ribavirin, and declined to 41% at 6-8 mg/kg/day, 27% at 8-10 mg/kg/day, 22% at 10-12 mg/kg/day and 11% in patients given ≥ 12 mg/kg/day. [62]

には，肝発癌抑止効果や生命予後改善が得られる．現時点で最も治療効果の高い抗ウイルス療法はPeg-IFN・RBV併用療法である．第二の目標として，ウイルス排除ができない場合には肝病変進展予防あるいは肝発癌予防を目指すことが重要である．

治療適応

治療適応については，C型肝疾患が患者の予後を規定し，薬剤の副作用への対処が可能であると考えられる症例を抗ウイルス療法の対象とする．このうち，良好な治療効果が予測される症例が，“良い適応”症例である．さらに，SVRの可能性が低い高齢者や線維化進展例でも，合併疾患がなければ抗ウイルス療法を提示す

べきであるということに88%の同意が得られた.

Consensus Statement 16:

C型肝疾患が患者の予後を規定し,薬剤の副作用への対処が可能であると考えられる症例は抗ウイルス療法の対象とする.(Level 6, Grade B/C)

たとえ,SVRの可能性が低い症例でも合併疾患がなければ抗ウイルス療法を提示すべきである.

Peg-IFN・RBV併用療法(薬剤投与量との関係)

Genotype1型におけるEVR(治療開始12週のHCV RNA陰性化)の達成には,Peg-IFN投与量が用量依存性に関与する(Fig. 3). Peg-IFNα2a投与量80%以上[60]あるいはPeg-IFNα2b平均投与量1.2 μg/kg/週以上[61]を目標とし,極力,減量投与開始は避ける(Level 2b/3, Grade B).また,Genotype1型のウイルス陰性化例における治療後再燃には,RBVが用量依存性に関与し,予定投与量の80%以上あるいは平均投与量10 mg/kg/日(可能であれば12 mg/kg/日)以上を目標とする[62](Level 2b/3, Grade B)(Fig. 4).一方,Genotype2/3型における減量(PegIFNα2a 135 μg/週 あるいはPegIFNα2b 1.0 μg/kg/週, RBV 400 mg/day)については,治療効果に有意な影響を及ぼさないものと考えられる(Level 2a, Grade B)[63)~65)].

ウイルス排除不能例・肝硬変例の治療

IFN療法を積極的に行うことが治療の第一選択であるが,IFN非適応例や無効例に対しては肝庇護療法や瀉血療法の重要性は認識されており,77%の同意が得られた.

Consensus Statement 17:

IFN非適応例やIFNでALT値やAFPの改善が得られない症例には,肝庇護剤による治療を行い,効果不十分な場合は,瀉血療法を併用する.(Level 3/6, Grade B/C)

次に,代償性肝硬変では,IFNを主体とした治療でHCV RNA排除を目指し,非代償性肝硬変では,肝予備能の改善や発癌予防を目標とした治療を行う.代償性肝硬変で発癌予防を目指す場合には,AST・ALT値,AFP値の改善を目標とし,IFNのみでなく肝庇護剤,瀉血療法,分岐鎖アミノ酸製剤を単独あるいは組み合わせて治療することが望ましい.AASLD guideline[1]では,"C型慢性肝疾患に対して抗ウイルス療法が奏功しない場合,肝移植を考慮する"という方針のみにとどまるのに対し,肝不全への進展,発癌予防に向けたわが国独特の肝庇護療法に対して,91%の同意が得られた.

Consensus Statement 18:

代償性肝硬変で発癌予防を目指す場合には,AST・ALT値,AFP値の改善を目標とし,IFNのみでなく肝庇護剤,瀉血療法,分岐鎖アミノ酸製剤を単独あるいは組み合わせて治療する.

新規抗ウイルス剤

新規抗ウイルス剤として,プロテアーゼ阻害剤,ポリメラーゼ阻害剤などが有効であり,PEG-IFN/RBVとの併用で著効率が向上する(Level 1b, B).特に,プロテアーゼ阻害剤であるTelaprevir(VX950)にPeg-IFN・RBVを加えた3者併用療法では,新規症例で6~7割[66],PEG-IFN・RBV併用療法の再燃例で約7割,無効例でも約4割にSVRを認めている.

今後の治療方針として,より早期のウイルス排除が期待される線維化進展例や高齢者ではPEG-IFN・RBV併用療法を行うが,それ以外の症例では新規治療を考慮に入れた治療選択が必要となる.

おわりに

わが国のC型肝炎は,欧米に比し高齢で組織進展例が多い.このため,年率発がん率が高く,IFN治療に対しても有効率が低く副作用の発現が多い.このような患者背景に即したわが国独自のエビデンスの確立が求められるが,現状では信頼度の高い情報が集積されていないものも多い.今回,アンサーパッドを用いて聴取したわが国の肝臓専門医の意見を基に,これらの多くをコンセンサスステートメントとしてまとめた.肝臓専門医の共感が得られた提言ではあるが,国際的に承認されるためには全国的な多施設研究による今後の検証が必要である.その一方で,わが国の患者の現状は欧米の患者の未来像を示している可能性が高いことから,日本の実情に即したガイドラインを海外にむけて発信し批判の俎上に載せることは,日本肝臓学会が果たすべき重要な役割と考える.このため,本論文にInformative statementやRecommendationを追記してHepatology Res.にも掲載する.

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JSH Consensus Kobe 2009: Diagnosis and Treatment of Hepatitis C

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BASIC STUDIES

Hepatitis C virus protein and iron overload induce hepatic steatosis through the unfolded protein response in mice

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Keywords
endoplasmic reticulum stress – hepatic steatosis – hepatitis C virus – iron – unfolded protein response

Abbreviations
ATF6, activating transcription factor 6; CHOP, CCAAT/enhancer-binding protein homology protein; CPTI, carnitine palmitoyl transferase I; ER, endoplasmic reticulum; FAS, fatty acid synthetase; HCC, hepatocellular carcinoma; HCV, hepatitis C virus; IRE1, inositol-requiring enzyme1; NAC, *N*-acetyl cysteine; p-eIF2α, phosphorylated eukaryotic initiation factor-2α; PERK, PKR-like ER kinase; PKR, RNA-activated protein kinase; ROS, reactive oxygen species; SCAP, SREBP cleavage-acting protein; SREBP, sterol-regulatory element-binding protein; XBP-1, X-box DNA-binding protein 1.

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Abstract

Background/Aim: Hepatic iron overload and steatosis play critical roles in the progression of hepatitis C virus (HCV)-associated chronic liver disease. However, how these two pathophysiological features affect each other remains unknown. The aim of this study was to investigate how hepatic iron overload contributes to the development of hepatic steatosis in the presence of HCV proteins. *Methods:* Male C57BL/6 transgenic mice expressing the HCV polyprotein and nontransgenic littermates were fed an excess-iron diet or a control diet. Mice in each group were assessed for the molecules responsible for fat accumulation in the liver. *Results:* Hepatic iron levels were positively correlated with triglyceride concentrations in the liver for all mice. As compared with the livers of nontransgenic mice fed the control diet, the livers of transgenic mice fed the iron-excess diet showed a lower expression of carnitine palmitoyl transferase I, a higher expression of sterol-regulatory element-binding protein 1 and fatty acid synthetase and an activated unfolded protein response indicated by a higher expression of unspliced and spliced X-box DNA-binding protein 1 (XBP-1), phosphorylated eukaryotic initiation factor-2α (p-eIF2α), CCAAT/enhancer-binding protein homology protein (CHOP) and abundant autophagosomes concomitant with increased production of reactive oxygen species. Six-month treatment with the anti-oxidant N-acetyl cysteine dramatically reduced hepatic steatosis in transgenic mice fed the iron-excess diet through decreased expression of unspliced and spliced XBP-1, p-eIF2α, and CHOP. *Results:* The iron-induced unfolded protein response appears to be one of the mechanisms responsible for fat accumulation in the liver in transgenic mice expressing the HCV polyprotein.

Hepatic steatosis and iron overload are not only the pathophysiological features of hepatitis C virus (HCV)-associated chronic liver disease (1, 2) but also risk factors for the development of hepatocellular carcinoma (HCC) (3, 4). Thus, these pathophysiological features appear to play critical roles in the pathogenesis of HCV-associated chronic liver disease. The mechanisms underlying HCV-related steatosis are diverse. HCV core protein has been demonstrated to inhibit microsomal transfer protein activity and very low-density lipoprotein secretion (5) and to upregulate the promoter activity of sterol-regulatory element-binding protein (SREBP) 1c, a transcription factor involved in lipid synthesis (6). Persistent activation of peroxisome proliferator-activated receptor α has also been reported to be essential for the development of hepatic steatosis in transgenic mice expressing the HCV core protein (7). As for hepatic iron overload, we have shown that HCV-induced reactive oxygen species (ROS) increase the hepatic iron concentration by reducing hepcidin transcription in transgenic mice expressing the HCV polyprotein (8), and that even modest iron supplementation results in the development of liver tumours,

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including HCC, through mitochondrial injury in these transgenic mice (9). However, it remains unknown how these two pathophysiological features affect each other in the progression of HCV-associated chronic liver disease. In our previous study, marked hepatic steatosis was observed at 6 months after commencement of iron overloading in transgenic mice, which was followed by the development of liver tumours. These results clearly indicated that hepatic iron overload was involved in the development of hepatic steatosis in the presence of HCV proteins. The aim of this study was to investigate how hepatic iron overload contributes to the development of hepatic steatosis in transgenic mice expressing the HCV polyprotein. In the present study, we report that iron-induced ROS-activated unfolded protein response may be postulated as one of the possible mechanisms of HCV-related fat accumulation in the liver.

Materials and methods

Animals

The transgene pAlbSVPA-HCV, containing the full-length polyprotein-coding region under the control of the murine albumin promoter/enhancer, was described in detail (10, 11). HCV polyprotein has been demonstrated to be processed into individual proteins in the liver and to be expressed at a biologically relevant level in which transcripts of RNA encoding the complete viral polyprotein are detectable only by a reverse-transcription polymerase chain reaction (11). 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 (11). Male FL-N/35 transgenic mice and age-matched C57BL/6 mice (control mice) were fed a normal rodent diet including carbonyl iron (45 mg/kg diet, control diet) or an iron-excess diet (carbonyl iron 225 mg/kg diet) at the age of 8 weeks, bred, maintained and killed by an intraperitoneal injection of 10% pentbarbital sodium preceded by 12-h fasting at 12 months after initiation of feeding according to the criteria outlined in the Guide for the Care and Use of Laboratory Animals. As another experiment, six FL-N/35 transgenic mice were fed the control diet for 6 months, and then they were divided into two groups: three fed the iron-excess diet for 6 months with administration of *N*-acetyl cysteine (NAC) and those without NAC. NAC was contained in drinking water (1 g/L).

Hepatic iron and triglyceride content

Iron concentrations in the livers were measured by atomic absorption spectrometry (Hitachi Z-6100, Hitachi Ltd., Tokyo, Japan), as described previously (9), and expressed as μg Fe/g of tissue (wet weight). Lipids were extracted from the homogenized liver tissue by the method of Bligh and Dyer (12). The triglyceride levels were measured with a TGE-test Wako kit (Wako Pure Chemicals, Tokyo, Japan) according to the manufacturer's instructions. The protein concentrations in the liver were determined by the method of Lowry *et al.* (13), using a DC protein assay kit (Bio-Rad Laboratories, Hercules, CA, USA).

Immunoblotting

Lysates of liver were separated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis. The proteins were transferred to polyvinylidene difluoride membranes (Millipore, Bedford, 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 an anti-human ferritin antibody (Dako, Glostrup, Denmark), anti-rabbit carnitine palmitoyl transferase I (CPT I) antibody, anti-rabbit CPT II antibody (Alpha Diagnostic International, San Antonio, TX, USA), anti-rabbit SREBP1 antibody (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA), anti-rabbit fatty acid synthetase (FAS) antibody (Cell Signaling Technology Inc., Boston, MA, USA), anti-mouse X-box DNA-binding protein 1 (XBP-1) antibody (Santa Cruz Biotechnology Inc.) or anti-bacterially expressed, mouse CCAAT/enhancer-binding protein homology protein (CHOP) fusion protein antibody (Abcam, Cambridge, England). Exceptionally, the proteins were blocked for 1 h at room temperature and subsequently incubated overnight at 4 °C with an anti-rabbit phosphorylated eukaryotic initiation factor-2α (p-eIF2α) antibody (Cell Signaling Technology Inc.).

Histological staining

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

Electron microscopy

Liver specimens were fixed in 2.1% glutaraldehyde, post-fixed in 1% osmium tetroxide, dehydrated in graded ethanol and propylene dioxide and embedded in Epok. Thick sections (1 μm in width) were stained with toluidine blue to identify steatosis by light microscopy. Thin sections were stained with uranyl acetate and lead citrate, and examined using a Hitachi-7000 transmission electron microscope (Hitachi Ltd.).

In situ detection of reactive oxygen species

In situ ROS production in the liver was assessed by staining with dihydroethidium, as described previously (8). In the presence of ROS, dihydroethidium (Invitrogen Corp., Carlsbad, CA, USA) is oxidized to ethidium bromide and stains nuclei bright red by intercalating with the DNA (14). Fluorescence intensity was quantified using NIH image analysis software for three randomly selected areas of digital images in each mouse.

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Statistical analysis

Quantitative values are expressed as mean ± SD. Two groups among multiple groups were compared by the rank-based, Kruskal–Wallis ANOVA test, followed by the Scheffé test. Data between two groups were compared by Student's *t*-test. The statistical significance of correlation was determined by the use of a simple regression analysis. A P value of < 0.05 was considered to be significant.

Results

Correlation between iron and triglyceride contents in the liver

Dietary intake and body weight were measured every 4 weeks until 12 months after commencement of iron loading, and these parameters did not differ significantly among any of the 4 groups. The hepatic iron content (267 ± 94 μg/g liver weight) of FL-N/35 transgenic mice fed the iron-excess diet was significantly greater than that of nontransgenic and FL-N/35 transgenic mice fed the control diet at 12 months after commencement of iron loading (Fig. 1A), and was comparable to that of a large number of patients with chronic hepatitis C in extensive studies (15, 16). The hepatic ferritin level of FL-N/35 transgenic mice fed the iron-excess diet was significantly greater than that of nontransgenic mice fed the control diet (Fig. 1B). The hepatic iron content was positively correlated with the hepatic triglyceride concentration when both parameters were compared for all mice ($r = 0.63$, $P = 0.002$, Fig. 1C). These results were consistent with our previous observation that FL-N/35 transgenic mice fed the iron-excess diet demonstrated the most severe steatosis in the liver among the four groups (9).

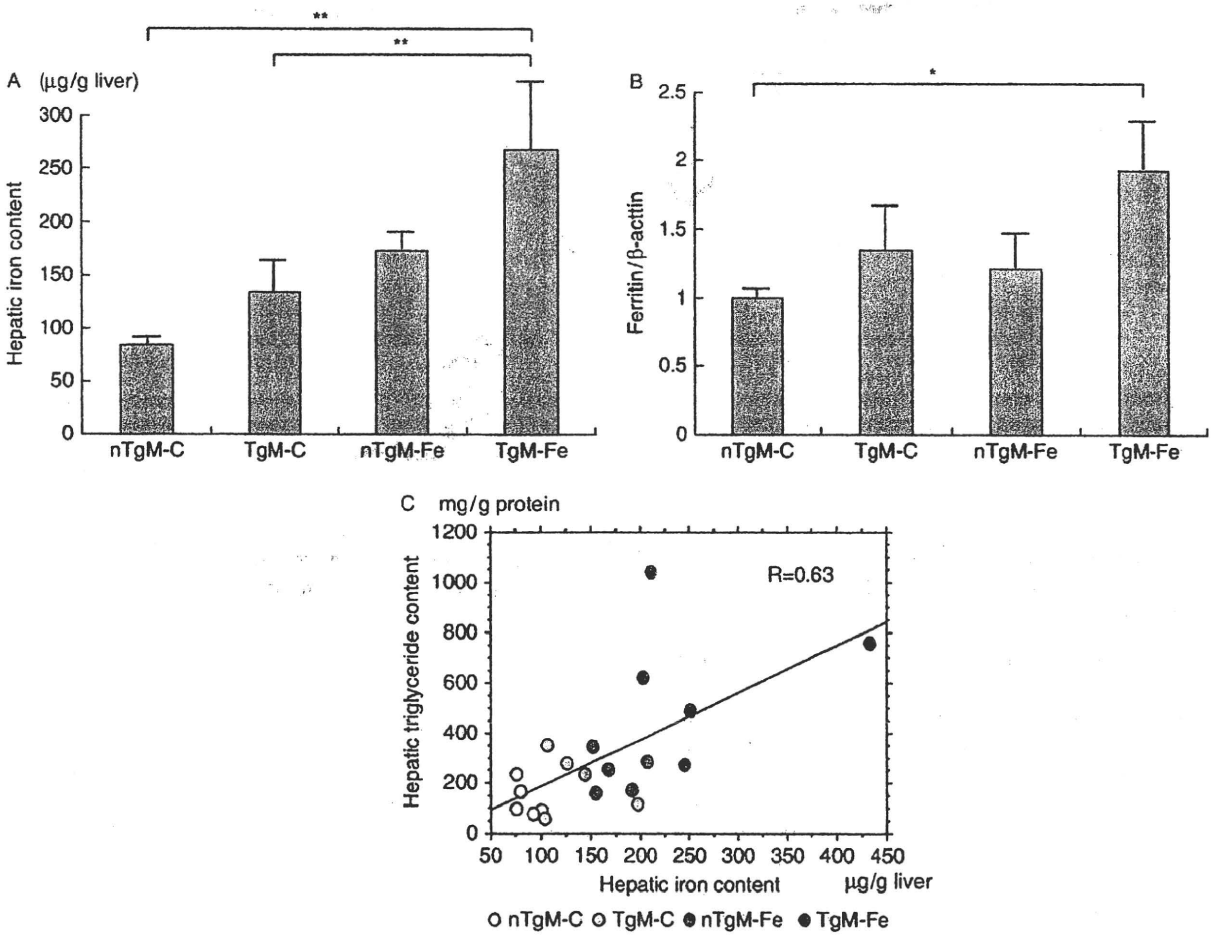


Fig. 1. Hepatic iron contents and ferritin levels, and correlation between iron and triglyceride contents in the liver. (A) The hepatic iron content was measured by atomic absorption spectrometry in five mice in each group at 12 months after initiation of iron loading. (B) Immunoblots for ferritin were performed using liver lysates obtained from four mice in each group. The protein expression was normalized with β-actin. (C) The correlation between hepatic iron and triglyceride levels was determined in 20 mice from four groups. nTgM-C: nontransgenic mice fed the control diet, nTgM-Fe: nontransgenic mice fed the iron-excess diet, TgM-C: FL-N/35 transgenic mice fed the control diet, TgM-Fe: FL-N/35 transgenic mice fed the iron-excess diet. $^{*}P < 0.05$, $^{**}P < 0.01$.

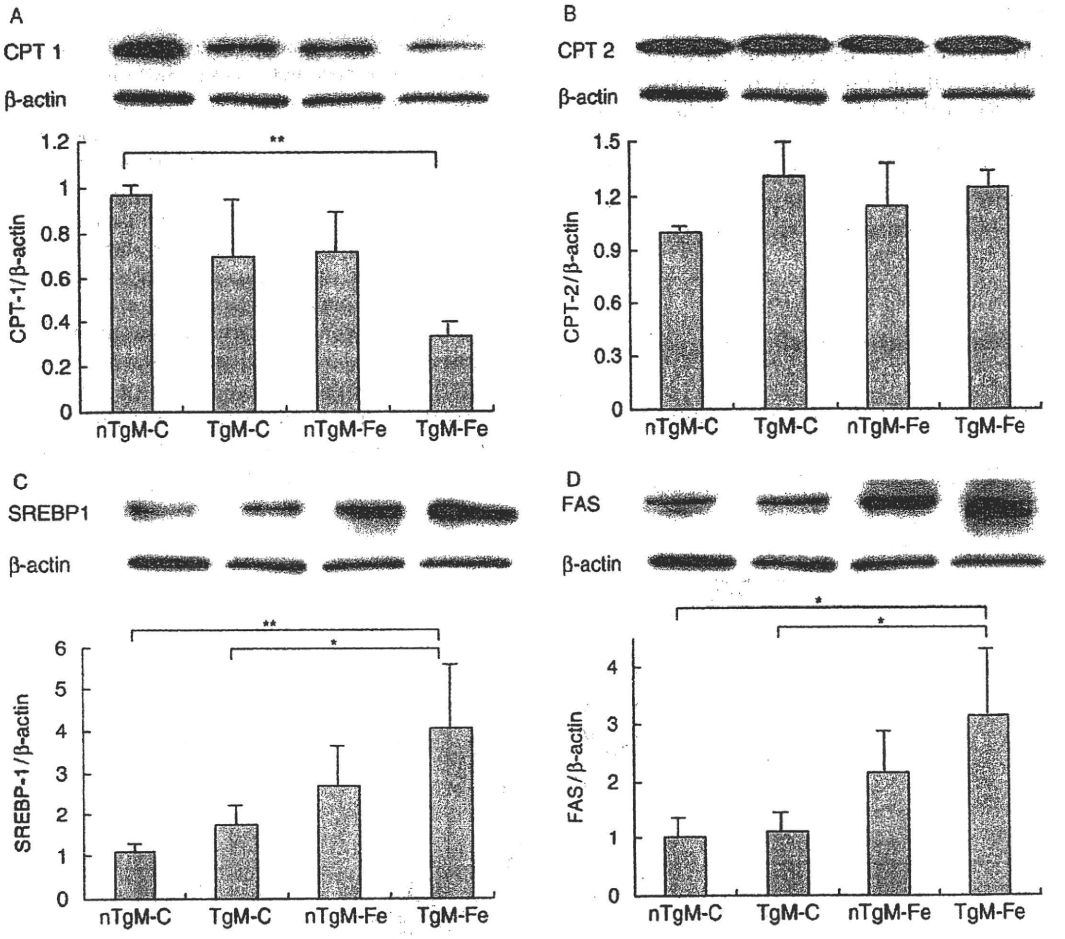


Fig. 2. Expression of carnitine palmitoyl transferase I (CPT1), carnitine palmitoyl transferase I (CPT2), sterol-regulatory element-binding protein I (SREBP1) and fatty acid synthetase (FAS) in the liver. Immunoblots for CPT1 (A), CPT2 (B), SREBP1 (C) and FAS (D) were performed using liver lysates obtained from four mice in each group at 12 months after initiation of iron loading. The protein expression was normalized with β-actin. $^{*}P < 0.05$, $^{**}P < 0.01$. nTgM-C, TgM-C, nTgM-Fe and TgM-Fe; see legend for Figure 1.

Decreased expression of carnitine palmitoyl transferase I and increased expression of sterol-regulatory element-binding protein 1

As we previously reported reduced oxidation activity of fatty acid in iron-overloaded transgenic mice (9), we first examined the expression levels of CPT1 and CPT2, which regulate oxidation of long-chain fatty acids in the mitochondria. The expression of CPT1, but not CPT2, was significantly reduced in FL-N/35 transgenic mice fed the iron-excess diet compared with the nontransgenic mice fed the control diet ($P = 0.0003$, Fig. 2A and B). We next examined the expression level of SREBP1, a transcription factor that activates the genes required for lipogenesis (17), and FAS, a target gene of SREBP1. As shown in Figures 2C and D, the expression of SREBP1 and FAS was significantly greater in FL-N/35 transgenic mice fed the iron-excess diet than in nontransgenic and FL-N/35 transgenic mice fed the control diet, suggesting the involvement of activated lipogenesis in hepatic steatosis in FL-N/35 transgenic mice fed the iron-excess diet. It should also be noted that modest iron supplementation significantly activated lipogenesis in FL-N/35 transgenic mice, but not in nontransgenic mice.

Activated unfolded protein response

Upon endoplasmic reticulum (ER) stress, the SREBP–SREBP cleavage-acting protein (SCAP) complex dissociates from the ER retention protein and subsequently translocates to the Golgi apparatus, where SREBP is cleaved and activated (18, 19). We therefore investigated whether increased expression of SREBP1 was related to ER stress. The unfolded protein response-signalling cascades are initiated by three ER-resident sensors: inositol-requiring enzyme 1 (IRE1), RNA-activated protein kinase (PKR)-like ER kinase (PERK) and activating transcription factor 6 (ATF6) (20). IRE1 activation splices unspliced XBP-1 (uXBP-1) to form spliced XBP-1 (sXBP-1) mRNA (21),

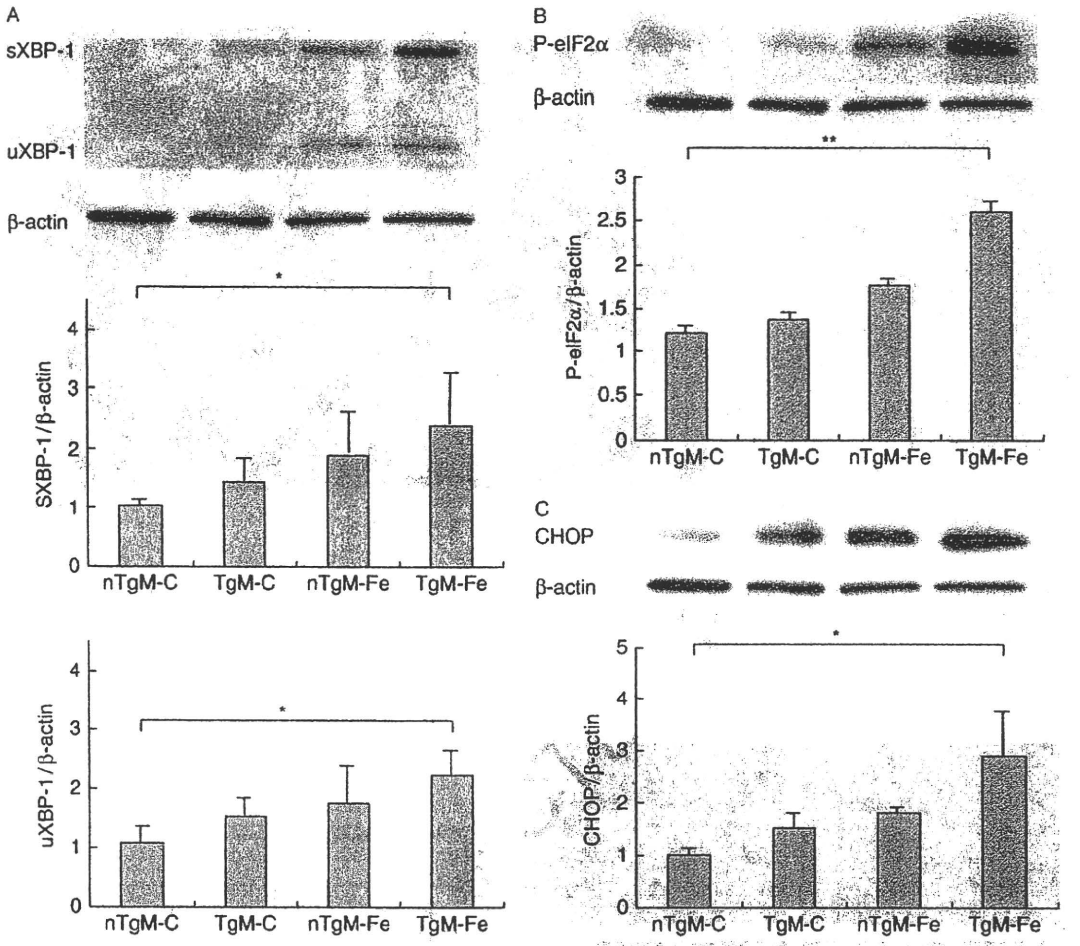


Fig. 3. Expression of spliced X-box DNA-binding protein 1 (sXBP-1), unspliced X-box DNA-binding protein 1 (uXBP-1), phosphorylated eukaryotic initiation factor-2α (p-eIF2α) and CCAAT/enhancer-binding protein homology protein (CHOP) in the liver. Immunoblots for sXBP-1 and uXBP-1 (A), p-eIF2α (B) and CHOP (C) were performed using liver lysates obtained from seven full-length HCV-N open reading frame (FL-N/35) transgenic mice fed the iron-excess diet and four mice in the three other groups at 12 months after initiation of iron loading. The protein expression was normalized with β-actin. $^{*}P < 0.05$, $^{**}P < 0.01$. nTgM-C, TgM-C, nTgM-Fe, and TgM-Fe; see legend for Figure 1.

and was assessed with the sXBP-1 protein level (22). PERK activation was evaluated by measurement of p-eIF2α and CHOP (23). ATF6 activation was assessed with uXBP-1 expression (24). The expression of uXBP-1, sXBP1, p-eIF2α and CHOP was significantly greater in FL-N/35 transgenic mice fed the iron-excess diet than that in nontransgenic mice fed the control diet (Fig. 3).

Autophagy

We next examined the formation of autophagosomes at the ultrastructural level to confirm the activation of the unfolded protein response, because autophagy has been shown to be induced by the unfolded protein response (25–27). As shown in Figure 4, autophagosomes (indicated by arrows) were abundantly found in the liver in FL-N/35 transgenic mice fed the iron-excess diet. In contrast, autophagosomes were not present in the liver of nontransgenic mice fed the iron-excess diet. These results were compatible with the increased expression of uXBP1, sXBP1, p-eIF-2α and CHOP in FL-N/35 transgenic mice fed the iron-excess diet, suggesting activation of the unfolded protein response. Thus, activation of the unfolded protein response appeared to be involved in the development of hepatic steatosis in FL-N/35 transgenic mice fed the iron-excess diet.

Reactive oxygen species generation and endoplasmic reticulum stress

Superoxide has been reported to be selectively involved in ER stress-mediated apoptosis (28). It is also reported that anti-oxidants reduce ER stress and improve protein secretion (29). These results suggest that ROS production induces ER stress. We evaluated *in situ* ROS production in the liver by staining with dihydroethidium and assessed whether treatment with an anti-oxidant reduced hepatic steatosis through inhibition of the unfolded protein response. ROS production was significantly higher in FL-N/35

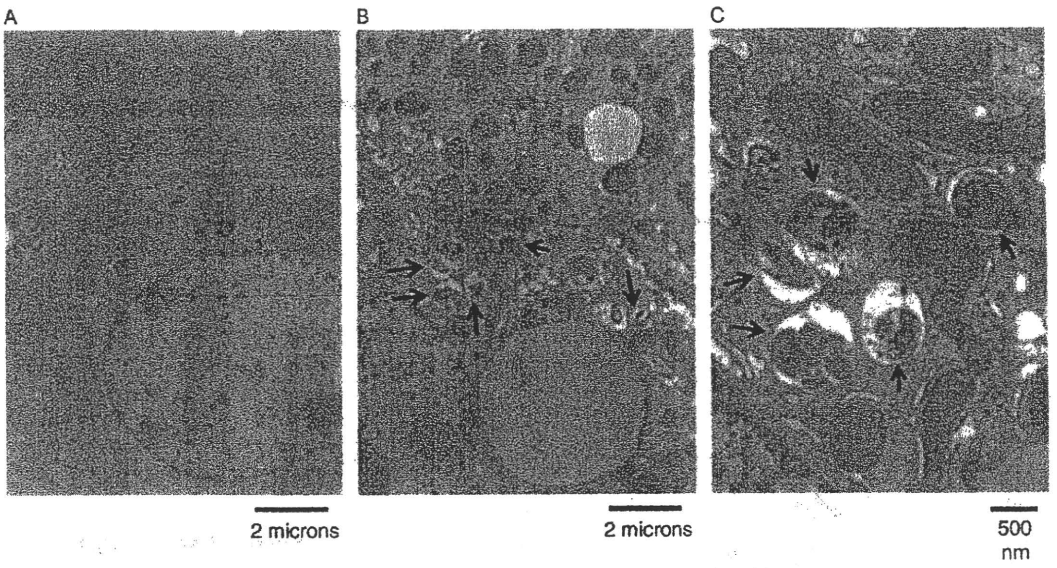


Fig. 4. Electron microscopy of the liver of an FL-N/35 transgenic mouse and a nontransgenic mouse, both of which were fed the iron-excess diet for 12 months. (A) Nontransgenic mouse, (B) full-length HCV-N open reading frame (FL-N/35) transgenic mouse and (C) Magnified picture of B. Autophagosomes (indicated by arrows) are abundantly found in the liver of the FL-N/35 transgenic mouse fed the iron-excess diet. Magnification scales are indicated below each picture.

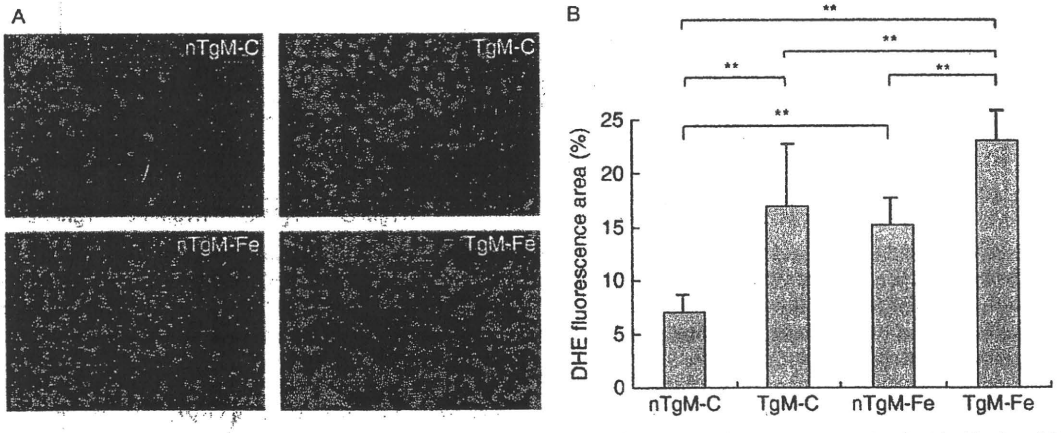
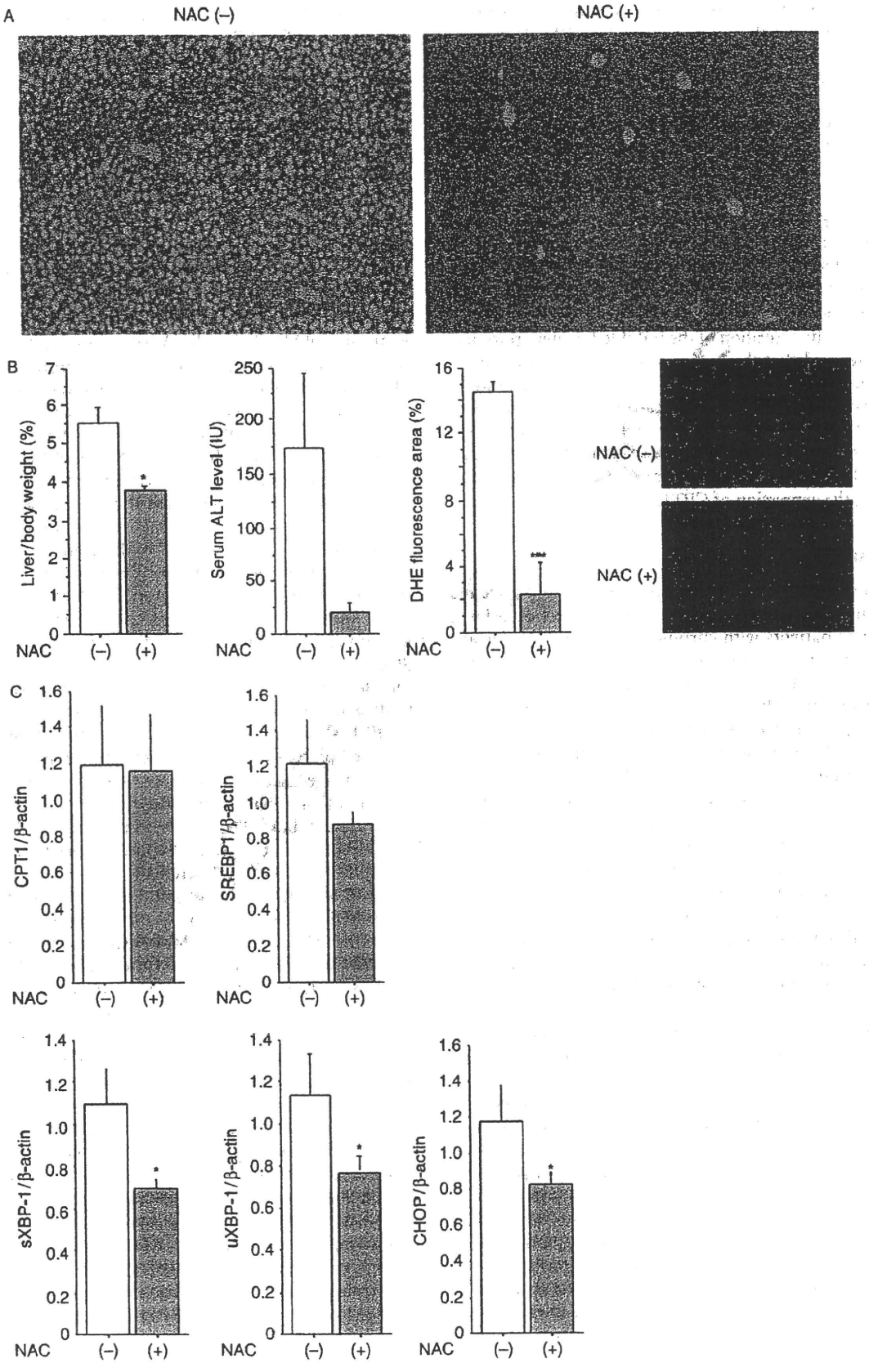


Fig. 5. Reactive oxygen species production in the liver. (A) Frozen liver sections of mice in each group were stained with dihydroethidium. (B) Fluorescence intensity was quantified by NIH image analysis software for three randomly selected areas of digital images for three mice in each group at 12 months after initiation of iron loading. $^{**}P < 0.01$. nTgM-C, TgM-C, nTgM-Fe, and TgM-Fe; see legend for Figure 1.

transgenic mice fed the iron-excess diet than in mice in the three other groups, even though abundant ROS production was found in all mice, except for nontransgenic mice fed the control diet (Fig. 5A and B). ROS production was significantly higher in transgenic mice than in nontransgenic mice irrespective of iron overloading. Iron overloading also significantly increased ROS production irrespective of whether the mice were transgenic or nontransgenic (Fig. 5B). FL-N/35 transgenic mice fed the iron-excess diet had the highest level of ROS production.

A six-month treatment with an anti-oxidant, NAC, dramatically reduced hepatic steatosis in FL-N/35 transgenic mice fed the iron-excess diet (Fig. 6A), together

Fig. 6. Liver histology, and the ratio of liver weight to body weight, serum alanine aminotransferase (ALT) levels, reactive oxygen specie's production and expression of carnitine palmitoyl transferase I (CPT1), sterol-regulatory element-binding protein I (SREBP1), spliced X-box DNA-binding protein 1 (sXBP1), unspliced X-box DNA-binding protein 1 (uXBP1) and CCAAT/enhancer-binding protein homology protein (CHOP) in the liver of full-length HCV-N open reading frame (FL-N/35) transgenic mouse fed the iron-excess diet with/without *N*-acetyl cysteine (NAC) treatment. (A) NAC treatment drastically reduced hepatic steatosis in mice. (B) Frozen liver sections of mice in each group were stained with dihydroethidium. Fluorescence intensity was quantified with the method described in the legend for Figure 5 in three mice in each group. (C)



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with a significant reduction in the ratio of liver weight to body weight and ROS production (Fig. 6B). The serum alanine aminotransferase level was also reduced by NAC, but this change was not statistically significant because of the large variance of the data. The expression of uXBP1, sXBP1 and CHOP was significantly reduced after treatment with NAC, suggesting an inhibitory effect of the anti-oxidant on the unfolded protein response (Fig. 6C). The expression of SREBP1 was also reduced by treatment with NAC, but this reduction was not statistically significant ($P = 0.08$). The expression of CPT1, which regulates oxidization of long-chain fatty acids in the mitochondria, did not change after NAC treatment. These results suggested that iron-induced ROS generation induced hepatic steatosis through the activation of the unfolded protein response. It also seemed that the increased lipogenesis through the activated unfolded protein response contributed more to the development of hepatic steatosis than the reduced β-oxidation activity in FL-N/35 transgenic mice fed the iron-excess diet, because the anti-oxidant almost completely inhibited the development of hepatic steatosis without affecting the expression of CPT1.

Discussion

The hepatic iron content of FL-N/35 transgenic mice fed the iron-excess diet was comparable to that of a large number of patients with chronic hepatitis C in extensive studies (15, 16). The positive correlation between the iron level and the triglyceride concentration in the liver was consistent with our previous observation that even modest iron supplementation enhanced hepatic steatosis in FL-N/35 transgenic mice (9), suggesting a potential role of iron in the development of HCV-related steatosis. Although previous studies revealed a direct contribution of HCV core protein to the development of hepatic steatosis (5–7), how iron overload, which is one of pathological features in chronic hepatitis C, affects hepatic steatosis remains unknown. The decreased expression of CPT I suggested reduced β-oxidation activity, because this transmembrane enzyme of the mitochondrial outer membrane has been shown to be the rate-limiting step in the β-oxidation of long-chain fatty acids (30). This result was consistent with our previous observation that the degradation activity of fatty acids *in vivo* was reduced in iron-overloaded transgenic mice. The decreased expression of CPT I may be related to the association of HCV core protein with the mitochondrial outer membrane (31). However, the decreased expression of CPT I seemed to reflect the rather increased synthesis of fatty acids because CPT I is negatively regulated by malonyl-CoA, an intermediate product in fatty acid synthesis, at the transcriptional level (30). In fact, the expression of FAS was significantly increased in FL-N/35 transgenic mice fed the iron-excess diet, which was presumably driven by upregulation of a transcription factor, SREBP1. We could not differentiate SREBP1c from SREBP1a at the protein level because of the lack of an adequate antibody; nevertheless, the expression of SREBP1 was assumed to mainly reflect that of SREBP1c, because the SREBP1c transcript extremely predominates over the SREBP1a transcript is considerably predominant in the mouse liver (32).

The regulation of SREBP activation occurs at two levels: transcriptional and post-transcriptional (17). Upregulation of SREBP1c promoter activity has been reported in HCV core gene-transgenic mice (6), but we did not find a significant difference in SREBP1 expression between transgenic and nontransgenic mice without iron overloading. This contradiction may have arisen from a difference in the transgenic mice used in the two studies. In addition, a recent report found no significant difference in the hepatic expression of SREBP1c mRNA between subjects with HCV infection and those with a histologically normal liver (33). HCV has been demonstrated to induce proteolytic cleavage of SREBP1 and 2 in HCV replicon cells (34). As described previously, modest iron supplementation restored a major phenotype of FL-N/35 transgenic mice marked by hepatic steatosis and liver tumour development (9). Thus, the present animal model was useful for understanding the critical role of iron overloading in the development of HCV-related hepatic steatosis. We therefore focused on the post-transcriptional regulation of SREBP1 by iron in the presence of HCV proteins. Upon ER stress, the SREBP–SCAP complex dissociates from the ER retention protein and subsequently translocates to the Golgi apparatus, where SREBP is cleaved and activated (18, 19). FL-N/35 transgenic mice fed the iron-excess diet showed the activated unfolded protein response, assessed by the increased expression of uXBP-1, sXBP-1, p-eIF2α and CHOP, suggesting that the unfolded protein response was activated by iron overloading in the presence of HCV proteins. On the other hand, it is demonstrated that the trans-activating activity of XBP-1 is suppressed, but ATF6 functions properly in HCV replicon cells (35), which is in part contradictory to the present results. Methodological differences (*in vivo* or *in vitro*, iron overload or not, etc.) in two studies may account for this contradiction. Thus, the role of the unfolded protein response in HCV infection alone is still a matter of debate.

To confirm activation of the unfolded protein response in FL-N/35 transgenic mice fed the iron-excess diet, we wanted to assess not only the activation of ER-resident sensors but also the morphological change induced by the unfolded protein response. Autophagy has been shown to play important roles in cell survival after ER stress (25–27). A double-membrane structure, which is called the autophagosome or the autophagic vacuole, is formed *de novo* to sequester cytoplasm. Then the vacuole membrane fuses with the lysosomal membrane to deliver the contents into the autolysosome, where they are degraded and the resulting macromolecules are recycled. Some studies demonstrated a critical role of IRE1 in inducing autophagy under ER stress (25, 36), whereas another study reported the involvement of the PERK-eIF2α signalling pathway, not IRE1, in autophagy induction by ER stress (37). Thus, it is still controversial as to

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which transducer is utilized for ER stress-induced autophagy in mammalian cells. The abundant presence of autophagosomes was consistent with the activation of both ER-resident sensors, IRE1 and PERK, in the liver in FL-N/35 transgenic mice fed the iron-excess diet. Although there is no direct link between induction of autophagy and hepatic steatosis in FL-N/35 transgenic mice fed the iron-excess diet, induction of autophagy seemed to support the ER stress-related hepatic steatosis because autophagy is one of the morphological changes under ER stress (25–27).

Iron overload is potentially one of multiple sources of ROS production, as represented in the iron-catalysed Fenton reaction (38). FL-N/35 transgenic mice fed the iron-excess diet had a significantly higher level of ROS production than mice in the three other groups, suggesting a cooperative role of HCV proteins and iron in inducing oxidative stress. ROS have been demonstrated to be involved upstream of the unfolded protein response (28). Anti-oxidants have also been shown to reduce the unfolded protein response and improve protein secretion (29). The present findings that the expression of uXBP1, sXBP1 and CHOP, but not CPT1, was significantly reduced with NAC treatment were consistent with these previous observations, suggesting that iron-induced ROS activated the unfolded protein response in the presence of HCV proteins. How then does ER stress activate SREBP1? There are several lines of evidence suggesting that one mechanism by which ER stress leads to activation of SREBPs is related to downregulation of insulin-induced genes. Downregulation of insulin-induced genes is associated with less retention of SREBPs in the ER, which leads to increased SREBP activation (39–41). As another mechanism, it has been shown that overexpression of glucose-regulated protein 78, one of the ER resident chaperone proteins, inhibits SREBP activation (42). Irrespective of how ER stress activates SREBP, the predominant role of SREBP1 in the ER stress-related hepatic steatosis in FL-N/35 transgenic mice fed the iron-excess diet was similar to that observed in a murine intragastric ethanol-feeding model (43). In conclusion, considering the complexity of the argument and the limited number of evaluated mechanisms, iron-induced ROS-activated unfolded protein response may be postulated as one of the possible mechanisms of HCV-related fat accumulation in the liver.

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Original Article

Standardized Prevalence Ratios for Chronic Hepatitis C Virus Infection Among Adult Japanese Hemodialysis Patients

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ABSTRACT

Background: Many studies have estimated the prevalence of anti-hepatitis C virus (HCV) antibody among hemodialysis (HD) patients; however, the prevalence of HCV core antigen—which indicates the presence of chronic HCV infection—is not known.

Methods: Standardized prevalence ratios (SPRs) for anti-HCV antibody and HCV core antigen among HD patients ($n = 1214$) were calculated on the basis of data from the general population ($n = 22\,472$) living in the same area.

Results: The prevalences of anti-HCV antibody and HCV core antigen were 12.5% and 7.8%, respectively, in male hemodialysis patients, and 8.5% and 4.1% in female hemodialysis patients. The SPRs (95% confidence interval) for anti-HCV antibody and HCV core antigen were 8.39 (6.72–10.1) and 12.9 (9.66–16.1), respectively, in males, and 5.42 (3.67–7.17) and 8.77 (4.72–12.8) in females.

Conclusions: The prevalences of chronic HCV infection among male and female HD patients were 13-fold and 9-fold, respectively, those of the population-based controls. Further studies should therefore be conducted to determine the extent of chronic HCV infection among HD patients in other populations and to determine whether chronic HCV infection contributes to increased mortality in HD patients.

Key words: hepatitis C virus infection; hemodialysis; standardized prevalence ratio (SPR); population-based study; cross-sectional analysis

INTRODUCTION

The prevalence of hepatitis C virus (HCV) infection in hemodialysis patients is very high.[1–15] Because hemodialysis patients are vulnerable to HCV infection due to the risk of HCV exposure associated with the dialysis procedure and blood transfusion,[16–18] infection control measures have been established to reduce the risks of HCV infection. Tests for detecting antibodies to HCV were first licensed by the Food and Drug Administration (FDA) in 1990[19] and are now used worldwide. The risk of HCV infection due to dialysis and blood transfusion has therefore dramatically decreased.

The estimated prevalence of HCV infection in hemodialysis patients, although lower than in the past, remains high in developed countries in Europe, despite measures to prevent transmission of HCV.[13,20,21] It has been suggested that HCV infection independently contributes to increased mortality among hemodialysis patients.[14,22–26] In order to reduce mortality associated with HCV infection among hemodialysis patients, the prevalence of HCV infection and the factors that predispose hemodialysis patients to HCV infection require immediate investigation.

The prevalence of anti-HCV antibody among hemodialysis patients has been estimated in many studies, but the prevalence of chronic HCV infection is not known. In

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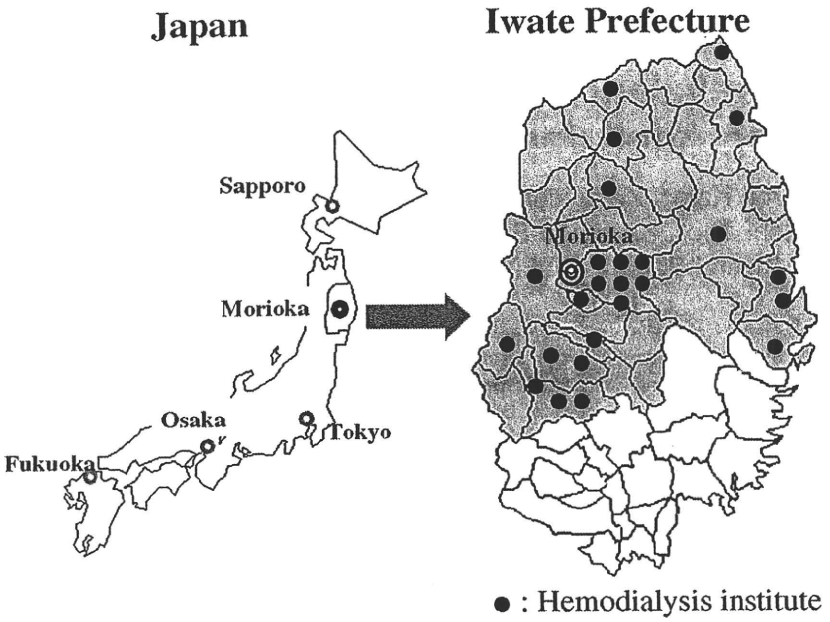

Figure 1. Maps of the KAREN Study area.
The maps show the location of Morioka (the capital of Iwate Prefecture), in northeastern Honshu island. The KAREN Study area (shaded area) covers approximately two-thirds of Iwate Prefecture, and includes 26 hemodialysis facilities; only 1 facility (in which 7 patients were treated) was not included in the study. Closed circles indicate the sites of the hemodialysis facilities.

general, patients who are anti-HCV antibody-positive include those who are chronically infected and those who have recovered from infection. However, all patients who are HCV core antigen-positive are considered chronically infected. Therefore, it is necessary to test for both anti-HCV antibody and HCV core antigen to accurately assess the extent of chronic HCV infection in hemodialysis patients.

We investigated the prevalences of anti-HCV antibody and HCV core antigen in hemodialysis patients. We then compared these prevalences with those of the general population and examined associations between the prevalences and hemodialysis vintage.

SUBJECTS AND METHODS

Subjects

We have conducted the "Kaleidoscopic Approaches to patients with end-stage RENal disease Study" (the KAREN Study) since 2003 in northern Japan (Figure 1). The KAREN Study is a population-based prospective study designed to determine the effects of risk factors on cardiovascular morbidity and mortality in end-stage renal disease (ESRD) patients.[27] A total of 1214 adult hemodialysis patients (80.6% of the total number of hemodialysis patients in the study area; age 22 to 95 years; 779 males and 435 females) are included in the KAREN Study. Figure 2 shows a flow chart of the procedure for selecting subjects participating in the KAREN Study.

Control subjects were recruited from the general population living in the same area, and comprised 22474 participants (7650 men and 14824 women) who underwent annual health check-ups in Iwate Prefecture and HCV screening tests in 2005.

This study was approved by the Medical Ethics Committee of Iwate Medical University and was conducted in accordance with the guidelines of the Declaration of Helsinki.

Measurements

The initial investigations in the KAREN Study were conducted from June 2003 through March 2004. These consisted of a questionnaire, review of medical records, measurements of blood pressure and anthropometric data, and blood tests. Anthropometrical examinations and blood pressure measurements were performed in a consistent manner. Self-administered questionnaires were used to collect individual information on demographic characteristics, history of cardiovascular disease, use of medication, alcohol consumption, and smoking status.[27]

Two medical doctors and 8 nurses visited 25 medical facilities and reviewed patients' medical records and treatment regimens. They recorded patient characteristics, such as age, sex, past history, family history, date when hemodialysis was initiated, length of hemodialysis sessions, number of hemodialysis sessions per week, prescribed dry weight, interdialysis weight gain at the beginning of the week, cause of ESRD, diabetes status, comorbid conditions, current medications, and history of other hemodialysis treatment.[27]

In the present study, information on anti-HCV antibody serology testing was collected by reviewing medical charts. All anti-HCV antibody serology tests at the 25 medical

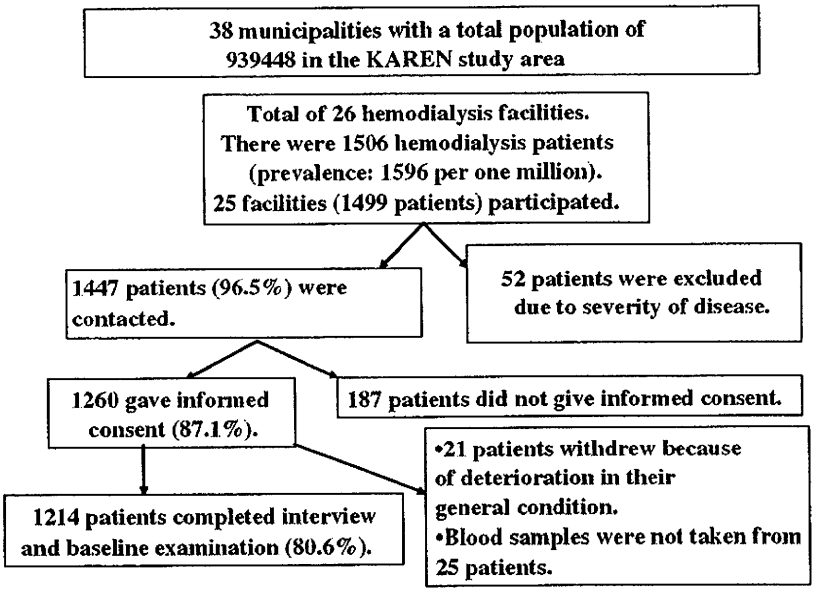

Figure 2. Flow chart for selecting subjects participating in the KAREN Study.
A total of 1506 adult patients were undergoing hemodialysis in 26 institutes in the study area. We were able to contact 1447 patients (96.5%); an additional 52 patients were excluded because of the severity of their condition. A total of 1260 patients (87.1%) gave written informed consent for participation in the study. Of these, 1214 (80.6%) completed the baseline examination.

facilities were performed by using a second- or third-generation assay.

Predialysis blood sampling was performed by dialysis nursing staff immediately before beginning hemodialysis sessions. Blood samples were drawn from arteriovenous fistulae or grafts through hemodialysis cannulae into vacuum tubes. The blood samples were transported to a laboratory (Mitsubishi Kagaku Bio-Clinical Laboratories, Inc., Morioka branch office), and biochemical measurements and combined blood counts were performed on the same day. Residual sera of each sample were collected and stored at −80 °C in our laboratory.

Results of anti-HCV antibody tests could not be obtained from 50 patients upon reviewing their medical charts. Frozen serum samples from those patients were unfrozen and anti-HCV antibody tests were performed using a second-generation assay (Architect HCV, Abbott, Japan). Frozen samples from patients who were positive for anti-HCV antibody (as confirmed by chart review or by HCV antibody determination using frozen samples) were unfrozen and HCV core antigen tests were performed using the Chemiluminescent Enzyme Immunoassay (CLEIA). Quantitative determination of HCV-RNA by reverse transcription polymerase chain reaction (RT-PCR) was not performed in hemodialysis patients who were positive for anti-HCV antibody and negative for HCV core antigen (Figure 3).

The HCV screening survey of the general population was conducted in Iwate Prefecture in 2005. All samples were transported to a laboratory (Iwate Health Service Association), and HCV antibody serology tests were performed by using an enzyme immunoassay (AxSYM HCV Dynapack II, Abbott Japan). Additional HCV core antigen tests were also performed using CLEIA in subjects who were positive for HCV antibody. A total of 236 samples from participants who were positive for anti-HCV antibody and negative for HCV core antigen were then used for qualitative determination of HCV-RNA by RT-PCR (AMPLICOR TM HCV test, Roche, Figure 4).

Statistical analysis

Hemodialysis patients and population-based control subjects were divided into sex- and age-specific groups (20–39, 40–49, 50–59, 60–69, and ≥70 years). Sex- and age-specific prevalences of anti-HCV antibody and HCV core antigen were determined both in hemodialysis patients and controls.

Among hemodialysis patients, the expected number of patients positive for anti-HCV antibody (or HCV core antigen) in each sex- and age-specific group was calculated by using the prevalence of each sex- and age-specific group from the population-based controls. The total number of expected patients positive for anti-HCV antibody (or HCV core antigen) among hemodialysis patients was calculated by summing the numbers of positive individuals expected in all age-specific groups. The ratio of the observed number of hemodialysis patients with anti-HCV antibody (or HCV core antigen) to the expected number was defined as the standardized prevalence ratio (SPR). We assumed that the data would have a Poisson distribution; therefore, the confidence intervals for the SPRs were estimated using standard errors.[28]